frontiers | Research Topics

Application of next generation sequencing (NGS) and CRISPR-cas systems in the diagnosis of infectious diseases

Edited by
Yanfei Chen, Yanjiao Zhou, George Weinstock, Lei Chen and Li Ang

Published in
Frontiers in Medicine
Frontiers in Cellular and Infection Microbiology
Frontiers in Public Health

FRONTIERS EBOOK COPYRIGHT STATEMENT

The copyright in the text of individual articles in this ebook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this ebook is the property of Frontiers.

Each article within this ebook, and the ebook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this ebook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or ebook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-83251-633-1
DOI 10.3389/978-2-83251-633-1

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Application of next generation sequencing (NGS) and CRISPR-cas systems in the diagnosis of infectious diseases

Topic editors

Yanfei Chen — Zhejiang University, China
Yanjiao Zhou — UCONN Health, United States
George Weinstock — Jackson Laboratory for Genomic Medicine, United States
Lei Chen — Shanghai Jiao Tong University, China
Li Ang — First Affiliated Hospital of Zhengzhou University, China

Citation

Chen, Y., Zhou, Y., Weinstock, G., Chen, L., Ang, L., eds. (2023). *Application of next generation sequencing (NGS) and CRISPR-cas systems in the diagnosis of infectious diseases*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-633-1

Table of contents

08 SARS-CoV-2 Direct Detection Without RNA Isolation With Loop-Mediated Isothermal Amplification (LAMP) and CRISPR-Cas12
Alfredo Garcia-Venzor, Bertha Rueda-Zarazua, Eduardo Marquez-Garcia, Vilma Maldonado, Angelica Moncada-Morales, Hiram Olivera, Irma Lopez, Joaquin Zuñiga and Jorge Melendez-Zajgla

17 Case Report: Metagenomics Next-Generation Sequencing Can Help Define the Best Therapeutic Strategy for Brain Abscesses Caused by Oral Pathogens
Zhonghui Ma, Su Yan, Haoxin Dong, Huifen Wang, Yonggang Luo and Xi Wang

22 Case Report: Proven Diagnosis of Culture-Negative Chronic Disseminated Candidiasis in a Patient Suffering From Hematological Malignancy: Combined Application of mNGS and CFW Staining
Yanqi Jin, Zhouhan Wang, Chunxia Zhu, Qing Yang, Yingfeng Lu, Xiaopeng Yu, Bao Hong, Xiaojing Wang and Yimin Zhang

28 Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of Talaromycosis of an Immunocompetent Patient
Jiejun Shi, Naibin Yang and Guoqing Qian

36 Case Report: Next-Generation Sequencing in Diagnosis of Pneumonia Due to *Pneumocystis jirovecii* and Cytomegalovirus in a Patient With HIV Infection
Yirui Xie, Bing Ruan, Lingxiao Jin and Biao Zhu

42 CRISPR/Cas System: A Potential Technology for the Prevention and Control of COVID-19 and Emerging Infectious Diseases
Ronghua Ding, Jinzhao Long, Mingzhu Yuan, Yuefei Jin, Haiyan Yang, Mengshi Chen, Shuaiyin Chen and Guangcai Duan

52 CRISPR-Based COVID-19 Testing: Toward Next-Generation Point-of-Care Diagnostics
Uyanga Ganbaatar and Changchun Liu

63 Case Report: A Severe and Multi-Site *Nocardia farcinica* Infection Rapidly and Precisely Identified by Metagenomic Next-Generation Sequencing
Mengfan Jiao, Xiang Deng, Hongfu Yang, Junqiang Dong, Jun Lv and Fang Li

68 Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of *Legionella pneumophila* Pneumonia in a Patient After Umbilical Cord Blood Stem Cell Transplantation
Yangyan Wang, Yuanyuan Dai, Huaiwei Lu, Wenjiao Chang, Fan Ma, Ziran Wang, Zhican Liu and Xiaoling Ma

73 Early Detection of *Legionella pneumophila* and *Aspergillus* by mNGS in a Critically Ill Patient With *Legionella* Pneumonia After Extracorporeal Membrane Oxygenation Treatment: Case Report and Literature Review
Ruiming Yue, Xiaoxiao Wu, Tianlong Li, Li Chang, Xiaobo Huang and Lingai Pan

79 Diagnosis of *Mycobacterium tuberculosis* Septic Shock in Patients With Anti-synthetase Syndrome Based on Next-Generation Sequencing: A Case Report and Literature Review
Limin Sun, Ziyue Yang, Fei Yang, Zhenhua Wang, Hongqiang Li, Huifen Wang and Tongwen Sun

85 Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of Disseminated Tuberculosis of an Immunocompetent Patient
Yuanting Ye, Naibin Yang, Jingying Zhou, Guoqing Qian and Jinguo Chu

91 Metagenomic Next-Generation Sequencing Can Clinch Diagnosis of Non-Tuberculous Mycobacterial Infections: A Case Report
He Zhu, Min Zhu, Jia-Hui Lei, Ya-Li Xiao and Li-Min Zhao

95 Case Report: *Tropheryma whipplei* Hide in an AIDS Patient With *Pneumocystis* Pneumonia
Jun Yan, Binhai Zhang, Zhongdong Zhang, Jinchuan Shi, Shourong Liu, Jianjiang Qi and Mengyan Wang

99 Homologous *Escherichia coli* Identified in Cerebrospinal Fluid and Bloodstream
Qingmiao Shi, Jun Zhang, Jinghui Wang, Lijuan Du, Zhaoyang Shi, Min Xu and Yonggang Luo

107 Metagenomic Next Generation Sequencing in the Detection of Pathogens in Cerebrospinal Fluid of Patients After Alternative Donor Transplantation: A Feasibility Analysis
Binglei Zhang, Jian Zhou, Ruirui Gui, Zhen Li, Yingling Zu, Juan Wang, Fengkuan Yu, Yanli Zhang, Huifang Zhao, Zhenyu Ji and Yongping Song

118 Case Report: Diagnostic Value of Metagenomics Next Generation Sequencing in Intracranial Infection Caused by Mucor
YuChen Liu, Jun Zhang, Bing Han, LiJuan Du, ZhaoYang Shi, ChunCheng Wang, Min Xu and YongGang Luo

124 Metagenomic Next-Generation Sequencing in the Diagnosis of HHV-1 Reactivation in a Critically Ill COVID-19 Patient: A Case Report
Lei Shi, Han Xia, Matthew D. Moore, Chao Deng, Na Li, Hui Ren, Yunru Chen, Jinfeng Liu, Fenjing Du, Gezhi Zheng, Jing Li, Qunying Han, Wanhu Fan, Feng Ye, Shumei Lin, Zhengwen Liu, Hongjuan Liu, Yawen Wang, Jian Yang, Qingguang Liu, Yingren Zhao and Tianyan Chen

129 A Retrospective Paired Comparison Between Untargeted Next Generation Sequencing and Conventional Microbiology Tests With Wisely Chosen Metagenomic Sequencing Positive Criteria
Hanyu Qin, Jinmin Peng, Ling Liu, Jing Wu, Lingai Pan, Xiaobo Huang, Man Huang, Haibo Qiu, Bin Du and the China Critical Care Clinical Trials Group (CCCCTG)

139 Case Report: Metagenomics Next-Generation Sequencing Can Be Performed for the Diagnosis of Disseminated Mucormycosis
Yi Sun, HuiLing Li, JiaJun Chen, ZhongHui Ma, Pin Han, YuChen Liu, Jing Wen, Fang Ren and XiaoXu Ma

145 Case Report: A *Candida* Meningitis in an Immunocompetent Patient Detected Through the Next-Generation Sequencing
Xiao-guang Cao, Chuang-wei Yu, Shu-sheng Zhou, Yu Huang and Chun-yan Wang

152 Severe Pneumonia Caused by Infection With *Tropheryma whipplei* Complicated With *Acinetobacter baumannii* Infection: A Case Report Involving a Young Woman
Sheng Wang, Demeng Xia, Jianghong Wu, Dandan Jia, Lei Li and Shuogui Xu

158 Case Report: Streptococcus Suis Meningitis Diagnosed in a HIV-Infected Patient With Cryptococcal Meningitis Using Next-Generation Sequencing
Yirui Xie, Bing Ruan, Guanjing Lang and Biao Zhu

163 The Early Diagnosis of Scrub Typhus by Metagenomic Next-Generation Sequencing
Xianghong Liu, Ye Zhang, Jun Zhang, Zheng Lou, Han Xia and Zhijuan Lu

170 Case Report: Fascioliasis Hepatica Precisely Diagnosed by Metagenomic Next-Generation Sequencing and Treated With Albendazole
Yaling Zhang, Huan Xu, Yi Liu, Juan Kang, Hairu Chen, Zhiyi Wang and Dachuan Cai

177 Metagenomic Analysis Identifying a Rare *Leishmania* Infection in an Adult With AIDS
Pingping Song, Shuai Chen, Xiaoyu Tan, Yanjun Gao, Juanjuan Fu, Zhiqing You, Chengtan Wang, Qigang Zhao and Feng Pang

184 Case Report: Metagenomics Next-Generation Sequencing for Diagnosing Cerebral Infarction and Infection Caused by Hematogenous Disseminated Mucormycosis in a Patient With Acute Lymphoblastic Leukemia
Bingbing Wen, Lisheng Cai, Yun Cai and Xin Du

190 Diffuse Large B-Cell Lymphoma of the Mandible Diagnosed by Metagenomic Sequencing: A Case Report
Kaili Liu, Yang Gao, Jiawei Han, Xu Han, Yingqi Shi, Chao Liu and Jie Li

195 Case Report: Next-Generation Sequencing in Diagnosis of Atypical Aspiration Pneumonia
Quan Zhang, Wei Xu, Fei Peng, Si Lei, Yanjun Zhong, Jun Yi and Shangjie Wu

201 Clinical Symptoms and Outcomes of Severe Pneumonia Caused by *Chlamydia psittaci* in Southwest China
Fuxun Yang, Jiajia Li, Bo Qi, Longfei Zou, Zongming Shi, Yu Lei, Jun Li, Xiaoxiu Luo, Fan Zeng, Sen Lu, Xiaobo Huang, Rongan Liu and Yunping Lan

209 Detection of *Nocardia* by 16S Ribosomal RNA Gene PCR and Metagenomic Next-Generation Sequencing (mNGS)
Juanjuan Ding, Bing Ma, Xupeng Wei and Ying Li

215 The Application of Whole–Exome Sequencing in Patients With FUO
Wanru Guo, Xuewen Feng, Ming Hu, Yanwan Shangguan, Jiafeng Xia, Wenjuan Hu, Xiaomeng Li, Zunjing Zhang, Yunzhen Shi and Kaijin Xu

222 Case Report: Metagenomic Next-Generation Sequencing for Diagnosis of Human Encephalitis and Endophthalmitis Caused by Pseudorabies Virus
Weiqian Yan, Zhiping Hu, Yingchi Zhang, Xiaomei Wu and Hainan Zhang

228 Metagenomic Next-Generation Sequencing vs. Traditional Microbiological Tests for Diagnosing Varicella-Zoster Virus Central Nervous System Infection
Yunqi Zhu, Miaomiao Xu, Chengyuan Ding, Zhihang Peng, Weixiao Wang, Binghu Sun, Jian Cheng, Chen Chen, Wei Chen, Hongxia Wei and Zhiliang Hu

235 Evaluations of Clinical Utilization of Metagenomic Next-Generation Sequencing in Adults With Fever of Unknown Origin
Zhang-fan Fu, Hao-cheng Zhang, Yi Zhang, Peng Cui, Yang Zhou, Hong-yu Wang, Ke Lin, Xian Zhou, Jing Wu, Hong-long Wu, Wen-hong Zhang and Jing-wen Ai

245 Next Generation Sequencing for Diagnosis of Leptospirosis Combined With Multiple Organ Failure: A Case Report and Literature Review
Juan Lu, Juan Hu, Shanshan Yu and Lanjuan Li

250 Case Report: Community-Acquired *Legionella gormanii* Pneumonia in an Immunocompetent Patient Detected by Metagenomic Next-Generation Sequencing
Cheng Lei, Xianglin Zhou, Shuizi Ding, Yingjie Xu, Binyi Yang, Wei Guo, Min Song, Min Yang, Yunan Jia and Hong Luo

257 Autoimmune Encephalitis With Multiple Auto-Antibodies With Concomitant Human Herpesvirus-7 and Ovarian Teratoma: A Case Report
Jianhua Yang, Pengcheng Wu, Xianghong Liu, Han Xia and Zhaohui Lai

262 Brain Abscess Caused by *Nocardia farcinica* and Diagnosed by Metagenomic Next-Generation Sequencing: A Case Report
Jianhua Yang, Shuhua Xie, Junda Li, Han Xia and Xianghong Liu

268 Characteristics and Prognostic Factors of Non-HIV Immunocompromised Patients With Pneumocystis Pneumonia Diagnosed by Metagenomics Next-Generation Sequencing
Jiali Duan, Jing Gao, Qiuhong Liu, Mengfei Sun, Yang Liu, Yingshuai Tan and Lihua Xing

276 Invasive Pulmonary Aspergillosis Diagnosis *via* Peripheral Blood Metagenomic Next-Generation Sequencing
Xiaoxu Ma, Suping Zhang, Haizhou Xing, Huiling Li, Jiajun Chen, Haijun Li, Mengfan Jiao, Qingmiao Shi, Aiguo Xu, Lihua Xing and Weijie Cao

285 Recurrent Pneumonia With Tuberculosis and *Candida* Co-infection Diagnosed by Metagenomic Next-Generation Sequencing: A Case Report and Literature Review
Ning Ma, Mei Chen, Jingyi Ding, Fang Wang, Jingbo Jin, Sitong Fan and Jiajia Chen

291 Multisystem Involvement Induced by Human Parvovirus B19 Infection in a Non-immunosuppressed Adult: A Case Report
Qihang Zou, Peisong Chen, Jiaxin Chen, Dingbang Chen, Han Xia, Ling Chen, Huiyu Feng and Li Feng

299 Case Report: An Ulceration With a Stalactite Appearance on the Index Finger
Liangliang Zhang, Yalan Dai, Mengting Lin, Qiuyun Xu, Tingting Lin, Ting Gong, Bo Cheng, Chao Ji and Donghua Cai

302 Potential Opportunities and Challenges of Deploying Next Generation Sequencing and CRISPR-Cas Systems to Support Diagnostics and Surveillance Towards Malaria Control and Elimination in Africa
Beatus M. Lyimo, Zachary R. Popkin-Hall, David J. Giesbrecht, Celine I. Mandara, Rashid A. Madebe, Catherine Bakari, Dativa Pereus, Misago D. Seth, Ramadhan M. Ngamba, Ruth B. Mbwambo, Bronwyn MacInnis, Daniel Mbwambo, Issa Garimo, Frank Chacky, Sijenunu Aaron, Abdallah Lusasi, Fabrizio Molteni, Ritha Njau, Jane A. Cunningham, Samwel Lazaro, Ally Mohamed, Jonathan J. Juliano, Jeffrey A. Bailey and Deus S. Ishengoma

frontiers in Medicine

ORIGINAL RESEARCH
published: 17 February 2021
doi: 10.3389/fmed.2021.627679

SARS-CoV-2 Direct Detection Without RNA Isolation With Loop-Mediated Isothermal Amplification (LAMP) and CRISPR-Cas12

*Alfredo Garcia-Venzor[1], Bertha Rueda-Zarazua[1], Eduardo Marquez-Garcia[2], Vilma Maldonado[3], Angelica Moncada-Morales[2], Hiram Olivera[4], Irma Lopez[4], Joaquin Zuñiga[2] and Jorge Melendez-Zajgla[1]**

[1] Cancer Functional Genomics Laboratory, Instituto Nacional de Medicina Genómica, Mexico City, Mexico, [2] Molecular Biology Unit, Instituto Nacional de Enfermedades Respiratorias, Mexico City, Mexico, [3] Epigenetics Laboratory, Instituto Nacional de Medicina Genómica, Mexico City, Mexico, [4] Instituto Nacional de Referencia Epidemiológica, Mexico City, Mexico

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Antonio Gennaro Nicotera,
University of Messina, Italy
Shuaiyin Chen,
Zhengzhou University, China

***Correspondence:**
Jorge Melendez-Zajgla
jmelendez@inmegen.gob.mx

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 09 November 2020
Accepted: 25 January 2021
Published: 17 February 2021

Citation:
Garcia-Venzor A, Rueda-Zarazua B, Marquez-Garcia E, Maldonado V, Moncada-Morales A, Olivera H, Lopez I, Zuñiga J and Melendez-Zajgla J (2021) SARS-CoV-2 Direct Detection Without RNA Isolation With Loop-Mediated Isothermal Amplification (LAMP) and CRISPR-Cas12. Front. Med. 8:627679. doi: 10.3389/fmed.2021.627679

As to date, more than 49 million confirmed cases of Coronavirus Disease 19 (COVID-19) have been reported worldwide. Current diagnostic protocols use qRT-PCR for viral RNA detection, which is expensive and requires sophisticated equipment, trained personnel and previous RNA extraction. For this reason, we need a faster, direct and more versatile detection method for better epidemiological management of the COVID-19 outbreak. In this work, we propose a direct method without RNA extraction, based on the Loop-mediated isothermal amplification (LAMP) and Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein (CRISPR-Cas12) technique that allows the fast detection of SARS-CoV-2 from patient samples with high sensitivity and specificity. We obtained a limit of detection of 16 copies/μL with high specificity and at an affordable cost. The diagnostic test readout can be done with a real-time PCR thermocycler or with the naked eye in a blue-light transilluminator. Our method has been evaluated on a small set of clinical samples with promising results.

Keywords: COVID-19, SARS-CoV-2, diagnosis, LAMP, CRISPR-Cas12a

INTRODUCTION

The COVID-19 global outbreak caused by the virus SARS-Cov-2 is one of the major global concerns due to its rapid spread around the world. The official numbers on November 07, 2020, are 49,636,193 confirmed cases and 1,247,187 deaths on the entire planet (Johns Hopkins University, https://coronavirus.jhu.edu/map.html; last access on November 07).

The precise and rapid screening of possible infected subjects is one of the major strategies to contain the COVID-19 outbreak, to reduce the impact on the mortality of the population and avoid hospital saturation. However, the current diagnostic strategies mainly consist of qRT-PCR analysis, which has drawbacks as it requires RNA isolation, and thus takes several hours to obtain a result. These aspects have hindered the applicability of qRT-PCR systems to efficiently contain the COVID-19 outbreak (1).

For these reasons, it is of capital importance to develop novel diagnostic techniques, that improve cost, increase the number of the diagnostic facilities able to perform assays, and the time needed to obtain a result. Loop-mediated Isothermal Amplification (LAMP) technology allows the specific amplification of a target DNA or RNA sequences at a single temperature (in contrast with the three steps required by PCR), allowing a continuous target amplification, making it more versatile than PCR. LAMP has been previously used in the diagnosis of different pathogens at low cost and with great sensitivity and specificity (2–5).

Recently, Zhang et al. (6) used LAMP technology for the specific detection of SARS-CoV-2, observing promising results that could have a direct application in the current COVID-19 outbreak. These results led us to take advantage of the versatility and sensitivity of LAMP to standardize a diagnostic kit for SARS-CoV-2 detection for limited-resource settings use.

In this work, we developed a practical SARS-CoV-2 diagnostic method using a LAMP reaction to amplify a specific region of SARS-CoV-2 N gene mRNA and tested three main detection methods: fluorescence accumulation, colorimetric detection, and LbCas12a-mediated detection. The results obtained showed that the best limit of detection (LOD) was obtained using a qPCR thermocycler to measure the fluorescence accumulation over time. Interestingly, the colorimetric approach gave comparable LOD than fluorescence mediated detection. However, its dependence on pH changes makes it unreliable to use with poor buffered samples such as saliva.

MATERIALS AND METHODS

Primers and Probes

We used two different primer sets for our diagnostic assay, one targeting SARS-CoV-2 N gene transcript, and another targeting human RNAseP POP7 mRNA. Primer sequencing was obtained from previous work, and the sequences used are listed in **Supplementary Table 1** (7). For controls, we synthesized a fragment of N gene mRNA and RNAseP POP7 mRNA as gene Blocks (gBlocks) (IDT, Cal., USA) (**Supplementary Table 2**). Additionally, to work with an RNA SARS-CoV-2 N gene control, the gBlock was *in vitro* transcribed using RiboMAX Large Scale RNA Production System kit and following manufacturer instructions (P1280, Promega, MA, USA). The N-gene RNA copy number was determined using its molar concentration and it was serially diluted to obtain a group of known copy number dilutions used for Limited of calculation (LOD) calculation.

For *Lachnospiraceae bacterium* Cas12a (LbCas12) mediated detection, we used two previously designed guide RNAs (gRNAs), targeting the SARS-CoV-2 N gene, and RNAseP POP7 amplicons. The gRNAs sequence is shown in **Supplementary Table 3**. Also, for LbCas12 fluorescence readout, we used a Taqman probe (5′FAM/3′BHQ1), which is intended to be degraded by the specific activation of LbCas12a.

LAMP Reactions

Fluorescent LAMP reactions were done using the WarmStart LAMP kit (E1700L, New England Biolabs, NE, USA), and Colorimetric WarmStart LAMP 2X Master Mix (M1800L, New England Biolabs, NE, USA) was used for colorimetric LAMP. All LAMP reactions were done following manufacturers' instructions, per 29 μL reaction we use 2.5 μL of a LAMP primer mix (F3, B3: 2 μM, LF, LB: 4 μM and FIP, BIP: 16 μM), 5 μL of sample or control and $MgSO_4$ concentration was increased to 6 mM by adding 1.13 μL per reaction, of a 100 mM solution. For colorimetric and fluorescent LAMP reactions, the amplification was carried by incubating at 65°C for 40 min. For real-time fluorescent detection of LAMP reactions, we used a QuantStudio 3 Real-Time PCR system (ThermoFisher Scientific, MA, USA) thermocycler, using a PCR program of 160 cycles of 15 s each. End-point fluorescence quantification was carried out in a Beckman Coulter DTX 880 multimode detector (Beckman Coulter, Nyon, Switzerland), using the FAM emission and excitation filters. Direct visualization of fluorescence was done in a tabletop transilluminator.

LbCas12a Detection

For LbCas12a mediated detection we used commercial LbaCas12a (EnGen LbaCas12a, M0653T, NEB, NE) and RNA was synthesized as RNA oligonucleotides by IDT (CA, USA). First, LbaCas12a-gRNAs complexes were generated by incubating LbaCas12a (50 nM, final concentration) and, gRNAs (62.5 nM), in 1X NEBuffer 2.1 at a final volume of 20 μL per reaction. The LbaCas12a-gRNA complexes were incubated 30 min at 37°C, after incubation, the Taqman probe was added at 500 nM final concentration and transferred to ice. After the LAMP reaction is done, 2 μL of each LAMP reaction was added to 20 μL of the LbaCas12a-gRNA complexes, and the reactions were incubated at 37°C for 10 min. The real-time fluorescence acquisition was done using the QuantStudio 3 Real-Time PCR system (ThermoFisher Scientific, MA, USA) thermocycler, using a qPCR program of 80 cycles of 15 s at 65°C. The visual inspection of fluorescence was done using a blue-light transilluminator (Invitrogen, CA, USA).

In silico Alignment

We used blastn from NCBI for the alignment *in silico*, we compare every primer sequence to the main virus and bacteria that causes symptoms similar to covid-19 or are related to the virus SARS-CoV-2 (Candida albicans taxid: 5476, Neisseria meningitidis taxid: 487, pseudomonas aeruginosa taxid: 287, Staphylococcus aureus taxid: 1280, Influenza A taxid: 11320, Influenza B taxid: 11520, bat MERS-like coronavirus taxid: 2664423, MERS-CoV taxid: 1335626, HCoV-SARS taxid: 694009, HCoV-229E taxid: 11137, HCoV-OC43 taxid: 31631, HCoV-NL63 taxid: 277944, and HCoV-HKU1 taxid: 290028).

ROC Curves and AUC Values

The receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) values were calculated using pROC R package (1.16.2) with the values of Cycle threshold (Ct) of the LOD curves or patient samples evaluation from fluorescent LAMP reactions or the qualitative values of the use of Cas12a variation. The non-amplified cases were considered as Ct = 160. An AUC of 50% is related to a positive result by chance,

while we consider a percentage above 90% as acceptable and an accurate result.

Sample Treatment and RNA Extraction

For the LOD determination, we added a known amount of N gene RNA copies to 8 μL of a healthy donor nasal swab, subsequently adding 2 uL of 5X lysis solution (0.5 M DTT and 5 mM EDTA). The samples were incubated at 42°C for 20 min, followed by 64°C for 5 min. These steps allowed nuclease inactivation and lysis of biological material releasing nucleic acid without extraction. Five microliter of these inactivated samples were added to 20 μL of LAMP reaction and processed as specified before. Also, this simple inactivation method was used for virus inactivation and lysis of samples from patients.

Patients

The Institutional Review Board (IRB) of the National Institute of Respiratory Diseases (INER) reviewed and approved the protocols for COVID-19 studies. This project was approved by the IRB under the registration number B09-20. All subjects or their legal responsible, particularly in case of critically ill patients, provided written informed consent for these studies, and they authorized the storage of their nasopharyngeal samples at INER repositories for this and future studies. In this study, we did not collect samples from minors/children, only young adults older than 17 years were included.

A total of 34 patient samples were collected at the Molecular Biology "Unidad de Biología Molecular", from the National Institute for Respiratory Diseases in Mexico City. We collected 31 nasal swabs and 3 saliva samples from individuals that attended to the emergency room of the institute between March 01 and May 31, 2020; coinciding with the rise of the COVID-19 outbreak in Mexico City. RNA extraction was done by the addition of 2 μL of Lysis solution (DTT 0.5 M and EDTA 5 mM) to 8 μL of the sample, followed by incubation at 42°C for 20 min, and 64°C for 5 min. For LAMP reactions, we used 5 μL of the extracted samples for SARS-CoV-2 N gene amplification and 5 μL for POP7 amplification. Real-time fluorescence measurement was done using a StepOnePlus Real-Time PCR system (ThermoFisher, MA, USA) for RT-qPCR and LAMP reactions. The presence of viral transcript was assessed first by a one-step RT-PCR with a modified Berlin method internally standardized, using specific oligonucleotide sequences for the E and RdRp genes (2019-nCoV) and confirmed with the LAMP test.

Assay for SARS-CoV-2 Detection by a LAMP-LbCas12a Method

The LAMP assay tube contained 24 μL of the mix with the LAMP primers and Bst enzyme. To this tube 5 μL of sample or control was added and incubated for 40 min at 65°C. After this time 2 μL of this reaction was added into the LbCas12a tube containing the enzyme-gRNA complex and the reporter. The reaction was incubated for 10 min at 37°C to observe or measure the fluorescence (**Figure 5**).

RESULTS

Sensitivity and Specificity of SARS-CoV-2 LAMP Detection

To detect the SARS-CoV-2 virus we used two previously published primer sets, one targeting a specific region of SARS-CoV-2 N-gene and a human control set targeting the RNAseP POP7 gene (3). The N-gene set detected the presence of the COVID-19 virus, whereas the POP7 set was a control for the presence of human transcripts. Alignment analysis was performed to evaluate the specificity of the LAMP amplification primers for N-gene. We considered the main pathogens causing respiratory symptoms like those of Covid-19. No cross alignment was found with any virus or bacteria, suggesting high specificity of the sequences used for detection. For amplification detection, we used two main systems: fluorescence, and colorimetry. For the fluorescent detection system, we added an ATTO Intercalating dye with similar absorption/emission spectra than SYBR-green to the LAMP reactions. Fluorescence increase was recorded using a real-time PCR thermocycler, and the final fluorescence with a multi-well plate fluorometer. The colorimetric assay is based on the acidification of the reaction buffer by the amplification system, which can be detected by a colorimetric pH sensor. This detection system has the advantage of no require any additional detection device than the human eye. Also, to improve the applicability of the method to the actual needs of physicians, we standardized a rapid method for sample inactivation and lysis, based on high temperatures and reducing conditions. To this end, we prepared a lysis solution at 5X concentration, added 1 μL of lysis solution per every 4 μL of a nasal swab, and heated the samples at 42°C for 20 min followed by 64°C for 5 min.

First, we wanted to determine the limit of detection of these assays, for this end, we used a synthetic SARS-CoV-2 N-gene RNA diluted to the specific copy number concentration using a 5 μL of sample volume per reaction (**Table 1**). Real-time detection of LAMP amplification in a qPCR thermocycler showed a 100% rate of detection (LOD) in samples with 66 copies/μL after 30 min of amplification (**Table 2**). To enhance the LOD, we increased the reaction time to 50 min. Even when we found a

TABLE 1 | The copy number concentration of samples.

Sample	cp/5 μL sample	cp/μL
NTC	0	0
S16.5	16.5	3.3
S33	33	6.6
S55	55	11
S82	85	17
S165	165	33
S330	330	66
S550	550	110
S1000	1,000	200
S1600	1,600	320

NTC, Non-template control; Cp, copies.

TABLE 2 | Limit of detection of LAMP reaction by real-time fluorescence with 30 and 50 min.

Samples	Signal Positivity					Det. at 30 min	Det. at 50 min
	N1	N2	N3	N4	N5		
NTC	–	–	–	–	–	0%	40%
S16.5	–	–	+	+	+	60%	80%
S33	+	+	+	–	+	80%	100%
S55	+	+	+	+	+	100%	100%
S82	+	+	+	+	+	100%	100%
S165	+	+	–	+	+	80%	100%
S330	+	+	+	+	+	100%	100%
S550	+	+	+	+	+	100%	100%
S1000	+	+	+	+	+	100%	100%
S1600	+	+	+	+	+	100%	100%

+ is a positive signal.
– is a negative signal.

TABLE 3 | Limit of detection of LAMP reaction by real-time fluorescence.

Sample	Signal of positivity (Ct values)																				
	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21	Det 40 min
NTC	NS	NS	NS	NS	–	NS	152	158	–	–	–	130	NS	NS	NS	157	–	–	–	–	–	–
S16.5	NS	NS	89	79	86	120	146	123	114	110	NS	102	124	NS	NS	NS	119	129	133	NS	126	67%
S55	78	84	109	96	77	140	123	102	113	NS	114	114	NS	NS	113	NS	129	115	NS	119	NS	71%
S82	95	109	99	106	91	114	99	112	160	120	138	NS	108	NS	142	100	127	NS	104	114	111	86%
S165	89	104	NS	87	115	NS	138	142	134	122	119	106	NS	114	108	107	121	110	NS	NS	129	81%
S330	101	111	118	106	96	130	121	131	104	115	98	95	94	100	95	100	95	106	68	104	104	100%
S550	103	95	85	106	114	130	142	90	118	120	110	106	105	97	107	101	105	85	124	105	77	100%
S1600	25	26	25	26	25	38	38	38	38	39	42	32	32	33	33	32	33	32	33	31	32	100%

NS, Non signal.

decreased LOD of 6.6 copies/μl, this modification also increased the probability of a false-positive detection, since the proportion of amplificated NTC increased to 40% (**Table 2**). For this reason, we expanded the number of samples, confirming the LOD with 66 copies/μL after 40 min of amplification (**Table 3**). The ROC curve analysis showed that the LOD of 66 copies/μL presents a good proficiency in terms of specificity and sensitivity (AUC of 99.4%, CI 95 = 98–100%) (**Figure 1**).

The specificity of the N-gene LAMP reaction was assessed using human total RNA samples (MCF-7) without N-gene transcripts (**Figure 2A**). We observed that MCF-7 samples behave as NTC samples (data not shown). As expected, we observed a specific amplification in MCF-7 samples using the POP7 primer set. Besides, we used a panel of controls, including influenza A/H1N1/pdm09, Influenza B, and other common human coronaviruses like HCoV-NL63, HCoV-HKU1, HCoV-OC43, and HCoV-229E to verify the specificity of the test. All cases were negative to SARS-CoV-2, so there is no cross-reactivity with these viruses.

Next, we analyzed the final fluorescence of LAMP reactions using a multi-well plate fluorometer, using the fluorescein isothiocyanate (FITC) filter. The results showed that final fluorescence detection was less sensitive than real-time fluorescence detection since the LOD obtained for final fluorescence detection was of 1,000 overall copies (**Table 4**). These results highlight that final fluorescence detection by fluorometer showed a strong downfall in sensitivity and reproducibility.

Since colorimetric reactions demonstrated to be easily and rapidly analyzed by eye. We next aimed to establish the limits of this detection system. We analyzed the LOD of colorimetric LAMP reactions. For this end, we spiked samples of a nasal swab with known numbers of N-gene synthetic transcripts, and inactivated and lysed these samples. The LOD obtained in these spiked samples was of 66 copies/μL, like the LOD obtained by real-time fluorescence (**Figure 2B**). We found that an N-gene reaction with 320 copies/μL changed the color of the reaction toward yellow, in contrast with the NTC reaction that remained pink (**Figure 2C**).

Finally, to decrease the limit of detection of the LAMP detection system we proved another fluorescence detection

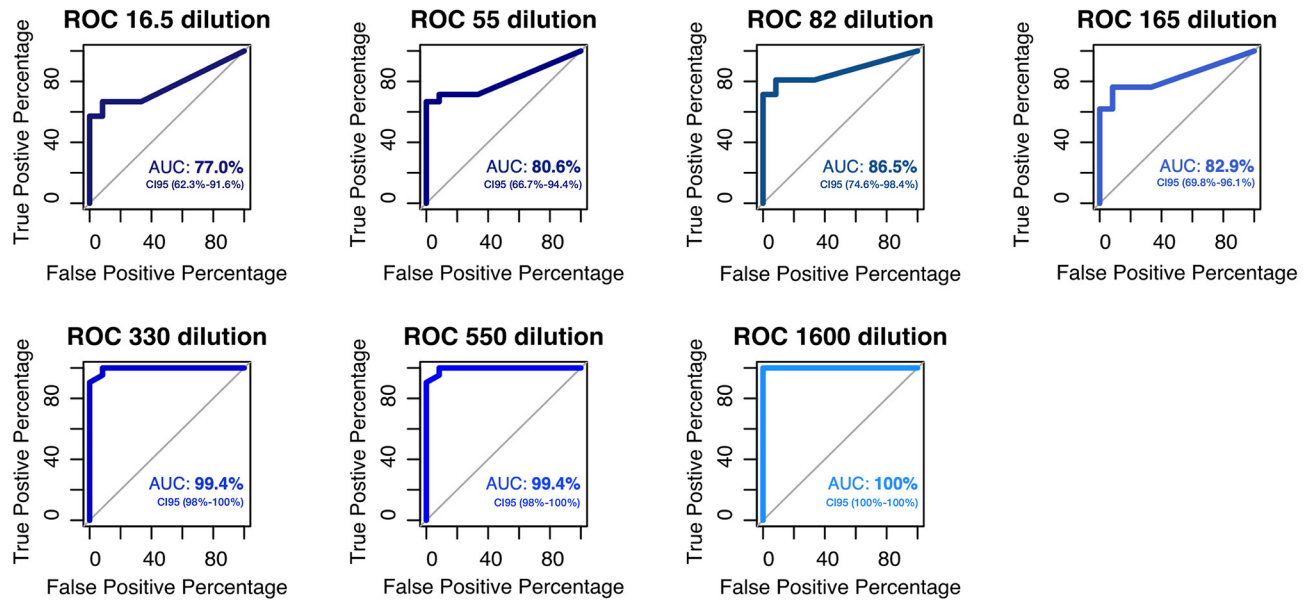


FIGURE 1 | ROC curves and AUC values for each copy number concentration spiked with SARS-CoV-2. The dataset used for the construction of ROC curves and AUC values corresponds to the LOD curves for fluorescent LAMP reactions analyzed using a Real-time thermocycler. From the dilution with 330 copies, we can observe an acceptable accuracy of the test (AUC > 90.0%).

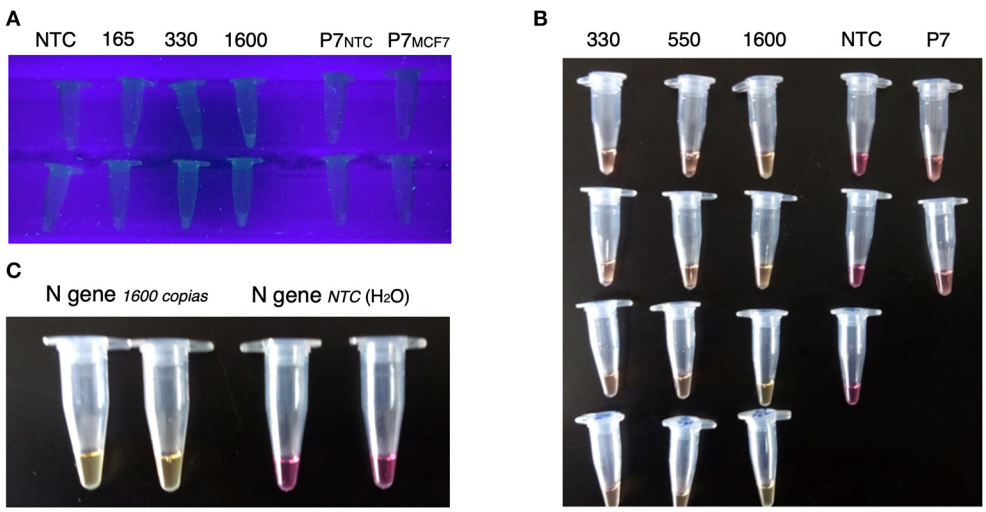


FIGURE 2 | Visual results of fluorescent and colorimetric LAMP systems. **(A)** Representative images of the visual inspection of LAMP fluorescent reactions using a UV transilluminator, fluorescence is perceived in the 66 and 320 copies/5 uL reactions. **(B)** Representative images of the LOD curves of colorimetric LAMP reactions, positive reactions change toward yellow, but negative ones remain pink. **(C)** Representative images of positive and negative colorimetric LAMP results.

system based on LbCas12a reactions. LbCas12a is a bacterial endonuclease that recognizes and hydrolyzes a target DNA sequence complementary to the sequence of a guide RNA (gRNA). Interestingly, once LbCas12a recognizes and hydrolyzes its target sequence, it activates an endonuclease domain that degrades any DNA molecule at hand (collateral nuclease activity). To use this property of LbCas12a we designed two gRNAs targeting N-gene and POP7, and we amplified the signal by the addition of a quenched DNA-probe, that fluoresce once degraded by activated LbCas12a. For LbCas12a reactions we added 5 μL of LAMP reactions previously incubated for 30 min. Interestingly, the recording of real-time fluorescence of LbCas12a showed that this detection system improves the LOD of the simple LAMP reaction measured by real-time PCR to 16 copies/μL (**Table 5**). It also improved the visual detection of the LAMP reaction using a blue-light transilluminator (**Figure 3**).

At last, we proved the efficiency of our SARS-CoV-2 detection system on patient samples previously analyzed by RT-qPCR. As shown in **Table 6**, LAMP reactions showed good proficiency in detecting SARS-CoV-2 viral transcripts on patient samples. However, using qPCR as a standard, we observed the fluorescent LAMP system failed to detect two of twelve negative samples. Furthermore, the fluorescent LAMP system showed an accuracy of ten of ten in positive samples with which we obtain an AUC value of the ROC curve of 90.8%, CI 95 = 77.6–100% (**Figure 4**).

The results obtained to this point suggest that LAMP reactions are a robust method for SARS-CoV-2 detection. Since amplification can be detected by several methods, being the colorimetric method the most versatile for field applications, and the fluorescent method in addition to the LbCas12a system the more sensible and easier to interpret if the hospital facility counts with a real-time thermocycler (**Table 7**). This system improves the ROC curve reaching an AUC value of 100.0%, CI 95 = 100–100% using patient samples previously validated with the standard method, qPCR (**Figure 4**). In addition, LAMP reactions are suitable for the direct use of samples from rapid lysis methods as the ones described here.

Our LAMP/Cas12 detection protocol is a simple and versatile method that has the advantage of obtaining a result in only 65 min, since the reception of the sample. The procedure consists in a sample treatment with lysis buffer at two temperatures that can be achieved by almost all thermoblocks (20 min at 42 C, and 5 min at 65 C). Followed by the amplification of SARS-CoV-2 N gene by LAMP reactions, which is accomplished in 30 min, and finally the detection of specific amplicons using a Cas12a/crRNA system that can be achieved in 10 min. The readout of the result can be done using a real-time thermocycler, or a blue-light lamp in obscure conditions (**Figure 5**).

DISCUSSION

The COVID-19 outbreak has become one of the most important public health problems at the global level. In the absence of an approved vaccine, early and reliable detection of the virus in the entire population is our best strategy to contain COVID-19 lethality and hospitable saturation. In the present work, we developed a new SARS-CoV-2 detection test which allows rapid

TABLE 4 | Limit of detection of LAMP reaction by final fluorescence.

	Signal positivity				
Samples	**N1**	**N2**	**N3**	**N4**	**% detection**
NTC	–	–	–	–	0%
S16.5	–	–	–	–	0%
S33	+	–	–	–	20%
S55	+	–	–	–	20%
S82	+	–	–	–	20%
S165	+	–	–	+	40%
S330	–	–	–	+	20%
S550	+	–	–	+	40%
S1000	+	+	+	+	100%
S1600	+	+	+	+	100%

+ is a positive signal.
– is a negative signal.

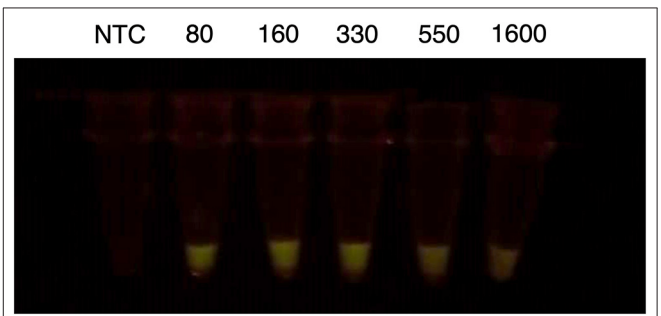


FIGURE 3 | Visual results of dCas12-mediated detection. The results show a representative image of the fluorescence signals in dCas12a reactions. As observed, positive signals glow with a green color under blue-light illumination. However, negative signals have a very faint glow.

TABLE 5 | Limit of detection of LbCas12a reaction by final fluorescence.

	Positivity of signal															
Sample	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**N7**	**N8**	**N9**	**N10**	**N11**	**N12**	**N13**	**N14**	**N15**	**% detection**
NTC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0%
S40	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	26%
S80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100%
S160	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100%
S330	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100%
S550	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100%
S1600	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100%

+ is a positive signal.
– is a negative signal.

TABLE 6 | LAMP efficiency in patient samples.

Sample	Origin	qPCR result	LAMP result
1	Extraction	Negative	Negative
2	Extraction	Negative	Negative
3	Nasal swab	Negative	Negative
4	Nasal swab	Negative	Negative
5	Nasal swab	Negative	Negative
6	Nasal swab	Negative	Negative
7	Nasal swab	Negative	Negative
8	Nasal swab	Negative	Positive
9	Nasal swab	Negative	Positive
10	Saliva	Negative	Negative
11	Saliva	Negative	Negative
12	Saliva	Negative	Negative
13	Extraction	Positive	Positive
14	Extraction	Positive	Positive
15	Nasal swab	Positive	Positive
16	Nasal swab	Positive	Positive
17	Nasal swab	Positive	Positive
18	Nasal swab	Positive	Positive
19	Nasal swab	Positive	Positive
20	Nasal swab	Positive	Positive
21	Nasal swab	Positive	Positive
22	Nasal swab	Positive	Positive

Nasal swab: Sample collected from nasal mucosa. Saliva: Collected from the saliva accumulation after 30 s of movements like chewing gum. Extraction: Samples extracted with a commercial kit before the process.

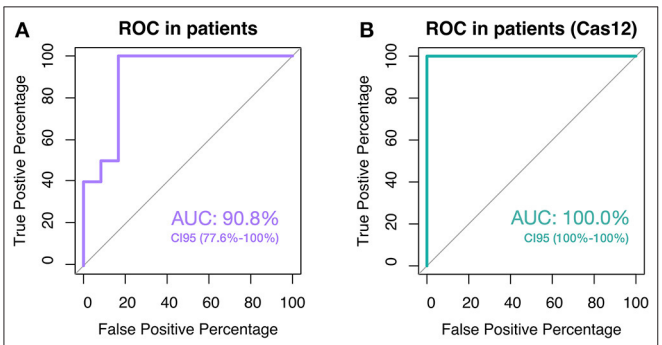


FIGURE 4 | ROC curves and AUC values in the test performed with patient samples. **(A)** The ROC curve and AUC value were calculated with the Ct values of the fluorescent LAMP reactions using patient samples previously validated by Real-time PCR method. **(B)** The ROC curve and AUC value consider the qualitative results of Cas12a enhancement used in validated patient samples.

detection of viral N gene transcript after a simple, fast and cheap RNA isolation procedure. Furthermore, LAMP technology enables a versatile diagnostic test, which can be done using any water bath able to warm at 65°C, and can be detected, in the best of cases with a real-time thermocycler, but also with the naked eye if the equipment is absent.

In this work, we tested several detection methods to develop a more versatile and sensitive diagnostic test for the COVID-19 virus. Based on fluorescence and colorimetric readouts we found that the colorimetric system is the more versatile and easily field-deployable strategy but has the drawbacks that in low-copy number samples the color change is difficult to perceive clearly, and its sensitiveness to bad buffered samples. However, real-time fluorescence detection gave the more easily interpretable results but requires the use of a real-time PCR thermocycler, which is almost impossible to obtain outside of a few facilities. Also, to increase the sensitivity of our diagnostic test, we evaluated the integration of LbCas12a to amplify the signal generated by the LAMP reaction. However, the results obtained showed that fluorescence increase due to LbCas12a activity (due to LAMP amplicons) has a linear behavior, which amplifies more the LAMP signals. Hence, the addition of LbCas12a improves the LOD obtained from LAMP reactions alone.

TABLE 7 | Cas12a efficiency in patient samples.

Sample	Origin	qPCR result	Cas12a result
1	Nasal swab	Negative	Negative
2	Nasal swab	Negative	Negative
3	Nasal swab	Negative	Negative
4	Nasal swab	Negative	Negative
5	Nasal swab	Negative	Negative
6	Nasal swab	Negative	Negative
7	Nasal swab	Positive	Positive
8	Nasal swab	Positive	Positive
9	Nasal swab	Positive	Positive
10	Nasal swab	Positive	Positive
11	Nasal swab	Positive	Positive
12	Nasal swab	Positive	Positive

Nasal swab: Sample collected from nasal mucosa.

In comparison with other diagnostic technologies assessed by other groups, our SARS-CoV-2 detection system outperforms almost all currently available diagnostic systems in terms of speed, since the inactivation protocol used decreased RNA-extraction time to 25 min. The developed LAMP system requires just 40 min to obtain a result and if we implement the LbCas12a system we add 10 more minutes to the protocol, much less time compared with the 4 h used in the CDC/WHO protocols (8, 9) and is similar to other LAMP protocols (30–45 min) (6, 10), or Crispr based protocols such as DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR) (30 min) or Specific High Sensitivity Enzymatic Reporter Unlocking (SHERLOCK) (58 min), these methods use a similar RNA extraction protocol (11). However, since the other diagnostic technologies use a time-expensive RNA-extraction protocol, our rapid-inactivation protocol to extract nucleic acids gives us a total time of 65–75 min to obtain a result, starting from sample reception. Also, the RNA extraction protocol required by all methods could represent a risk of exposure and contamination. In this case, we reduce sample processing by using the sample directly.

It is important to note, that the colorimetric LAMP system has several advantages concerning other systems in terms of its applicability. The incubation temperatures used in the entire protocol can be achieved using a common water bath, and

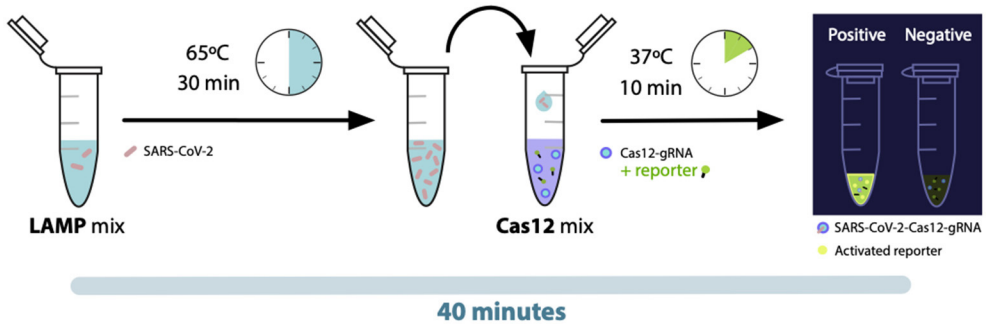


FIGURE 5 | LAMP-Cas12 diagnostic method for SARS-CoV-2 detection. Schema shows the steps to perform the virus detection. From a previous inactivated clinical sample, we add the LAMP mix containing specific primers that target the virus N-gene and amplify it, if is present on the sample. At the same time, the LbCas12a enzyme is incubated with a N-gene specific gRNA, after 30 min the reporter containig a fluorophore is added. When the LbCas12a-gRNA complex finds its target, it has a non-specific cleavage of the reporter that releases the fluorophore and emits fluorescence.

TABLE 8 | Recent SARS-CoV2 detection method comparison.

	Our work	**Wang et al. (16)**	**Pang et al. (14)**	**Lalli et al. (15)**
Methods	RT-LAMP/Cas12a	RT-LAMP/Cas12a	RT-LAMP/Cas12a	RT-LAMP
Time	40 min	45 min	40 min	25 min
LOD	330 cp/rxn (66 cp/μL)	5 cp/μL	150 cp/rxn (30 cp/μL)	59 cp/rxn
Clinical samples	34	50	100 swabs	30 saliva
RNA extraction	No	Yes	Yes	No
Visualization	Colorimetric and fluorescence	Blue light	UV light and cellphone	Colorimetric and fluorescence
Target	N gene	S-gene	N and E gene	N gene and Orflab
Brief description	We use HUDSON (25 min) Both reactions at the same time in separate tubes and mixing the last 10 min.	Both reactions in the same tube and the same time. The LAMP mix in the bottom and the Cas12 mix on the lid for the first incubation, then they mix and do the second incubation.	Similar to Wang without the oil and with different times and volumes	There are two temperature incubation of the samples (65°/15 min and 95°/5 min) followed by an incubation of 25 min.
Volumes	24 μL RT-LAMP + 20 μL Cas12 + 5 μL sample	40 μL RT-LAMP + 20 μL Cas12 + 1 μL sample	25 μL RT-LAMP + 10 μL Cas12 + 2-10 μL sample	20 μL RT-LAMP + 3 μL sample

the final readout of the test can be easily obtained by visual inspection. Also, our experience revealed that colorimetric LAMP master-mixes (with primers) can be stored at 4°C for up to a week. These advantages make colorimetric LAMP tests a promising technology to improve viral diagnosis in the field, easily adaptable to physicians (3) needs. However, since colorimetric LAMP readout is achieved by a pH decrease, these reactions are sensitive to samples without a good buffering system, giving rise to increased false-negative rates.

The SARS-CoV-2 LAMP diagnostic system developed showed a sensibility of 66 copies per μL, which is comparable to the LOD of DETECTR (70–300 copies per μL) (3) or SHERLOCK (10–100 copies per μL) (11). And if we use the LbCas12a system, this LOD improves to 16 copies per μL. In comparison with other LAMP-based diagnostic methods, our system has a similar sensitivity, since Zhang et al., reported a sensitivity of 24 copies/uL (2, 11, 12), and Yu et al. reported a sensitivity of 10 copies/mL (13) which makes these colorimetric tests almost as sensitive as qPCR. However, the RT-qPCR protocol (CDC/WHO) is still the most sensitive diagnostic method, the limit of detection is about 1–3 copies per μL (4, 8). Furthermore, the LOD value achieved in our diagnostic system showed a good proficiency for the detection of the SARS-CoV-2 virus in patient samples and showed similar results than the qRT-PCR system in the same set. Also, even when the LOD is higher than the conventional method, it is enough to detect infection from clinical samples in which the copy number on average is 676 copies per μL (12). The present method has the advantage of combining two assays without RNA extraction, which, in our hands gives a more robust result that either one separately, as it is more sensitive and specific, while conserving specific technical benefits useful for deploying in the daily lab. For example, the recently published Pang et al. method (14) uses the same tube for the reaction but requires previous RNA extraction and a higher level of manipulation, since each tube has to hold the Cas12 mix in the lid, which is prone to errors when working with multiple samples. The recently published assay by Lalli et al. (15) does not need previous RNA isolation, but starts with saliva, which, in our hands at least, have a reproducibility and specificity issues, as we have found that the additional use of Cas12a decrease false positives. In addition, their method requires the addition of proteinase K or guanidine isothiocyanate, adding to the cost of the assay. Finally, in terms of

specificity the SARS-Cov-2 LAMP detection system we present showed no amplification in samples containing human RNA, and the genomes of other viruses causing respiratory diseases. A comparison between assays is provided in **Table 8**.

In conclusion, these results imply that SARS-CoV-2 LAMP detection system showed an average sensitivity in terms of LOD value, but with a proficiency enough to detect the virus in patient samples, and with a high specificity that does not cross with human transcriptomes, or other viral genomes., SARS-CoV-2 LAMP detection system offers a rapid, sensitive and specific option with minimal sample processing or equipment requirements for the detection of SARS-CoV-2.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Instituto Nacional de Medicina Genómica Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AG-V, BR-Z, EM-G, AM-M, and HO performed all the experiments and including samples' analysis. VM, IL, and JM-Z coordinated the study. AG-V, BR-Z, VM, and JM-Z wrote the article. AG-V, BR-Z, EM-G, VM, and JM-Z analyzed the data. All authors contributed to the article and approved the submitted version.

FUNDING

This project was funded by Consejo Nacional de Ciencia y Tecnología (CONACyT), Project FORDECYT 03/2020.

ACKNOWLEDGMENTS

Thanks to Dr. Gisela Ceballos and Dr. Magali Espinosa for the help and support during plasmid subcloning. Thanks to CONACyT for the special funding, that made possible this project. We thank Dr. Gustavo Reyes-Terán for his support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.627679/full#supplementary-material

REFERENCES

1. Center J. S. H. C. R. *Coronavirus COVID-19 global cases by the center for systems science and engineering (CSSE) at John Hopkins 2020*. Baltimore: John Hopkins Center (2020).
2. Mori Y, Notomi T. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J Infect Chemother.* (2009) 15:62–9. doi: 10.1007/s10156-009-0669-9
3. Nzelu CO, Kato H, Peters NC. Loop-mediated isothermal amplification (LAMP): an advanced molecular point-of-care technique for the detection of Leishmania infection. *PLoS Negl Trop Dis.* (2019) 13:e0007698. doi: 10.1371/journal.pntd.0007698
4. World Health Organization. *The Use of Loop-Mediated Isothermal Amplification (TB-LAMP) for the Diagnosis of Pulmonary Tuberculosis: Policy Guidance*. Geneva: World Health Organization (2016).
5. Seki M, Kilgore PE, Kim EJ, Ohnishi M, Hayakawa S, Kim DW. Loop-mediated isothermal amplification methods for diagnosis of bacterial meningitis. *Front Pediatr.* (2018) 6:57. doi: 10.3389/fped.2018.00057
6. Zhang Y, Odiwuor N, Xiong J, Sun L, Nyaruaba RO, Wei H, et al. Rapid molecular detection of SARS-CoV-2 (COVID-19) virus RNA using colorimetric LAMP. *medRxiv [preprint].* (2020). doi: 10.1101/2020.02.26.20028373
7. Broughton JP, Deng X, Yu G, Fasching CL, Singh J, Streithorst J, et al. CRISPR-Cas12-based detection of SARS-CoV-2. *Nat Biotechnol.* (2020) 38:870–4. doi: 10.1038/s41587-020-0513-4
8. Centers for Disease Control and Prevention. *CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Centers for Disease Control and Prevention*. Atlantla: Centers for Disease Control and Prevention (2020).
9. Ching L, Chang SP, Nerurkar VR. COVID-19 special column: principles behind the technology for detecting SARS-CoV-2, the cause of COVID-19. *Hawaii J Health Soc Welf.* (2020) 79:136–142.
10. Huang WE, Lim B, Hsu C-C, Xiong D, Wu W, Yu Y, et al. RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microb Biotechnol.* (2020) 13:950–61. doi: 10.1111/1751-7915.13586
11. Zhang F, Abudayyeh OO, Gootenberg JS. *A Protocol for Detection of COVID-19 Using CRISPR Diagnostics*. Vol. 8. Cambridge, MA: The Broad's Institute (2020).
12. Wölfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Müller MA, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature.* (2020) 581:465–9. doi: 10.1038/s41586-020-2196-x
13. Yu L, Wu S, Hao X, Li X, Liu X, Ye S, et al. Rapid colorimetric detection of COVID-19 coronavirus using a reverse tran-scriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic plat-form: iLACO. *medRxiv [preprint].* (2020). doi: 10.1101/2020.02.20.20025874
14. Pang B, Xu J, Liu Y, Peng H, Feng W, Cao Y, et al. Isothermal amplification and ambient visualization in a single tube for the detection of SARS-CoV-2 using loop-mediated amplification and CRISPR technology. *Anal Chem.* (2020) 92:16204–12. doi: 10.1021/acs.analchem.0c04047
15. Lalli MA, Langmade JS, Chen X, Fronick CC, Sawyer CS, Burcea LC, et al. Rapid and extraction-free detection of SARS-CoV-2 from saliva by colorimetric reverse-transcription loop-mediated isothermal amplification. *Clin Chem.* (2020) 62. doi: 10.1093/clinchem/hvaa267
16. Wang R, Qian C, Pang Y, Li M, Yang Y, Ma H, et al. opvCRISPR: One-pot visual RT-LAMP-CRISPR platform for SARS-cov-2 detection. *Biosens Bioelectron.* (2021) 172:112766. doi: 10.1016/j.bios.2020.112766

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Garcia-Venzor, Rueda-Zarazua, Marquez-Garcia, Maldonado, Moncada-Morales, Olivera, Lopez, Zuñiga and Melendez-Zajgla. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 22 February 2021
doi: 10.3389/fmed.2021.644130

Case Report: Metagenomics Next-Generation Sequencing Can Help Define the Best Therapeutic Strategy for Brain Abscesses Caused by Oral Pathogens

Zhonghui Ma[1,2], *Su Yan*[2,3], *Haoxin Dong*[1,2], *Huifen Wang*[2,4], *Yonggang Luo*[5]* and *Xi Wang*[1,2]*

[1] Department of Stomatology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Precision Medicine Center, Gene Hospital of Henan Province, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Health Management Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5] Department of Intensive Care Unit, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Guoqing Qian,
Ningbo First Hospital, China
Beiwen Zheng,
Zhejiang University, China

***Correspondence:**
Yonggang Luo
luoyg_514@126.com
Xi Wang
fccwangx1@zzu.edu.cn

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 20 December 2020
Accepted: 28 January 2021
Published: 22 February 2021

Citation:
Ma ZH, Yan S, Dong HX, Dong HF, Luo YG and Wang X (2021) Case Report: Metagenomics Next-Generation Sequencing Can Help Define the Best Therapeutic Strategy for Brain Abscesses Caused by Oral Pathogens. Front. Med. 8:644130. doi: 10.3389/fmed.2021.644130

Brain abscesses are associated with an increased long-term risk of new seizures and increased mortality within several years after infection. Common microorganisms that cause brain abscesses include bacteria, fungi, and mycoplasma. We report a 75-year-old man with a brain abscess caused by *Prevotella denticola*, an oral pathogen. Based on the clinical condition, we suspected that the patient had a blood-borne brain abscess, and he received antibiotics and systemic supportive treatment. The patient developed shock for the second time after negative Gram-staining results. Metagenomics next-generation sequencing showed one strain from the oral microbiome, confirming our hypothesis, and targeted antibiotic treatment was administered quickly. Thus, we report a case in which genomic analysis was the critical factor in determining the best antimicrobial therapy for administration.

Keywords: brain abscesses, oral microbiome, gram stain, metagenomics next-generation sequencing, precision treatment

INTRODUCTION

Brain abscesses are focal infections of the brain, which are associated with an increased long-term risk of new-onset epilepsy and mortality within several years after infection (1). In immunocompetent patients, the microbiome is responsible for most brain abscesses, and the most common pathogenic bacteria are *Streptococcus* and *Staphylococcus* bacteria (2). These bacteria enter the brain either through contiguous spread or hematogenous dissemination, especially in cases of infective endocarditis, or as a consequence of distant infectious foci (e.g., tooth infection) (3, 4).

Changes in the abundance of species disrupt host microbial homeostasis and lead to inflammatory disease (5). Metagenomic next-generation sequencing (mNGS) is used to detect microbial nucleic acids contained in samples via the genomics method, and its most significant advantage is the lack of requirement for culture and premise hypothesis (6, 7).

Herein, we report a case of a brain abscess due to *Prevotella denticola*, an oral periodontitis pathogen half a month after tooth extraction. The patient was diagnosed with intracranial infection

based on the clinical indications. The symptoms were significantly relieved after symptomatic treatment for 2 days, followed by sudden and persistent high fever and increased white blood cells. mNGS was used to detect the cerebrospinal fluid samples of the patients and quickly identify the pathogenic microorganisms to administer precision treatment and quickly alleviate the pain in the patient.

CASE DESCRIPTION

We report a 75-year-old man who presented with an unexplained headache persisting for 1 month. Three days prior to admission, the patient presented with dizziness and an aggravated headache, and he did not report any trauma history prior to the onset.

Initially, the patient was admitted to the Department of Neurosurgery. Physical examination showed that his underarm temperature was 38°C, pulse was 105 beats/min, and nervous system showed negative signs. Blood routine examination (BRE) revealed 33,700 leukocytes/μL (91% neutrophils), and brain computed tomography (CT) showed lesions in the left temporal lobe. Hence, he was preliminarily diagnosed with a brain abscess. Treatment with biapenem (0.3 g, twice a day) and vancomycin [1 g, three times a day administered via intravenous (IV) drip] was initiated. One day later, the patient was transferred to the intensive care unit (ICU) as he presented with sudden shock without obvious inducement (low blood pressure, fast heart rate, and abnormal heart rhythm).

On arrival, the patient's body temperature reached 38.1°C and blood pressure was 105/65 mmHg. The patient had poor consciousness and poor spirit, and BRE showed C-reactive protein (CRP) 61.10 mg/L and 9,950 leukocytes/μL (93.7% neutrophils). The cerebrospinal fluid (CSF) was pale yellow and clear, and Gram staining of CSF displayed until 4 days later showed no bacterial growth. Therefore, the treatment regimen included vancomycin (1 g, IV, twice a day), antithrombotic and anti-heart failure agents, and systemic nutritional support. Three days later, his body temperature increased and heart rate was 67 beats/min, while his consciousness was clear, BRE showed a C-reactive protein (CRP) level of 8.35 mg/L, and his leukocyte count returned to normal. However, the patient developed a sudden and persistent high fever on day 6 of ICU arrival. BRE of the patient showed 10,110 leukocytes/μL (16.7% monocytes, 2.9% basophils) and 7,420 neutrophils/μL. Meanwhile, the erythrocyte count decreased to 3,410,000/μL. The CSF presented with a yellow and muddy appearance, with cytology showing 860 leukocytes/μL (25% lymphocytes, 3% monocytes, 72% neutrophils) and a CRP level of 6.1 mg/L.

Meanwhile, mNGS technology was used to detect DNA pathogenic microorganisms in the CSF, and the results showed that the relative abundances of the two oral bacteria were remarkably elevated (**Table 1**).

After repeated questioning, the patient finally admitted to having undergone a tooth extraction 1 month prior. Brain magnetic resonance imaging (MRI) revealed a left maxillary sinus cyst in addition to the brain abscess (**Figure 1**). Therefore, the doctor speculated that this was the primary cause of the most recent brain abscess. In general, after tooth extraction, *P. denticola* and *Fusobacterium nucleatum* in the oral cavity enter the blood circulation through the wounds of tooth extraction, return to the intracranial region, and finally cause brain abscesses. After identifying the new pathogenic bacteria, vancomycin was discontinued. The antibiotic treatment was changed to ornidazole (500 mg, IV, twice a day), piperacillin (4.5 g, IV, four times a day), and rifampicin (0.30 g, IV, once a day). One day later, the patient's temperature returned to normal, symptoms improved significantly, BRE showed that the leukocyte count returned to normal, and specialists suggested that the patient could be transferred to an ordinary ward after 2-day infection control. On the 7th day, the patient's brain MRI showed that a sheet low-density shadow in the left temporal lobe of the brain was decreasing (**Figure 2**). Finally, the patient was followed up to ensure that he did not relapse after 10 days, 1 month, and 3 months. The patient was in good mental condition, had normal physical signs, and did not develop recurrence through January 10, 2021.

DISCUSSION

Brain abscesses are frequently caused by the continuous spread of concomitant infection areas (e.g., the middle ears, mastoids, and sinuses), and the most common clinical manifestations are headache and fever, which can cause shock in severe cases (2). The most frequent sources of infection in brain abscesses are *Staphylococcus* and *Streptococcus* bacteria. In recent years, some studies have demonstrated other sources of infection, including anaerobes, Gram-negative bacilli, oral *Methanobrevibacter*, associated *Actinobacillus*, bacillus, clostridium, and fungi (8–11).

Currently, CT with contrast enhancement and MRI are the clinical diagnostic methods implemented for brain abscesses. The former provides a rapid means to detect the size, number, and location of abscesses (2). Meanwhile, combined with diffusion-weighted and dispersion coefficient images, MRI is a valuable diagnostic tool for differentiating brain abscesses from primary, cystic, or necrotic tumors (12). At present, abscess puncture, neurosurgery, and antimicrobial agents are the main means of treatment. A stereotaxic navigation system based on volumetric CT or MRI can be applied to the puncture and drainage of abscesses through carefully planned trajectories (13). This system is especially suitable for patients with abscess diameters >1 cm, and abscess puncture is a necessary condition for determining pathogenic bacteria in neurosurgery (3). Neurosurgery is mainly used for superficial abscesses, abscesses that cause brain displacement, or herniation (3).

Antibiotic choice and duration depend on the type of pathogen and severity of infection (8). Brouwer et al. suggested that the selection of initial antimicrobial therapy should be based on the most likely causal organisms of the disease, basis of the mechanisms of infection, patient's predisposing condition, patterns of antimicrobial susceptibility, and ability of the antimicrobial agent to penetrate the abscess (3). In addition, due to the poor sensitivity of current techniques for identifying microorganisms, many experts advocate that antibiotics should

TABLE 1 | Pathogenic microorganisms isolated in this study.

Type	Genus		Species			
	Name	Sequence number	Name	Sequence number	Relative abundance	Attention
G-	Prevotella	36	*Prevotella denticola*	32	9.80%	High
G-	Fusobacterium	7	*Fusobacterium nucleatum*	3	0.26%	Low

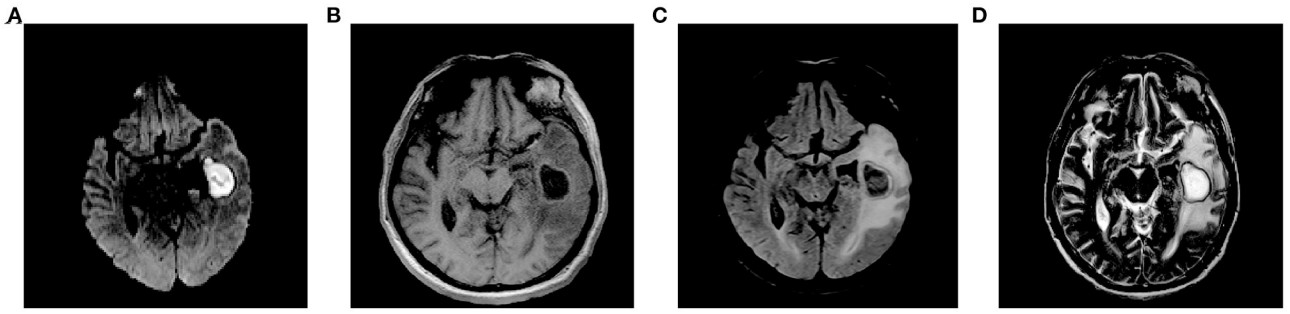


FIGURE 1 | Brain MRI before mNGS. **(A)** DWI; **(B)** T1WI; **(C)** T2-FLAIR; **(D)** T2WI. MRI, magnetic resonance imaging; mNGS, metagenomic next-generation sequencing; DWI, diffusion-weighted imaging; T1WI, T1-weighted imaging; FLAIR, fluid-attenuated inversion recovery; T2WI, T2-weighted imaging.

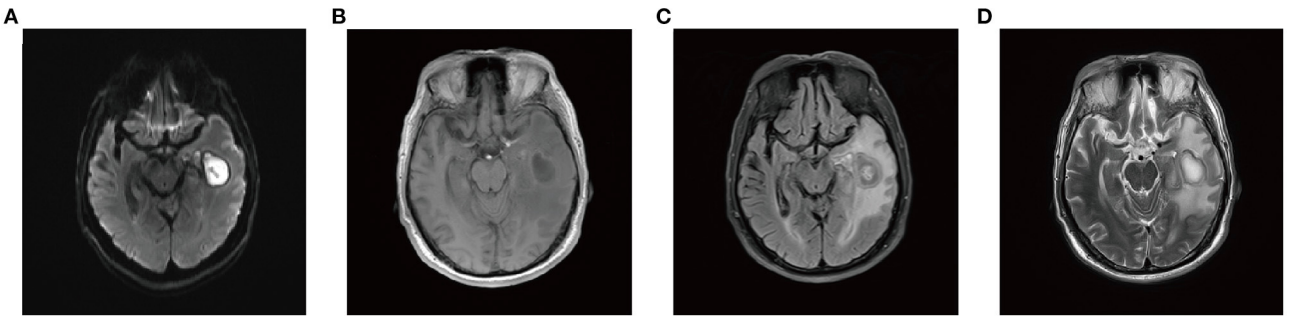


FIGURE 2 | Brain MRI 10 days after mNGS. **(A)** DWI; **(B)** T1WI; **(C)** T2-FLAIR; **(D)** T2WI. MRI, magnetic resonance imaging; mNGS, metagenomic next-generation sequencing; DWI, diffusion-weighted imaging; T1WI, T1-weighted imaging; FLAIR, fluid-attenuated inversion recovery; T2WI, T2-weighted imaging.

cover anaerobes, pending organism identification and the results of *in vitro* susceptibility testing. The combination of third-generation cephalosporin and metronidazole for 6 weeks may treat community-acquired brain abscesses in most immunocompetent patients (14). It is worth mentioning that, besides these two antibiotics, vancomycin should be added to treat potential staphylococcal infection in patients with brain abscesses due to hematogenous spread (8).

Currently, the conventional methods for determining the microorganisms in CSF, blood, or abscesses include aerobic and anaerobic cultures and Gram staining. However, the rapid diagnosis of pathogens in brain abscesses remains a challenge. Experts suggest that the current Gram staining technique is not sufficiently sensitive (14). Al Masalma et al. found that when this test was performed on aspirates from brain abscesses in 71 patients, the positive cultures only corresponded to 30 (42%) (9). Similarly, in this case, negative Gram-staining results prevented the patient from receiving precision antibiotic treatment, leading to the recurrence of the brain abscess. In a previous report, based on the patient's history of present illness, although a bacterial brain abscess was strongly suspected, the culture result was negative, and polymerase chain reaction (PCR)-based 16S ribosomal RNA sequencing provided a definitive etiological diagnosis, allowing for targeted antimicrobial therapy (15). Over the past two decades, the diagnosis rate of pathogenic microorganisms in patients with encephalopyosis has remained poor, while the detection technology (e.g., PCR, antigen assays) of pathogen microorganisms has made remarkable progress (16).

mNGS refers to the interrogation of all the genetic material in an environmental sample. It can be used to identify multiple pathogens (viruses, bacteria, fungi, or parasites) from cultures or directly from clinical samples based on uniquely identifiable DNA and/or RNA sequences (17). Over the past few years, with the increasing maturity and extensive promotion of mNGS technology, the time required to complete initial data analysis has

been dramatically reduced from a few weeks to 5–20 min (16, 18). To date, clinical applications of metagenomics have included the diagnosis of infectious diseases for various syndromes and sample types, microbiome analysis in diseases and health control, identification of human host responses to infection through transcriptomics, and identification of tumor-related viruses and their genomic integration sites (6). Thus, RNA and DNA extracted from CSF and brain tissue provide an alternative strategy for the diagnosis of neurological infections via mNGS technology.

In this case, the patient was initially diagnosed with a brain abscess on admission. Meanwhile, based on the patient's physical condition being classified as an emergency, he was initially treated with vancomycin and biapenem as antimicrobial agents. Specially, the bacterial culture results of CSF were negative, and there are two reasons that may be speculated. First, antibiotics might have eliminated *Streptococcus* and *Staphylococcus* as common pathogens; second, we speculate that the limitation of Gram staining technology may have led to the absence of other pathogenic bacteria.

Afterward, the Gram-negative bacteria *P. denticola* and *F. nucleatum* that caused shock were quickly identified through mNGS technology detection, and the patient was saved by antibiotics targeted at the pathogenic bacteria. *Prevotella peridotica* is a Gram-negative bacterium, which is generally considered to be an oral colonization bacterium with conditional pathogenicity that usually causes periodontitis and other diseases. In addition, there have also been cases of headaches after tooth extraction in healthy men, and *Prevotella* was found in blood culture, suggesting that *Prevotella* may represent the etiology of brain abscesses in patients with fever, stroke, and a history of tooth extraction (19). Therefore, we speculated that the Gram-negative result may be attributed to problems with the microbial detection technology. Compared with the success rate of mNGS technology detection, the current Gram staining technology, as a clinical detection technology for microorganisms, has a higher failure rate and is more time-consuming. Finally, the most suitable time for treatment is delayed, which confers many disadvantages to patients, clinical diagnosis, and treatment.

In general, mNGS technology has the advantages of speed and precision, although the current price may be slightly high for some patients. With a significant decrease in the cost of mNGS technology and improvements in data analysis efficiency, mNGS may accelerate the transition from laboratory technology to clinical applications in the future. This suggests that, in addition to authenticating the microorganisms that cause brain abscesses, mNGS may also be applied to the identification of microbes corresponding to other symptoms in the future, such as secondary periprosthetic joint infection and respiratory tract infection caused by unknown microorganisms.

DATA AVAILABILITY STATEMENT

MNGS was performed at Illumina NextSeq 550 platform in Gene Hospital of Henan Province. Metagenomics whole genome shotgun sequences have been uploaded to EMBL accession: PRJEB41159.

ETHICS STATEMENT

The study was approved by the institutional review board of the First Affiliated Hospital of Zhengzhou University. The ethics approval number is 2019-KY-330. The patients provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MZH, LYG, and WX analyzed and interpreted the patient data. YS, DHX, and WHF performed the experiments. MZH and WX analyzed the genomics data. MZH and LYG wrote the manuscript. All authors have read and approved the final manuscript.

REFERENCES

1. Bodilsen J, Dalager-Pedersen M, Van De Beek D, Brouwer MC, Nielsen H. Long-term mortality and epilepsy in patients after brain abscess: a nationwide population-based matched cohort study. *Clin Infect Dis.* (2020) 71:2825–32. doi: 10.1093/cid/ciz1153
2. Brouwer MC, Coutinho JM, Van De Beek D. Clinical characteristics and outcome of brain abscess: systematic review and meta-analysis. *Neurology.* (2014) 82:806–13. doi: 10.1212/WNL.0000000000000172
3. Brouwer MC, Tunkel AR, Mckhann GM, Van De Beek D. Brain abscess. *N Engl J Med.* (2014) 371:447–56. doi: 10.1056/NEJMra1301635
4. Mathis S, Dupuis-Girod S, Plauchu H, Giroud M, Barroso B, Ly KH, et al. Cerebral abscesses in hereditary haemorrhagic telangiectasia: a clinical and microbiological evaluation. *Clin Neurol Neurosurg.* (2012) 114:235–40. doi: 10.1016/j.clineuro.2011.10.036
5. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe.* (2011) 10:497–506. doi: 10.1016/j.chom.2011.10.006
6. Chiu CY, Miller SA. Clinical metagenomics. *Nat Rev Genet.* (2019) 20:341–55. doi: 10.1038/s41576-019-0113-7
7. Han D, Li Z, Li R, Tan P, Zhang R, Li J. mNGS in clinical microbiology laboratories: on the road to maturity. *Crit Rev Microbiol.* (2019) 45:668–85. doi: 10.1080/1040841X.2019.1681933
8. Kuppalli K, Livorsi D, Talati NJ, Osborn M. Lemierre's syndrome due to *Fusobacterium necrophorum*. *Lancet Infect Dis.* (2012) 12:808–15. doi: 10.1016/S1473-3099(12)70089-0
9. Al Masalma M, Lonjon M, Richet H, Dufour H, Roche PH, Drancourt M, et al. Metagenomic analysis of brain abscesses identifies specific bacterial associations. *Clin Infect Dis.* (2012) 54:202–10. doi: 10.1093/cid/cir797
10. Nkamga VD, Lotte R, Chirio D, Lonjon M, Roger PM, Drancourt M, et al. *Methanobrevibacter oralis* detected along with Aggregatibacter actinomycetemcomitans in a series of community-acquired brain abscesses. *Clin Microbiol Infect.* (2018) 24:207–8. doi: 10.1016/j.cmi.2017.08.021
11. Snyder JW, Gibson B. Photo quiz: frontal brain abscesses in an immunocompetent patient. *J Clin Microbiol.* (2016) 54:2215. doi: 10.1128/JCM.01750-14

12. Reddy JS, Mishra AM, Behari S, Husain M, Gupta V, Rastogi M, et al. The role of diffusion-weighted imaging in the differential diagnosis of intracranial cystic mass lesions: a report of 147 lesions. *Surg Neurol.* (2006) 66:246–50; discussion 50–1. doi: 10.1016/j.surneu.2006.03.032
13. Barlas O, Sencer A, Erkan K, Eraksoy H, Sencer S, Bayindir C. Stereotactic surgery in the management of brain abscess. *Surg Neurol.* (1999) 52:404–10; discussion 11. doi: 10.1016/s0090-3019(99)00118-4
14. Sonneville R, Ruimy R, Benzonana N, Riffaud L, Carsin A, Tadié JM, et al. An update on bacterial brain abscess in immunocompetent patients. *Clin Microbiol Infect.* (2017) 23:614–20. doi: 10.1016/j.cmi.2017.05.004
15. Al Masalma M, Armougom F, Scheld WM, Dufour H, Roche PH, Drancourt M, et al. The expansion of the microbiological spectrum of brain abscesses with use of multiple 16S ribosomal DNA sequencing. *Clin Infect Dis.* (2009) 48:1169–78. doi: 10.1086/597578
16. Wilson MR, Sample HA, Zorn KC, Arevalo S, Yu G, Neuhaus J, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. *N Engl J Med.* (2019) 380:2327–40. doi: 10.1056/NEJMoa1803396
17. Lefterova MI, Suarez CJ, Banaei N, Pinsky BA. Next-generation sequencing for infectious disease diagnosis and management: a report of the Association for Molecular Pathology. *J Mol Diagn.* (2015) 17:623–34. doi: 10.1016/j.jmoldx.2015.07.004
18. Wilson MR, Naccache SN, Samayoa E, Biagtan M, Bashir H, Yu G, et al. Actionable diagnosis of neuroleptospirosis by next-generation sequencing. *N Engl J Med.* (2014) 370:2408–17. doi: 10.1056/NEJMoa1401268
19. Wu PC, Tu MS, Lin PH, Chen YS, Tsai HC. Prevotella brain abscesses and stroke following dental extraction in a young patient: a case report and review of the literature. *Intern Med.* (2014) 53:1881–7. doi: 10.2169/internalmedicine.53.1299

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Ma, Yan, Dong, Wang, Luo and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 24 February 2021
doi: 10.3389/fmed.2021.627166

Case Report: Proven Diagnosis of Culture-Negative Chronic Disseminated Candidiasis in a Patient Suffering From Hematological Malignancy: Combined Application of mNGS and CFW Staining

Yanqi Jin[1†], Zhouhan Wang[1†], Chunxia Zhu[1], Qing Yang[2], Yingfeng Lu[1], Xiaopeng Yu[1], Bao Hong[3], Xiaojing Wang[3] and Yimin Zhang[1,3]*

[1] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [2] Laboratory Department, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [3] Department of Infectious Diseases of Haining Campus, The First Affiliated Hospital, College of Medicine, Zhejiang University, Haining, China

OPEN ACCESS

Edited by:
George Weinstock,
Jackson Laboratory for Genomic Medicine, United States

Reviewed by:
Yingli He,
Xi'an Jiaotong University, China
Yu-Chen Fan,
Shandong University, China

***Correspondence:**
Yimin Zhang
1307020@zju.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 08 November 2020
Accepted: 20 January 2021
Published: 24 February 2021

Citation:
Jin Y, Wang Z, Zhu C, Yang Q, Lu Y, Yu X, Hong B, Wang X and Zhang Y (2021) Case Report: Proven Diagnosis of Culture-Negative Chronic Disseminated Candidiasis in a Patient Suffering From Hematological Malignancy: Combined Application of mNGS and CFW Staining. Front. Med. 8:627166. doi: 10.3389/fmed.2021.627166

Chronic disseminated candidiasis (CDC) is a severe complication with high morbidity and mortality in patients with hematological malignancies who have undergone chemotherapy. Blood or sterile liver biopsy cultures are negative due to recurrent empirical antifungal therapy. With the escalating resistance to azole-based antifungal drugs in infection by *Candida* species, pathogen identification is becoming increasingly important for determining definitive diagnosis and treatment strategy. In this case report, we present, for the first time, diagnostic confirmation of a culture-negative CDC case with *Candida tropicalis* infection using a combination of metagenomics next-generation sequencing and calcofluor white staining.

Keywords: chronic disseminated candidiasis, metagenomics next-generation sequencing, *Candida tropicalis*, calcofluor white staining, hematological malignancies, proven diagnosis

INTRODUCTION

Chronic disseminated candidiasis (CDC; also known as "hepatosplenic candidiasis") is a unique manifestation of invasive candidiasis. CDC carries significant morbidity and mortality in patients with hematological malignancies who have undergone chemotherapy (1–3). The previous diagnostic criteria were positive histology plus culture evidence for *Candida* species from blood or a sterile liver biopsy (4, 5). The chance of positive findings in blood cultures with an onset of the clinical and radiological picture suggestive of CDC is poor (3). The prevalence of a proven diagnosis is quite low due to the difficulty of liver puncture and recurrent use of empirical antifungal prophylaxis after chemotherapy.

Development of metagenomics next-generation sequencing (mNGS) has enabled identification of causative pathogens in infectious diseases (6) and has been applied for the diagnosis of CDC. We report a culture-negative CDC case with *Candida tropicalis* (C. tropicalis) infection diagnosed by mNGS and calcofluor white (CFW) staining.

Description of this case study was approved (2020IIT 593) by the Ethics Committee of the First Affiliated Hospital of Zhejiang University (Hangzhou, China) and by the patient.

CASE PRESENTATION

A 62-year-old woman with acute myeloid leukemia-M2 received remission-induction chemotherapy (day-0). An outline of the following episodes is described in **Figure 1**. From day-0, recurrent fever occurred (**Figure 2**). Empirical treatment was undertaken with broad-spectrum antibiotics. The patient experienced neutropenia from day-2 to day-19. Antifungal prophylaxis was not employed until day-18, and yielded a positive result for the (1,3)-β-D-glucan (BDG) test (470 pg/mL). At that time, the results of a series of blood cultures were negative from day-16 to day-18 (**Figure 1**). Computed tomography (CT) of the abdomen showed multiple low-density lesions in the liver, spleen, and kidneys in day-28.

Caspofungin (CAP) from day-18 to day-28 and voriconazole (VRC) from day-28 to day-38 were used as antifungal therapy after a positive result for the BDG test on day-18 (**Figure 1**). However, the recurrent fever persisted without remission for the lesions on the liver, spleen, and kidneys even after recovery from neutropenia.

Contrast-enhanced CT of the abdomen showed multiple lesions with hypodensity in the liver, spleen, and kidneys as characterized by marked rim enhancement (**Figure 1A**). Ultrasound of the abdomen, enhanced magnetic resonance imaging (MRI) of the abdomen, ^{18}F-fluorodeoxyglucose-positron emission tomography/computed tomography, and contrast-enhanced ultrasound confirmed the result of contrast-enhanced CT of the abdomen (**Figure 3**). While a follow-up BDG test produced a negative result on day-43 and on several subsequent occasions (**Figure 1**). Expectedly, several cultures of blood and feces on different days failed to grow any pathogen.

On day-54, ultrasound-mediated liver puncture and biopsy was undertaken (**Figure 2**). A specimen was obtained between the boundary of lesions and normal liver tissues. Histology showed very few epithelioid cells, possibly a tumor, but no evidence of fungal infection. The antifungal agent was changed from VRC to CAP due to suspicion of CDC (**Figure 2**). However, her condition did not improve significantly, and recurrent fever persisted (**Figure 2**).

Another liver biopsy was done 1-week later (day-62). The puncture needle passed through the entire lesion to reach its center, and tissues were sampled. Histology revealed granulomatous inflammation with necrosis and no evidence of malignancy (**Figures 1B,C**). Immunohistochemistry showed positive in TB (FISH) (only one found) but negative in fungal (FISH). The serum T-SPOT®.TB interferon-γ release assay for active tuberculosis was negative. No pathogen was cultured from the two liver-biopsy tissues.

The samples from the second liver puncture were tested using mNGS (**Figure 1**). The results showed 55 total reads of *C. tropicalis*, accounting for 98.21% total fungal reads which was sentenced as responsible pathogen in accordance with other

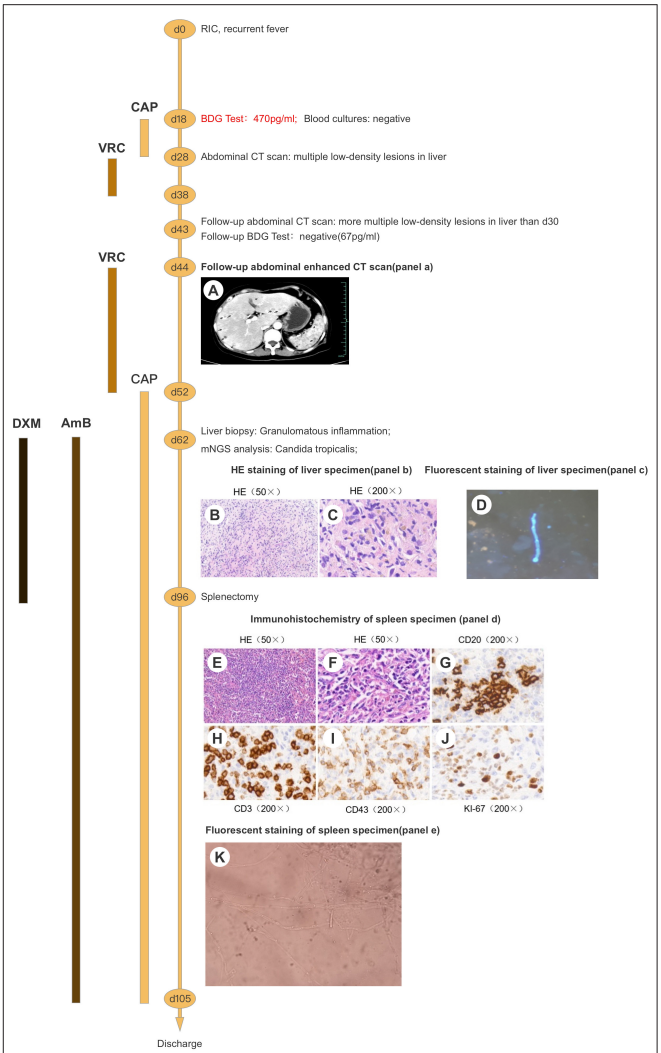


FIGURE 1 | Clinical course of the patient (schematic). **(A)** Contrast-enhanced CT of the liver shows multiple nodules with marked rim-enhancement. **(B,C)** HE staining of liver specimen shows granulomatous inflammation with necrosis. **(D)** Pseudohyphae were found under fluorescence microscopy using CFW staining. **(E,F)** HE staining of spleen specimen shows granulomatous inflammation with necrosis. **(G-J)** Immunohistochemical staining of spleen specimen showed positivity for CD20 (B cells), CD3/43 (T cells), and Ki-67 (30%). **(K)** Budding cells with pseudohyphae were discovered under microscopy. RIC, remission-induction chemotherapy; BDG, (1,3)-β-D-glucan; CT, computed tomography; CAP, caspofungin; VRC, voriconazole; AmB, amphotericin B; DXM, dexamethasone; mNGS, metagenomics next-generation sequencing; HE, haematoxylin and eosin; CD, cluster of differentiation.

clinical evidences. Interestingly, pseudohyphae were found under fluorescence microscopy using CFW staining (**Figure 1D**) to confirm the mNGS result. After consideration of detection by mNGS and the microscopic appearance, we decided to add the antifungal agent (amphotericin B (AmB), 25 mg per day, i.v.) and adjuvant corticosteroid (dexamethasone (DXM), 7.5 mg per day, i.v.) for further treatment (**Figure 2**). After adjusting the

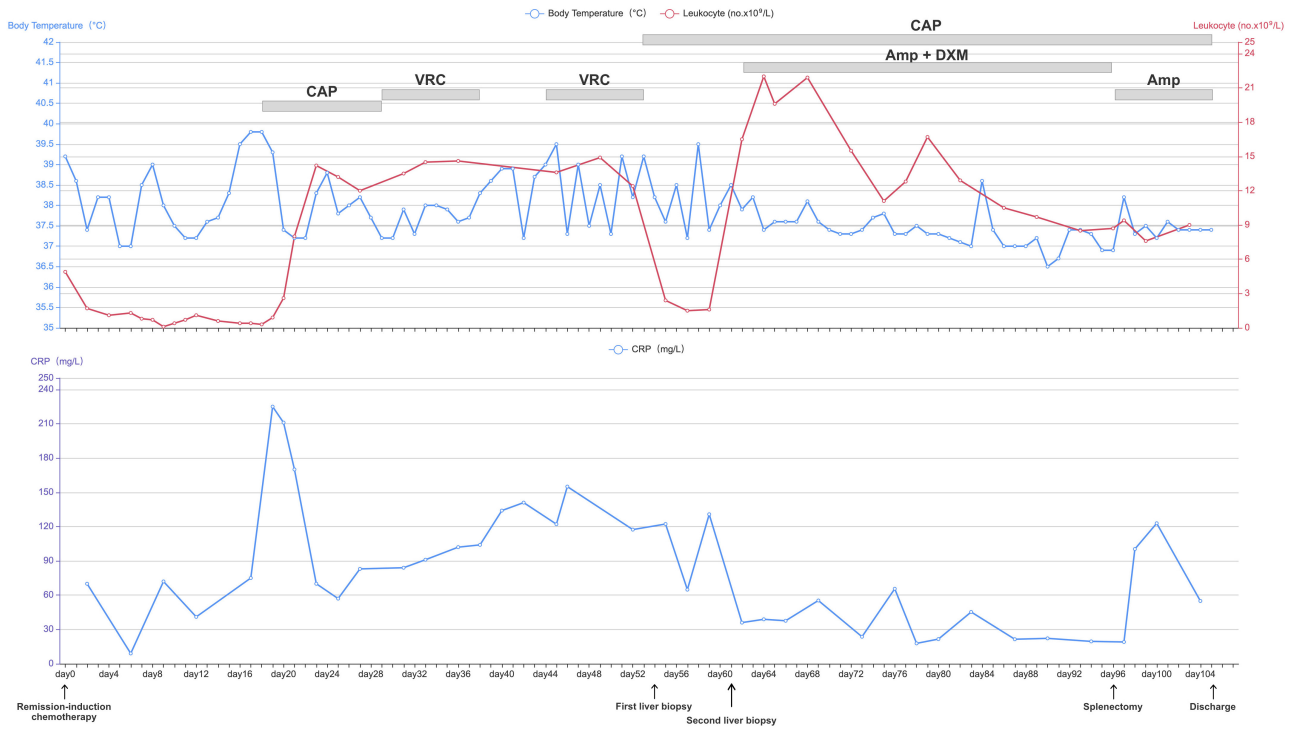


FIGURE 2 | Daily course of the patient's treatment; curves of body temperature, leukocyte counts and CRP. Major events are indicated with arrows. Red line shows the leukocyte counts in peripheral blood. Blue line on the top shows body-temperature values. Blue line on the bottom shows CRP values. Horizontal thick gray lines show the medications administered: CAP, caspofungin; VRC, voriconazole; AmB, amphotericin B; DXM, dexamethasone; CRP, C-reactive protein.

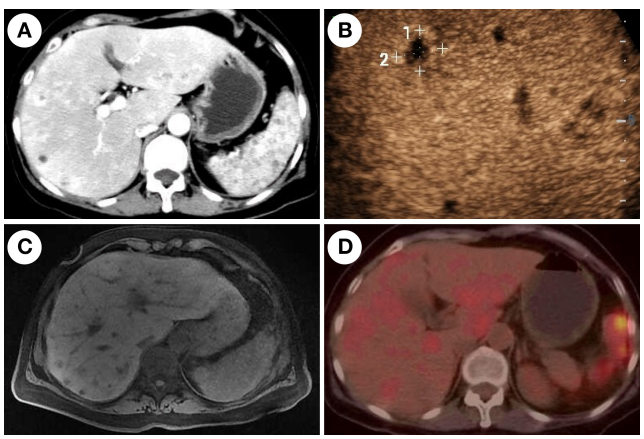


FIGURE 3 | Imaging features of chronic disseminated candidiasis. **(A)** Contrast-enhanced computed tomography of the abdomen shows multiple nodules with marked rim-enhancement. **(B)** Contrast-enhanced ultrasound shows multiple hypo-echoic lesions in the liver. **(C)** Magnetic resonance imaging shows multiple-sized circular abnormal signal lesions in the liver and spleen. **(D)** ^{18}F-fluorodeoxyglucose-positron emission tomography/computed tomography (^{18}F-FDG-PET/CT) shows multiple lesions in the liver and spleen with increased metabolism of FDG.

medication regimen, the body temperature dropped to a normal level (**Figure 2**).

CAP+AmB+DXM therapy was continued for >6 weeks while patient had a normal temperature but improvement in imaging features (lesions on the liver, spleen, and kidneys) was not observed. On day-96, splenectomy was undertaken after consultation with the multidisciplinary team (**Figure 1**). Multiple dense abscesses were found in the entire spleen, and granulomatous inflammation with necrosis was observed in splenic tissue under microscopy (**Figures 1E,F**). Budding cells with pseudohyphae consistent with infection by *Candida* species were observed under microscopy (**Figure 1K**). Immunohistochemistry showed leukocyte common antigen and positivity for cluster of differentiation (CD)3/43 (T cells), CD20 (B cells), and Ki-67 (30%), whereas a methenamine silver stain was negative (**Figures 1G–J**). After splenectomy, the patient symptoms were alleviated with a decrease of CRP level and white blood cell count (**Figure 2**). Her body temperature remained normal and we discontinued use of DXM (**Figure 2**). On day-105, the patient was discharged with continuing antifungal therapy of CAP and AmB. Cytological examination of the bone marrow showed complete remission. The patient was transferred to local hospital to continue antifungal therapy and was suggested to follow-up with CT scan every 2 months.

DISCUSSION

The high morbidity and mortality in CDC patients necessitates rapid diagnostic. Few cases are confirmed by culture, this is supported by a retrospective single-center study from 2008 to 2013 (7). It showed very low positive results from both of blood

culture (∼20%) and liver biopsy(10–20%) culture from patients undergoing chemotherapy and administrated with empirical antifungal therapy (7–9).

Cultures are limited further by slow turnaround times (typically requiring 2–3 days for growth to be evident), and the fact that they often turn positive late in the course of infection (10). Hence, according to previous "gold standard" diagnostic criteria, the conventional culture-based method has limitations, and definitive treatment is often delayed (10).

MRI, CT, and ultrasound are useful for the diagnosis of CDC. However, these imaging methods do not always succeed in distinguishing fungal abscesses in the active phase from possible (even if rare) bacterial and tubercular causes (11, 12).

With the development of molecular-biology methods, diagnosis using nucleic acids in tissue was introduced in the 2020 European Organization for Research and Treatment of Cancer/Mycoses Study Group guideline of invasive fungal disease (6) compared with recommendations from the 2008 version (4). The guideline of 2020 version (6) recommended each of the following investigations as essential for the diagnosis of CDC: microscopic analyses from sterile material (including histopathologic, cytopathologic, or direct microscopic examination of a specimen); culture from sterile material; blood culture; nucleic-acid diagnosis from tissue.

A conventional method of direct microscopic examination, CFW, was introduced to the diagnosis of CDC. The *rationale* of CFW staining is to detect (non-specifically) β(1–3) and β(1–4) polysaccharides of the chitin-rich areas found in the cell walls of *Candida* species and most other fungi (13). Also, addition of CFW to preparations of potassium hydroxide can improve detection of cells from *Candida* species by increasing the contrast from background debris (14). Compared with time-consuming conventional cultures, fluorescent staining of biopsy tissues requires only ≤1 min for specimen generation and ∼10 min to read samples, and is a more economical option (15). However, the disadvantage of CFW is the lack of ability to identify the species of fungus.

mNGS was an emerging culture-independent, high-throughput, microbiological diagnostic approach (16). It is relatively effective in cataloging and detecting pathogens, especially for uncommon or non-cultivable species (17). mNGS offers a faster and less biased non-culture-based methodology of pathogen detection through sequencing of extracted DNA from the sample directly (18). Therefore, mNGS has been applied to detect causal pathogens in deep-seated tissue samples (18, 19), such as in tissue from the lungs, (20), liver (21), brain (22), and cardiac-valve vegetation (23). We reported, for the first time, CDC diagnosed by mNGS. In this way, we showed that mNGS is a fast and non-culture-dependent diagnostic method.

The duration of CDC treatment can be 3–6 months (24). Usually, fluconazole is chosen after induction by CAP or AmB (25). With the escalating resistance of *Candida* species to azole (26–28), identification of *Candida* species has become important for the antifungal strategy in CDC patients. For the *C. tropicalis*, it appeared to be sensitive to fluconazole. However, drug-susceptibility testing of 20,788 strains of *Candida* species from 39 countries showed increased resistance to fluconazole for *C. tropicalis* of 2.5% in 1997 to 4.9% in 2014 (28). A survey of patients in intensive care units in China showed that the prevalence of resistance of *C. tropicalis* to fluconazole was 19.3%, but there was no resistance to CAP or AmB (29). Pathogen identification is becoming increasingly important in culture-negative CDC patients, and mNGS is a good choice.

Patients with CDC appear to occur after recovering from neutropenia, which suggests that CDC is a manifestation of immune reconstitution inflammatory syndrome (IRIS). Compared with use of an antifungal agent alone, adjuvant therapy using corticosteroids for a short course and of medium dose could benefit for patients with CDC by hastening improvement of clinical symptoms (30). Therefore, if empirical use of antifungal agents is not efficacious, identification of the pathogen in CDC is crucial to distinguish if it is caused by drug resistance or IRIS. Hence, development and validation of non-culture diagnostic methods for CDC is a top medical priority.

We combined the results from mNGS with those of CFW staining from liver biopsies. This strategy enabled confirmation of the diagnosis of CDC and guided antifungal treatment and subsequent adjuvant therapy using the corticosteroid DXM.

The histopathology manifestations of CDC are associated with three hepatic patterns: necrosis, abscess, and granuloma (31). Acute histopathology is manifested as a minimal inflammatory reaction in the neutropenic stage, whereas microabscesses with an intense inflammatory reaction occur after recovery from neutropenia. The budding cells of *Candida* species with pseudohyphae or hyphae can be seen in the center of necrotic tissue. Chronic histopathology manifests as granulomatous lesions. These are characterized by fibrosis or central necrosis surrounded by giant-cell granulomatous tissue with fibroblasts and/or macrophages arranged in palisade (9). There were only a few budding cells of *Candida* species with pseudohyphae or hyphae in the lesions in our patient. Therefore, when carrying out ultrasound-guided liver biopsy, the needle should reach the lesion center to have a greater chance of obtaining the pathogen. This strategy also explains why this specific histology feature was detected in the second liver biopsy but not in the first liver biopsy.

A combination of mNGS and CFW staining may be an alternative option for a culture-negative diagnosis of fungal infection. Nevertheless, the absence of detection of antibiotic resistance by mNGS was a limitation in this case study.

CONCLUSIONS

mNGS testing of a liver-biopsy sample along with CFW staining is a fast and reliable method for obtaining the proven diagnosis of culture-negative CDC. Early identification of the infective pathogens in culture-negative CDC can guide pathogen-targeted antifungal chemotherapy and subsequent adjuvant therapy with corticosteroids and/or splenectomy if indicated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. The patient provided the written informed consent to participate in this study. Written informed consent was obtained from the patient for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YZ designed research. YJ, ZW, and YZ analyzed and collected data, and wrote the manuscript. CZ, BH, XW, QY, YL, and XY collected clinical data. All authors read and approved the final manuscript.

FUNDING

This work was supported by Fundamental Research Funds for the Central Universities (2019FZA7001), Zhejiang Education Department 2020 Special Project Against COVID-19 (Zhejiang University 94), China National Science and Technology Major Project (2017ZX10202202), Youth Fund Project of Zhejiang Provincial Natural Science Foundation, (LQ17H030002), and Haining Science and Technology Project (201805).

ACKNOWLEDGMENTS

We thank Dr. Dewi Andayani for her critical language editing.

REFERENCES

1. Sallah S, Semelka RC, Wehbie R, Sallah W, Nguyen NP, Vos P. Hepatosplenic candidiasis in patients with acute leukaemia. *Br J Haematol*. (1999) 106:697–701. doi: 10.1046/j.1365-2141.1999.01592.x
2. Anttila VJ, Elonen E, Nordling S, Sivonen A, Ruutu T, Ruutu P. Hepatosplenic candidiasis in patients with acute leukemia: incidence and prognostic implications. *Clin Infect Dis*. (1997) 24:375–80. doi: 10.1093/clinids/24.3.375
3. Pagano L, Mele L, Fianchi L, Melillo L, Martino B, D'Antonio D, et al. Chronic disseminated candidiasis in patients with hematologic malignancies. Clinical features and outcome of 29 episodes. *Haematologica*. (2002) 87:535–41. doi: 10.1016/S0301-472X(02)00785-3
4. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis*. (2008) 46:1813–21. doi: 10.1086/588660
5. Masood A, Sallah S. Chronic disseminated candidiasis in patients with acute leukemia: emphasis on diagnostic definition and treatment. *Leuk Res*. (2005) 29:493–501. doi: 10.1016/j.leukres.2004.10.003
6. Donnelly JP, Chen SC, Kauffman CA, Steinbach WJ, Baddley JW, Verweij PE, et al. Revision and update of the Consensus Definitions of Invasive Fungal Disease From the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium. *Clin Infect Dis*. (2020) 71:1367–76. doi: 10.1093/cid/ciz1008
7. Chen CY, Cheng A, Tien FM, Lee PC, Tien HF, Sheng WH, et al. Chronic disseminated candidiasis manifesting as hepatosplenic abscesses among patients with hematological malignancies. *BMC Infect Dis*. (2019) 19:635. doi: 10.1186/s12879-019-4260-4
8. Anttila VJ, Ruutu P, Bondestam S, Jansson SE, Nordling S, Farkkila M, et al. Hepatosplenic yeast infection in patients with acute leukemia: a diagnostic problem. *Clin Infect Dis*. (1994) 18:979–81. doi: 10.1093/clinids/18.6.979
9. Kontoyiannis DP, Luna MA, Samuels BI, Bodey GP. Hepatosplenic candidiasis. A manifestation of chronic disseminated candidiasis. *Infect Dis Clin North Am*. (2000) 14:721–39. doi: 10.1016/S0891-5520(05)70128-8
10. Clancy CJ, Nguyen MH. Finding the "missing 50%" of invasive candidiasis: how nonculture diagnostics will improve understanding of disease spectrum and transform patient care. *Clin Infect Dis*. (2013) 56:1284–92. doi: 10.1093/cid/cit006
11. Pagano L, Larocca LM, Marra R, Pizzigallo E, Leone G. A leukemic patient with hepatosplenic abscesses due to coagulase-negative staphylococci. *Clin Infect Dis*. (1992) 14:364–5. doi: 10.1093/clinids/14.1.364
12. Lee DG, Choi JH, Kim YJ, Lee S, Min CK, Kim DW, et al. Hepatosplenic tuberculosis mimicking disseminated candidiasis in patients with acute leukemia. *Int J Hematol*. (2001) 73:119–21. doi: 10.1007/BF02981913
13. Pringle JR, Preston RA, Adams AE, Stearns T, Drubin DG, Haarer BK, et al. Fluorescence microscopy methods for yeast. *Methods Cell Biol*. (1989) 31:357–435. doi: 10.1016/s0091-679x(08)61620-9
14. Perry JL, Miller GR. Quality control slide for potassium hydroxide and cellufluor fungal preparations. *J Clin Microbiol*. (1989) 27:1411–2.
15. Harrington BJ, Hageage GJ. Calcofluor white: a review of its uses and applications in clinical mycology and parasitology. *Lab Med*. (2003) 34:361–7. doi: 10.1309/eph2tdt8335gh0r3
16. Lefterova MI, Suarez CJ, Banaei N, Pinsky BA. Next-generation sequencing for infectious disease diagnosis and management: a report of the Association for Molecular Pathology. *J Mol Diagn*. (2015) 17:623–34. doi: 10.1016/j.jmoldx.2015.07.004
17. Besser J, Carleton HA, Gerner-Smidt P, Lindsey RL, Trees E. Next-generation sequencing technologies and their application to the study and control of bacterial infections. *Clin Microbiol Infect*. (2018) 24:335–41. doi: 10.1016/j.cmi.2017.10.013
18. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis*. (2018) 67:S231–40. doi: 10.1093/cid/ciy693
19. Zhang HC, Ai JW, Cui P, Zhu YM, Li YJ, Zhang WH. Incremental value of metagenomic next generation sequencing for the diagnosis of suspected focal infection in adults. *J Infect*. (2019) 79:419–25. doi: 10.1016/j.jinf.2019.08.012
20. Li H, Gao H, Meng H, Wang Q, Li S, Chen H, et al. Detection of pulmonary infectious pathogens from lung biopsy tissues by metagenomic next-generation sequencing. *Front Cell Infect Microbiol*. (2018) 8:205. doi: 10.3389/fcimb.2018.00205
21. Ai JW, Li Y, Cheng Q, Cui P, Wu HL, Xu B, et al. Diagnosis of local hepatic tuberculosis through next-generation sequencing: smarter, faster and better. *Clin Res Hepatol Gastroenterol*. (2018) 42:178–81. doi: 10.1016/j.clinre.2018.04.007
22. Salzberg SL, Breitwieser FP, Kumar A, Hao H, Burger P, Rodriguez FJ, et al. Next-generation sequencing in neuropathologic diagnosis of infections of the nervous system. *Neurol Neuroimmunol Neuroinflamm*. (2016) 3:e251. doi: 10.1212/nxi.0000000000000251
23. Fukui Y, Aoki K, Okuma S, Sato T, Ishii Y, Tateda K. Metagenomic analysis for detecting pathogens in culture-negative infective endocarditis. *J Infect Chemother*. (2015) 21:882–4. doi: 10.1016/j.jiac.2015.08.007
24. Pappas PG, Rex JH, Sobel JD, Filler SG, Dismukes WE, Walsh TJ, et al. Guidelines for treatment of candidiasis. *Clin Infect Dis*. (2004) 38:161–89. doi: 10.1086/380796
25. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al. Clinical practice guideline for the management of candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin Infect Dis*. (2016) 62:e1–50. doi: 10.1093/cid/civ933

26. Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, et al. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *J Clin Microbiol*. (2010) 48:1366–77. doi: 10.1128/jcm.02117-09
27. Xiao M, Fan X, Chen SC, Wang H, Sun ZY, Liao K, et al. Antifungal susceptibilities of *Candida glabrata* species complex, *Candida krusei*, *Candida parapsilosis* species complex and *Candida tropicalis* causing invasive candidiasis in China: 3 year national surveillance. *J Antimicrob Chemother*. (2015) 70:802–10. doi: 10.1093/jac/dku460
28. Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN. Twenty years of the SENTRY Antifungal Surveillance Program: results for *Candida* species from 1997–2016. *Open Forum Infect Dis*. (2019) 6:S79–94. doi: 10.1093/ofid/ofy358
29. Liu W, Tan J, Sun J, Xu Z, Li M, Yang Q, et al. Invasive candidiasis in intensive care units in China: *in vitro* antifungal susceptibility in the China-SCAN study. *J Antimicrob Chemother*. (2014) 69:162–7. doi: 10.1093/jac/dkt330
30. Legrand F, Lecuit M, Dupont B, Bellaton E, Huerre M, Rohrlich PS, et al. Adjuvant corticosteroid therapy for chronic disseminated candidiasis. *Clin Infect Dis*. (2008) 46:696–702. doi: 10.1086/527390
31. Rammaert B, Desjardins A, Lortholary O. New insights into hepatosplenic candidosis, a manifestation of chronic disseminated candidosis. *Mycoses*. (2012) 55:e74–84. doi: 10.1111/j.1439-0507.2012.02182.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Jin, Wang, Zhu, Yang, Lu, Yu, Hong, Wang and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 29 March 2021
doi: 10.3389/fmed.2021.656194

Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of Talaromycosis of an Immunocompetent Patient

Jiejun Shi†, Naibin Yang† and Guoqing Qian†*

Department of Infectious Diseases, Ningbo First Hospital, Ningbo University, Ningbo, China

Background: Talaromycosis is a serious fungal infection which is rare in immunocompetent people. Since its clinical manifestations lack specificity, it is easy to escape diagnosis or be misdiagnosed leading to high mortality and poor prognosis. It is necessary to be alert to the disease when broad-spectrum antibiotics do not work well in immunocompetent patients.

Case Presentation: A 79-year-old man was admitted to our Infectious Diseases Department for recurrent fever and cough. Before admission he has been treated with piperacillin-tazobactam, moxifloxacin followed by antituberculous agents in other hospitals while his symptoms were not thoroughly eased. During the first hospitalization in another hospital, he has been ordered a series of examination including radionuclide whole body bone imaging, transbronchial needle aspiration for subcarinal nodes. However, the results were negative showing no neoplasm. After being admitted to our hospital, he underwent various routine examinations. The initial diagnosis was bacterial pneumonia, and he was given meropenem injection and tigecycline injection successively, but there were no improvement of symptoms and inflammatory indicators. In the end, the main pathogen *Talaromyces marneffei* was confirmed using Metagenomic Next-Generation Sequencing (mNGS), and his clinical symptoms gradually relieved after targeted antifungal treatment using voriconazole.

Conclusion: When empirical anti-infective treatment is ineffective, it is necessary to consider the possibility of opportunistic fungal infections on immunocompetent patients. mNGS, as a new generation of pathogenic testing methods, can often detect pathogenic bacteria faster than traditional methods, providing important help for clinical decision-making.

Keywords: talaromyces marneffei, immunocompetent, next-generation sequencing, anti-fungal treatment, case report

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Wenjun Du,
Shandong Public Health Clinical Center, China
Liang Li,
Fudan University, China

***Correspondence:**
Guoqing Qian
bill.qian@outlook.com

†ORCID:
Jiejun Shiorcid
orcid.org/0000-0003-0911-2773
Naibin Yang
orcid.org/0000-0001-7439-723X
Guoqing Qian
orcid.org/0000-0003-2427-8042

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *20 January 2021*
Accepted: *05 March 2021*
Published: *29 March 2021*

Citation:
Shi J, Yang N and Qian G (2021) Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of Talaromycosis of an Immunocompetent Patient. Front. Med. 8:656194. doi: 10.3389/fmed.2021.656194

INTRODUCTION

Talaromycosis is an opportunistic fungal infection caused by *Talaromyces marneffei* (*T. marneffei*), the only thermal dimorphic fungus of Penicillium, which is endemic to Southeast Asia but rare in the Ningbo city of Zhejiang province. Excrement of Rhizomys that were carrying *T. marneffei* may contaminates surroundings, leading to infections.

Susceptible people may be infected through alimentary tract, respiratory tract and broken skin or mucosa contact (1). Since the clinical manifestations lack specificity, it is easy to escape diagnosis and be misdiagnosed especially amongst immunocompetent patients. It is one of the severe opportunistic infections in HIV patients. Recently a small but increasingly number of HIV-negative patients with abnormal immunity, such as patients of connective tissue disease, organ transplant recipients, and long-term users of immunosuppressive agents, were reported to be infected. It rarely infects immunocompetent people, though when it happens the mortality were higher, maybe due to delayed diagnosis (2, 3). Here we presented an immunocompetent Ningbo native suffered from *T. marneffei* which was hard to identify by conventional detection methods. Finally, the fungus was confirmed by mNGS and prolonged sputum culture.

CASE REPORT

A 79-year-old man was presented to our hospital with 1-month cough and fever. He has visited two other local hospitals for more than three times where a series of examinations were performed. His chest computerized tomography (CT) scan revealed ground-glass nodules in the upper lobe of the right lung with multiple enlarged lymph nodes in the mediastinum which were suspected of tumors (**Figure 1A**). In order to clarify the nature of the lesion he further took a needle biopsy of lymph nodes under bronchoscope while pathological results showed

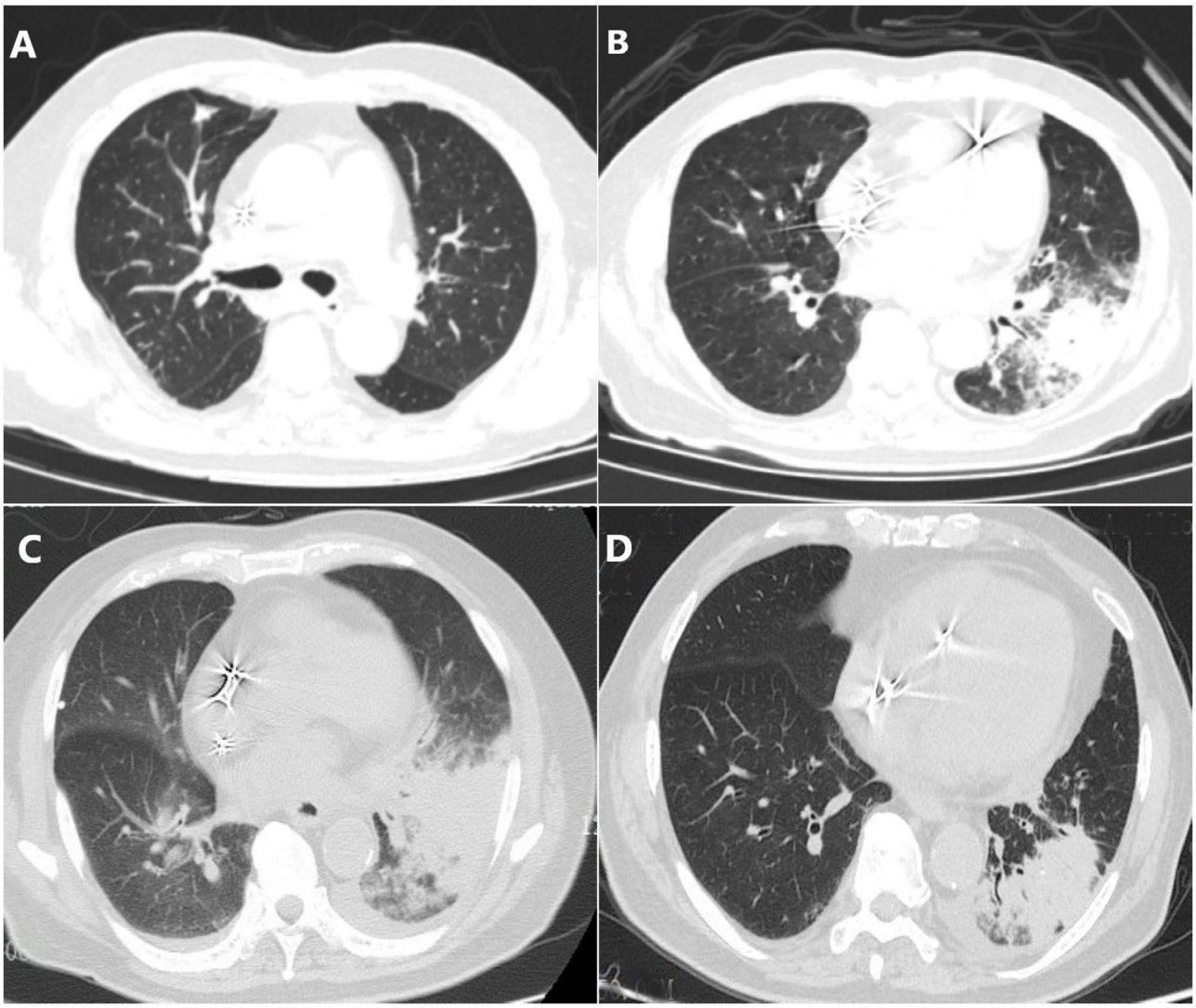


FIGURE 1 | Chest CT scans in different phases. **(A)** Ground-glass nodules in the upper lobe of the right lung with multiple enlarged lymph nodes in the mediastinum which were suspected of tumors. **(B)** Infectious lesions in both lungs, left hilar shadow increased, and mediastinal lymph nodes slightly enlarged. **(C)** The area of pneumonia was larger than before and pleural fluid appeared. **(D)** Pneumonia and pleural effusion were both absorbed obviously.

no evidence of tumors. Presumed to be pulmonary infection, he was then given piperacillin-tazobactam, moxifloxacin, and empirical anti-tuberculosis treatment successively. His body temperature gradually returned to normal, but the cough still exists. One week later, his cough worsened without fever. Outpatient blood routine examination revealed that white cells count (WBC) 13.1 x $10^9/L$, C-reactive protein (CRP) 183.51 mg/L. Re-examination of chest CT showed infectious lesions in both lungs, left hilar shadow increased, and mediastinal lymph nodes slightly enlarged (**Figure 1B**). For further treatment, he was admitted to the Department of Infectious Diseases of Ningbo First hospital. His initial diagnosis was bacterial pneumonia.

He has not taken any immunosuppressants or glucocorticoids before. He had hypertension for more than 10 years treated by amlodipine and bisoprolol, diabetes mellitus for 3 years treated by insulin with unsatisfactory glycemic control, and eczema for 10 years without therapy. He did not have chronic airway disease although he had smoked for 20 years and quitted smoking for more than 20 years. Three years ago, he had undergone Permanent Cardiac Pacemaker implantation. As a Ningbo native, he had retired from a Metal Instrument Factory where he worked as an installer of metallic parts for more than 20 years. And he hasn't been to the wild recently. He has no prior family history of malignancy, infectious diseases or psychosis. Physical examination showed large eczema-like scars on two legs and found no other positive signs.

After admission, we examined his cellular immune function for lymphocyte subsets analysis (**Table 1**), immunoglobulins, cytokines, and found no obvious immune deficiency. And his tuberculosis T-SPOT. TB test, human immunodeficiency virus (HIV) test, tumor biomarkers (including carcinoembryonic antigen, carbohydrate associated antigen 199, alpha-fetoprotein, and cancer antigen 125), Antinuclear antibody (ANA) and Antineutrophil autoantibodies (ANCA), serum cryptococcal antigen colloidal gold test, and serological test for syphilis were all negative.

TABLE 1 | Lymphocyte subsets analysis.

Items	Results	Normal range
CD3	65.57%	58.4-81.56
CD4	39.72%	31-60
CD8	27.23%	13-41
CD4/CD8	1.5	0.8-4.2
NK	27.09%	14-40
CD19	6.31%	6-25

TABLE 2 | The details of therapy strategies.

Period	Therapy
Oct 25-Oct 28	Meropenem 1 g q8h ivgtt
Oct 28-Nov 1	Tigecycline 50 mg q12h ivgtt
Nov 1-Nov 9	Caspofungin 50 mg qd ivgtt (70mg for first dose)
Nov 2-Nov 9	Linezolid 0.6 g q12h ivgtt
Nov 9-Nov 15	Piperacillin and Tazobactam 4.5 g q8h ivgtt
Nov 9-Nov 26	Voriconazole 200 mg q12h ivgtt
Nov 9-Nov 23	Ganciclovir 250 mg qd ivgtt
Nov 15-Nov 26	Levofloxacin 0.5 g qd ivgtt

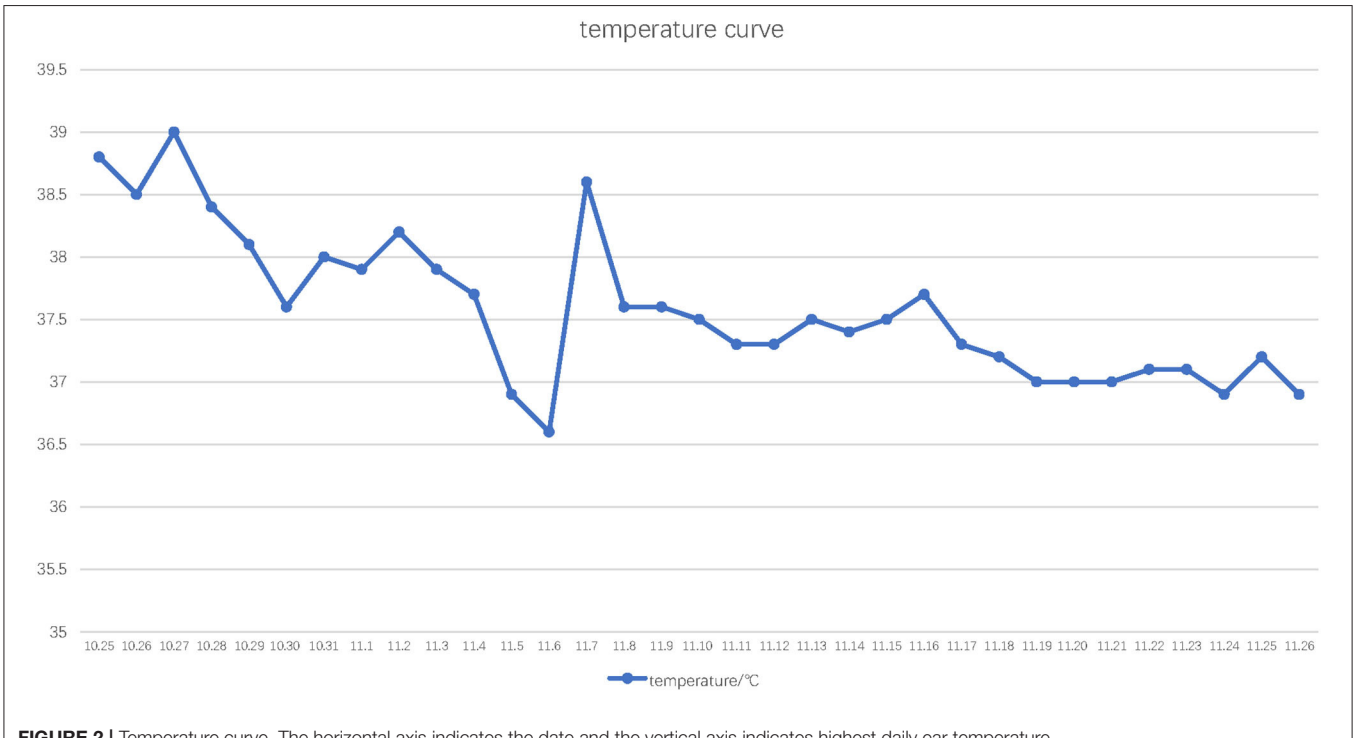


FIGURE 2 | Temperature curve. The horizontal axis indicates the date and the vertical axis indicates highest daily ear temperature.

TABLE 3 | Routine blood test, hsCRP and procalcitonin (PCT).

	hsCRPmg/L	WBC*10^9/L	NE%	RBC*10^12/L	Hb g/L	PLT *10^9/L	PCT ng/ml
Oct 25	176.73	13.91	75.1	2.95	81	440	0.42
Oct 27	138.67	14.4	73.7	3.06	80	449	-
Oct 30	173.42	11.64	70.7	3.1	82	527	-
Nov 1	142.18	15.47	71.1	3.2	88	511	-
Nov 3	131.76	17.21	75.1	3.26	88	569	0.33
Nov 5	119.37	18.53	75.2	2.98	86	527	-
Nov 7	80.59	21.89	92.1	3.12	81	478	-
Nov 11	114.77	14.82	78.3	2.89	76	347	-
Nov 14	74.05	11.89	63.9	2.6	70	386	-
Nov 17	63.47	11.47	71.1	2.74	69	550	-
Nov 19	48.72	10.15	73.9	2.72	72	745	-
Nov 22	36.8	10.86	69.9	2.68	72	768	-
Nov 24	31.57	10.85	67.5	2.41	75	761	-
Nov 26	25.41	9.53	66.5	2.54	77	563	-

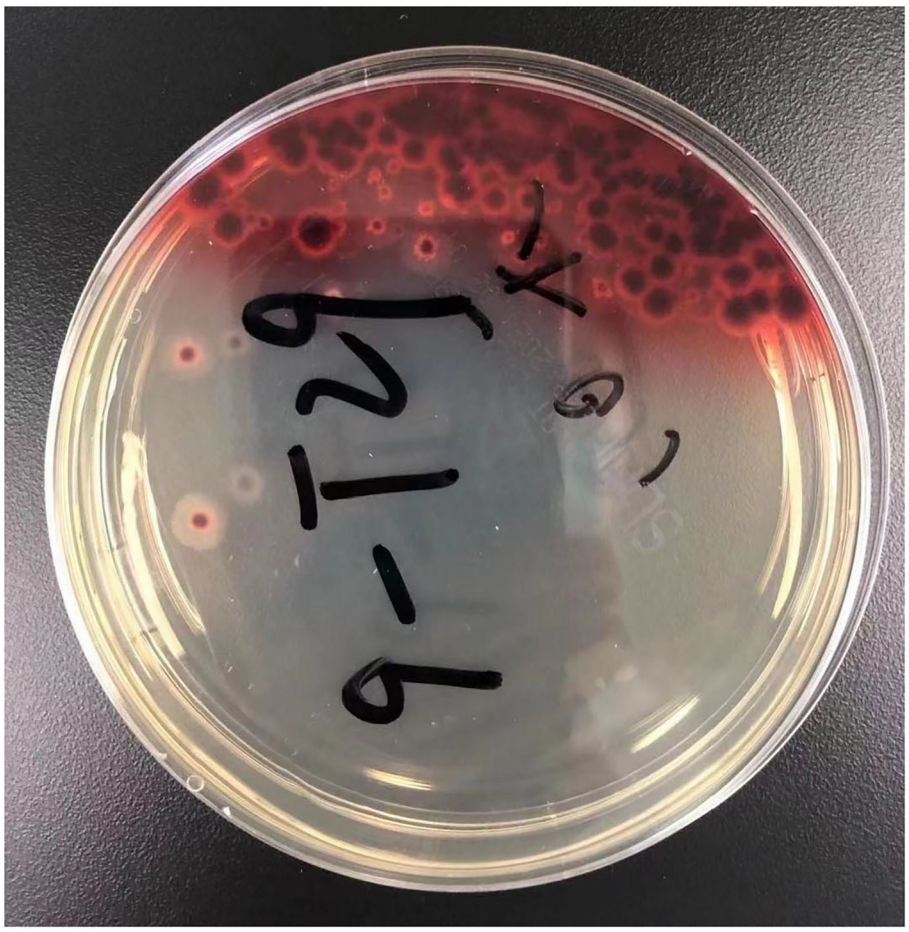


FIGURE 3 | The result of prolonged sputum culture. A diffusible red pigment produced by *Talaromyces marneffei*, diffuses to the surrounding medium.

Since he has used broad-spectrum antibiotics with poor efficacy, we performed meropenem, tigecycline consecutively (**Table 2**). However, the peak temperature did not drop (**Figure 2**), and the inflammation indexes did not improve (**Table 3**). During hospitalization, his galactomannan (GM) was positive in serum. Thus, we empirically add caspofungin for

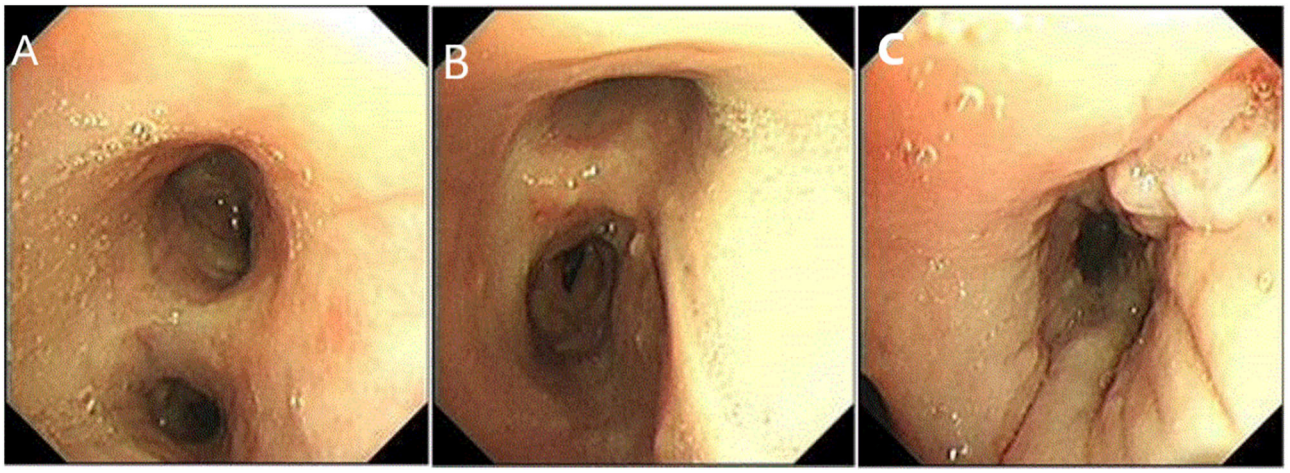


FIGURE 4 | Fiberoptic bronchoscopy. **(A,B)** showed the mucosa of the left and right main bronchus is scattered with nodules. **(C)** Longitudinal change of mucosa in the basal segment of the left lower lobe.

TABLE 4 | Results of mNGS of BALF and blood.

Sample	Type	Name	Sequence number	Confidence
BALF	G-	*Klebsiella pneumonia*	28	High
BALF	G-	*Pseudomonas aeruginosa*	20	High
BALF	dsDNA	Human betaherpesvirus 5	2	Medium
BALF	Fungi	Talaromycesmarneffei	38	High
BALF	Fungi	*Candida albicans*	5	High
Blood	G-	*Pseudomonas aeruginosa*	66	Medium
Blood	G+	*Staphylococcus aureus*	4	Medium
Blood	dsDNA	Human betaherpesvirus 5	159	High
Blood	dsDNA	Human gammaherpesvirus 4	7	Medium
Blood	Fungi	Talaromycesmarneffei	1	Medium

antifungal treatment. Meanwhile, we replaced tigecycline with linezolid. Since then, the fever seems to have gradually eased but WBC increased slowly (**Figure 3**). We ordered another chest CT scan which indicated the lesions of pneumonia were larger than before and pleural fluid appeared (**Figure 1C**). On November 5, he underwent a fiberoptic bronchoscopic examination (**Figure 4**) and lavage of the basal segment of the left lower lobe. Bronchoalveolar Lavage fluid (BALF) was tested for mNGS, culture, liquid-based cytology. At the same time, blood sample was sent for mNGS examination.

Four days later, we changed antibiotics to intravenous voriconazole and piperacillin-tazobactam since mNGS suggested Penicillium marneffei infection (**Table 4**). Moreover, we prolonged sputum culture time to one week and the fungus was further validated by culture (**Figure 3**). After he was prescribed targeted anti-fungal agent of voriconazole 200 mg twice daily, the clinical presentations and laboratory indicators were gradually returning to normal (**Table 3**). The whole process was concluded (**Figure 5**).

Prior to follow-up, he carried on taking voriconazole 200 mg twice daily regularly without relapse. On December 9th, his repeated chest CT scan showed that pneumonia was cured and pleural effusion were absorbed obviously (**Figure 1D**).

DISCUSSION

As the only thermal dimorphic fungus of Penicillium, *Talaromyces marneffei* grows as yeast *in vivo* at 37°C as pathogenic mode and as mold *in vitro* at 25°C (4). It was originally taken from the hepar of Rhizomys in 1956 while three years later, the first case of human infected by *Talaromyces marneffei* was found derived from the laboratory (1, 2). *Talaromyces marneffei* proliferates in macrophages and spreads through reticuloendothelial system. T cells, especially CD4+ Cells, are vital to remove *Talaromyces marneffei*. In theory, *Talaromyces marneffei* can be cleared in 2–3 weeks in healthy hosts while it may be fatal to immunocompromised patients (1). Thus, it tends to be found on hosts with cellular immune deficiency. The most common hosts are HIV patients followed by other immunocompromised hosts, such as patients who suffer from connective tissue diseases or hematopathy, organ transplant recipients, and long-term users of immunosuppressants. It rarely persists in immunocompetent hosts.

The pathological changes caused by talaromycosis may be divided into three groups as granuloma, reactive necrosis and purulent inflammation (1). The most common clinical symptoms are fever, coughing, anemia, wasting, and characteristic umbilicated skin lesion (5, 6) which are usually nonspecific and of little help to the judgment. Chest CT scan of pulmonary talaromycosis often lacks specificity. As is reported, it can be multiple cavities with uneven thick wall and clear edge, enlarged mediastinal lymph nodes, ground glass opacity, or pleural effusion (1, 7). Thus, without enough suspicion, it is easy to be misdiagnosed as bacterial pneumonia, cancer, tuberculosis, and other fungal infection.

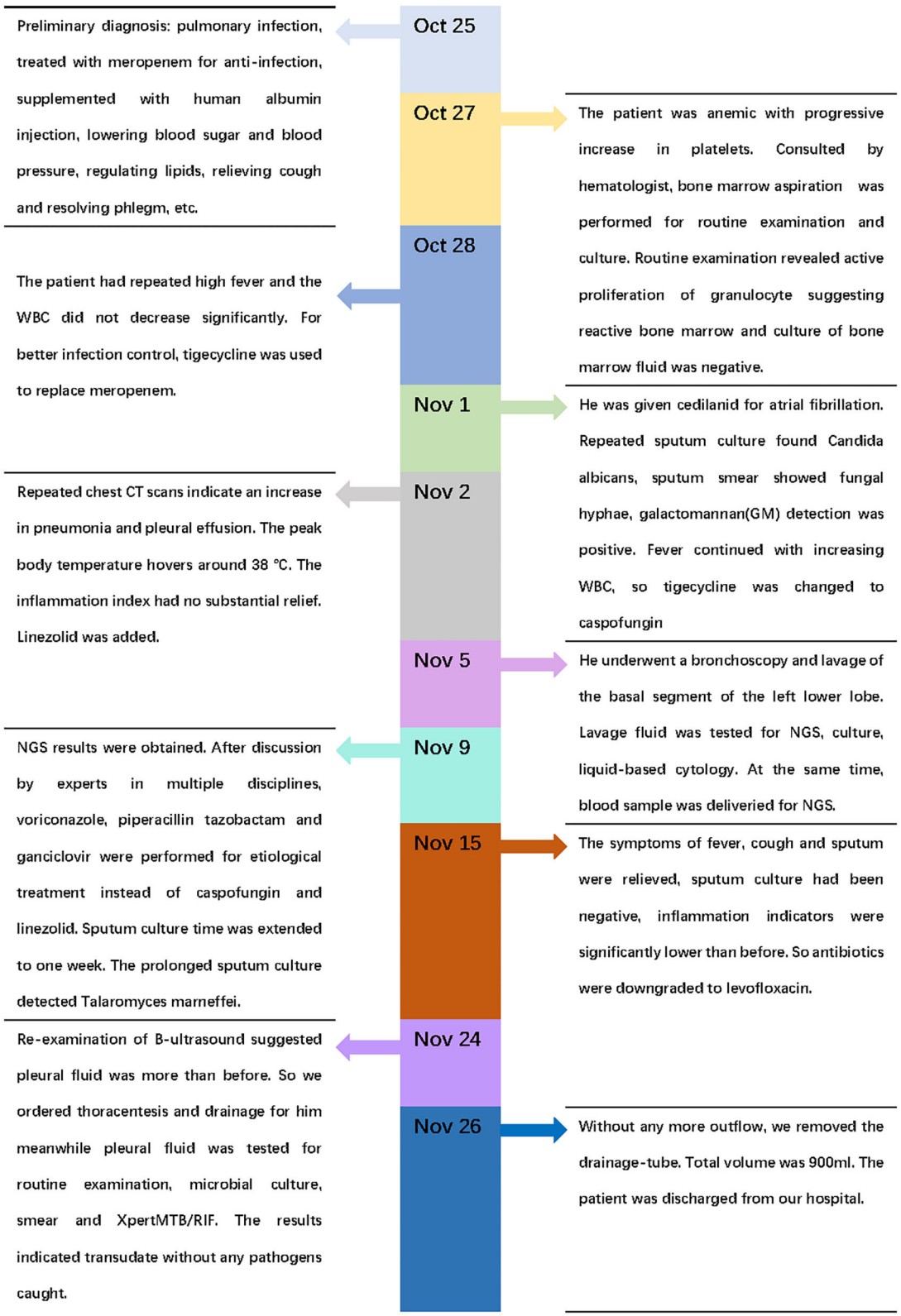


FIGURE 5 | Timeline of the case.

In recent decades, increasing incidents of talaromycosis in healthy hosts are reported (8). The fatality rate of talaromycosis in non-HIV patients is higher than HIV-infected patients which is probably due to delayed diagnoses and treatments (2, 3). Without timely diagnosis and targeted therapy, the mortality rate of talaromycosis can be above 90% (9). We herein reported a case of HIV-negative talaromycosis with pleural effusion from nonendemic region, and without history of recent contact with wildlife. The old man suffered from diabetes with unsatisfactory blood glucose control, cardiac disease and eczema which maybe risk factors of talaromycosis. However, lymphocyte subsets analysis showed his CD3, CD4/CD3, CD8, and CD4/CD8 were all normal in addition to the negative HIV test. Like many other immunocompetent talaromycosis patients, he had been misdiagnosed as bacterial pneumonia and tuberculosis until mNGS cleared away the confusion. Furthermore, prolonged sputum culture verified the pathogen showing bright red surrounding molds which is the unique feature of *Talaromyces marneffei* as it can product a soluble red pigment (3) whose essence maybe monascorubramine encoded by pks3 and other four genes (10). Blood dissemination can be determined from the mNGS of blood. According to literature, disseminated talaromycosis usually has worse prognosis than localized ones since it may be accompanied with multiple organ dysfunction (1). We speculated his anemia maybe related to *Talaromyces marneffei* (11) although it needs further investigation and verification.

Amphotericin B is the first line agent for talaromycosis with HIV suggested by guideline (12). However, severe nephrotoxicity limits the clinical use of amphotericin B. As a broad-spectrum antifungal agent, voriconazole was reported to be effective for talaromycosis (13, 14). Therefore, we chose voriconazole to treat this patient since it is safe, strong and within his economic affordability. The result was satisfactory.

There are some take-away lessons from this case. Since routine culture often cannot cover all bacteria and fungi, timely communications between clinicians and microbiologists is necessary when a particular pathogen is suspected. Microbiologists may then adjust culture methods to improve detection rate of rare pathogens. When traditional anti-infection treatments are not effective and the pathogen is difficult to be detected by routine examinations, mNGS is recommended since it may detect some rare pathogen faster than culture. Then the patient may get the appropriate treatments sooner and have a better prognosis.

Some limitations were discovered in this case. First, in the course of empirical medication, we changed agents frequently which may be influenced by patient's anxiety and high expectations. Second, we lacked suspicion to this rare fungus and initially did not have close communication with microbiology experts which we later rectified. Last, we still don't understand if his thrombocytosis and refractory pleural effusions are related to talaromycosis when his albumin concentration was above 30 g/L.

In conclusion, we should be alert of rare fungi when routine anti-infective therapies were useless even in immunocompetent hosts. As an advanced test method, mNGS may detect pathogens fast and exactly.

DATA AVAILABILITY STATEMENT

The datasets have been uploaded into EMBL database, accession number PRJEB43434.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JS analyzed data and drafted the manuscript. NY collected the data. GQ revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was supported by Key Program of Natural Science Foundation of Ningbo under Grant No. 202003N4019.

ACKNOWLEDGMENTS

We thank Dr. Ada Hoi Yan Ma for polishing the manuscript.

REFERENCES

1. Wang YG, Cheng JM, Ding HB, Lin X, Chen GH, Zhou M, et al. Study on the clinical features and prognosis of *Penicilliosis marneffei* without human immunodeficiency virus infection. *Mycopathologia.* (2018) 183:551–8. doi: 10.1007/s11046-017-0236-3
2. Chan JF, Lau SK, Yuen KY Woo PC. Talaromyces (Penicillium) marneffei infection in non-HIV-infected patients. *Emerg Microbes Infect.* (2016) 5:e19. doi: 10.1038/emi.2016.18
3. Kawila R, Chaiwarith R Supparatpinyo K. Clinical and laboratory characteristics of penicilliosis marneffei among patients with and without HIV infection in Northern Thailand: a retrospective study. *BMC Infect Dis.* (2013) 13:464. doi: 10.1186/1471-2334-13-464
4. Zhou F, Bi X, Zou X, Xu Z, Zhang T. Retrospective analysis of 15 cases of *Penicilliosis marneffei* in a southern China hospital. *Mycopathologia.* (2014) 177:271–9. doi: 10.1007/s11046-014-9737-5
5. Du Q Tong CK. Talaromyces (Penicillium) marneffei infection. *IDCases.* (2018) 13:e00428. doi: 10.1016/j.idcr.2018.e00428
6. Furusawa H, Miyazaki Y, Sonoda S, Tsuchiya K, Yaguchi T, Kamei K, et al. Penicilliosis marneffei complicated with interstitial pneumonia. *Intern Med.* (2014) 53:321–3. doi: 10.2169/internalmedicine.53.1465
7. Li X, Hu W, Wan Q, Lei Q, Sun C, Hou Z, et al. Non-HIV talaromycosis: Radiological and clinical analysis. *Medicine.* (2020) 99:e19185. doi: 10.1097/MD.0000000000019185
8. Ye F, Luo Q, Zhou Y, Xie J, Zeng Q, Chen G, et al. Disseminated penicilliosis marneffei in immunocompetent patients: a report of two

cases. *Indian J Med Microbiol.* (2015) 33:161–5. doi: 10.4103/0255-0857.148433
9. Liyan X, Changming L, Xianyi Z, Luxia W Suisheng X. Fifteen cases of penicilliosis in Guangdong, China. *Mycopathologia.* (2004) 158:151–5. doi: 10.1023/b:myco.0000041842.90633.86
10. Tam EW, Tsang CC, Lau SK Woo PC. Polyketides, toxins and pigments in Penicillium marneffei. *Toxins.* (2015) 7:4421–36. doi: 10.3390/toxins7114421
11. Sirisanthana V Sirisanthana T. Disseminated *Penicillium marneffei* infection in human immunodeficiency virus-infected children. *Pediatr Infect Dis J.* (1995) 14:935–40. doi: 10.1097/00006454-199511000-00003
12. Larsson M, Nguyen LH, Wertheim HF, Dao TT, Taylor W, Horby P, et al. Clinical characteristics and outcome of *Penicillium marneffei* infection among HIV-infected patients in northern Vietnam. *AIDS Res Ther.* (2012) 9:24. doi: 10.1186/1742-6405-9-24
13. Ge Y, Xu Z, Hu Y Huang M. Successful voriconazole treatment of *Talaromyces marneffei* infection in an HIV-negative patient with osteolytic lesions. *J Infect Chemother.* (2019) 25:204–7. doi: 10.1016/j.jiac.2018.08.007
14. Wang P, Chen Y, Xu H, Ding L, Wu Z, Xu Z, et al. Acute disseminated *Talaromyces marneffei* in an immunocompetent patient. *Mycopathologia.* (2017) 182:751–4. doi: 10.1007/s11046-017-0127-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Shi, Yang and Qian. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 29 March 2021
doi: 10.3389/fmed.2021.653294

Case Report: Next-Generation Sequencing in Diagnosis of Pneumonia Due to *Pneumocystis jirovecii* and Cytomegalovirus in a Patient With HIV Infection

Yirui Xie[1]*, *Bing Ruan*[1], *Lingxiao Jin*[2,3] and *Biao Zhu*[1]*

[1] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The Department of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, School of Medicine, The First Affiliated Hospital, Zhejiang University, Hangzhou, China, [2] Department of Infectious Diseases, People's Hospital of Pujiang County, Jinhua, China, [3] School of Medicine, Pujiang Branch of the First Affiliated Hospital, Zhejiang University, Hangzhou, China

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Hossein Zarrinfar,
Mashhad University of Medical Sciences, Iran
Shusaku Haranaga,
University of the Ryukyus, Japan

***Correspondence:**
Yirui Xie
1312019@zju.edu.cn
Biao Zhu
zhubiao1207@zju.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 14 January 2021
Accepted: 02 March 2021
Published: 29 March 2021

Citation:
Xie Y, Ruan B, Jin L and Zhu B (2021) Case Report: Next-Generation Sequencing in Diagnosis of Pneumonia Due to Pneumocystis jirovecii and Cytomegalovirus in a Patient With HIV Infection. Front. Med. 8:653294. doi: 10.3389/fmed.2021.653294

Background: Pulmonary infections remain a significant cause of morbidity and mortality in immunocompromised patients. The pathogens spectrum of pulmonary infection that can affect patients with human immunodeficiency virus (HIV) is wide such as bacterial, fungal, viral, parasitic organisms, and so on. The risk of multi-pathogenic pneumonia is higher in HIV-infected patients. However, the fast and accurate diagnosis of multi-pathogenic pneumonia is challenging because of the limitations of current conventional tests.

Case Presentation: Here, we report a case of pneumonia due to *Pneumocystis jirovecii* and cytomegalovirus (CMV) in a 22-year-old male with newly diagnosed HIV infection. Blood tests revealed a low CD4 count, a chest computed tomography (CT) scan showed extensive ground-glass opacities in the bilateral lung with multiple cavity lesions in the left upper lung. Microscopic examination of stained sputum and bronchoalveolar lavage fluid (BALF) smear specimens did not find any pathogens. There was also no evidence of pathogens known to cause pneumonia in bacteria and fungi culture tests and virus antibodies such as EBV, CMV, and COVID-19. The nucleic acid of CMV in blood was reported by quantitative PCR. Next-generation sequencing (NGS) analysis of BALF specimens identified a large number of *P. jirovecii* and CMV reads, and confirmed the diagnosis of pneumonia due to *P. jirovecii* and CMV. Following the patient's treatment with anti-PCP and anti-CMV, the patient was cured and discharged.

Conclusions: This case highlights the combined application of NGS in the clinical diagnosis of multi-pathogenic pneumonia in an HIV-infected patient. NGS is proposed as an important adjunctive diagnostic approach for identifying pathogens of multi-pathogenic pneumonia in HIV-infected patients.

Keywords: next-generation sequencing, *Pneumocystis jirovecii*, cytomegalovirus, bronchoalveolar lavage fluid, pneumonitis, HIV

INTRODUCTION

Pulmonary infections can be caused by a variety of microbes including bacteria, fungi, and viruses, which can be severe and fatal particularly among immunocompromised individuals (1–3). The risk of mixed pulmonary infection is high in immunocompromised patients such as patients with HIV infection (4, 5). Compared to patients with monomicrobial pulmonary infection, patients with mixed pulmonary infection may have a more severe clinical manifestation and are difficult to diagnose. The diagnosis of multi-pathogenic pneumonia must be as fast and accurate as possible, because severe clinical manifestation and combined treatment have many potential side effects. However, fast and accurate diagnosis of multi-pathogenic pneumonia is challenging because of the limitations of current conventional tests (6). Occasionally, we still cannot draw a correct conclusion according to these tests. Next-generation sequencing (NGS), a highly sensitive method for analyzing the microbiome, could provide additional valuable information for the detection of pathogens. Here, we report a patient with newly diagnosed HIV infection who developed multi-pathogenic pneumonia revealed by NGS of bronchoalveolar lavage fluid (BALF).

CASE REPORT

A 22-year-old Chinese male form Zhejiang province initially presented to the local community hospital. He complained of 10 days of intermittent fever with a temperature of up to 38.7°C and 3 days of progressive dyspnea and dry cough. He was given moxifloxacin at the local community hospital. However, he contracted chest thrush after using moxifloxacin, so this treatment was stopped and he was given ambroxol hydrochloride. Due to a history of male sexual partners and weight loss, an HIV test was obtained which returned positive. Then he came to our institution, the infectious disease department of the First Affiliated Hospital, School of Medicine, Zhejiang University, and hospitalized.

Upon presentation at our institution, he still had fever, dyspnea, fatigue, cough with a small amount of pale sputum, and occasionally pectoralgia and dyspnea during activity. Physical examination found a body temperature of 37.4°C, blood pressure of 113/50 mmHg, a pulse of 115 beats per minute, a respiration rate of 23 breaths per minute, and SpO_2 93% using fingertip pulse oximetry. The bilateral lung presented moist rales. The patient did not have a skin rash. The laboratory results were as follows: white blood cell count 7.7×10^9/L, with a neutrophil ratio of 65.7%, hemoglobin 133 g/L, and platelet count 365×10^9/L; C-reactive protein (CRP) 8.5 mg/L; erythrocyte sedimentation rate (ESR) 5 mm/h; and procalcitonin 0.08 ng/mL. Blood biochemical index results showed that lactate dehydrogenase content increased to 401 U/L (reference range 150–250 U/L), and -hydroxybutyrate dehydrogenase content increased to 326 U/L (reference range 72–182 U/L). The 1,3-beta-D-glucan test (G test) result (251.64 pg/ml) was positive, while galactomannan test (GM test) (0.1 μg/L) and cryptococcus capsular antigen test results were negative. His CD4 count was 16 cells/μL. Arterial blood gas analysis (with O_2 3 L/min *via* nasal catheter) showed pH 7.51; pCO_2 27 mmHg; pO_2 98 mmHg; HCO_3 −21.9 mmol/L; SBE −0.8 mmol/L; lactate 0.8 mmol/L; and O_2 saturation 99%. A chest computed tomography (CT) scan showed extensive ground-glass opacities in the bilateral lung with multiple thin-walled cavity lesions in the left upper lung (**Figures 1A,B**). The IgG and IgM antibodies of coronavirus disease 2019 (COVID-19) were negative. The nucleic acid detection of COVID-19 was negative. Nucleic acid detection of common respiratory pathogens (including Mycoplasma pneumoniae, Chlamydia pneumoniae, adenovirus, respiratory syncytial virus, parainfluenza virus, and influenza A and B) was negative. The IgG antibodies of Epstein-Barr virus (EBV) and human *cytomegalovirus* (CMV) were positive, while the IgM antibodies of EBV and CMV were negative. The nucleic acid of CMV in the blood was reported at 1.86×10^4 copies/mL by quantitative PCR. Mycobacterium tuberculosis (MTB) was negative based on blood test with T-SPOT.TB as well as acid-fast staining of sputum smear and GeneXpert. The blood, sputum, and urine smear and culture of bacteria and fungi were negative. We also sent the sputum sample to perform Gomori-Grocott methenamine silver nitrate staining (GMS) for *Pneumocystis jirovecii* detection and obtained a negative result. Given the patient's hypoxia and demonstration of diffuse ground-glass opacities on CT of the chest, a clinical diagnosis of *P. jirovecii* pneumonia (PCP) was made. Oral trimethoprim-sulfamethoxazole (TMP-SMX, TMP 0.24 g /SMX 1.2 g, q8h), intravenous clindamycin (0.6 g, q12h), and methylprednisolone (40 mg daily) were administered, which resulted in the improvement of general conditions and abatement of fever and dyspnea in 3 days.

To verify pathogen-induced pneumonia, the patient underwent a bronchoscopy examination with a collection of BALF specimens on day 5 of admission. The BALF was sent to the laboratory for conventional tests and an NGS test [using the MGISeq 2000 platform (MGI Tec Co. Ltd, Shenzhen, China)]. The BALF Gram stain and acid-fast stain were negative. The G test and GM test of BALF were negative. In addition, a BALF culture of fungal and bacteria showed no organisms. Microbiological tests of BALF were negative for MTB by the smear and GeneXpert MTB/RIF assay. Two days later, the NGS results showed that *P. jirovecii* (1,000,078 reads) and CMV (4,192 reads) were found in the BALF. The other microorganisms detected by NGS were bacteria and are listed in **Table 1**, but they were not regarded as responsible for the invasive pulmonary infection. Based on the report, the diagnosis of multi-pathogenic pneumonia due to PCP and CMV was made in this patient, and intravenous ganciclovir (0.25, Q12H) was started.

After 2 weeks of hospitalization, his symptoms disappeared, general conditions returned to normal, and laboratory findings improved. The chest CT scan showed that lesions had been significantly absorbed except for a faint patchy opacity in the lateral segment and thin-walled cavity lesions in the left upper lung (**Figures 1C,D**). So, the patient began antiretroviral therapy with Truvada (emtricitabine and tenofovir disoproxil fumarate tablets, 500 mg QD) and dolutegravir (50 mg QD). He was discharged to complete 1 week of TMP-SMX and 2 weeks of oral ganciclovir (500 mg, three times daily) at home, and then switch

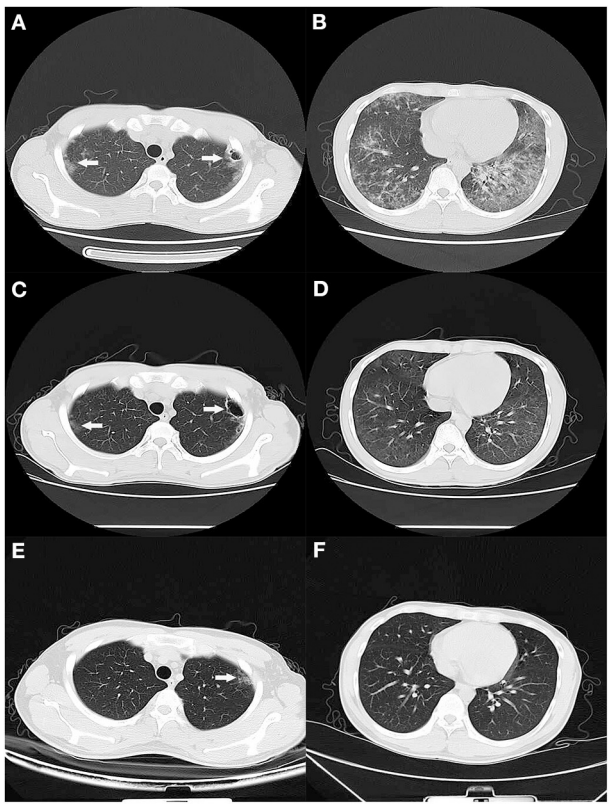


FIGURE 1 | CT scan of the lung. **(A,B)** On the 1st day of admission, a CT scan showed diffuse bilateral ground-glass opacities with thin-walled cavity lesions (white arrow) in the left upper lung. **(C,D)** After 2 weeks of treatment, a CT scan showed that lesions were significantly absorbed except for a faint patchy opacity in the lateral segment and thin-walled cavity lesions (white arrow) in the left upper lung. **(E,F)** Two months later, a CT scan exhibited no abnormal finding except for a faint patchy opacity (white arrow) in the left upper lateral segment.

TABLE 1 | The other microorganisms detected by next-generation sequencing (NGS) in the BALF of the case.

Genus		Species	
Name	**Sequence number[#]**	**Name**	**Sequence number[#]**
Staphylococcus	172	*S. epidermidis*	130
Prevotella	105	*Prevotella melaninogenica*	65
Streptococcus	44	*S. sanguis*	15
		S. mitis	3
Cutiibacterium	13	*C. acnes*	7
Rothia	10	*R. mucilaginosa*	10
Veillonella	7	*V. atypical*	4
Moraxella	5	*M. osloensis*	5

[#]*The sequence number of the strict comparison of the microorganism detected at the level of genus/species.*

to prophylactic doses of TMP-SMX until CD4 count recovery. At his 2-week follow-up visit, he had no symptoms. At his 2-month follow up, he appeared healthy with no symptoms and a chest CT scan exhibited no abnormal findings except for a faint patchy opacity in the left lateral segment (**Figures 1E,F**).

DISCUSSION

With the widespread use of highly active anti-retroviral therapy (HAART), the incidence of AIDS-related opportunity infections has declined dramatically (7–9). However, a rapid growth of HIV-infected cases, poor adherence to medications, and the emergence of virological and immunological failure have caused infections to thrive (10, 11). The risk of multi-pathogenic pneumonia is higher in HIV-infected patients and the spectrum of pathogens is broad including bacterial, fungal, viral, and parasitic organisms (7, 12). So, consideration of the possibility of a multi-pathogenic pneumonia is essential in HIV-infected patients and early bronchoscopy is recommended. Conventional tests including microscopy, specific antigen or antibody detection, PCR amplification, and culture have been utilized in clinical work. However, these conventional tests have limitations in terms of sensitivity, speed, spectrum for pathogen detection, and simultaneous multi-pathogen detection (6, 12). So, the prompt appropriate diagnosis of multi-pathogenic pneumonia in HIV-infected patients is still challenging.

Next-generation sequencing (NGS) is a highly sensitive, culture-independent, and unbiased method that can identify all potential known and new or unexpected pathogens (13, 14). NGS has been successfully used as a diagnostic tool for various infectious diseases in immunocompromised hosts (5, 15). Its application to diagnose multi-pathogenic pneumonia due to PCP and CMV in HIV-infected patients has never been reported until now. Here we report a case of PCP and CMV multi-pathogenic pneumonia diagnosed by NGS of BALF following negative results in multiple conventional diagnostic tests.

PCP is serious and sometimes critical infections caused by *P. jirovecii* occur in immune-suppressed patients (16, 17). Up until now, the definitive diagnosis of PCP is confirmed upon finding *P. jirovecii* in respiratory secretion, tissue, or a BALF sample. A Wright-Giemsa stained smear was the most common conventional diagnostic method in the past few decades and other methods such as methenamine silver and cresyl echt violet fluorescence staining and nucleic acid amplification testing may further increase the detection sensitivity. Despite high incidence of PCP in HIV-infected patients, the diagnosis of PCP remains challenging due to its non-specific signs and symptoms and inadequate performance of conventional diagnostic methods (18). CMV pneumonitis is uncommon, and its diagnosis is challenging as the clinical manifestations and radiological features of CMV pneumonia are also non-specific (7, 10, 18, 19). The challenges of prompt appropriate diagnosis of PCP and CMV-mixed pulmonary infection are well-reflected in the present case report. Consistent with our patient, HIV-infected patients with a CD4 count <100 cells are at higher risk for CMV infection and/or reactivation. As a result of viral shedding, it is common for CMV to present in the BALF of HIV-infected patients; thus, its presence is

TABLE 2 | Reports of PCP and CMV cases detected by next-generation sequencing (NGS).

Patient ID	Underlying disease	Pulmonary disorders	Smear results	Culture results	Pathology results	NGS-based diagnosis (number of unique reads)	Reference no.
No. 1	Immunological anemia	Pulmonary infection Type I respiratory failure	Negative	Negative	Alveolar septal fibrous tissue hyperplasia, Inflammatory cell infiltration	*P. jirovecii (28)* *H. cytomegalovirus (72)*	(5)
No. 2	Acute myeloid leukemia	Pulmonary infection	Negative	Negative	Fibrous tissue hyperplasia, Inflammatory cell infiltration	*P. jirovecii (50916)* *H. cytomegalovirus (10)*	(5)
No. 3	Autoimmune hemolytic anemia	Pulmonary infection	Gram-negative bacilli	Negative	Chronic inflammation of the mucosa, a small amount of neutrophil infiltration, partial alveolar septum widening with fibrous tissue hyperplasia	*P. jirovecii (64), H. cytomegalovirus (23282), Rhizopus microspores (6)*	(5)
No. 4	Acute lymphocytic leukemia	Pulmonary infection	Negative	*P. jirovecii*	Lymphocyte, plasma cell, and neutrophil infiltration	*P. jirovecii (1058), H. cytomegalovirus (154)*	(5)
No. 5	Lymphoma	Pulmonary infection	Gram-positive cocci, Gram-negative bacilli	*Staphylococcus epidermidis*	Alveolar fibrous tissue hyperplasia	*P. jirovecii (303572), H. cytomegalovirus (1626)*	(5)
No. 6	None	Pulmonary infection	Gram-positive cocci	*Acinetobacter baumannii, P. jirovecii*	Inflammatory cell infiltration and interstitial fibrous tissue hyperplasia	*P. jirovecii (4522), H. cytomegalovirus (4), Aspergillus fumigatus (6), Acinetobacter baumannii (338)*	(5)
No. 7	None	Pulmonary infection	Negative	*P. jirovecii*	Inflammatory cell infiltration and interstitial fibrous tissue hyperplasia	*P. jirovecii (104054), H. cytomegalovirus (29)*	(5)
No. 8	Lymphoma	Pulmonary infection	Negative	*P. jirovecii*	Mild hyperplasia of fibrous tissue, a little lymphocyte infiltration	*P. jirovecii (86), H. cytomegalovirus (4)*	(5)
No. 9	Lymphoma	Pulmonary infection	Fungi	Candida	–	*P. jirovecii (427), H. cytomegalovirus (652), Candida albicans (2), Staphylococcus aureus (2)*	(15)
No. 10	Lymphoma	Pulmonary infection	Negative	Negative	–	*P. jirovecii (5), H. cytomegalovirus (28)*	(15)
No. 11	Thymoma; pemphigus	Pulmonary infection	Negative	Negative	–	*P. jirovecii (9), H. cytomegalovirus (187), Nocardia cyriacigeorgica (42)*	(15)
No. 12	Breast cancer; liver cirrhosis; diabetes; renal insufficiency; cardiac insufficiency	Pulmonary infection	Negative	Negative	–	*P. jirovecii (5), H. cytomegalovirus (38), Candida tropicalis (3791), Aspergillus fumigatus (3)*	(15)

P. jirovecii, Pneumocystis jirovecii; H. cytomegalovirus, human cytomegalovirus.

insufficient for the diagnosis of invasive CMV pneumonitis. The definitive diagnosis of CMV pneumonitis relies on BALF cytology or a lung biopsy demonstrating cells with inclusion bodies, after excluding other etiologies (7, 20). So, CMV pneumonitis was reported in only 5–8% of HIV patients undergoing BALF testing (19). Quantitative polymerase chain reaction (PCR) of CMV viral load has been investigated as a marker of invasive CMV disease, with a sensitivity of 86% and specificity of 87% when the cut-off is applied at >5,000 copies/ml, but mainly in transplant recipients (21–23).

CMV and PCP coinfection has been previously reported in the literature (10). However, there have only been two study reports of 12 cases on the successful use of NGS to diagnose pulmonary infections involving PCP and CMV in BALF or blood or lung biopsy (**Table 2**) until now (5, 15). The study reported

that NGS is more sensitive than conventional methods (non-PCR based). However, the numbers of sequence reads for *P. jirovecii* (5–303,572) and CMV (4–23,282) are highly variable among cases (5, 15). So, the cut-off reads of NGS to be used as a marker of invasive PCP and CMV disease is uncertain. A study suggested that specific reads of *P. jirovecii* ranking among the top 15 or its relative reads proportion in fungi higher than 85% might be satisfactory cut-off values for clinical diagnosis of PCP (18). In patients with PCP, the co-presence of CMV in BALF has traditionally been presumed to be non-invasive and there were no difference in outcome between anti-CMV-treated and CMV-untreated groups (24). However, several reports have highlighted a poor outcome among HIV patients who were co-infected with PCP and CMV (25, 26). A study reported a 2-fold higher mortality at 3 months in patients with PCP who also had CMV cultured from BALF with corticosteroids treatment (27). In our case, the *P. jirovecii* and CMV reads were 1,000,078 and 4,192, respectively, and the patient was treated with corticosteroids. So, additional antiviral therapy with ganciclovir was given and the patient was treated successfully according to the diagnosis based on NGS. This case demonstrates that the addition of anti-CMV therapy may benefit such patients in the right clinical scenario. Furthermore, NGS might be beneficial to HIV-infected patients who develop pneumonia and are more likely to be infected by multiple pathogens. Nevertheless, it is necessary to investigate which cut-off reads should be used as a diagnosis marker of invasive PCP and CMV disease. Multi-pathogenic pneumonia in severely immunocompromised patients presents a diagnostic and management dilemma. Consideration of multiple etiologies in immunocompromised patients is necessary, and early bronchoscopy and NGS are recommended. The resulting interpretation of NGS needs caution, along with other laboratory, radiological, and clinical findings. Furthermore, the application of NGS to clinical diagnosis is still challenging due to barriers in cost-effectiveness and the standardization of the entire process from sample collection to result interpretation (28). Clearly, all these issues need investigation in the future.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Written informed consent was obtained from the patient for the publication of this case report.

AUTHOR CONTRIBUTIONS

YX collected data and wrote the manuscript. LJ and BZ analyzed and interpreted patient data. BR and BZ reviewed the manuscript. All authors read through and approved the final manuscript.

FUNDING

This study was funded by the Natural Science Foundation of China (Young Scientist Fund, 81500491) and the National Science Foundation of China (Major Research Plan, 2018ZX10715-014). These funding agencies have no role in the design, data collection, analysis or interpretation of the research, or in the writing of the manuscript.

REFERENCES

1. Zarrinfar H, Saber S, Kordbacheh P, Makimura K, Fata A, Geramishoar M, et al. Mycological microscopic and culture examination of 400 bronchoalveolar lavage (BAL) samples. *Iran J Public Health.* (2012) 41:70–6.
2. Zarrinfar H, Mirhendi H, Fata A, Khodadadi H, Kordbacheh P. Detection of *Aspergillus flavus* and *A. fumigatus* in bronchoalveolar lavage specimens of hematopoietic stem cell transplants and hematological malignancies patients by real-time polymerase chain reaction, nested PCR and mycological assays. *Jundishapur J Microbiol.* (2015) 8:e13744. doi: 10.5812/jjm.13744
3. Zarrinfar H, Kaboli S, Dolatabadi S, Mohammadi R. Rapid detection of *Candida* species in bronchoalveolar lavage fluid from patients with pulmonary symptoms. *Brazil J Microbiol.* (2016) 47:172–6. doi: 10.1016/j.bjm.2015.02.001
4. Di Pasquale MF, Sotgiu G, Gramegna A, Radovanovic D, Terraneo S, Reyes LF, et al. Prevalence and etiology of community-acquired pneumonia in immunocompromised patients. *Clin Infect Dis.* (2019) 68:1482–93. doi: 10.1093/cid/ciy723
5. Wang J, Han Y, Feng J. Metagenomic next-generation sequencing for mixed pulmonary infection diagnosis. *BMC Pulm Med.* (2019) 19:252. doi: 10.1186/s12890-019-1022-4
6. Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM, et al. Community-acquired pneumonia requiring hospitalization among U.S. adults. *N Engl J Med.* (2015) 373:415–27. doi: 10.1056/NEJMoa1500245
7. Skalski JH, Limper AH. Fungal, viral, and parasitic pneumonias associated with human immunodeficiency virus. *Semin Respir Crit Care Med.* (2016) 37:257–66. doi: 10.1055/s-0036-1578802
8. Crothers K, Thompson BW, Burkhardt K, Morris A, Flores SC, Diaz PT, et al. HIV-associated lung infections and complications in the era of combination antiretroviral therapy. *Proc Am Thoracic Soc.* (2011) 8:275–81. doi: 10.1513/pats.201009-059WR
9. Huang L, Crothers K. HIV-associated opportunistic pneumonias. *Respirology.* (2009) 14:474–85. doi: 10.1111/j.1440-1843.2009.01534.x
10. Fei MW, Kim EJ, Sant CA, Jarlsberg LG, Davis JL, Swartzman A, et al. Predicting mortality from HIV-associated Pneumocystis pneumonia at illness presentation: an observational cohort study. *Thorax.* (2009) 64:1070–6. doi: 10.1136/thx.2009.117846
11. Ismail T, Lee C. HIV associated opportunistic pneumonias. *Med J Malaysia.* (2011) 66:76–82.
12. Labelle AJ, Arnold H, Reichley RM, Micek ST, Kollef MH. A comparison of culture-positive and culture-negative health-care- associated pneumonia. *Chest.* (2010) 137:1130–7. doi: 10.1378/chest.09-1652
13. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol Mech Dis.* (2019) 14:319–38. doi: 10.1146/annurev-pathmechdis-012418-012751
14. Camargo JF, Ahmed AA, Lindner MS, Morris MI, Anjan S, Anderson AD, et al. Next-generation sequencing of microbial cell-free DNA for rapid noninvasive diagnosis of infectious diseases in immunocompromised hosts. *F1000Research.* (2020) 8:1194. doi: 10.12688/f1000research.19766.4

15. Zhang Y, Ai JW, Cui P, Zhang WH, Wu HL, Ye MZ. A cluster of cases of Pneumocystis pneumonia identified by shotgun metagenomics approach. *J Infect.* (2019) 78:158–69. doi: 10.1016/j.jinf.2018.08.013
16. Özmen A, Mistik R, Alver O, Coşkun F, Ursavaş A, Uzaslan E. The *Pneumocystis jirovecii* colonization in bronchoalveolar lavage (BAL) and bronchial washing and the comparison of methods which are used in diagnosis. *Tuberk Toraks.* (2013) 61:303–11. doi: 10.5578/tt.2954
17. Izadi M, Jafari NJ, Sadraei J, Poornaki AM, Rezavand B, Zarrinfar H, et al. The prevalence of *Pneumocystis jiroveci* in bronchoalveolar lavage specimens of lung transplant recipients examined by the nested PCR. *Jundishapur J Microbiol.* (2014) 7:e13518. doi: 10.5812/jjm.13518
18. Shah K, Cherabuddi K, Beal SG, Kalyatanda G. Refractory acute respiratory failure due to *Pneumocystis jiroveci* (PCP) and cytomegalovirus (CMV) pneumonitis: a case report and review of literature. *IDCases.* (2017) 10:42–5. doi: 10.1016/j.idcr.2017.08.011
19. Poh KC, Zheng S. A rare case of CMV pneumonia in HIV-infection. *Respir Med Case Rep.* (2019) 28:100945. doi: 10.1016/j.rmcr.2019.100945
20. Kaplan JE, Benson C, Holmes KK, Brooks JT, Pau A, Masur H, et al. Guidelines for the prevention and treatment of opportunistic infections in adults and adolescents with HIV recommendations from the centers for disease control and prevention. *MMWR Recomm Rep.* (2009) 58:1–198.
21. Dioverti MV, Razonable RR. Clinical utility of cytomegalovirus viral load in solid organ transplant recipients. *Curr Opin Infect Dis.* (2015) 28:317–22. doi: 10.1097/QCO.0000000000000173
22. Humar A, Gregson D, Caliendo AM, Mcgeer A, Malkan G, Krajden M, et al. Clinical utility of quantitative cytomegalovirus viral load determination for predicting cytomegalovirus disease in liver transplant recipients. *Transplantation.* (1999) 68:1305–11. doi: 10.1097/00007890-199911150-00015
23. Toyoda M, Carlos JB, Galera OA, Galfayan K, Zhang X, Sun Z, et al. Correlation of cytomegalovirus DNA levels with response to antiviral therapy in cardiac and renal allograft recipients. *Transplantation.* (1997) 63:957–63. doi: 10.1097/00007890-199704150-00009
24. Jacobson MA, Mills J, Rush J, Peiperl L, Seru V, Mohanty PK, et al. Morbidity and mortality of patients with AIDS and first-episode *Pneumocystis carinii* pneumonia unaffected by concomitant pulmonary cytomegalovirus infection. *Am Rev Respir Dis.* (1991) 144:6–9. doi: 10.1164/ajrccm/144.1.6
25. Acar J. *Pneumocystis jirovecii* and cytomegalovirus co-infection in AIDS patients. *J Med Cases.* (2013) 4:402–6. doi: 10.4021/jmc1249w
26. Simon D, Greyling KE, Irusen EM, Rigby J, Taljaard JJ, Koegelenberg CFN. *Pneumocystis jiroveci* and cytomegalovirus co-infection in an immunocompromised patient. *South African Respir J.* (2016) 22:20. doi: 10.7196/SARJ.2016.v22i1.40
27. Jensen AMB, Lundgren JD, Benfield T, Nielsen TL, Vestbo J. Does cytomegalovirus predict a poor prognosis in *Pneumocystis carinii* pneumonia treated with corticosteroids? A note for caution. *Chest.* (1995) 108:411–4. doi: 10.1378/chest.108.2.411
28. Miller S, Chiu C, Rodino KG, Miller MB. Point-counterpoint: should we be performing metagenomic next-generation sequencing for infectious disease diagnosis in the clinical laboratory? *J Clin Microbiol.* (2020) 58:e01739–19. doi: 10.1128/JCM.01739-19

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Xie, Ruan, Jin and Zhu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 23 April 2021
doi: 10.3389/fcimb.2021.639108

CRISPR/Cas System: A Potential Technology for the Prevention and Control of COVID-19 and Emerging Infectious Diseases

Ronghua Ding[1], Jinzhao Long[1], Mingzhu Yuan[1], Yuefei Jin[1], Haiyan Yang[1], Mengshi Chen[2], Shuaiyin Chen[1] and Guangcai Duan[1,3]**

[1] College of Public Health, Zhengzhou University, Zhengzhou, China, [2] Hunan Provincial Key Laboratory of Clinical Epidemiology, Xiangya School of Public Health, Central South University, Changsha, China, [3] Key Laboratory of Molecular Medicine in Henan Province, Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UCONN Health,
United States

Reviewed by:
He Kangxin,
Zhejiang University, China
Changchun Liu,
UCONN Health,
United States

***Correspondence:**
Guangcai Duan
gcduan@zzu.edu.cn
Shuaiyin Chen
sychen@zzu.edu.cn

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *08 December 2020*
Accepted: *08 April 2021*
Published: *23 April 2021*

Citation:
Ding R, Long J, Yuan M, Jin Y, Yang H, Chen M, Chen S and Duan G (2021) CRISPR/Cas System: A Potential Technology for the Prevention and Control of COVID-19 and Emerging Infectious Diseases. Front. Cell. Infect. Microbiol. 11:639108. doi: 10.3389/fcimb.2021.639108

The continued global pandemic of coronavirus disease 2019 (COVID-19) poses a serious threat to global public health and social stability and it has become a serious global public health problem. Unfortunately, existing diagnostic and therapeutic approaches for the prevention and control of COVID-19 have many shortcomings. In recent years, the emerging CRISPR/Cas technology can complement the problems of traditional methods. Biological tools based on CRISPR/Cas systems have been widely used in biomedicine. In particular, they are advantageous in pathogen detection, clinical antiviral therapy, drug, and vaccine development. Therefore, CRISPR/Cas technology may have great potential for application in the prevention and control of COVID-19 and emerging infectious diseases in the future. This article summarizes the existing applications of CRISPR/Cas technology in infectious diseases with the aim of providing effective strategies for the prevention and control of COVID-19 and other emerging infectious diseases in the future.

Keywords: CRISPR/Cas, SARS-CoV-2, pathogen detection, clinical therapy, drug and vaccine development

INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) first broke out in Wuhan, China, and rapidly grew into a global pandemic, seriously endangering human health and social development. By February 23, 2021, SARS-CoV-2 had become a global pandemic in 180 countries and regions, with the cumulative number of infections exceeding 110 million and the cumulative number of deaths exceeding 2.48 million. More importantly, with the ongoing mutation of SARS-CoV-2 and the coming winter and spring, these numbers may continue to increase (Grubaugh et al., 2020; Korber et al., 2020). In response to the SARS-CoV-2 pandemic, it is urgent to strengthen pathogen detection capacity and accelerate the development of vaccines and specific antiviral drugs. At present, the traditional nucleic acid

detection method, mainly quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), is recommended by WHO and some medical departments to detect pathogens. However, some researchers found that the results are unstable, and the sensitivity is even as low as 42.10%, and the sample processing requirements are very strict (Bustin and Nolan, 2020; Tesija Kuna et al., 2021). More importantly, the therapeutic effect of antiviral drugs has not achieved the expected effect, and symptomatic treatment is still the main treatment at present (Khan et al., 2020). Vaccine development is still in the clinical evaluation stage (Folegatti et al., 2020; Gao Q. et al., 2020; Tu et al., 2020), it will take some time before it can be applied, and even the mutation of the virus may make the vaccine less effective than expected (Becerra-Flores and Cardozo, 2020; Li Q. et al., 2020). Moreover, the vaccine may not be able to meet the huge global demand.

CRISPR (clustered regularly interspaced short palindromic repeats) consists of a gene encoding a Cas-related protein and the CRISPR array, which is a repetitive sequence within the prokaryotic genome, an immune weapon produced by the struggle between bacteria and phages throughout the history of evolution (Bondy-Denomy et al., 2013; Koonin et al., 2017). Therefore, bacteria can protect themselves by using the CRISPR/Cas system to eliminate foreign invading phage genes. Since 1987, CRISPR/Cas was discovered by Ishino in *E. coli*, scientists have been developing several biotechnologies based on this basic principle (Ishino et al., 1987). The development and application of the CRISPR/Cas system have given new impetus to the development of life sciences and biotechnology (Knott and Doudna, 2018). Especially in medical science, CRISPR/Cas has made remarkable achievements in gene editing, nucleic acid detection, functional gene screening, and so on (Zhou et al., 2014; Kurata et al., 2018; Pickar-Oliver and Gersbach, 2019; Wang et al., 2019; Yan et al., 2019). Also, in recent years, scientists have used the CRISPR/Cas system broadly in the prevention and control of infectious diseases. In the article, we reviewed recent advances regarding the application of the CRISPR/Cas system in the prevention and control of COVID-19 and other infectious diseases, such as in molecular diagnostics, infectious disease treatment, the antiviral drug, and vaccine development. And provide effective strategies for the prevention and control of COVID-19 and other future emerging infectious diseases.

APPLICATION OF CRISPR/CAS IN PATHOGEN DETECTION

Inadequacy of Existing Pathogen Detection

Pathogen detection is crucial for the prevention and control of infectious diseases. Early identification of pathogens enables identification of the source of infection and timely quarantine, so that appropriate anti-infective protocols can be developed to improve patient prognosis, especially for severe emerging infectious diseases such as COVID-19. However, the current detection of SARS-CoV-2 is mainly by qRT-PCR, which is time-consuming and dependent on specialized equipment and operators, which may not be available in developing countries or remote rural hospitals, and is not suitable for field testing (Corman et al., 2020; Lu et al., 2020). Besides, limited sensitivity may prevent the detection of asymptomatic infected persons, or some patients during the time-window of viral replication, who are likely to contribute to further transmission without medical treatment (Li Y. et al., 2020; Younes et al., 2020; Yu et al., 2020). overcome the shortcomings of qRT-PCR technology, some experts have suggested using a combined antibody assay to detect SARS-CoV-2 (Li Z. T. et al., 2020). Although enzyme-linked immunosorbent assay (ELISA) has been successfully used to detect SARS-CoV-2, specific antibodies need to be produced after more than 7 days of infection with the virus (Lin et al., 2020; Lisboa Bastos et al., 2020; Wang Q. et al., 2020). In addition, antibody detection can produce cross-reactivity, and people with poor immunity may produce fewer antibodies, which can affect test results (Liu W. et al., 2020). Furthermore, isothermal amplification detection technology has a great prospect of application because of its simple operation and can be applied to field detection. However, the current isothermal amplification technology has its inherent defects in sensitivity, specificity, and anti-interference. Therefore, highly sensitive and specific, field-applicable pathogen detection is necessary for the detection of emerging infectious diseases (**Table 1**).

TABLE 1 | Available diagnostic methods for COVID-19.

Test	Detection type	Positive rate	Time	Characteristics	Limitations	References
qRT-PCR	nucleic acid detection	38%	>2h	Currently the most common detection method, simple quantitative detection.	Time-consuming, poor sensitivity	(Liu R. et al., 2020)
ELISA	Immunological detection	80.4%	>2h	Fast, easy to operate, low sample volumes	Cross-reactivity, Antibodies are produced about 7-10 days after infection.	(Liu W. et al., 2020)
RT-LAMP	nucleic acid detection	92.9%	40–60 min	Highly sensitive, fast, and convenient field testing	Prone to false positives	(Baek et al., 2020)
CRISPR-based detection	nucleic acid detection	96%	30-60min	Highly sensitive, high specificity, fast and convenient for field testing., low-cost	Not yet widespread and in clinical trials	(Patchsung et al., 2020)
ddPCR	nucleic acid detection	40%	NA	Absolute quantification, high sensitivity	Expensive equipment, poor accessibility, and complexity of the operation	(Suo et al., 2020)
CT Scan	Radiological Screening	NA	NA	Check for disease progression, easy access, rapid detection	Unable to identify the pathogen of infection, clinical assistant tests	(Dong et al., 2020)

Application of CRISPR/Cas-Based Systems for Rapid, Accurate Point-of-Care Diagnostics

In recent years, emerging nucleic acid detection technologies based on CRISPR/Cas developments have opened up new opportunities for pathogen detection. SHERLOCK (Cas13a), DETECTR (Cas12a), CDetection (Cas12b) and Cas14-DETECTR, which have successfully performed rapid, highly sensitive and accurate detection of a variety of pathogens (Gootenberg et al., 2017; Chen et al., 2018a; Chen et al., 2018b; Harrington et al., 2018; Teng et al., 2019) (**Figure 1**). Compared to traditional assays, CRISPR/Cas system has advantages in terms of rapidity, low cost, portability, ease of operation and extension, while maintaining high sensitivity and specificity (Zhou et al., 2018). In addition, the low sample quality requirements and high interference resistance mean that rapid sample pre-processing such as HUDSON can be combined to enable nucleic acid extraction on site without relying on specialized equipment (Gootenberg et al., 2017; Wang M. et al., 2020). In combination with lateral flow test strip technology for visualizing point-of-care diagnostics with the naked eye (Dai et al., 2020; Van Dongen et al., 2020). Fluorescence readout also can achieve point-of-care diagnostics with portable fluorescence collectors or colorimetric analysis (Yuan et al., 2020; Cheng et al., 2021).

CRISPR/Cas based detection of SARS-CoV-2 has been actively researched by scientists since the COVID-19 outbreak (**Table 2**). For example, James P. Broughton can successfully detect SARS-COV-2 within 30-40 minutes using the CRISPR-Cas12a-based method with a limit of detection (LoD) of up to 10 copies/μL, and the combination of lateral flow strip technology enables visible detection results in the field (Broughton et al., 2020b). Similarly, based on the CRISPR/Cas13a method, the SHERLOCK platform has been successfully validated in 534 clinical samples with a LoD of 42 copies/μL and 100% sensitivity for fluorescence readout and 97% for lateral-flow detection (Patchsung et al., 2020). Feng Zhang's team developed the SARS-CoV-2 detection platform STOPCovid.v2 based on AapCas12b, which enables point-of-care diagnostics to be performed quickly and easily within 1 hour, without relying on specialized equipment. Also, it has obtained the FDA (US Food and Drug Administration) emergency use right of SARS-CoV-2 detection (Guglielmi, 2020; Joung et al., 2020). Besides, researchers using the Cas12a-based opvCRISPR platform were able to visualize fluorescence detection of SARS-CoV-2 with the naked eye in 45 min (Wang et al., 2021). It is worth noting that CRISPR/Cas technology allows not only rapid detection but also massively multiplexed nucleic acid detection. For example, Cheri M. Ackerman developed Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (CARMEN) based on CRISPR/Cas13, which allows for the simultaneous differentiation of 169 viruses associated with humans, as well as the multiple identifications of various subtypes and even drug resistance mutations, thereby increasing detection rates while reducing detection costs (Ackerman et al., 2020).

Moreover, CRISPR/Cas technology has the unique ability to detect single nucleotide polymorphisms (SNP) compared to other above-mentioned detection technologies. Compared with conventional sequencing for SNP detection, CRISPR/Cas technology is simpler, faster, and cheaper, and more importantly, can detect low-frequency mutations. Faced with SARS-CoV-2 mutations such as the D614G and N501Y locus mutation. These mutations may increase the infectivity of SARS-COV-2 and even pose a significant threat to existing therapies and vaccines (Li Q. et al., 2020; Yurkovetskiy et al., 2020). So the identification of mutant strains based on high-specificity CRISPR/Cas technology has great significance. Although no researchers have yet performed CRISPR/Cas-based detection of the SARS-COV-2 mutation site, some scientists have successfully used Cas13a to detect the HBV drug resistance site rt204 (Gootenberg et al., 2017; Wang S. et al., 2020).

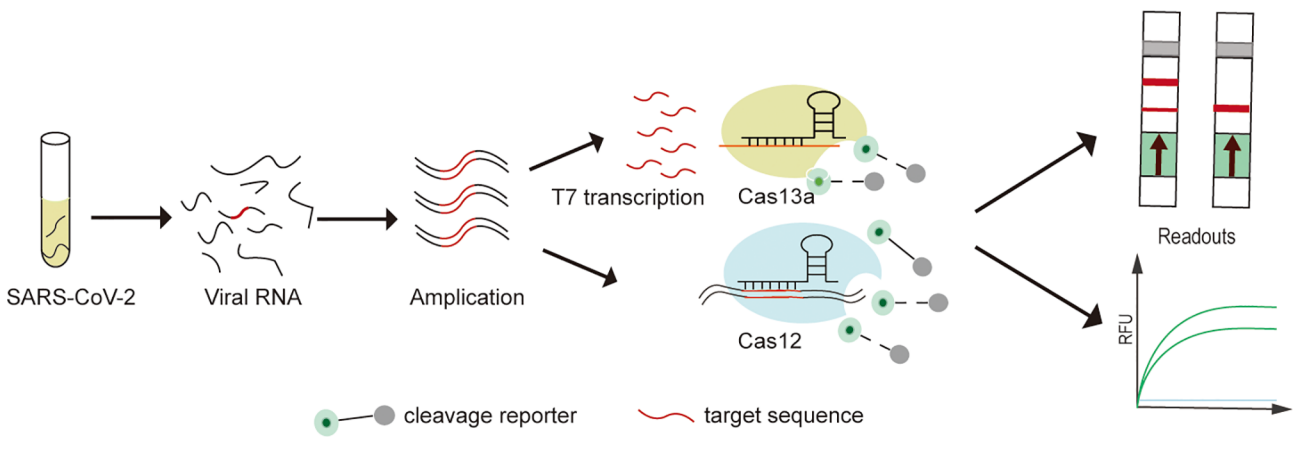


FIGURE 1 | Experimental protocol for SARS-CoV-2 detection on CRISPR/Cas detection platform. (1) The extracted SARS-CoV-2 viral RNA is first pre-amplified (RT-RPA and RT-LAMP) into double-stranded DNA (dsDNA). (2) For Cas13a-SHERLOCK assay, dsDNA is first T7 transcribed into single-stranded RNA (ssRNA), followed by activation of the Cas13a cleavage reporter. (3) For Cas12a or Cas12b assays, direct detection of pre-amplified dsDNA enables activation of the Cas12a or Cas12b cleavage reporter. (4) Visualization of CRISPR/Cas assay results by fluorescence and lateral flow readouts.

TABLE 2 | Summary of the CRISPR/Cas platform for SARS-CoV-2 detection.

Detection platforms	CRISPR/ Cassystem	Limit of detection	Time	Characteristics	References
CREST	Cas13a	10copies/μL	30-50min	Combined with RT-PCR technology, CRISPR detection is stable. However, there may be drawbacks to field detection.	(Rauch et al., 2020)
SHERLOCK	Cas13a	42 copies per reaction	70min	Combination of RT-RPA amplification and lateral flow assay for rapid field testing.	(Patchsung et al., 2020)
SHINE	Cas13a	10copies/μL	50min	Improved sample pre-processing (HUDSON) combined with a one-step SHERLOCK for fast field inspection.	(Arizti-Sanz et al., 2020)
DETECTR	Cas12a	10copies/μL	45min	Combined with LAMP to solve the problem of unstable RPA amplification enables faster and more sensitive detection in the field.	(Broughton et al., 2020a)
STOPCovid	Cas12b	100 copy per reaction	<60min	Integrated LAMP and Cas12b detection enables one-step detection, making it easier and faster to operate.	(Joung et al., 2020)
CARMEN	Cas13a	attomolar	NA	Enables large-scale pathogen detection and simultaneous identification of 165 human-associated viruses.	(Ackerman et al., 2020)

CRISPR/CAS IN CLINICAL ANTIVIRAL THERAPY

Application of Cas9 Gene-Editing to Clinical Antiviral Therapy

In the face of emerging infectious diseases such as SARS, MERS, and COVID-19, existing antiviral drugs are often ineffective. However, developing an effective antiviral drug is an arduous process. Therefore, clinical treatment is mainly supportive and to prevent complications, but in the absence of specific antivirals, this approach may result in a higher case fatality rate. The scientific community has been searching for ways to eliminate viruses more quickly and accurately, which could have significant implications for early source prevention and control of emerging infectious diseases. In recent years, experts using CRISPR/Cas9 gene-editing technology to treat many clinically refractory diseases. For one thing, the researchers used CRISPR/Cas9 targeting virus-specific sequences for gene editing to directly eliminate the virus to treat the disease. For example, The Edward M. Kennedy in the cell-free system trial used Cas9 to specifically target the HPV E6E7 gene to eliminate HPV infection to treat cervical cancer (Kennedy et al., 2014). Zhen has validated in animal studies that Cas9-targeted elimination of covalently closed circular DNA (cccDNA) is effective in the treatment of hepatitis B (Zhen et al., 2015). In addition, scientists also successfully used Cas9 to accurately eliminate Cytomegalovirus, Epstein-Barr virus infections *in vitro* (Ebina et al., 2013; Hu et al., 2014; Huo and Hu, 2019; Chen S. J. et al., 2020). For another thing, some researchers have used Cas9 to engineer T cells with the ability to treat specific diseases and thus indirectly eliminate viral infections (Zhao et al., 2018; Stadtmauer et al., 2020). For example, Cas9 was developed to improve CAR-T (Chimeric Antigen Receptor T-Cell Immunotherapy) therapy to treat incurable cancers such as B-cell leukemias and lymphomas (Brentjens et al., 2011; Porter et al., 2016; Eyquem et al., 2017; Labanieh et al., 2018). Moreover, many *in vivo* trial studies show Cas9 gene editing modifies CD4+ T cells to be effective in treating HIV infection, which may provide a new approach to refractory infectious diseases (Ebina et al., 2013; Liu et al., 2017; Hultquist et al., 2019; Xiao et al., 2019). With SARS-CoV-2 and other difficult to treat emerging infectious disease, CRISPR/Cas9 gene editing is likely to be one of the best treatments in the future (Grifoni et al., 2020) (**Figure 2**). In addition, with ongoing research and development, Cas12a and Cas14 also hold great potential for gene editing to treat infectious diseases in the future (Gao Z. L. et al., 2020).

Cas13 Specifically Targets RNA Viruses for Degradation

Compared to Cas9, gene editing of Cas13-targeted RNA may offer a new approach to RNA virus therapy (Freije et al., 2019). LshCas13a system specifically targeted E6E7mRNA of HPV has a positive effect on inhibiting human cervical cancer *in vivo* testing (Chen Y. et al., 2020). Besides, Hao Li used Cas13a cleavage of the Dengue virus NS3 gene to efficiently inhibits viral replication *in vivo* testing (Li H. et al., 2020). Interestingly, Timothy R. Abbott used Cas13d to develop the PAC-MAN platform (prophylactic antiviral CRISPR in human cells) for viral inhibition that can effectively degrade RNA from SARS-CoV-2 sequences and live influenza A virus (IAV) in human lung epithelial cells. Notably, this approach provides a novel treatment strategy for SARS-CoV-2 and influenza virus, among others (Abbott et al., 2020; Nguyen et al., 2020) (**Figure 2**).

APPLICATION OF CRISPR/CAS SYSTEMS IN VACCINE AND DRUG DEVELOPMENT

CRISPR/Cas9 Assisted Drug Development and Target Screening

The rapid development of effective antiviral drugs is also essential for the treatment of COVID-19 (**Table 3**). However, existing antiviral drugs are often ineffective in the treatment of emerging infectious diseases, mainly due to the lack of effective targets for the treatment of emerging viruses. Unfortunately, the main reason for the failure of antiviral drug development is also the difficulty in identifying effective targets, which makes development more difficult, expensive, and time-consuming. Moreover, host factors associated with viral action are critical to the success of treatment and drug development (Li B. et al., 2020; Wei et al., 2020). Therefore, how to identify functional targets more quickly and accurately is one of the challenges plaguing drug development. Although RNAi is undoubtedly a

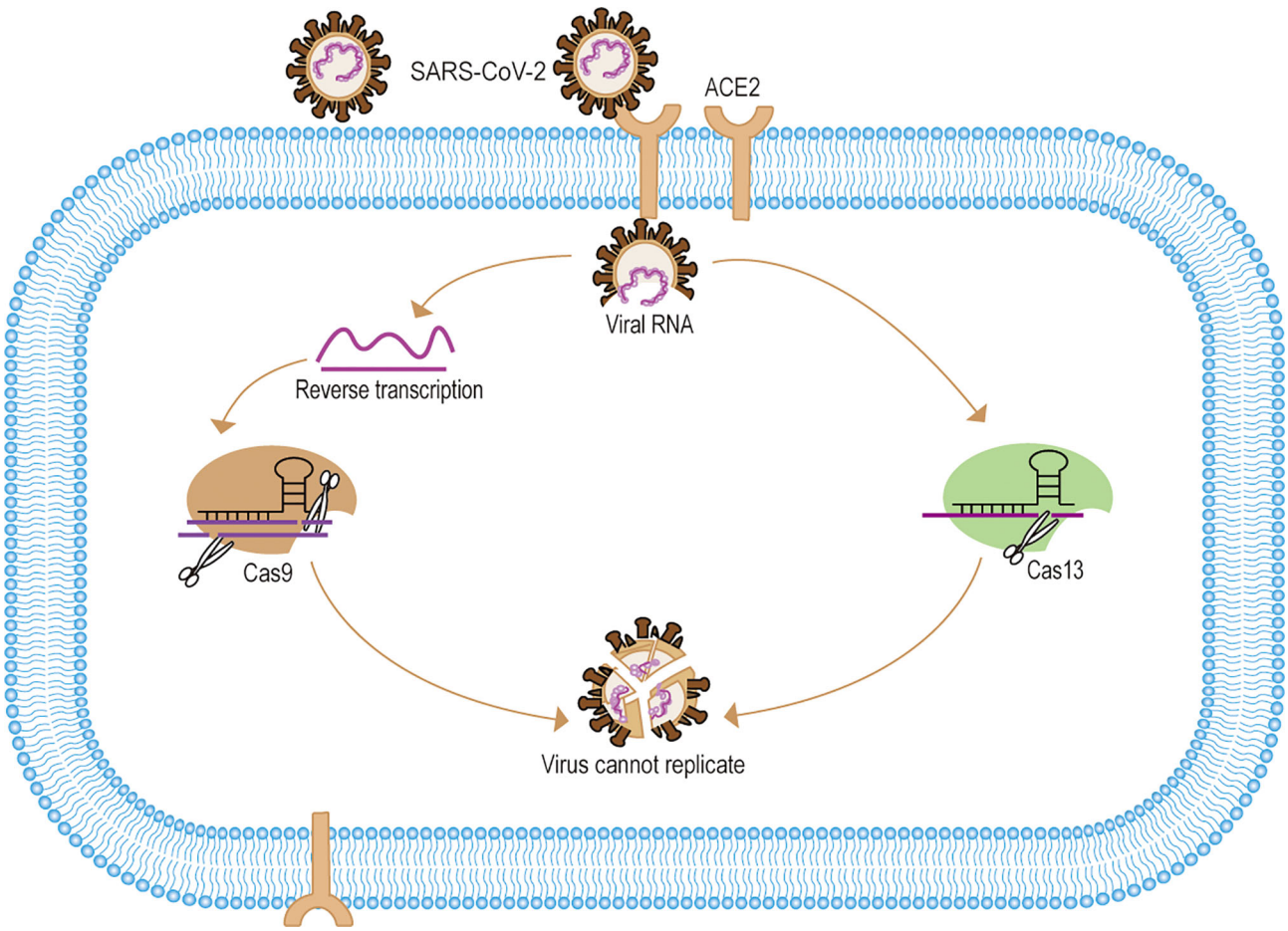


FIGURE 2 | Mechanisms of CRISPR/Cas systematic elimination of SARS-CoV-2. (1) SARS-CoV-2 targets the ACE2 receptor to release viral RNA into cells. (2) After reverse transcription of the viral RNA to form dsDNA, the pre-designed sgRNA-Cas9 identified and specifically cleaved the viral DNA. (3) For Cas13 the system can directly identify and eliminate viral RNA. (4) The CRISPR/Cas system eliminates the SARS-CoV-2 gene, thus making the viral RNA unable to replicate and the viral gene unable to be expressed, and eventually making SARS-CoV-2 unable to replicate.

TABLE 3 | Summary of current antiviral drugs or approaches to treat COVID-19.

Type	Candidate therapies	Mechanism of action	Clinical efficacy	References
Antiviral	Remdesivir	Inhibition of viral RNA replication	Efficacy is controversial, Under clinical trial for SARS-CoV-2	(Grein et al., 2020; Wang Y. et al., 2020)
Antiviral	Lopinavir-Ritonavir	Viral Protease Inhibitors, inhibits 3CLpro	Under clinical trial for SARS-CoV-2	(Cao et al., 2020)
Antiviral	Hydroxy-chloroquine, Chloroquine	Increasing endosomal pH prevents membrane fusion between virus and host cells	Under clinical trial for SARS-CoV-2	(Gautret et al., 2020; Pereira, 2020)
Antiviral	Favipiravir	RNA polymerase inhibitors	Under clinical trial for SARS-CoV-2	(Cai et al., 2020; Du and Chen, 2020)
Antiviral	PAC-MAN method based on CRISPR-Cas13d, CRISPR-Cas9	Specific degradation of SARS-COV-2	Although not in clinical trials, its unique advantages provide therapeutic strategies for refractory, poorly treated viruses	(Abbott et al., 2020)
Symptomatic therapeutic	Bevacizumab, SARS-CoV-2-Specific Neutralizing Antibodies	Attenuating the Inflammatory Response	Under clinical trial for SARS-CoV-2	(Thickett et al., 2001; Zhou and Zhao, 2020)

widely recognized and powerful method, it may produce misleading results due to off-target effects. As a result, the off-target effect may affect many functionally important genes (Settleman et al., 2018; Haley and Roudnicky, 2020). The emergence and development of CRISPR/Cas technology can more easily and quickly find potential drug targets to inhibit the replication of viruses, and provide safer and more feasible strategies for the treatment and prevention of diseases (Behan et al., 2019) (**Figure 3**). For instance, Flint used CRISPR/Cas technology to screen the genome-wide library and found that N-acetylglucosamine-1-phosphate transferase subunits alpha and beta (GNPTAB) were potential targets for the anti-Ebola virus (Flint et al., 2019). Interestingly, researchers on the bat cells of genome-wide screening explore the target of widespread antiviral treatment, after screening detects MTHFD1 targets is a potential target for broad-spectrum antiviral drugs. MTHFD1 inhibitor caprolactone broad antiviral activities against the Zika virus, mumps virus, and importantly, SARS-CoV-2 (Anderson et al., 2020). Furthermore, CRISPR/Cas screening can reveal host genes that regulate SARS-CoV-2 infection, such as SRSF protein kinases 1 and 2, ACE2, and HMGB1 (Heaton et al., 2020; Hoffmann et al., 2020; Wang R. et al., 2020; Wei et al., 2020). This provides insight into the signaling pathways underlying viral action. Although these articles have not been peer-reviewed, these ideas provide good insights and thoughts for exploring responses to emerging infectious diseases such as SARS-COV-2.

Application of CRISPR/Cas9 in Vaccine Development

The prevention and control of outbreaks and epidemics of COVID-19 and other emerging infectious diseases require not only specific antiviral drugs but also vaccines. Vaccination is one of the most economical and effective public health interventions for the prevention and control of infectious diseases and one of the effective ways to prevent the large-scale spread of infectious diseases. However, the development of a vaccine is a long, complex process, and expensive. How to develop specific vaccines quickly and easily is a difficult problem for scientists. In response to emerging infectious diseases, CRISPR/Cas9 could boost progress in vaccine development. For example, Mustafa Ozan Atasoy developed

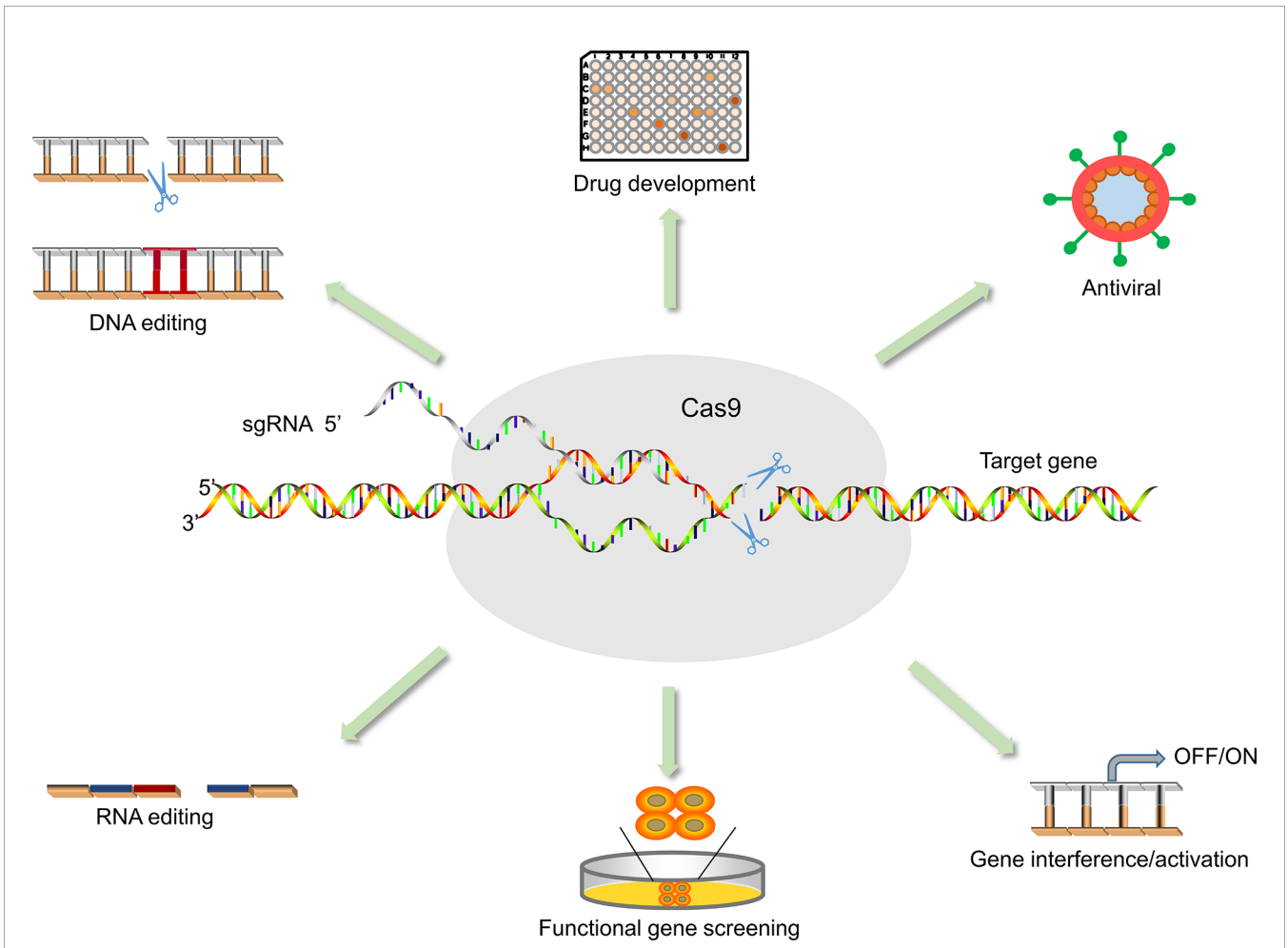


FIGURE 3 | Application of CRISPR/Cas9 in medical sciences. The application of Cas9 is not only for antiviral but also for functional gene screening, drug development, animal model vector construction, and signaling pathway research.

and applied a highly efficient and rapid NHEJ-CRISPR/Cas9 (non-homologous end-joining, NHEJ) and Cre–Lox-mediated genome-editing approach for simultaneous deletion of virulence factors and insertion of antigens into the infectious laryngotracheitis virus to generate recombinant, multivalent, and safer vaccine vectors (Atasoy et al., 2019). Homology-directed repair (HDR) CRISPR/Cas9 and erythrocyte binding be used for the rapid generation of recombinant turkey herpesvirus-vectored avian influenza virus vaccines (Chang et al., 2019). In addition to Manuel used the CRISPR/Cas9 gene-editing system to produce recombinant African swine fever virus (ASFVs) (Borca et al., 2018). And the recombinant ASFV has great significance for the development of the live attenuated vaccine. Significantly, the researchers generated mouse models expressing human angiotensin-converting enzyme II (hACE2) using the Cas9 knock-in technique, which was used to study the transmission and pathogenesis of SARS-CoV-2 and to provide a useful tool for evaluating COVID-19 vaccines and therapeutic agents (Sun et al., 2020) (**Figure 3**).

LIMITATIONS AND IMPROVEMENTS OF THE CRISPR/CAS SYSTEM

Despite the many advantages of the emerging CRISPR/Cas system mentioned above, it also has some limitations. For example, Cas9 may have limited specificity of the recognition site during gene editing, which may cause a high off-target effect and eventually lead to permanent damage to the genome, such as cancer (Fu et al., 2013). In addition, Cas12 and Cas9 also need to recognize protospacer adjacent motif (PAM) sequences before activation, and PAM sequences may limit targeting and affect editing efficiency and flexibility (Mojica et al., 2009; Marraffini and Sontheimer, 2010). More importantly, the transport vector of the CRISPR/Cas system is also a major problem (Komor et al., 2017; Glass et al., 2018). Currently, viruses are mainly used as transport vectors, which may cause potential harm to the human body. In addition, the human body may be immune to sgRNA and Cas proteins, thus affecting the proper role of the CRISPR/Cas system (Kim et al., 2018). Beyond that, it is noteworthy that the CRISPR/Cas system may have implications for medical ethics (Brokowski and Adli, 2019).

As the CRISPR/Cas system has been deeply researched, some limitations have been addressed. Jennifer Doudna's team used the anti-CRISPR protein AcrllA4 to reduce the incidence of off-target effects by a factor of four, without any disruption to gene editing (Chen et al., 2017; Harrington et al., 2017; Jiang et al., 2019). In terms of optimizing the delivery carrier of the CRISPR/Cas system, scientists have made gradual progress in using nanocarriers to replace virus carriers (Lee et al., 2017). Another approach is to use smaller Cas proteins such as Cas14 (Harrington et al., 2018). Even more exciting is that although the CRISPR/Cas system needs to recognize specific PAM sequences, recent SCIENCE reports that using near-PAMless engineered can almost eliminate the limitations of PAM. Thus, high precision targeting can be realized in the application of genome editing, while further reducing off-target effect (Nishimasu et al., 2018).

CONCLUSIONS

Although the clinical application of the CRISPR/Cas system is still in its infancy, its emergence provides many possibilities in the biomedical field. We should be more supportive and inclusive of emerging technologies. With continued in-depth research and clinical validation, we believe that the application of CRISPR/Cas system will play an irreplaceable role in the prevention and control of emerging infectious diseases in the future.

AUTHOR CONTRIBUTIONS

RD and GD designed the study. RD and SC wrote the paper. JL, MY, YJ, HY, MC, and SC provided writing and revision suggestions. All authors contributed to the article and approved the submitted version.

FUNDING

The work was supported by the National Science and Technology Specific Projects (2018ZX10301407), Hunan Provincial Key Laboratory of Clinical Epidemiology (2020ZNDXLCL003), and The Key Scientific Research Projects in Colleges and Universities of Henan Province (20A330004). Henan Province University Science and Technology Innovation Talent Projects (17HASTIT045).

REFERENCES

Abbott, T. R., Dhamdhere, G., Liu, Y. X., Lin, X. Q., Goudy, L., Zeng, L. P., et al. (2020). Development of CRISPR as an Antiviral Strategy to Combat SARS-Cov-2 and Influenza. *Cell* 181, 865–86+. doi: 10.1016/j.cell.2020.04.020

Ackerman, C. M., Myhrvold, C., Thakku, S. G., Freije, C. A., Metsky, H. C., Yang, D. K., et al. (2020). Massively Multiplexed Nucleic Acid Detection With Cas13. *Nature* 582, 277–282. doi: 10.1038/s41586-020-2279-8

Anderson, D. E., Cui, J., Ye, Q., Huang, B., Zu, W., Gong, J., et al. (2020). Orthogonal Genome-Wide Screenings in Bat Cells Identify MTHFD1 as a Target of Broad Antiviral Therapy. *bioRxiv* 2020.2003.2029.014209. doi: 10.1101/2020.03.29.014209

Arizti-Sanz, J., Freije, C. A., Stanton, A. C., Petros, B. A., Boehm, C. K., Siddiqui, S., et al. (2020). Streamlined Inactivation, Amplification, and Cas13-Based Detection of SARS-Cov-2. *Nat. Commun.* 11, 5921. doi: 10.1101/2020.05.28.119131

Atasoy, M. O., Rohaim, M. A., and Munir, M. (2019). Simultaneous Deletion of Virulence Factors and Insertion of Antigens Into the Infectious Laryngotracheitis Virus Using NHEJ-CRISPR/Cas9 and Cre-Lox System for Construction of a Stable Vaccine Vector. *Vaccines* 7 (4), 207. doi: 10.3390/vaccines7040207

Baek, Y. H., Um, J., Antigua, K. J. C., Park, J. H., Kim, Y., Oh, S., et al. (2020). Development of a Reverse Transcription-Loop-Mediated Isothermal Amplification as a Rapid Early-Detection Method for Novel SARS-Cov-2. *Emerg. Microbes Infect.* 9, 998–1007. doi: 10.1080/22221751.2020.1756698

Becerra-Flores, M., and Cardozo, T. (2020). SARS-Cov-2 Viral Spike G614 Mutation Exhibits Higher Case Fatality Rate. *Int. J. Clin. Pract.* 74 (8), e13525. doi: 10.1111/ijcp.13525

Behan, F. M., Iorio, F., Picco, G., Goncalves, E., Beaver, C. M., Migliardi, G., et al. (2019). Prioritization of Cancer Therapeutic Targets Using CRISPR-Cas9 Screens. *Nature* 568, 511–51+. doi: 10.1038/s41586-019-1103-9

Bondy-Denomy, J., Pawluk, A., Maxwell, K. L., and Davidson, A. R. (2013). Bacteriophage Genes That Inactivate the CRISPR/Cas Bacterial Immune System. *Nature* 493, 429–U181. doi: 10.1038/nature11723

Borca, M. V., Holinka, L. G., Berggren, K. A., and Gladue, D. P. (2018). CRISPR-Cas9, a Tool to Efficiently Increase the Development of Recombinant African Swine Fever Viruses. *Sci. Rep.* 8 (1), 3154. doi: 10.1038/s41598-018-21575-8

Brentjens, R. J., Riviere, I., Park, J. H., Davila, M. L., Wang, X. Y., Stefanski, J., et al. (2011). Safety and Persistence of Adoptively Transferred Autologous CD19-Targeted T Cells in Patients With Relapsed or Chemotherapy Refractory B-Cell Leukemias. *Blood* 118, 4817–4828. doi: 10.1182/blood-2011-04-348540

Brokowski, C., and Adli, M. (2019). CRISPR Ethics: Moral Considerations for Applications of a Powerful Tool. *J. Mol. Biol.* 431, 88–101. doi: 10.1016/j.jmb.2018.05.044

Broughton, J. P., Deng, X., Yu, G., Fasching, C. L., Servellita, V., Singh, J., et al. (2020a). CRISPR–Cas12-Based Detection of SARS-Cov-2. *Nat. Biotechnol.* 38, 870–874. doi: 10.1038/s41587-020-0513-4

Broughton, J. P., Deng, X. D., Yu, G. X., Fasching, C. L., Servellita, V., Singh, J., et al. (2020b). CRISPR-Cas12-Based Detection of SARS-Cov-2. *Nat. Biotechnol.* 38, 870–U854. doi: 10.1038/s41587-020-0513-4

Bustin, S. A., and Nolan, T. (2020). RT-Qpcr Testing of SARS-Cov-2: A Primer. *Int. J. Mol. Sci.* 21 (8), 3004. doi: 10.3390/ijms21083004

Cai, Q., Yang, M., Liu, D., Chen, J., Shu, D., Xia, J., et al. (2020). Experimental Treatment With Favipiravir for COVID-19: An Open-Label Control Study. *Eng. (Beijing)* 6, 1192–1198. doi: 10.1016/j.eng.2020.03.007

Cao, B., Wang, Y., Wen, D., Liu, W., Wang, J., Fan, G., et al. (2020). A Trial of Lopinavir-Ritonavir in Adults Hospitalized With Severe Covid-19. *N Engl. J. Med.* 382, 1787–1799. doi: 10.1056/NEJMoa2001282

Chang, P. X., Ameen, F., Sealy, J. E., Sadeyen, J. R., Bhat, S., Li, Y. Q., et al. (2019). Application of HDR-CRISPR/Cas9 and Erythrocyte Binding for Rapid Generation of Recombinant Turkey Herpesvirus-Vectored Avian Influenza Virus Vaccines. *Vaccines* 7 (4), 192. doi: 10.3390/vaccines7040192

Chen, J. S., Dagdas, Y. S., Kleinstiver, B. P., Welch, M. M., Sousa, A. A., Harrington, L. B., et al. (2017). Enhanced Proofreading Governs CRISPR-Cas9 Targeting Accuracy. *Nature* 550, 407–410. doi: 10.1038/nature24268

Cheng, M., Xiong, E., Tian, T., Zhu, D., Ju, H. Q., and Zhou, X. (2021). A CRISPR-Driven Colorimetric Code Platform for Highly Accurate Telomerase Activity Assay. *Biosens. Bioelectron* 172, 112749. doi: 10.1016/j.bios.2020.112749

Chen, Y., Jiang, H., Wang, T., He, D., Tian, R., Cui, Z., et al. (2020). In Vitro and in Vivo Growth Inhibition of Human Cervical Cancer Cells Via Human Papillomavirus E6/E7 Mrnas' Cleavage by CRISPR/Cas13a System. *Antiviral Res.* 178, 104794. doi: 10.1016/j.antiviral.2020.104794

Chen, J. S., Ma, E., Harrington, L. B., Da Costa, M., Tian, X., Palefsky, J. M., et al. (2018a). CRISPR-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded Dnase Activity. *Science* 360, 436–439. doi: 10.1126/science.aar6245

Chen, J. S., Ma, E. B., Harrington, L. B., Da Costa, M., Tian, X. R., Palefsky, J. M., et al. (2018b). CRISPR-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded Dnase Activity. *Science* 360, 436–43+. doi: 10.1126/science.aar6245

Chen, S. J., Wang, S. C., and Chen, Y. C. (2020). Antiviral Agents as Therapeutic Strategies Against Cytomegalovirus Infections. *Viruses-Basel* 12 (1), 21. doi: 10.3390/v12010021

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., et al. (2020). Detection of 2019 Novel Coronavirus, (2019-Ncov) by Real-Time RT-PCR. *Euro Surveill.* 25 (3), 2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045

Dai, Y., Wu, Y., Liu, G., and Gooding, J. J. (2020). CRISPR Mediated Biosensing Toward Understanding Cellular Biology and Point-of-Care Diagnosis. *Angew. Chem. Int. Ed. Engl.* 59, 20754–20766. doi: 10.1002/anie.202005398

Dong, X., Cao, Y. Y., Lu, X. X., Zhang, J. J., Du, H., Yan, Y. Q., et al. (2020). Eleven Faces of Coronavirus Disease 2019. *Allergy* 75, 1699–1709. doi: 10.1111/all.14289

Du, Y. X., and Chen, X. P. (2020). Favipiravir: Pharmacokinetics and Concerns About Clinical Trials for 2019-Ncov Infection. *Clin. Pharmacol. Ther.* 108, 242–247. doi: 10.1002/cpt.1844

Ebina, H., Misawa, N., Kanemura, Y., and Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 System to Disrupt Latent HIV-1 Provirus. *Sci. Rep.* 3, 2510. doi: 10.1038/srep02510

Eyquem, J., Mansilla-Soto, J., Giavridis, T., Van Der Stegen, S. J., Hamieh, M., Cunanan, K. M., et al. (2017). Targeting a CAR to the TRAC Locus With CRISPR/Cas9 Enhances Tumour Rejection. *Nature* 543, 113–117. doi: 10.1038/nature21405

Flint, M., Chatterjee, P., Lin, D. L., Mcmullan, L. K., Shrivastava-Ranjan, P., Bergeron, E., et al. (2019). A Genome-Wide CRISPR Screen Identifies N-Acetylglucosamine-1-Phosphate Transferase as a Potential Antiviral Target for Ebola Virus. *Nat. Commun.* 10 (1), 285. doi: 10.1038/s41467-018-08135-4

Folegatti, P. M., Ewer, K. J., Aley, P. K., Angus, B., Becker, S., Belij-Rammerstorfer, S., et al. (2020). Safety and Immunogenicity of the Chadox1 Ncov-19 Vaccine Against SARS-Cov-2: A Preliminary Report of a Phase 1/2, Single-Blind, Randomised Controlled Trial. *Lancet* 396, 467–478. doi: 10.1016/S0140-6736(20)31604-4

Freije, C. A., Myhrvold, C., Boehm, C. K., Lin, A. E., Welch, N. L., Carter, A., et al. (2019). Programmable Inhibition and Detection of RNA Viruses Using Cas13. *Mol. Cell* 76 (5), 826–837.e11. doi: 10.1016/j.molcel.2019.09.013

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K., et al. (2013). High-Frequency Off-Target Mutagenesis Induced by CRISPR-Cas Nucleases in Human Cells. *Nat. Biotechnol.* 31, 822–826. doi: 10.1038/nbt.2623

Gao, Q., Bao, L. L., Mao, H. Y., Wang, L., Xu, K. W., Yang, M. N., et al. (2020). Development of an Inactivated Vaccine Candidate for SARS-Cov-2. *Science* 369, 77–7+. doi: 10.1126/science.abc1932

Gao, Z. L., Fan, M. H., Das, A. T., Herrera-Carrillo, E., and Berkhout, B. (2020). Extinction of All Infectious HIV in Cell Culture by the CRISPR-Cas12a System With Only a Single Crrna. *Nucleic Acids Res.* 48, 5527–5539. doi: 10.1093/nar/gkaa226

Gautret, P., Lagier, J. C., Parola, P., Hoang, V. T., Meddeb, L., Mailhe, M., et al. (2020). Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *Int. J. Antimicrob. Agents* 56, 105949. doi: 10.1016/j.ijantimicag.2020.105949

Glass, Z., Lee, M., Li, Y., and Xu, Q. (2018). Engineering the Delivery System for CRISPR-Based Genome Editing. *Trends Biotechnol.* 36, 173–185. doi: 10.1016/j.tibtech.2017.11.006

Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., et al. (2017). Nucleic Acid Detection With CRISPR-Cas13a/C2c2. *Science* 356, 438–43+. doi: 10.1126/science.aam9321

Grein, J., Ohmagari, N., Shin, D., Diaz, G., Asperges, E., Castagna, A., et al. (2020). Compassionate Use of Remdesivir for Patients With Severe Covid-19. *New Engl. J. Of Med.* 382, 2327–2336. doi: 10.1056/NEJMoa2007016

Grifoni, A., Weiskopf, D., Ramirez, S. I., Mateus, J., Dan, J. M., Moderbacher, C. R., et al. (2020). Targets of T Cell Responses to SARS-Cov-2 Coronavirus in Humans With COVID-19 Disease and Unexposed Individuals. *Cell* 181 (7), 1489–1501.e15. doi: 10.1016/j.cell.2020.05.015

Grubaugh, N. D., Hanage, W. P., and Rasmussen, A. L. (2020). Making Sense of Mutation: What D614G Means for the COVID-19 Pandemic Remains Unclear. *Cell* 182, 794–795. doi: 10.1016/j.cell.2020.06.040

Guglielmi, G. (2020). First CRISPR Test for the Coronavirus Approved in the United States. *Nature*. doi: 10.1038/d41586-020-01402-9, https://www.nature.com/articles/d41586-020-01402-9

Haley, B., and Roudnicky, F. (2020). Functional Genomics for Cancer Drug Target Discovery. *Cancer Cell* 38, 31–43. doi: 10.1016/j.ccell.2020.04.006

Harrington, L. B., Burstein, D., Chen, J. S., Paez-Espino, D., Ma, E., Witte, I. P., et al. (2018). Programmed DNA Destruction by Miniature CRISPR-Cas14 Enzymes. *Science* 362, 839–842. doi: 10.1126/science.aav4294

Harrington, L. B., Doxzen, K. W., Ma, E., Liu, J. J., Knott, G. J., Edraki, A., et al. (2017). A Broad-Spectrum Inhibitor of CRISPR-Cas9. *Cell* 170 (6), 1224–1233.e15. doi: 10.1016/j.cell.2017.07.037

Heaton, B. E., Trimarco, J. D., Hamele, C. E., Harding, A. T., Tata, A., Zhu, X., et al. (2020). SRSF Protein Kinases 1 and 2 are Essential Host Factors for Human Coronaviruses Including SARS-Cov-2. *bioRxiv* 2020.2008.2014.251207. doi: 10.1101/2020.08.14.251207

Hoffmann, H.-H., Schneider, W. M., Sánchez-Rivera, F. J., Luna, J. M., Ashbrook, A. W., Soto-Feliciano, Y. M., et al. (2020). Functional Interrogation of a SARS-Cov-2 Host Protein Interactome Identifies Unique and Shared Coronavirus Host Factors. *Cell Host Microbe* 29, 267–280.e265. doi: 10.1101/2020.09.11.291716

Hu, W., Kaminski, R., Yang, F., Zhang, Y., Cosentino, L., Li, F., et al. (2014). RNA-Directed Gene Editing Specifically Eradicates Latent and Prevents New HIV-1 Infection. *Proc. Natl. Acad. Sci. U. S. A.* 111, 11461–11466. doi: 10.1073/pnas.1405186111

Hultquist, J. F., Hiatt, J., Schumann, K., Mcgregor, M. J., Roth, T. L., Haas, P., et al. (2019). CRISPR-Cas9 Genome Engineering of Primary CD4(+) T Cells for the Interrogation of HIV-Host Factor Interactions. *Nat. Protoc.* 14, 1–27. doi: 10.1038/s41596-018-0069-7

Huo, H. F., and Hu, G. H. (2019). CRISPR/Cas9-Mediated LMP1 Knockout Inhibits Epstein-Barr Virus Infection and Nasopharyngeal Carcinoma Cell Growth. *Infect. Agents And Cancer* 14, 30. doi: 10.1186/s13027-019-0246-5

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., and Nakata, A. (1987). Nucleotide Sequence of the Iap Gene, Responsible for Alkaline Phosphatase Isozyme Conversion in Escherichia Coli, and Identification of the Gene Product. *J. Bacteriol.* 169, 5429–5433. doi: 10.1128/JB.169.12.5429-5433.1987

Jiang, F., Liu, J. J., Osuna, B. A., Xu, M., Berry, J. D., Rauch, B. J., et al. (2019). Temperature-Responsive Competitive Inhibition of CRISPR-Cas9. *Mol. Cell* 73 (3), 601–610.e5. doi: 10.1016/j.molcel.2018.11.016

Joung, J., Ladha, A., Saito, M., Kim, N. G., Woolley, A. E., Segel, M., et al. (2020). Detection of SARS-Cov-2 With SHERLOCK One-Pot Testing. *N Engl. J. Med.* 383, 1492–1494. doi: 10.1056/NEJMc2026172

Kennedy, E. M., Kornepati, A. V. R., Goldstein, M., Bogerd, H. P., Poling, B. C., Whisnant, A. W., et al. (2014). Inactivation of the Human Papillomavirus E6 or E7 Gene in Cervical Carcinoma Cells by Using a Bacterial CRISPR/Cas RNA-Guided Endonuclease. *J. Of Virol.* 88, 11965–11972. doi: 10.1128/JVI.01879-14

Khan, S., Siddique, R., Shereen, M. A., Ali, A., Liu, J., Bai, Q., et al. (2020). Emergence of a Novel Coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2: Biology and Therapeutic Options. *J. Clin. Microbiol.* 58 (5), e00187-20. doi: 10.1128/JCM.01297-20

Kim, S., Koo, T., Jee, H. G., Cho, H. Y., Lee, G., Lim, D. G., et al. (2018). CRISPR Rnas Trigger Innate Immune Responses in Human Cells. *Genome Res* 8 (3), 367–373. doi: 10.1101/gr.231936.117

Knott, G. J., and Doudna, J. A. (2018). CRISPR-Cas Guides the Future of Genetic Engineering. *Science* 361, 866–869. doi: 10.1126/science.aat5011

Komor, A. C., Badran, A. H., and Liu, D. R. (2017). CRISPR-Based Technologies for the Manipulation of Eukaryotic Genomes. *Cell* 169, 559. doi: 10.1016/j.cell.2017.04.005

Koonin, E. V., Makarova, K. S., and Zhang, F. (2017). Diversity, Classification and Evolution of CRISPR-Cas Systems. *Curr. Opin. Microbiol.* 37, 67–78. doi: 10.1016/j.mib.2017.05.008

Korber, B., Fischer, W. M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., et al. (2020). Tracking Changes in SARS-Cov-2 Spike: Evidence That D614G Increases Infectivity of the COVID-19 Virus. *Cell* 182, 812–81+. doi: 10.1016/j.cell.2020.06.043

Kurata, M., Yammoto, K., Moriarity, B. S., Kitagwa, M., and Largaespada, D. A. (2018). CRISPR/Cas9 Library Screening for Drug Target Discovery. *J. Hum. Genet.* 63 (2), 179–186. doi: 10.1038/s10038-017-0376-9

Labanieh, L., Majzner, R. G., and Mackall, C. L. (2018). Programming CAR-T Cells to Kill Cancer. *Nat. Biomed. Eng.* 2, 377–391. doi: 10.1038/s41551-018-0235-9

Lee, K., Conboy, M., Park, H. M., Jiang, F., Kim, H. J., Dewitt, M. A., et al. (2017). Nanoparticle Delivery of Cas9 Ribonucleoprotein and Donor DNA in Vivo Induces Homology-Directed DNA Repair. *Nat. BioMed. Eng.* 1, 889–901. doi: 10.1038/s41551-017-0137-2

Li, B., Clohisey, S. M., Chia, B. S., Wang, B., Cui, A., Eisenhaure, T., et al. (2020). Genome-Wide CRISPR Screen Identifies Host Dependency Factors for Influenza a Virus Infection. *Nat. Commun.* 11, 164. doi: 10.1038/s41467-019-13965-x

Li, H., Wang, S., Dong, X., Li, Q., Li, M., Li, J., et al. (2020). CRISPR-Cas13a Cleavage of Dengue Virus NS3 Gene Efficiently Inhibits Viral Replication. *Mol. Ther. Nucleic Acids* 19, 1460–1469. doi: 10.1016/j.omtn.2020.01.028

Li, Q., Wu, J., Nie, J., Zhang, L., Hao, H., Liu, S., et al. (2020). The Impact of Mutations in SARS-Cov-2 Spike on Viral Infectivity and Antigenicity. *Cell* 1821284-1294, e1289. doi: 10.1016/j.cell.2020.07.012

Li, Y., Yao, L., Li, J., Chen, L., Song, Y., Cai, Z., et al. (2020). Stability Issues of RT-PCR Testing of SARS-Cov-2 for Hospitalized Patients. *J. Med. Virol.* 92 (7), 903–908. doi: 10.1002/jmv.25786

Li, Z. T., Yi, Y. X., Luo, X. M., Xiong, N., Liu, Y., Li, S. Q., et al. (2020). Development and Clinical Application of a Rapid Igm-Igg Combined Antibody Test for SARS-Cov-2 Infection Diagnosis. *J. Of Med. Virol.* 92, 1518–1524. doi: 10.1002/jmv.25727

Lin, D. C., Liu, L., Zhang, M. X., Hu, Y. L., Yang, Q. T., Guo, J. B., et al. (2020). Evaluations of the Serological Test in the Diagnosis of 2019 Novel Coronavirus (SARS-Cov-2) Infections During the COVID-19 Outbreak. *Eur. J. Of Clin. Microbiol. Infect. Diseases* 39, 2271–2277. doi: 10.1007/s10096-020-03978-6

Lisboa Bastos, M., Tavaziva, G., Abidi, S. K., Campbell, J. R., Haraoui, L. P., Johnston, J. C., et al. (2020). Diagnostic accuracy of serological tests for covid-19: systematic review and meta-analysis. *BMJ* 370, m2516. doi: 10.1136/bmj.m2516

Liu, Z., Chen, S., Jin, X., Wang, Q., Yang, K., Li, C., et al. (2017). Genome Editing of the HIV Co-Receptors CCR5 and CXCR4 by CRISPR-Cas9 Protects CD4(+) T Cells From HIV-1 Infection. *Cell Biosci.* 7, 47. doi: 10.1186/s13578-017-0174-2

Liu, R., Han, H., Liu, F., Lv, Z., Wu, K., Liu, Y., et al. (2020). Positive Rate of RT-PCR Detection of SARS-Cov-2 Infection in 4880 Cases From One Hospital in Wuhan, China, From Jan to Feb 2020. *Clin. Chim. Acta* 505, 172–175. doi: 10.1016/j.cca.2020.03.009

Liu, W., Liu, L., Kou, G., Zheng, Y., Ding, Y., Ni, W., et al. (2020). Evaluation of Nucleocapsid and Spike Protein-Based Enzyme-Linked Immunosorbent Assays for Detecting Antibodies Against SARS-Cov-2. *J. Clin. Microbiol.* 58 (6), e00461-20. doi: 10.1128/JCM.00461-20

Lu, X., Wang, L., Sakthivel, S. K., Whitaker, B., Murray, J., Kamili, S., et al. (2020). US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2. *Emerg. Infect. Dis.* 26 (8), 1654–1665. doi: 10.3201/eid2608.201246

Marraffini, L. A., and Sontheimer, E. J. (2010). Self Versus Non-Self Discrimination During CRISPR RNA-Directed Immunity. *Nature* 463, 568–571. doi: 10.1038/nature08703

Mojica, F. J. M., Diez-Villasenor, C., Garcia-Martinez, J., and Almendros, C. (2009). Short Motif Sequences Determine the Targets of the Prokaryotic CRISPR Defence System. *Microbiol. (Reading)* 155, 733–740. doi: 10.1099/mic.0.023960-0

Nguyen, T. M., Zhang, Y., and Pandolfi, P. P. (2020). Virus Against Virus: A Potential Treatment for 2019-Ncov (SARS-Cov-2) and Other RNA Viruses. *Cell Res.* 30, 189–190. doi: 10.1038/s41422-020-0290-0

Nishimasu, H., Shi, X., Ishiguro, S., Gao, L., Hirano, S., Okazaki, S., et al. (2018). Engineered CRISPR-Cas9 Nuclease With Expanded Targeting Space. *Science* 361, 1259–1262. doi: 10.1126/science.aas9129

Patchsung, M., Jantarug, K., Pattama, A., Aphicho, K., Suraritdechachai, S., Meesawat, P., et al. (2020). Clinical Validation of a Cas13-Based Assay for the Detection of SARS-Cov-2 RNA. *Nat. Biomed. Engineering* 4, 1140–1149. doi: 10.1038/s41551-020-00603-x

Pereira, B. B. (2020). Challenges and Cares to Promote Rational Use of Chloroquine and Hydroxychloroquine in the Management of Coronavirus Disease 2019 (COVID-19) Pandemic: A Timely Review. *J. Of Toxicol. And Environ. Health Part B Critical Rev.* 23, 177–181. doi: 10.1080/10937404.2020.1752340

Pickar-Oliver, A., and Gersbach, C. A. (2019). The Next Generation of CRISPR-Cas Technologies and Applications. *Nat. Rev. Mol. Cell Biol.* 20 (8), 490–507. doi: 10.1038/s41580-019-0131-5

Porter, D. L., Levine, B. L., and Kalos, M. (2016). Chimeric Antigen Receptor-Modified T Cells in Chronic Lymphoid Leukemia (Vol 3652011). *New Engl. J. Of Med.* 374, 998–998, pg 725. doi: 10.1056/NEJMx160005

Rauch, J. N., Valois, E., Solley, S. C., Braig, F., Lach, R. S., Audouard, M., et al. (2020). A Scalable, Easy-to-Deploy, Protocol for Cas13-Based Detection of SARS-Cov-2 Genetic Material. *J Clin Microbiol* 59, e02402–20. doi: 10.1101/2020.04.20.052159

Settleman, J., Sawyers, C. L., and Hunter, T. (2018). Challenges in Validating Candidate Therapeutic Targets in Cancer. *Elife* 7, e32402. doi: 10.7554/eLife.32402

Stadtmauer, E. A., Fraietta, J. A., Davis, M. M., Cohen, A. D., Weber, K. L., Lancaster, E., et al. (2020). CRISPR-Engineered T Cells in Patients With Refractory Cancer. *Science* 367, 1001–100+. doi: 10.1126/science.aba7365

Sun, S. H., Chen, Q., Gu, H. J., Yang, G., Wang, Y. X., Huang, X. Y., et al. (2020). A Mouse Model of SARS-Cov-2 Infection and Pathogenesis. *Cell Host Microbe* 28 (1), 124–133.e4. doi: 10.1016/j.chom.2020.05.020

Suo, T., Liu, X., Feng, J., Guo, M., Hu, W., Guo, D., et al. (2020). Ddpcr: A More Accurate Tool for SARS-Cov-2 Detection in Low Viral Load Specimens. *Emerg. Microbes Infect.* 9, 1259–1268. doi: 10.1080/22221751.2020.1772678

Teng, F., Guo, L., Cui, T., Wang, X. G., Xu, K., Gao, Q., et al. (2019). Cdetection: CRISPR-Cas12b-Based DNA Detection With Sub-Attomolar Sensitivity and Single-Base Specificity. *Genome Biol.* 20, 132. doi: 10.1186/s13059-019-1742-z

Tesija Kuna, A., Hanzek, M., Vukasovic, I., Nikolac Gabaj, N., Vidranski, V., Celap, I., et al. (2021). Comparison of diagnostic accuracy for eight SARS-CoV-2 serological assays. *Biochem Med (Zagreb)* 31, 010708. doi: 10.11613/BM.2021.010708

Thickett, D. R., Armstrong, L., Christie, S. J., and Millar, A. B. (2001). Vascular Endothelial Growth Factor May Contribute to Increased Vascular Permeability in Acute Respiratory Distress Syndrome. *Am. J. Of Respir. And Crit. Care Med.* 164, 1601–1605. doi: 10.1164/ajrccm.164.9.2011071

Tu, Y. F., Chien, C. S., Yarmishyn, A. A., Lin, Y. Y., Luo, Y. H., Lin, Y. T., et al. (2020). A Review of SARS-Cov-2 and the Ongoing Clinical Trials. *Int. J. Mol. Sci.* 21 (7), 2657. doi: 10.3390/ijms21072657

Van Dongen, J. E., Berendsen, J. T. W., Steenbergen, R. D. M., Wolthuis, R. M. F., Eijkel, J. C. T., and Segerink, L. I. (2020). Point-of-Care CRISPR/Cas Nucleic Acid Detection: Recent Advances, Challenges and Opportunities. *Biosens. Bioelectron* 166, 112445. doi: 10.1016/j.bios.2020.112445

Wang, F., Wang, L., Zou, X., Duan, S., Li, Z., Deng, Z., et al. (2019). Advances in CRISPR-Cas Systems for RNA Targeting, Tracking and Editing. *Biotechnol. Adv.* 37, 708–729. doi: 10.1016/j.biotechadv.2019.03.016

Wang, M., Zhang, R., and Li, J. (2020). CRISPR/Cas Systems Redefine Nucleic Acid Detection: Principles and Methods. *Biosens. Bioelectron* 165, 112430. doi: 10.1016/j.bios.2020.112430

Wang, Q., Du, Q., Guo, B., Mu, D., Lu, X., Ma, Q., et al. (2020). A Method To Prevent SARS-CoV-2 IgM False Positives in Gold Immunochromatography and Enzyme-Linked Immunosorbent Assays. . *J. Clin. Microbiol.* 58(6), e00375-20. doi: 10.1128/JCM.00375-20

Wang, R., Qian, C., Pang, Y., Li, M., Yang, Y., Ma, H., et al. (2021). Opvcrispr: One-Pot Visual RT-LAMP-CRISPR Platform for SARS-Cov-2 Detection. *Biosens. Bioelectron* 172, 112766. doi: 10.1016/j.bios.2020.112766

Wang, R., Simoneau, C. R., Kulsuptrakul, J., Bouhaddou, M., Travisano, K., Hayashi, J. M., et al. (2020). Functional Genomic Screens Identify Human Host Factors for SARS-Cov-2 and Common Cold Coronaviruses. *Cell* 184, 106–119 e114. doi: 10.1101/2020.09.24.312298

Wang, S., Li, H., Kou, Z., Ren, F., Jin, Y., Yang, L., et al. (2020). Highly Sensitive and Specific Detection of Hepatitis B Virus DNA and Drug Resistance. *Clin. Microbiol. Infect.* 29, S1198–743X(20)30226-3. doi: 10.1016/j.cmi.2020.04.018.

Wang, Y., Zhang, D., Du, G., Du, R., Zhao, J., Jin, Y., et al. (2020). Remdesivir in Adults With Severe COVID-19: A Randomised, Double-Blind, Placebo-Controlled, Multicentre Trial. *Lancet* 395, 1569–1578. doi: 10.1016/S0140-6736(20)31022-9

Wei, J., Alfajaro, M. M., Deweirdt, P. C., Hanna, R. E., Lu-Culligan, W. J., Cai, W. L., et al. (2020). Genome-Wide CRISPR Screens Reveal Host Factors Critical for SARS-Cov-2 Infection. *Cell* 184, 76–91 e13. doi: 10.1016/j.cell.2020.10.028

Xiao, Q., Chen, S., Wang, Q., Liu, Z., Liu, S., Deng, H., et al. (2019). CCR5 Editing by Staphylococcus Aureus Cas9 in Human Primary CD4(+) T Cells and Hematopoietic Stem/Progenitor Cells Promotes HIV-1 Resistance and CD4(+) T Cell Enrichment in Humanized Mice. *Retrovirology* 16, 15. doi: 10.1186/s12977-019-0482-1

Yan, F., Wang, W., and Zhang, J. (2019). CRISPR-Cas12 and Cas13: The Lesser Known Siblings of CRISPR-Cas9. *Cell Biol. Toxicol.* 35, 489–492. doi: 10.1007/s10565-019-09489-1

Younes, N., Al-Sadeq, D. W., Al-Jighefee, H., Younes, S., Al-Jamal, O., Daas, H. I., et al. (2020). Challenges in Laboratory Diagnosis of the Novel Coronavirus SARS-Cov-2. *Viruses* 12 (6), 582. doi: 10.3390/v12060582

Yuan, C., Tian, T., Sun, J., Hu, M., Wang, X., Xiong, E., et al. (2020). Universal and Naked-Eye Gene Detection Platform Based on the Clustered Regularly Interspaced Short Palindromic Repeats/Cas12a/13a System. *Anal. Chem.* 92, 4029–4037. doi: 10.1021/acs.analchem.9b05597

Yurkovetskiy, L., Wang, X., Pascal, K. E., Tomkins-Tinch, C., Nyalile, T. P., Wang, Y., et al. (2020). Structural and Functional Analysis of the D614G SARS-Cov-2 Spike Protein Variant. *Cell* 183, 739–751 e738. doi: 10.1016/j.cell.2020.09.032

Yu, F., Yan, L., Wang, N., Yang, S., Wang, L., Tang, Y., et al. (2020). Quantitative Detection and Viral Load Analysis of SARS-Cov-2 in Infected Patients. *Clin. Infect. Dis.* 71, 793–798. doi: 10.1093/cid/ciaa345

Zhao, J. J., Lin, Q. D., Song, Y. P., and Liu, D. L. (2018). Universal Cars, Universal T Cells, and Universal CAR T Cells. *J. Of Hematol. Oncol.* 11 (1), 132. doi: 10.1186/s13045-018-0677-2

Zhen, S., Hua, L., Liu, Y. H., Gao, L. C., Fu, J., Wan, D. Y., et al. (2015). Harnessing the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-Associated Cas9 System to Disrupt the Hepatitis B Virus. *Gene Ther.* 22, 404–412. doi: 10.1038/gt.2015.2

Zhou, L., Peng, R., Zhang, R., and Li, J. (2018). The Applications of CRISPR/Cas System in Molecular Detection. *J. Cell Mol. Med.* 22, 5807–5815. doi: 10.1111/jcmm.13925

Zhou, G. Y., and Zhao, Q. (2020). Perspectives on Therapeutic Neutralizing Antibodies Against the Novel Coronavirus SARS-Cov-2. *Int. J. Of Biol. Sci.* 16, 1718–1723. doi: 10.7150/ijbs.45123

Zhou, Y., Zhu, S., Cai, C., Yuan, P., Li, C., Huang, Y., et al. (2014). High-Throughput Screening of a CRISPR/Cas9 Library for Functional Genomics in Human Cells. *Nature* 509, 487–491. doi: 10.1038/nature13166

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Ding, Long, Yuan, Jin, Yang, Chen, Chen and Duan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 30 April 2021
doi: 10.3389/fcimb.2021.663949

CRISPR-Based COVID-19 Testing: Toward Next-Generation Point-of-Care Diagnostics

Uyanga Ganbaatar[1,2] *and Changchun Liu*[1*]

[1] Department of Biomedical Engineering, University of Connecticut Health Center, Farmington, CT, United States,
[2] Department of Biomedical Engineering, University of Connecticut, Storrs, CT, United States

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Xianbo Qiu,
Beijing University of Chemical Technology, China
Michael Mauk,
University of Pennsylvania, United States

***Correspondence:**
Changchun Liu
chaliu@uchc.edu

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 04 February 2021
Accepted: 16 April 2021
Published: 30 April 2021

Citation:
Ganbaatar U and Liu C (2021) CRISPR-Based COVID-19 Testing: Toward Next-Generation Point-of-Care Diagnostics. Front. Cell. Infect. Microbiol. 11:663949. doi: 10.3389/fcimb.2021.663949

As the COVID-19 pandemic continues, people are becoming infected at an alarming rate, individuals are unknowingly spreading disease, and more lives are lost every day. There is an immediate need for a simple, rapid, early and sensitive point-of-care testing for COVID-19 disease. However, current testing approaches do not meet such need. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)-based detection methods have received substantial attention for nucleic acid-based molecular testing due to their simplicity, high sensitivity and specificity. This review explores the various CRISPR-based COVID-19 detection methods and related diagnostic devices. As with any emerging technology, CRISPR/Cas-based nucleic acid testing methods have several challenges that must be overcome for practical applications in clinics and hospitals. More importantly, these detection methods are not limited to COVID-19 but can be applied to detect any type of pathogen, virus, and fungi that may threaten humans, agriculture, and food industries in resource-limited settings. CRISPR/Cas-based detection methods have the potential to become simpler, more reliable, more affordable, and faster in the near future, which is highly important for achieving point-of-care diagnostics.

Keywords: COVID-19, coronavirus, SARS-CoV-2, CRISPR-based nucleic acid detection, point-of-care testing

INTRODUCTION

The relatively recent outbreak of coronavirus disease 2019 (COVID-19) in Wuhan, China has now become a global pandemic. COVID-19 is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), better known as coronavirus. Yet, SARS-CoV-2 is not the only virus that has recently emerged. Although coronavirus is the most recent case, one virus has emerged each year (Howard and Fletcher, 2012), threatening public health, the global economy, and even personal freedom. The spread of coronavirus primarily occurs *via* local community transmission (Liu et al., 2020), and this global pandemic has shown that most countries were not prepared for an outbreak due to a lack of rapid, reliable, point-of-care detection and treatment, which has enabled further

spreading of the virus. As of February 2021, more than 102 million people around the globe have been infected, with over two million deaths due to this virus ("WHO Coronavirus Disease (COVID-19) Dashboard | WHO Coronavirus Disease (COVID-19) Dashboard" n.d.). Thus, there is an immediate need for simple, rapid, and affordable testing for every facility around the world, from developing to first-world nations.

Coronaviruses are single-stranded RNA (ssRNA) viruses that belong to the family of *Coronaviridae*. These viruses have eight accessory proteins and four major structural proteins. Different laboratories around the world have targeted different components of SARS-CoV-2 to enable its detection, including the spike protein (S), small envelope protein (E), nucleocapsid protein (N), and RNA-dependent RNA polymerase (RdRp) gene of the ORF1ab sequence (Wang H. et al., 2020).

Numerous detection methods exist for COVID-19, including molecular, antigen, and antibody tests, each with their own benefits and drawbacks ("Coronavirus Testing Basics | FDA" n.d.). Currently, the most common detection method is the molecular test, also known as the nucleic acid detection, which determines whether patient sample (e.g., nasopharyngeal swab, saliva) has SARS-CoV-2 genetic material (Lisboa Bastos et al., 2020). To perform this test, viral RNA is first isolated and extracted from patient samples. Then, the purified RNA is subjected to reverse transcription quantitative polymerase chain reaction (RT-qPCR), which converts the virus RNA to DNA and amplifies the DNA to produce millions of copies. Finally, a detection probe can determine whether the patient has viral genetic material ("Coronavirus Testing Basics | FDA" n.d.). While the RT-PCR method is considered as the gold standard method, it does have some drawbacks. For instance, this test can produce false negative results (Chen Z. et al., 2020), there are shortages of test kits (American Society of Microbiology, 2020), and a period of several hours to few days is needed to obtain results. Moreover, this method requires expensive equipment, a molecular laboratory, and a trained scientist (Rao et al., 2020). Having a simple, reliable point-of-care testing (POCT) method will help physicians and patients fill in the gaps of our current testing for SARS-CoV-2.

Recently, clustered regularly interspaced short palindromic repeats (CRISPR), has received substantial attention for nucleic acid detection due to its simplicity, speed, high sensitivity and specificity. With the help of CRISPR RNA (crRNA), CRISPR proteins can specifically cut the target region that is complemented with the crRNA sequence. There are two components in the CRISPR detection: first, the CRISPR-RNA complex will cut the target region, this activates the next step, collateral cleavage of the surrounding nucleic acids. CRISPR Cas12, Cas13, and Cas14 effector proteins have a unique collateral cleavage ability, which enables these tools to indiscriminately cleave surrounding nucleic acid once they bind to the target site (**Figure 1**). With regard to cleavage activity, Cas12 recognizes double-stranded DNA (dsDNA) more efficiently than single-stranded DNA (ssDNA), but it still exhibits collateral activity for ssDNA. Cas14 recognizes ssDNA more effectively than dsDNA with respect to cleavage activity and also exhibits collateral activity for ssDNA. Cas13 is unique because it recognizes and exhibits collateral activity for ssRNA (van Dongen et al., 2020). By introducing appropriate nucleic acid reporters (ssDNA, ssRNA, dsDNA), different detection signals (e.g., fluorescent, colorimetric, electrochemical) can be specifically detected. Because of this collateral cleavage activity, CRISPR can be combined with isothermal nucleic acid amplification to simplify the detection method by visualizing the result of positive or negative samples with the naked eye, LED or UV lamps, or by observing the lateral flow strips. Traditional methods like PCR or RT-qPCR need expensive, bulky equipment, especially PCR needs to either run on an agarose gel for visualization of the target DNA. This takes longer, requires labor-intensive work and opening the amplified tube increases the contamination rate. Moreover, isothermal amplification like recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) alone is not specific enough and single-nucleotide polymorphisms (SNPs) cannot be discriminated (van Dongen et al., 2020). However, combination of isothermal amplification and CRISPR improves the sensitivity and specificity because the crRNA only binds to the target region and can even detect SNPs for human genotyping (Gootenberg et al., 2017).

POCT methods can rapidly generate results at a location close to the patient (Vashist, 2017), which reduces the duration for which a patient must stay at a clinic. Demand for POCT has increased over the last 40 years (St John and Price, 2014) and is especially high today because of the current global pandemic. Popular POCT technologies, such as glucose biosensor strips and lateral flow assays, have been widely used for home healthcare and disease detection. These devices eliminate the need to return to the doctor's office multiple times, thus saving the patient money (St John and Price, 2014), which is especially important in countries without affordable healthcare. These devices are also extremely beneficial for remote regions and cities that do not possess extensive, costly diagnostic tools (Tideman et al., 2014). In particular, there is substantial interest in integrating CRISPR-based molecular testing into lateral flow detection and microfluidic technology, enabling simple, rapid and reliable point-of-care diagnostics.

In this review, we focus on CRISPR-based diagnostic tools as well as their benefits and drawbacks. We review CRISPR as a nucleic acid detection tool for SARS-CoV-2 and report on research efforts occurring worldwide to improve the specificity, sensitivity, and speed of methods for detecting this virus. For those tools that are not ready for POCT, we discuss current challenges and future directions.

CAS12-EFFECTOR-BASED COVID-19 DIAGNOSTIC TOOLS

The breakthrough of using Cas12a in nucleic acid detection was first reported by Chen et al. who developed DNA endonuclease-targeted CRISPR trans reporter (DETECTR) for human

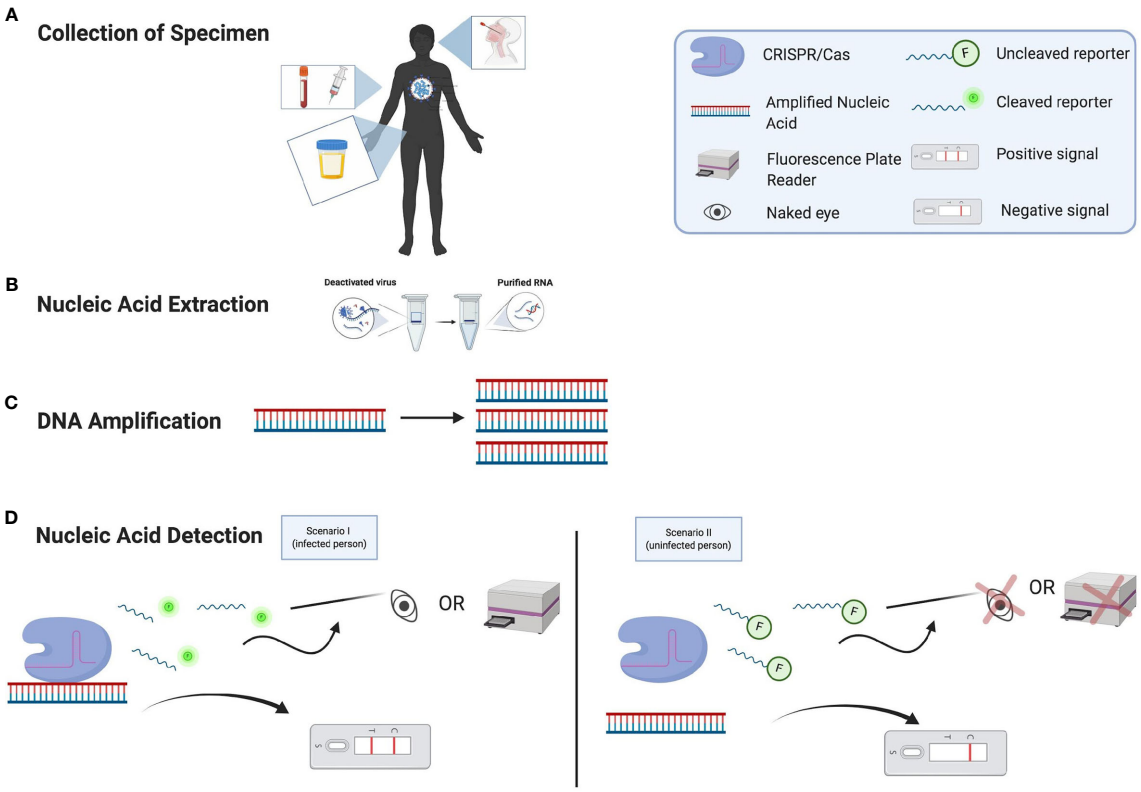


FIGURE 1 | Nucleic Acid Detection of SARS-CoV-2 Using CRISPR/Cas Assays. **(A)** Patient specimens can be collected from different types of clinical samples. **(B)** RNA is extracted from the specimen. **(C)** From the nucleic acid extraction, the DNA must be amplified. **(D)** The nucleic acid of SARS-CoV-2 can now be detected. If a person has COVID-19 (Scenario I), then the CRISPR/Cas complex will bind to the target region of the amplified nucleic acid and collateral cleavage activity can occur by cleaving the nearby fluorescence reporter nucleic acids. This can be detected by either by using the naked eye under specific light, a fluorescence plate reader, or a lateral flow assay that can indicate the presence of the virus's nucleic acid. If a person does not have COVID-19 (Scenario II), then the CRISPR/Cas complex will not bind to the target region of the amplified nucleic acid and collateral cleavage activity will not be initiated; this means that there will not be any viral signal (glow) from the sample observed by the naked eye, a plate reader, or a lateral flow assay.

papillomavirus (HPV) in 2018 (Chen J. S. et al., 2018). Due to the discovery of collateral activity in Cas12a, Chen et al. have explored its potential as a diagnostic tool. DETECTR was developed using crRNA and ssDNA fluorophore quencher (ssDNA-FQ) reporter by combining recombinase polymerase amplification RPA with Cas12a-based fluorescence detection. RPA conveniently eliminates the need for a labor-intensive, temperature-sensitive PCR method because RPA operates under isothermal conditions (e.g., ~37°C). With RPA assay, this tool is close to POCT. However, the fluorescence detection by using a fluorescence plate reader is not ideal for POCT applications. Recently, Broughton et al. adapted DETECTR to lateral flow assay for SARS-CoV-2 detection (Broughton et al., 2020). This version utilizes reverse transcription loop-mediated isothermal amplification (RT-LAMP) instead of RPA because the patient samples consist of RNA from nasopharyngeal or oropharyngeal swabs. The RT-LAMP-DETECTR reaction performs well at 62°C, which is less convenient than the RPA-DETECTR for the HPV virus, which worked at 37°C. Broughton et al. designed the crRNA around the N and E gene regions of SARS-CoV-2, because the detection methods utilized by the World Health Organization and US Centers for Disease Control (CDC) and Prevention operate around those regions. The detection results can be obtained more quickly with this method than with previous DETECTR detection methods (30-40 min vs. >1 h). In particular, they employed a lateral flow strip instead of a fluorescence plate reader, bringing this method closer to POCT and eliminating the need for bulky instrumentation. Broughton et al. compared the lateral flow strip method against the currently most commonly used CDC assay to test their limit of detection (LoD). The CDC assay could detect the virus at levels as low as 1 viral copy per μL while DETECTR could detect the virus at levels as low as 10 viral copies per μL using *in vitro* transcribed viral nucleoprotein RNA (Broughton et al., 2020). The authors then tested 11 clinical respiratory samples using this device. One of the samples had a quality control failure, and another gave the wrong result, but the results for the remaining nine samples matched the clinical test results. Thus, the performance is promising, but not perfect. The reason that two of their samples failed is because the samples contained fewer than 10 viral copies per μL. These researchers increased their sample size and compared results for 60

nasopharyngeal swab samples with CDC RT-qPCR assay results and claimed to obtain similar results. Interestingly, their crRNA was designed to target the E gene of SARS-CoV-2 in order to detect other coronavirus strains. But this may influence the detection specificity. The researchers tested the sensitivity of this assay at femtomolar (fM) levels rather than attomolar (aM) levels, as had been previously done. It would be interesting to determine whether aM detection sensitivity is achieved for this specific virus as well.

Several other groups have designed detection methods very similar to DETECTR for SARS-CoV-2. One such method is the CRISPR-based fluorescent detection system (CRISPR-FDS); however, this method requires a micro-plate fluorescent reader, which is not a substantial improvement in terms of POCT (Huang et al., 2020). Another group developed the *in vitro* specific CRISPR-based assay for nucleic acid detection (iSCAN) (Ali et al., 2020). The authors also developed this CRISPR Cas12a-based detection method with RT-LAMP. Their LoD was 10 RNA copies per reaction, which is difficult to compare with that of DETECTR, because these two LoD are in different units. The E gene employed in SARS-CoV-2 detection had a low sensitivity (38% accurate for positive, 100% accurate for negative SARS-CoV-2 samples), while the N gene detection was better (86% accurate for positive, 100% accurate for negative SARS-CoV-2 samples). To bring this method closer to POCT, the authors developed the assay as a one-pot technique. For this purpose, they replaced Cas12a with Cas12b because Cas12b can function at the same temperature as RT-LAMP; however, the efficiency of one-pot assay was low. To this end, they added CRISPR/Cas12b on the tube wall as a droplet, which remained until the RT-LAMP process was completed. They then mixed the sample and finally obtained a similar sensitivity (86% accurate for positive, 100% accurate for negative SARS-CoV-2 samples). Another group developed a similar method by adding mineral oil on top of the RT-LAMP sample to act as a barrier and then added the CRISPR Cas12a reagent to the top of the microcentrifuge tube (Chen Y. et al., 2020). The authors shook the samples by hand after RT-LAMP was performed. Adding CRISPR-Cas12b as a droplet can be risky because if the droplet is not sufficiently large, the CRISPR reagents can evaporate during the 30-min amplification step. In addition, the droplet could slide down and mix with the sample. As an advantage, the likelihood of introducing contamination is lower than cases in which the tube is reopened to add enzymes. However, the efficiency of this assay is not sufficient to replace current RT-qPCR CDC assays.

Two different groups independently developed an improved CRISPR-based detection method to visualize fluorescence signals using the naked eye. This feature is a great advantage because it can eliminate the need for an ultraviolet (UV) light source, which can be bulky and expensive. CRISPR/Cas12a-based detection with the naked eye readout (CRISPR/Cas12a-NER) utilizes reagents similar to those for other methods, with the exception of reagents for reverse-transcription recombinase-aided amplification (RT-RAA) (Wang X. et al., 2020). This method is a two-pot assay because the RT-RAA occurs at 39°C while the CRISPR/Cas12a process operates at 37°C. Conversely, the samples can be visualized under a blue light-emitting diode (LED). Another group developed AIOD-CRISPR (all-in-one dual CRISPR-Cas12a), which can also be visualized under blue LED light or UV light and, in some cases, even under ambient light without excitation (Ding et al., 2020). These researchers used RT-RPA, which occurs at 37°C, along with the CRISPR/Cas12a activity, making this assay a true one-pot approach. More importantly, this method utilizes two crRNAs that are not limited by a protospacer adjacent motif (PAM) site. This is significant because Broughton et al., 2020 stated they could not design crRNAs in the N1 or N3 regions of SARS-CoV-2 due to the lack of suitable PAM site (Broughton et al., 2020). However, Ding et al. demonstrated the feasibility of designing crRNAs without being limited by the PAM site. Additionally, the authors showed that the use of two crRNAs could improve the assay sensitivity (Ding et al., 2020). Ding et al. reported that although the use of PAM-limited crRNA is the fastest method for detecting SARS-CoV-2 plasmid, the combination of two crRNAs that are not limited by the PAM could still be useful for detection. This finding can greatly help scientists in the design of crRNAs for detection methods, indicating that these designs do not need to be restricted by the availability of the PAM site in the targeted region. By eliminating the separate pre-amplification step, the authors created a one-pot assay. Out of 28 clinical samples, this assay detected 8 RNA extracts as positive for SARS-CoV-2. This device needs to be performed on larger, more diverse clinical samples and some improvements on nucleic acid sample preparation are still needed for POCT. Further improvement may be achieved by integrating simple nucleic acid preparation with their AIOD-CRISPR assay, enabling simple, rapid, and "sample to answer" SARS-CoV-2 detection.

Similar to Y. Chen et al., R. Wang et al. developed one-pot visual RT-LAMP-CRISPR (opvCRISPR), which combines a one-pot assay based on mineral oil separation between the nucleic acid amplification and CRISPR/Cas12a detection steps. The authors used blue-light naked-eye detection, as employed by the two groups mentioned above. Wang et al. used RT-LAMP, which occurs at 65°C at the bottom of the tube, while the CRISPR/Cas12a complex is maintained on the lid and separated by mineral oil (Wang R. et al., 2021). Although one-pot assays are superior to two-pot assays due to the lower potential for contamination, one must consider the possibility of the reagent on the lid evaporating or mixing with the reagent underneath in order to avoid any interference with the assay specificity.

Another group proposed the use of dual crRNAs to increase detection. These researchers added divalent cations to improve the sensitivity of this method. They demonstrated that using Mn^{2+} increased the efficiency and, most importantly, the specificity. Thus, they termed this technique manganese-enhanced Cas12a (MeCas12a) (Ma et al., 2020). It has been found that Mn^{2+} helps to stabilize the most stable bond between CRISPR and target DNA (Sundaresan et al., 2017). The addition of Mn^{2+} aided in detecting virus in clinical samples from patients with both SARS-CoV-2 and Middle East respiratory syndrome-

related coronavirus (MERS-CoV). These viruses share highly conserved regions in their nucleic acid and differ by only a few single-nucleotide polymorphisms. Therefore, the addition of Mn^{2+} improved the detection of both viruses, without false detection of one virus over the other. Notably, the authors showed that Mn^{2+} helped improve the efficiency of *Lachnospiraceae bacterium* (LbCas12a), but not *Acidaminococcus* sp. (AsCas12a) in this paper. For AsCas12a, Mn^{2+} activated Cas12a even in the absence of target DNA, increasing the nonspecificity. This detection technology was not designed for POCT applications, but this higher-specificity method can be applied to future studies for POCT.

Guo et al. developed CASdetec (CRISPR-assisted detection), which has a LoD of 10 RNA copies per μL (Guo et al., 2020). This study was unique because of their careful study of each platform parameter. As a prime example, the authors found an optimal combination of crRNA concentration and ssDNA-FQ reporter length with the best fluorescence signal; in contrast, other studies are lacking in this regard. Similar to others, Guo et al. attempted to develop a one-pot reaction by placing the CRISPR complex in the cap of the tube, which carries the risk of premixing prior to the reaction, as stated above. As another unique aspect, the authors based their detection method on the RdRp locus of the virus, in contrast to the other studies. Other studies showed that RdRp tends to mutate more than other regions of the virus nucleic acid (Eskier et al., 2020), which could interfere with the detection sensitivity. Moreover, this study lacked clinical data, which would be useful to obtain.

Another group modified a component of the CRISPR-based assay to improve its sensitivity. This method was called ENHANCE (enhanced analysis of nucleic acids with crRNA extensions) and was developed to find the optimal length of crRNA extension to increase the collateral cleavage activity of Cas12a (Nguyen et al., 2020). The authors discovered that a 3' DNA with 7-mer extensions showed the brightest fluorescence for some orthologs of Cas12a. They studied the crystal structure of Cas12a to form a hypothesis regarding crRNA extension and its influence on collateral cleavage activity. Interestingly, this study produced results that contradicted those of Ma et al., 2020. The authors found out Mn^{2+} inhibited the LbCas12a activity, in contrast to Mg^{2+}, which increased the activity. This contradiction should be further studied with the same controls to determine the reason behind the two different results. This discrepancy may have arisen because the crRNAs were designed for different targets. All of the assays mentioned in this section are summarized below in **Table 1**.

CAS13 EFFECTOR-BASED COVID-19 DIAGNOSTIC TOOL

Even before Cas12 was used as a detection method, scientists had been exploring the potential of Cas13 as a diagnostic tool. One of the first Cas13 nucleic acid detection methods was developed by Zhang and colleagues in 2017. This method, called Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK), has been adapted by many different labs for various uses, such as the detection of the white spot syndrome virus in shrimp (Sullivan et al., 2019), the Ebola and Lassa viruses (Barnes et al., 2020), and malaria (Lee et al., 2020). As previously mentioned, Cas13 is a unique protein that can target an RNA sample, rather than a DNA sample like Cas12. This is advantageous because the SARS-CoV-2 is a single-stranded RNA virus (Carter et al., 2020). In fact, the investigators who originally developed SHERLOCK received FDA approval for COVID testing under emergency use purposes (Guglielmi, 2020). This was a big step toward the development and improvement of CRISPR-based detection and opened the door for many other efforts. SHERLOCK uses RPA technology with Cas13 and can detect aM sensitivity in synthetic virus samples (Gootenberg et al., 2017). However, minimal clinical data have been presented. Moreover, SHERLOCK requires a plate reader to detect the fluorescence reading, which is not ideal for POCT. Thus, the same researchers developed SHERLOCK version 2 (SHERLOCK v2) to detect multiple diseases from a sample and make the technology more applicable to POCT (Gootenberg et al., 2018).

Another lab has demonstrated that SHERLOCK can detect SARS-CoV-2 clinical samples (Patchsung et al., 2020). This group used RT-RPA to amplify the viral gene and designed their primers around the S, N, and ORF1ab regions. The results showed that detection of the S gene using SHERLOCK was the most sensitive, both with fluorescence readouts and a lateral flow strip, with a LoD of 42 RNA copies per reaction. We note that this sensitivity is not as good as the Cas12 nucleic acid detection mentioned earlier. Interestingly, this group had access to a large number of clinical samples and used 154 nasopharyngeal samples to test the S region of the virus. Their detection method was able to detect 100% (73/73) of the negative samples using either the fluorescence readout or lateral-flow strip readout; detection of the positive samples was 96% (78/81) and 88% (71/81), respectively. This reduced sensitivity may be due to a low amount of viral RNA in the samples. The researchers then implemented SHERLOCK as a quick (70 minute) pre-operative screening tool for patients in Thailand entering the operating room. However, the samples were all negative, due to overall low numbers of COVID cases in this country following nationwide lockdown to reduce viral spread. As a result, this method has not yet been tested in patients who are positive for SARS-CoV-2.

Another group developed a CRISPR-COVID test by targeting the ORF1ab and N genes of SARS-CoV-2 and demonstrated better sensitivity and specificity with ORF1ab detection (Hou et al., 2020). The LoD of this method was 7.5 RNA copies per reaction, which is an improvement. This group also presented clinical data with good specificity and sensitivity.

Recently, researchers developed another Cas13a-based detection method using a similar design approach as others, except without the nucleic acid amplification step (eliminating a step in **Figure 1C**). This technology made it possible to detect the virus in 30 minutes, although the LoD was 100 RNA copies/μL (Fozouni et al., 2021). This technology utilizes Cas13a and a quenched fluorescent RNA reporter, making it very simple and

TABLE 1 | Nucleic acid detection of SARS-CoV-2 using CRISPR/Cas12-based assays.

Assay Name	CRISPR Protein	Nucleic Acid Amplification	Target Region	LoD[x]	Clinical Samples	Type of clinical sample	Other major components	Testing time	One pot *vs.* two pots	References
DETECTR	Cas12a	RT-LAMP	N and E gene	10 RNA copies/ μL	11	Nasopharyngeal and oropharyngeal	ssDNA probe, lateral flow assay, 62°C water bath.	30-40 minutes	two pots	Broughton et al., 2020
CRISPR-FDS	Cas12a	RT-RPA	ORF1ab and N gene	2 RNA copies	29	Nasal swab	Fluorescent probe, fluorescence plate reader, 42° C water	50 minutes	two pots	Huang et al., 2020
iSCAN	Cas12a for 2 pots or Cas12b for one pot assay	RT-LAMP	N and E gene	10 RNA copies/ reaction	31	Nasopharyngeal	ssDNA-FQ reporter or lateral flow assay, 62°C water bath and fluorescence plate reader	1 hour	both	Ali et al., 2020
Y. Chen et al. assay	Cas12a	RT-LAMP	ORF gene, N gene and E gene	20 RNA copies/ reaction	10	Unknown	ssDNA probes, mineral oil, portable 3D printing or smartphone to detect fluorescence, 65°C water bath	40 minutes	one pot	Chen Y. et al., 2020
CRISPR/ Cas12a-NER	Cas12a	RT-RAA	E gene	10 RNA copies	31	Unknown	ssDNA-FQ reporter, 39°C water bath, blue light with a wavelength of 485 nm	45 minutes	two pots	Wang X .et al., 2020
AIOD-CRISPR	Cas12a with 2 crRNAs	RPA	N gene	5 RNA copies	28	Nasal swab	ssDNA-FQ reporter, UV and blue LED light	40 minutes	one pot	Ding et al., 2020
opvCRISPR	Cas12a	RT-LAMP	S gene	5 RNA copies	50	Nasopharyngeal	ssDNA reporter, mineral oil, air column, blue LED light, 65°C water bath	45 minutes	one pot	Wang R. et al., 2021
MeCas12a	Cas12a with 2 crRNAs	RT-RAA	E gene	5 RNA copies	24	Nasopharyngeal	ssDNA-FQ reporter, Mn^{2+}, 39° C water bath, wavelength of 485 nm light.	45 minutes	two pots	Ma et al., 2020
CASdetec	Cas12b	RT-RAA	RdRp locus	10 RNA copies/ μL	0	NA	ssDNA-FQ reporter, blue LED light	1 hour	one pot	Guo et al., 2020
ENHANCE	Cas12a with 3'DNA7-modified crRNA	RT-LAMP	N gene	3-300 RNA copies	0	NA	FITC reporter, lateral flow assay, 63°C water bath, Mg^{2+}	30 minutes	two pots	Nguyen et al., 2020

[x]*unit is different between studies. Some did not mention the units.*
37°C degree water bath can be replaced by body heat, and therefore was not included.
NA, not applicable.

unique. The tool combines three crRNAs to efficiently detect the N and E gene of SARS-CoV-2, even though the samples are not amplified. To make their assay closer to POCT, these researchers used a mobile phone camera as a portable plate reader to eliminate the need for a fluorescence plate reader. They tested this intriguing idea on five clinical samples and demonstrated a LoD of 200 RNA copies/μL when using a mobile phone. Despite the potential of this approach, the current study has many flaws. The sample size of five clinical samples is small, and 200 even 100 RNA copies/μL is too high for a clinical test. Arnaout et al. showed in their preprint article that increasing LoD by a factor of 10 could increase the rate of false negatives by 13% (Arnaout et al., 2020). Perhaps combining this triple crRNA approach with isothermal amplification and a lateral-flow assay for measurement would improve the sensitivity and make substantial strides toward POCT.

Finally, researchers have designed Cas13-based, rugged, equitable, scalable testing (CREST) to be cost effective and widely accessible to the public. In this article, they opted for the miniPCR and P51 fluorescence visualizer, which are both portable and small devices. This method is cheaper and more effective at detecting the virus compared with other PCR methods (Rauch et al. 2021). Notably, a miniPCR is compatible with mobile devices, creating the possibility that clinics could acquire a patient's data using a mobile phone and upload the data directly to the cloud. However, this assay is a 'two-pot' assay and must be performed by highly trained scientists. All the Cas13 effector-based COVID-19 diagnostic tools mentioned in this section are summarized in **Table 2**.

CAS9 EFFECTOR-BASED COVID-19 DIAGNOSTIC TOOL

The Cas9 effector is well known for its applications in genome editing, though not for its ability to detect nucleic acids. This is because Cas9 lacks collateral activity. However, one group used Francisella novicida Cas9 (FnCas9) to develop an FnCas9 editor-linked uniform detection assay (FELUDA) to detect the presence of SARS-CoV-2 in their preprint article (Azhar et al., 2020). Because FnCas9 has a high specificity for mismatches, it can improve the assay's specificity over the use of Streptococcus pyogenes (SpCas9), which is a very popular Cas9 ortholog used

TABLE 2 | Nucleic acid detection of SARS-CoV-2 using CRISPR/Cas13 and Cas9-based assays.

Assay Name	CRISPR Protein	Nucleic Acid Amplification	Target Region	LoD[x]	Clinical Samples	Type of clinical sample	Other major components	Testing time	One pot *vs.* two pots	References
Patchsung et al. assay	Cas13a	RT-RPA	S gene	42 RNA copies/ reaction	154	Nasopharyngeal and throat swab	ssRNA reporter, Lateral flow assay, RNase-responsive RNA reporter, 42°C water bath	1 hour	two pots	Patchsung et al., 2020
CRISPR-COVID	Cas13a	RT-RPA	ORF1ab	7.5 copies/ reaction	114	Unknown	ssRNA reporter, 42°C water bath, fluorescence plate reader	40 minutes	two pots	Hou et al., 2020
Fozouni et al. assay	Cas13a with 3 crRNAs	No amplification	N and E gene	100 copies/μL	5	Nasal swab	ssRNA-FQ reporter, mobile phone to detect fluorescence or plate reader	30 minutes	one pot	Fozouni et al., 2021
CREST	Cas13a	MiniPCR bio	N1 and N2 region	10 RNA copies/μL	159	Nasopharyngeal and oropharyngeal	ssRNA-FQ reporter, fluorescence LED visualizer	50 minutes	two pots	Rauch et al. 2021
FELUDA	Cas9	RT-PCR or RT-RPA	N gene	Unknown	Unknown	Unknown	FAM reporter, Lateral flow assay, 95°C water bath	45 minutes	two pots	Azhar et al., 2020

[x]*unit is different between studies. Some did not mention the units.*

37°C degree water bath can be replaced by body heat, and therefore was not included.

for gene editing (Chen F. et al., 2017). The findings using FELUDA are intriguing; however, this approach has not yet been compared to the other nucleic acid detection assays mentioned above (**Tables 1** and **2**). The results did not mention a LoD or how many clinical samples were used for testing **(Table 2)**. It will be intriguing to see more data in the future, especially if this assay ultimately shows significantly higher specificity than the Cas12- and Cas13-based nucleic acid detection methods. More importantly, for a CRISPR-based diagnostic tool to reach the market, it must have as much if not more specificity and sensitivity than the current detection method.

MICROFLUIDIC DEVICE FOR COVID-19 DIAGNOSIS BY CRISPR

In this section, we review microfluidic diagnostic devices that are beyond lateral flow strips. These approaches are more 'advanced' but also more complex. Some quantifiably measure SARS-CoV-2 using digital chip while others use engineering technologies such as microfluidic devices to detect the virus.

As the majority of the CRISPR-based diagnostic tools mentioned above lack the ability to measure the quantity of the virus, one group developed a RApid DIgital CRISPR Approach (RADICA) in their preprint article to quantifiably measure SARS-CoV-2 like RT-qPCR. This one-pot assay works in one hour using RPA, CRISPR-Cas12a, an ssDNA-FQ reporter, chip-based digital PCR, and a Clarity™ Reader for fluorescence detection (Wu et al., 2020). This device can analyze the proportion of positive to negative signals by partitioning a patient's sample into 10,000 portions on a chip. The specificity is good, and the device can even differentiate SARS-CoV-2 from a similar coronavirus. It is important that the device can perform reverse transcription, because SARS-CoV-2 is an RNA virus. However, since the RPA/RT-RPA can be initiated at room temperature, their reaction mixture needs to be prepared on ice and loaded into chips within one minute to minimize premature target amplification.

To prevent undesired premature amplification and accurately quantify nucleic acid, another group developed a digital warm-start CRISRP (WS-CRISPR) assay, which is only initiated at above 50°C. In this preprint article, they use reverse transcription dual-priming mediated isothermal amplification (RT-DAMP), Mg^{2+}, and pyrophosphatase (PPase) (Ding et al., 2021). The researchers take advantage of the mediation role of pyrophosphatase and phosphorothioate primers to develop a low-temperature RT-DAMP assay. Also, they optimize each concentration and the amount of each component to achieve higher specificity. This assay is clinically validated by quantitatively detect 35 clinical samples (32 swab samples and three saliva samples), showing 100% agreement with RT-PCR results. However, the detection process is labor intensive as it relies on fluorescence microscopy, and ImageJ software to count the number of positive spots on the chip compared to using a fluorescence reader like RADICA. By applying a fluorescence-automated reader, this device could be improved and simpler in the future.

With a similar goal of achieving faster, more accurate quantitative analysis, digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOVID) was developed using a similar approach as the devices mentioned above (**Figure 2A**). This device is faster but requires more components like WS-CRISPR (Park et al., 2020). The researchers used 0.1% Tween-20 to reduce the viscosity of the reaction mix to be compatible with digital PCR. They also used bovine serum albumin (BSA) to improve the reaction performance by reducing the adsorption of enzymes to the reaction tube. One interesting finding was that deCOVID's reaction time stayed constant even when there was a small amount of RNA present in the sample, unlike other assays. Future work could simplify the device, and possibly incorporate the use of a mobile phone instead of fluorescence microscopy.

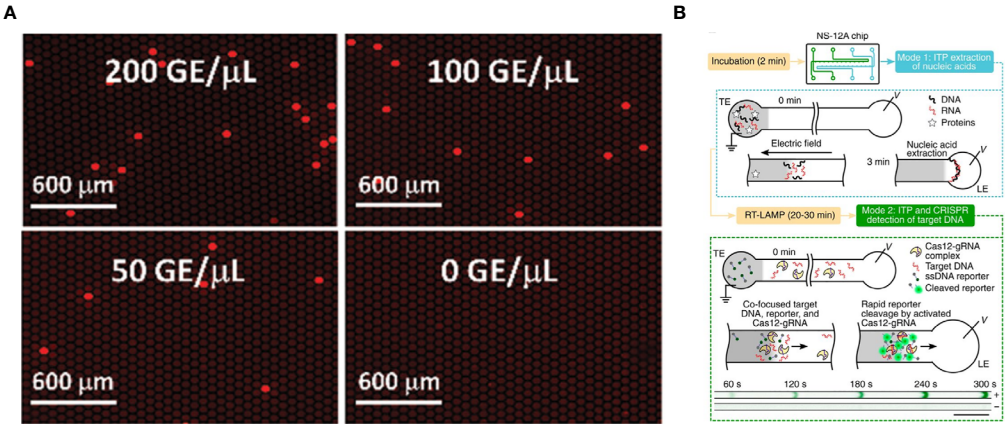


FIGURE 2 | SARS-CoV-2 detection on microfluidic diagnostic devices using CRISPR/Cas technology. **(A)** Fluorescence images of digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOVID) on microfluidic digital chips for SARS-CoV-2 detection (Digital CRISPR/Cas-assisted assay for rapid and sensitive detection of SARS-CoV-2 by Park et al., 2020 is licensed under CC BY 4.0). **(B)** Working principle of isotachophoresis-CRISPR (ITP-CRISPR) assay. The microfluidic chip has two channels for ITP extraction of nucleic acids (mode 1) and ITP–CRISPR detection (mode 2). In the mode 1, when an electric field is applied, nucleic acids selectively focus within the electromigrating LE–TE interface, leaving behind impurities. After off-chip RT-LAMP of ITP-extracted nucleic acids, in mode 2, ITP is used to detect target DNA by using a CRISPR-Cas12. A positive sample shows a strong fluorescent signal, while negative control not (Electric field-driven microfluidics for rapid CRISPR-based diagnostics and its application to detection of SARS-CoV-2 by Ramachandran et al., 2020 is licensed under CC BY 4.0).

All the devices mentioned above lack the ability to extract and purify nucleic acid from nasopharengeal or any other clinical samples. Rather, these devices rely on samples that are already extracted and ready to use. This is a big remaining hurdle because nucleic acid extraction and purification usually takes an hour and is an important step for accurate detection (Ramachandran et al., 2020). To this end, one group developed isotachophoresis-CRISPR (ITP-CRISPR) using microfluidics and an on-chip electric field (**Figure 2B**). ITP can be applied for nucleic acid extraction and purification from biological samples and the on-chip electric field enables the detection of SARS-CoV-2 by CRISPR. ITP-CRISPR is remarkably fast, with the nucleic acid extraction and purification and detection of the sample taking less than 40 minutes (Ramachandran et al., 2020). Another advantage of this assay is a reasonable reaction volume requirement, making it cost effective for the reagent that is used. Using a large number of clinical samples, ITP-CRISPR was able to detect 30 out of 32 positive samples (93.8%) and 32 out of 32 negative samples (100%). But this assay required off-chip manual steps for RT-LAMP and sample lysis. Off-chip steps potentially increase the risk of contamination, especially if tasks are not performed by someone who is well trained. Nonetheless, it is important to consider nucleic acid extraction, as it is the initial step for fully integrated nucleic acid detection. Several microfluidic diagnostic devices mentioned in this section are summarized in **Table 3**.

CHALLENGES AND PERSPECTIVES

Faster nucleic acid-based molecular detection methods are needed during this pandemic, but it is especially important that these detection methods are POCT so that they can be easily applied in developing countries and to people who live hours away from clinics and hospitals. Faster and more accurate detection methods could improve the speed of contact tracing and help control the pandemic (Koetter et al., 2020). Reduced costs and waiting times for results would also be beneficial to both national economies and patients' medical bills. Many groups have designed assays that are faster, easier to use, and more applicable to POCT of SARS-CoV-2. RT-qPCR is time consuming, and requires specific temperatures, bulky instruments, and highly trained scientists. However, it is not yet possible to replace RT-qPCR due to the trade-off with other technologies between being POCT friendly and having reduced sensitivity. All the emerging CRISPR approaches detailed in this paper must be tested using clinical data from different sources, such as blood, saliva, and oropharyngeal specimens, in order to prove their specificity, sensitivity, and duration. The assays will also need to be tested on clinical samples of SARS, MERS, and other coronaviruses to prove that they can distinguish between similar viruses despite shared conserved genomic regions. Moreover, not all the assays have shown aM sensitivity, which will be required before replacing RT-qPCR.

It appears that the performance of the CRISPR protein can vary depending on which organism it is isolated from, (Gootenberg et al., 2018; Ali et al., 2020; Nguyen et al., 2020). For example, addition of Mn^{2+} does not improve the sensitivity of all Cas12a proteins, because of the differing function depending on which organism the Cas12a is isolated from (Ma et al., 2020). There have also been contradictions between some studies. One study mentioned that Mn^{2+} improved the sensitivity of LbCas12a, whereas another study stated that it inhibited the activity of LbCas12a (Ma et al., 2020; Nguyen et al., 2020). It is important to choose the right organism, the right protein, and the right kind of modification. Several of the studies above also

TABLE 3 | SARS-CoV-2 detection on microfluidic diagnostic devices using CRISPR technology.

Assay Name	CRISPR Protein	Nucleic Acid Amplification	Target Region	LoD[x]	Clinical Samples	Type of clinical sample	Other Major components	Testing time	One pot *vs.* two pots	References
RADICA	Cas12a	RPA	N gene	LoQ*-2.2 DNA copies/ μL	0	NA	ssDNA-FQ reporter, chip-based digital PCR, Clarity^{TM} Reader for detecting fluorescence, 42°C incubation, ice	1 hour	one pot	Wu et al., 2020
WS-CRISPR	Cas12a	RT-DAMP	N gene	5 RNA copies/ μL in the reaction	35	Saliva and unknown clinical swab	ssDNA-FQ reporter, QuantStudio 3D digital PCR, Mg^{2+}, pyrophosphatase, 52°C incubation, fluorescence microscopy with camera, ImageJ software	50 minutes	one pot	Ding et al., 2021
deCOVID	Cas12a	RT-RPA	N gene	1 GE^/ μL	4	Unknown	ssDNA reporter, QuantStudio 3D digital PCR, 0.01 mg/mL BSA, 0.1% Tween-20, 42°C incubation, fluorescence microscopy with camera, QCapture software, ImageJ software	30 minutes	one pot	Park et al., 2020
ITP-CRISPR	Cas12	RT-LAMP	N and E gene	10 copies/ μL	64	Nasopharyngeal	ssDNA reporter, 62°C incubation, lysis buffer, on-chip ITP, fluorescence microscope, sourcemeter, camera,	40 minutes	two pots	Ramachandran et al., 2020

[x]unit is different between studies. Some did not mention the units.
37°C degree water bath can be replaced by body heat, and therefore was not included.
**LoQ-Limit of quantification.*
^GE-genome equivalent.
NA, not applicable.

demonstrated that using multiple crRNAs is more effective than using one. There are other proteins similar to CRISPR that have been developed. For example, *Thermus thermophilus (TtAgo)* is guided by ssDNA to cut the target DNA (Song et al., 2020). It does not get limited by the PAM site and uses DNA rather than RNA for the guide, which makes it more versatile. However, it is still new to the nucleic acid detection field, more studies are needed to compare this with CRISPR in terms of its efficiency.

We also note the importance of potential contamination and the presence of ribonuclease (RNase) in preparing an assay to be market ready. Especially in a clinical setting, the presence of RNase can interfere with the result, since they can degrade crRNAs and RNA. Such degradation could lead to a false signal (Patchsung et al., 2020). Therefore, researchers should consider adding RNase inhibitors and implement methods to detect the presence of RNase, such as the assay developed by Patchsung et al. In general, it is imperative to avoid any contamination, especially for assays that will need to be manufactured at large quantities and distributed globally. For these reasons, 'one-pot' assays are preferable over 'two-pot' assays to avoid the need for opening a tube multiple times.

To improve these assays and bring us closer to POCT, lyophilization of reagents could improve transportation and storage and prevent protein and RNA damage, because protein and RNA are temperature sensitive. Qian et al. also showed that using trehalose and pullulan to dehydrate Cas12a protein makes it possible to store the protein for more than a month at room temperature (Qian et al., 2020).

Even with all these improvements, DNA/RNA extraction is the biggest hurdle for bringing nucleic acid detection to POCT, because few nucleic acid extraction methods are POCT friendly. Not only does the extraction step require an extensive multi-step process, trained technicians and bulky equipment, but most of the extracted DNA/RNA needs to be purified before the amplification step to remove potential inhibitors that could further interfere with the downstream application (Zou et al., 2017). As a result, patients still need to visit a clinic for COVID-19 testing, because nucleic acid extraction must happen before CRISPR can be used on the samples. Complicating things further, the extraction method varies depending on the origin of the samples, because the component that is required is different for each sample. It would be convenient to use raw samples right away for detection without extracting and purifying it, but there are still remaining challenges for accurate diagnostics. Depending on which samples are used, some of the samples need to be diluted, for example blood and mucus samples (Paul et al., 2020). Others such as, saliva or nasopharyngeal samples need enough of the virus or pathogen genetic material present in it to be detected. Without the nucleic acid extraction method, most of the detection assays mentioned above would not be able to meet clinical diagnostic requirements due to reduced sensitivity and specificity.

Additionally, manufacturing the CRISPR protein could be a hurdle in the development of CRISPR-based testing. Currently Cas13 and Cas14 proteins are not commercially available ("Cas13 — Zhang Lab" n.d.). This means that laboratories are currently expressing and purifying these proteins themselves, which could lead to variations between labs. Manufacturing CRISPR using good manufacturing practice (GMP) and

making it widely available would hasten the improvement of CRISPR-based detection assays and the time to market.

In summary, CRISPR-based nucleic acid detection is an innovative approach that has progressed rapidly and has the potential for further improvement. Ultimately, if this technology became available in a hospital setting, patients could potentially learn their COVID-19 testing result within an hour at little cost, which would be a great resource during this pandemic. Testing could save lives, because as much as 81% of the people who test positive can be asymptomatic virus carriers, according to testing that was performed in passengers of an Argentinian expedition cruise ship (Ing et al., 2020). It is crucial to perform testing as early as possible to prevent asymptomatic people from spreading to the virus unknowingly to others. If testing is affordable and has a fast turn-around time with good accuracy, then millions of lives could be saved. Following the necessary improvements laid out in this paper, CRISPR-based detection could hopefully become available in a clinical setting or even as an at-home detection kit in the near future. Importantly, this assay would be useful for detecting many other viruses and pathogens at the point of care.

AUTHOR CONTRIBUTIONS

UG designed the structure of, wrote, and critically reviewed the manuscript. UG also designed the figures using Biorender.com. CL designed the structure of and critically reviewed the manuscript, and approved its final version. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported, in part, by NIH R01EB023607, R61AI154642, and R01CA214072.

REFERENCES

Ali, Z., Aman, R., Mahas, A., Rao, G. S., Tehseen, M., Marsic, T., et al. (2020). Iscan: An Rt-Lamp-Coupled CRISPR-Cas12 Module for Rapid, Sensitive Detection of SARS-Cov-2. *Virus Res.* 288, 198129. doi: 10.1016/j.virusres.2020.198129

American Society of Microbiology (2020). *Asm Expresses Concern About Coronavirus Test Reagent Shortages*. Available at: https://asm.org/Articles/Policy/2020/March/ASM-Expresses-Concern-about-Test-Reagent-Shortages (Accessed September 28, 2020).

Arnaout, R., Lee, R., Lee, G. R., Callahan, C., Yen, C., Smith, K., et al. (2020). SARS-Cov2 Testing: The Limit of Detection Matters. *BioRxiv: Preprint Server Biol.*, 617–667. doi: 10.1101/2020.06.02.131144

Azhar, M., Phutela, R., Ansari, A. H., Sinha, D., Sharma, N., Kumar, M., et al. (2020). Rapid, Field-Deployable Nucleobase Detection and Identification Using Fncas9. *Europepmc*. doi: 10.1101/2020.04.07.028167

Barnes, K. G., Lachenauer, A. E., Nitido, A., Siddiqui, S., Gross, R., Beitzel, B., et al. (2020). Deployable CRISPR-Cas13a Diagnostic Tools to Detect and Report Ebola and Lassa Virus Cases in Real-Time. *Nat. Commun.* 11 (1), 4131. doi: 10.1038/s41467-020-17994-9

Broughton, J. P., Deng, X., Yu, G., Fasching, C. L., Servellita, V., Singh, J., et al. (2020). Crispr–Cas12-Based Detection of SARS-Cov-2. *Nat. Biotechnol.* 38 (7), 870–874. doi: 10.1038/s41587-020-0513-4

Carter, L. J., Garner, L. V., Smoot, J. W., Li, Y., Zhou, Q., Saveson, C. J., et al. (2020). Assay Techniques and Test Development for COVID-19 Diagnosis. *ACS Cent. Sci.* 6 (5), 591–605. doi: 10.1021/acscentsci.0c00501

Cas13 — Zhang Lab. Available at: https://zlab.bio/cas13 (Accessed December 29, 2020).

Chen, F., Ding, X., Feng, Y., Seebeck, T., Jiang, Y., and Davis, G. D. (2017). Targeted Activation of Diverse Crispr-Cas Systems for Mammalian Genome Editing Via Proximal Crispr Targeting. *Nat. Commun.* 8 (1), 14958. doi: 10.1038/ncomms14958

Chen, Z., Li, Y., Wu, B., Hou, Y., Bao, J., and Deng, X. (2020). A Patient With Covid-19 Presenting a False-Negative Reverse Transcriptase Polymerase Chain Reaction Result. Korean Journal of Radiology. *Korean Radiol. Soc.* 21, 623–624. doi: 10.3348/kjr.2020.0195

Chen, J. S., Ma, E., Harrington, L. B., Costa, M. D., Tian, X., Palefsky, J. M., et al. (2018). Crispr-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded Dnase Activity. *Science* 360 (6387), 436–439. doi: 10.1126/science.aar6245

Chen, Y., Shi, Y., Chen, Y., Yang, Z., Wu, H., Zhou, Z., et al. (2020). Contamination-Free Visual Detection of SARS-CoV-2 With CRISPR/Cas12a: A Promising Method in the Point-of-Care Detection. *Biosens. Bioelectron.* 169, 112642. doi: 10.1016/j.bios.2020.112642

Coronavirus Testing Basics | FDA. Available at: https://www.fda.gov/consumers/consumer-updates/coronavirus-testing-basics (Accessed September 28, 2020).

Ding, X., Yin, K., Li, Z., Lalla, R. V., Ballesteros, E., Sfeir, M. M., et al. (2020). Ultrasensitive and Visual Detection of SARS-CoV-2Using All-in-One DualCRISPR-Cas12a Assay. *Nat. Commun.* 11 (1), 4711. doi: 10.1038/s41467-020-18575-6

Ding, X., Yin, K., Li, Z., Sfeir, M. M., and Liu, C. (2021). Sensitive Quantitative Detection of SARS-CoV-2 in Clinical Samples Using Digital Warm-Start CRISPR Assay. *Biosens Bioelectron* 184. doi: 10.1016/j.bios.2021.113218

Eskier, D., Karakülah, G., Suner, A., and Oktay, Y. (2020). Rdrp Mutations are Associated With SARS-CoV-2 Genome Evolution. *PeerJ* 8, e9587. doi: 10.7717/peerj.9587

Fozouni, P., Son, S., Doudna, J. A., and Fletcher, D. A. (2021). Amplification-Free Detection of SARS-CoV-2 With CRISPR-Cas13a and Mobile Phone Microscopy. *CellPress* 184, 1–11. doi: 10.1016/j.cell.2020.12.001

Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., et al. (2017). Nucleic Acid Detection With CRISPR-Cas13a/C2c2. *Science* 356 (6336), 438–442. doi: 10.1126/science.aam9321

Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., and Zhang, F. (2018). Multiplexed and PortableNucleic Acid DetectionPlatform With Cas13, Cas12aand Csm6. *Science* 360 (6387), 439–44. doi: 10.1126/science.aaq0179

Guglielmi, G. (2020). First CRISPR Test for the Coronavirus Approved in the United States. *Nat. NLM (Medline)*. doi: 10.1038/d41586-020-01402-9

Guo, L., Sun, X., Wang, X., Liang, C., Jiang, H., Gao, Q., et al. (2020). Cell Discovery SARS-Cov-2 Detection With CRISPR Diagnostics. *Cell Discovery* 6, 34. doi: 10.1038/s41421-020-0174-y

Hou, T., Zeng, W., Yang, M., Chen, W., Ren, L., Ai, J., et al. (2020). Development and Evaluation of a Rapid Crispr-Based Diagnostic for COVID-19. *PloS Pathog.* 16 (8), e1008705. doi: 10.1371/journal.ppat.1008705

Howard, C. R., and Fletcher, N. F. (2012). Emerging Virus Diseases: Can We Ever Expect the Unexpected? *Emerg. Microbes Infect.* 1, e46. doi: 10.1038/emi.2012.47

Huang, Z., Tian, D., Liu, Y., Lin, Z., Lyon, C. J., Lai, W., et al. (2020). Ultra-Sensitive and High-Throughput CRISPR-P Owered Covid-19 Diagnosis. *Biosens. Bioelectron.* 164, 112316. doi: 10.1016/j.bios.2020.112316

Ing, A. J., Cocks, C., and Green, J. P. (2020). Covid-19: In the Footsteps of Ernest Shackleton. *Thorax* 75 (8), 693–694. doi: 10.1136/thoraxjnl-2020-215091

Koetter, P., Pelton, M., Gonzalo, J., Du, P., Exten, C., Bogale, K., et al. (2020). Implementation and Process of a COVID-19 Contact Tracing Initiative: Leveraging Health Professional Students to Extend the Workforce During a Pandemic. *Am. J. Infect. Control* 48 (12), 1451. doi: 10.1016/j.ajic.2020.08.012

Lee, R. A., Puig, H. D., Nguyen, P. Q., Angenent-Mari, N. M., Donghia, N. M., McGee, J. P., et al. (2020). Ultrasensitive CRISPR-Based Diagnostic for Field-Applicable Detection of Plasmodium Species in Symptomatic and Asymptomatic Malaria. *Proc. Natl. Acad. Sci. U. S. A.* 117 (41), 25722–25731. doi: 10.1073/pnas.2010196117

Lisboa Bastos, M., Tavaziva, G., Abidi, S. K., Campbell, J. R., Haraoui, L. P., Johnston, J. C., et al. (2020). Diagnostic Accuracy of Serological Tests for Covid-19: Systematic Review and Meta-Analysis. *BMJ* 370, m2516. doi: 10.1136/bmj.m2516

Liu, J., Liao, X., Qian, S., Yuan, J., Wang, F., Liu, Y., et al. (2020). Community Transmission of Severe Acute Respiratory Syndrome Coronavirus 2, Shenzhen, Chin. *Emerg. Infect. Dis.* 26 (6), 1320–1323. doi: 10.3201/eid2606.200239

Ma, P., Meng, Q., Sun, B., Zhao, B., Dang, L., Zhong, M., et al. (2020). MeCas12a, a Highly Sensitive and Specific System for COVID-19 Detection. *Adv. Sci.* 7 (20), 2001300. doi: 10.1002/advs.202001300

Nguyen, L. T., Smith, B. M., and Jain, P. K. (2020). Enhancement of Trans-Cleavage Activity of Cas12a With Engineered Crrna Enables Amplified Nucleic Acid Detection. *Nat. Commun.* 11 (1), 4906. doi: 10.1038/s41467-020-18615-1

Park, J. S., Hsieh, K., Chen, L., Kaushik, A., Trick, A. Y., and Wang, T.-H. (2020). Digital CRISPR/Cas-Assisted Assay for Rapid and Sensitive Detection of SARS-Cov-2. *Adv. Sci.* 8, 2003564. doi: 10.1002/advs.202003564

Patchsung, M., Jantarug, K., Pattama, A., Aphicho, K., Suraritdechachai, S., Meesawat, P., et al. (2020). Clinical Validation of a Cas13-Based Assay for the Detection of SARS-CoV-2 Rna. *Nat. Biomed. Eng.* 4, 1140–1149. doi: 10.1038/s41551-020-00603-x

Paul, R., Ostermann, E., and Wei, Q. (2020). Biosensors and Bioelectronics Advances in Point-of-Care Nucleic Acid Extraction Technologies for Rapid Diagnosis of Human and Plant Diseases. *Biosens. Bioelectron.* 169, 112592. doi: 10.1016/j.bios.2020.112592

Qian, C., Wu, H., Shi, Y., Wu, J., and Chen, H. (2020). Dehydrated CRISPR-Mediated Dna Analysis for Visualized Animal-Borne Virus Sensing in the Unprocessed Blood Sample. *Sens. Actuators B: Chem.* 305, 127440. doi: 10.1016/j.snb.2019.127440

Ramachandran, A., Huyke, D. A., Sharma, E., Sahoo, M. K., Huang, C. H., Banaei, N., et al. (2020). Electric Field-Driven Microfluidics for Rapid Crispr-Based Diagnostics and Its Application to Detection of SARS-Cov-2. *Proc. Natl. Acad. Sci. U. S. A.* 117 (47), 29518–29525. doi: 10.1073/pnas.2010254117

Rao, G.G., Agarwal, A., and Batura, D. (2020). Testing Times in Coronavirus Disease (Covid-19): A Tale of Two Nations. *Med. J. Armed Forces India* 76, 243–239. doi: 10.1016/j.mjafi.2020.05.014

Rauch, J., Valois, E., Solley, S., Braig, F., Lach, R., Audouard, M., et al. (2021). A Scalable, Easy-to-Deploy Protocol for Cas13-Based Detection of SARS-CoV-2 Genetic Material. *J. Clinical Microbiol.* 59 (4), e02402–20. doi: 10.1128/JCM

Song, J., Hegge, J. W., Mauk, M. G., Chen, J., Till, J. E., Bhagwat, N., et al. (2020). Highly Specific Enrichment of Rare Nucleic Acid Fractions Using Thermus Thermophilus Argonaute With Applications in Cancer Diagnostics. *Nucleic Acids Res.* 48 (4), e19. doi: 10.1093/nar/gkz1165

St John, A., and Price, C. P. (2014). Existing and Emerging Technologies for Point-of-Care Testing. *Clinical Biochemist Reviews* 35 (3), 155–167.

Sullivan, T. J., Dhar, A. K., Cruz-Flores, R., and Bodnar, A. G. (2019). Rapid, CRISPR-Based, Field-Deployable Detection of White Spot Syndrome Virus In Shrimp. *Sci. Rep.* 9 (1), 19702. doi: 10.1038/s41598-019-56170-y

Sundaresan, R., Parameshwaran, H. P., Yogesha, S. D., Keilbarth, M. W., and Rajan, R. (2017). Rna-Independent DNA Cleavage Activities of Cas9 and Cas12a. *Cell Rep.* 21 (13), 3728–3739. doi: 10.1016/j.celrep.2017.11.100

Tideman, P. A., Tirimacco, R., Senior, D. P., Setchell, J. J., Huynh, L. T., Tavella, R., et al. (2014). Impact of a Regionalised Clinical Cardiac Support Network on Mortality Among Rural Patients With Myocardial Infarction. *Med. J. Aust.* 200 (3), 157–160. doi: 10.5694/mja13.10645

van Dongen, J. E., Berendsen, J. T. W., Steenbergen, R. D. M., Wolthuis, R. M. F., Eijkel, J. C. T., and Segerink, L. I. (2020). Point-of-Care CRISPR/Cas Nucleic Acid Detection: Recent Advances, Challenges and Opportunities. *Biosens. Bioelectron.* 166, 112445. doi: 10.1016/j.bios.2020.112445

Vashist, S. K. (2017). Point-of-Care Diagnostics: Recent Advances and Trends. *Biosensors* 7, 62. doi: 10.3390/bios7040062. MDPI AG.

Wang, H., Li, X., Li, T., Zhang, S., Wang, L., Wu, X., et al. (2020). The Genetic Sequence, Origin, and Diagnosis of SARS-Cov-2. *Eur. J. Clin. Microbiol. Infect. Dis.* 1–7. doi: 10.1007/s10096-020-03899-4. Springer Science and Business Media Deutschland GmbH.

Wang, R., Qian, C., Pang, Y., Li, M., Yang, Y., Ma, H., et al. (2021). Opvcrispr: One-Pot Visual RT-LAMP-CRISPR Platform for SARS-Cov-2 Detection. *Biosens. Bioelectron.* 172, 112766. doi: 10.1016/j.bios.2020.112766

Wang, X., Zhong, M., Liu, Y., Ma, P., Dang, L., Meng, Q., et al. (2020). Rapid and Sensitive Detection of COVID-19 Using CRISPR/Cas12a-Based Detection With Naked Eye Readout, Crispr/Cas12a-Ner. *Sci. Bull.* 65 (17), 1436–1439. doi: 10.1016/j.scib.2020.04.041

WHO Coronavirus Disease (COVID-19) Dashboard | Who Coronavirus Disease (Covid-19) Dashboard. Available at: https://covid19.who.int/ (Accessed February 2nd, 2020).

Wu, X., Chan, C., Lee, Y. H., Springs, S. L., Lu, T. K., and Yu, H. (2020). A Digital Crispr-Based Method for the Rapid Detection and Absolute Quantification of Viral Nucleic Acids. *MedRxiv*. doi: 10.1101/2020.11.03.20223602

Zou, Y., Mason, M. G., Wang, Y., Wee, E., Turni, C., Blackall, P. J., et al. (2017). Nucleic Acid Purification From Plants, Animals and Microbes in Under 30 Seconds. *PloS Biol.* 15 (11), e2003916. doi: 10.1371/journal.pbio.2003916. Edited by Tom Misteli.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Ganbaatar and Liu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 24 May 2021
doi: 10.3389/fmed.2021.669552

Case Report: A Severe and Multi-Site *Nocardia farcinica* Infection Rapidly and Precisely Identified by Metagenomic Next-Generation Sequencing

Mengfan Jiao[1,2†], *Xiang Deng*[3†], *Hongfu Yang*[4], *Junqiang Dong*[5], *Jun Lv*[1*] and *Fang Li*[3*]

[1] Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Precision Medicine Center, Gene Hospital of Henan Province, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Department of Integrated Intensive Care Unit, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5] Department of Imaging and Nuclear Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Nocardia genus is an aerobic, gram-positive, and opportunistic pathogen, which mainly affects cell-mediated immunosuppressed patients. Early diagnosis and treatment greatly improve prognosis. However, the limitation of golden standard-bacterial culture exists. Here, we report a 61-year-old male with pneumonia, sepsis and intermuscular abscesses induced by *Nocardia farcinica*. Venous blood culture reported negative results. Former improper diagnosis and treatment did not improve his condition. With the assistant of metagenomic next-generation sequencing, the pathogen was identified as *Nocardia farcinica*. He was then applied with accurate treatment and had a remarkable clinical and radiological improvement.

Keywords: case report, *Nocardia*, metagenomics next-generation sequencing, precise treatment, infectious disease

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Chia-Jui Yang,
Far Eastern Memorial Hospital (FEMH), Taiwan
Limin Zhao,
Henan Provincial People's Hospital, China
Li-Ying Sun,
Capital Medical University, China

***Correspondence:**
Jun Lv
fcclvj@zzu.edu.cn
Fang Li
fcclif6@zzu.edu.cn

[†] *These authors share first authorship*

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *19 February 2021*
Accepted: *29 April 2021*
Published: *24 May 2021*

Citation:
Jiao M, Deng X, Yang H, Dong J, Lv J and Li F (2021) Case Report: A Severe and Multi-Site Nocardia farcinica Infection Rapidly and Precisely Identified by Metagenomic Next-Generation Sequencing. Front. Med. 8:669552. doi: 10.3389/fmed.2021.669552

INTRODUCTION

Nocardia genus is an aerobic and gram-positive rod bacterium which belongs to the class: *Actinobacteria*, order: *Actinomycetales*, Family: *Nocardiaceae* and is an opportunistic pathogen (1, 2). *Nocardia* is widely distributed in plants, gardens and soil, and can be divided into more than 80 *Nocardia* species, of which more than 50 species can cause human diseases (3, 4).

Nocardiosis is the infection caused by *Nocardia* species, to which cell-mediated immunosuppressed patients are more susceptible (3, 5). Occasionally, the infection may occur in normal people. Inhalation of a spore or fragment of broken hyphae results in pulmonary nocardiosis, which is the most common primary nocardiosis (6).

Clinical manifestation of nocardiosis is a variable spectrum which makes nocardiosis difficult to diagnose. The traditional diagnosis of Nocardiosis is culture but with some limitations such as limited sensitivity (7). In recent days, a new approach, metagenomic next-generation sequencing (mNGS), is increasingly applied with high sensitivity and specificity and advantages of identification rare and difficult-to-detect pathogens from clinical samples, presenting superiority in infectious diseases (8).

Here we present a case of pneumonia, sepsis, intermuscular abscesses, and pneumonia infected by *Nocardia farcinica* and was identified by mNGS. With the quick and high sensitivity diagnosis method, the patient received prompt treatment and finally recovered.

CASE DESCRIPTION

On October 20, 2020, a 61-years-old male patient from Henan province, China, was admitted to intensive care unit (ICU) of our hospital due to "swelling and pain of skin for 7 days and listless for 1 day." His blood pressure was 80/55 mgHg with the highest body temperature 38.4°C 1 day before. On examination, his neck, back, and left buttocks were multiple local tissue swelling and significant tenderness, with no skin damage and wound. His families reported no recent injury. He developed edema in his eyelids with a moon face and buffalo back.

Ten days before the admission, the patient had cough and yellow sputum but relieved. The magnetic resonance imaging (MRI) in the local hospital revealed multiple intermuscular abscesses on the neck, back, and left buttocks. The patient received treatment at the local hospital including carbapenem (detailed treatment remained unknown) but with no improvement even severer pain.

The patient had previously been diagnosed with diabetes for 2 months and nephrotic syndrome for 5 months, and had been taken corticosteroid drugs (prednisolone) and tacrolimus for 5 months.

The abnormal laboratory test results were as follows: leukocyte count, 14.4×10^9 cells/L; red blood cell count, 3.16×10^{12} cells/L; hemoglobin, 95 g/L; neutrophils, 13.42×10^9 cells/L; activated partial thromboplastin time, 19.6 s; fibrinogen, 4.48 g/L; D-Dimer, 3.18 mg/L; fibrin degradation product, 41.32 mg/L; PH, 7.471; total protein, 58 g/L; albumin, 30.5 g/L; Hemoglobin A1C, 9.6%; blood glucose, 13.4 mmol/L; Interleukin 6 8.76 pg/ml. A computed tomography (CT) scan thorax indicated bilateral interstitial pneumonia, bilateral interlobular septal thickening, small nodular shadow and pleural effusion in both lungs (**Figure 1**). Venous blood cultures for 5 days but reported no bacterial growth. Considering the patient's clinical manifestations, laboratory tests and the history of corticosteroid drugs use, this patient was firstly considered as the infection of opportunistic pathogens. A treatment with linezolid intravenous 0.6 g twice a day and piperacillin/tazobactam intravenous 4.5 g three times a day was initiated to control the infection.

At the same time, the blood sample was collected to perform metagenomic next-generation sequencing (mNGS) on the first day and detected *Nocardia farcinica* on the second day (**Table 1**). Taking into account the patient's clinical manifestations, history of glucocorticoid use, and the mNGS test results, the patient was diagnosed with *Nocardia* infection. Then anti-infective therapy was changed into compound trimethoprim-sulfamethoxazole (TMP-SMX) tablets 2 pills twice a day, intravenous linezolid, and piperacillin/tazobactam. After consultation in the nephrology department, tacrolimus was discontinued and corticosteroid drugs were gradually discontinued.

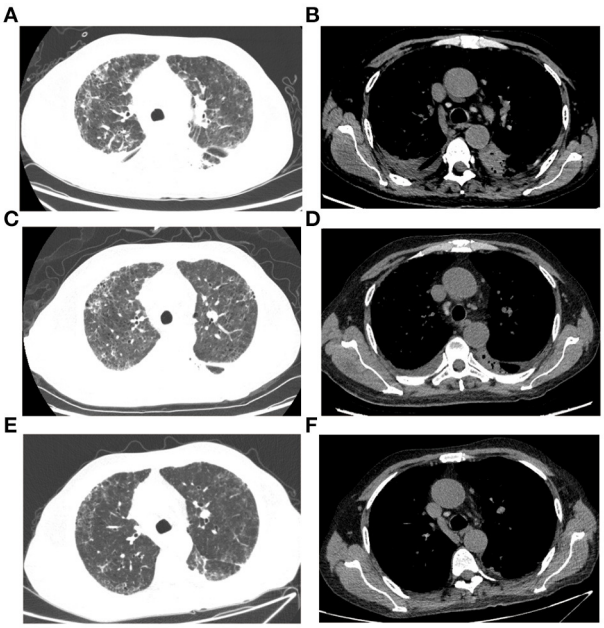


FIGURE 1 | Chest CT imaging of the patient. **(A,B)** CT imaging on October 22, 2020; **(C,D)** CT imaging on November 3, 2020; **(E,F)** CT imaging on December 6, 2020.

Four days after the usage of TMP-SMX, the patient's temperature and leukocyte returned to normal and his symptoms improved significantly.

On the 7th day, bronchoalveolar lavage fluid (BALF) was collected for mNGS and suggested two bacteria, *Pseudomonas aeruginosa* and *Nocardia farcinica* (**Table 2**). On the 13th day, bronchoscopy showed that a moderate amount of thin secretion in the bronchus of the lower left lobe (**Figure 2**). On the 14th day, the puncture was performed under ultrasound guidance (**Figure 2**). Cyst puncture fluid culture for 2 days reported negative results. The patient's swelling and pain of the skin improved and pulmonary infection and sepsis were gradually under control. His mental state was markedly improved. Also, the laboratory tests and CT scan were in remission (**Figure 1**). He was discharged after 16 days of treatment. On December 6, 2020, 1 month after the discharge, the patient was admitted again for the review. CT scan showed the disappearance of purulent secretion (**Figure 1**). He made a great recovery. The patient's timeline was shown (**Figure 3**).

DISCUSSION

The common type species of *Nocardia* include *Nocardia asteroides, Nocardia brasiliensis*, *Nocardia farcinica*, *Nocardia otitidiscaviarum*, etc., all of which may cause a variety of diseases in both humans and animals. *Nocardia* is an opportunistic pathogen and mainly affects immunosuppression patients (3, 5), such as patients with acquired immune deficiency syndrome (AIDS) (9), chronic granulomatous disease (10, 11),

TABLE 1 | Pathogenic microorganism identified in blood sample.

	Genus		Species		
Type	**Name**	**Sequence number**	**Name**	**Sequence number**	**Relative abundance**
G+	*Nocardia*	188	*Nocardia farcinica*	129	2.667%

TABLE 2 | Pathogenic microorganism identified in BALF.

	Genus		Species		
Type	**Name**	**Sequence number**	**Name**	**Sequence number**	**Relative abundance**
G–	*Pseudomonas*	621	*Pseudomonas aeruginosa*	503	40.38%
G+	*Nocardia*	17	*Nocardia farcinica*	11	0.745%

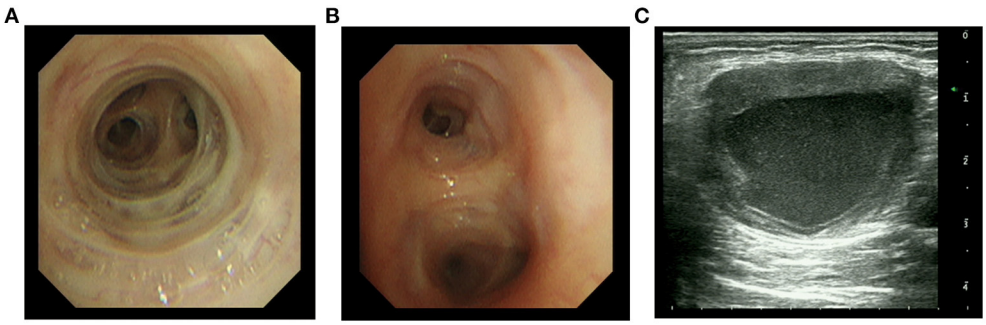


FIGURE 2 | Bronchoscopy and ultrasound imaging of an abscess. **(A,B)** Right middle and left lower bronchoscopy, respectively. **(C)** Ultrasound imaging of an abscess in the left upper arm.

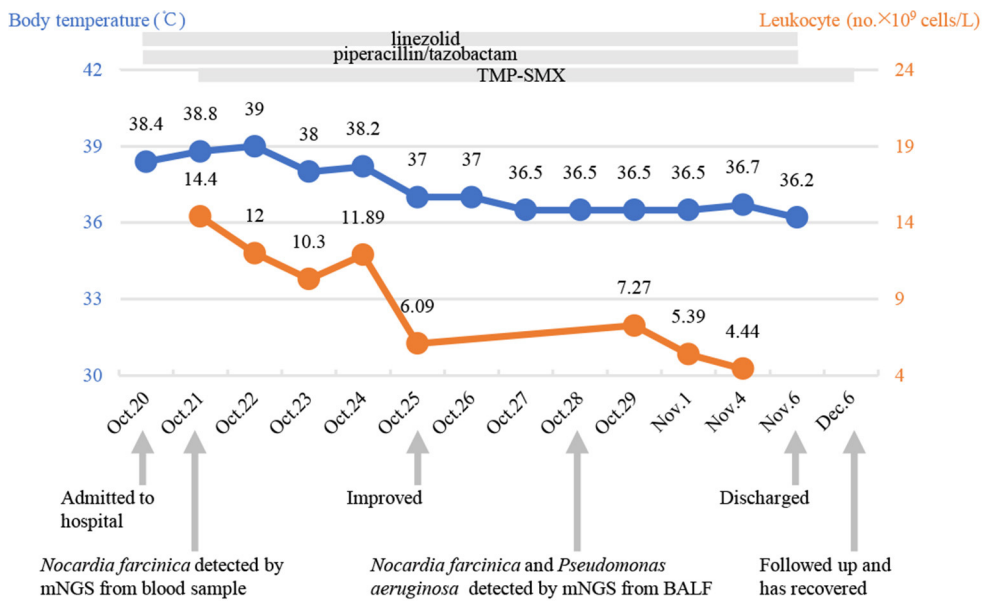


FIGURE 3 | Timeline with relevant data from the case in our hospital; curves of body temperature and leukocyte counts. The arrows below indicate major events. Blue line shows body temperature values. Orange line shows leukocyte counts. TMP-SMX, trimethoprim-sulfamethoxazole; mNGS, metagenomic next-generation sequencing.

transplant recipients (12, 13), and with drug-induced systemic immunosuppression (14). It is also found that one-third of nocardiosis patients are immunocompetent (15).

Pulmonary nocardiosis is the most common primary infection, through the inhalation of airborne spores or mycelial fragments from the environment, and is more common in immunosuppression individuals (16). Pulmonary nocardiosis can present as cough, fever, chest pain, and is similar with other pulmonary infection (6). Chest radiographic manifestations are variable, showing as large irregular nodules, nodules, pleural effusions (17), etc., in which pleural effusions account for one-third of all the manifestations (5). Other common *Nocardia* infections have also been reported as central nervous system (CNS) nocardiosis (18) and cutaneous nocardiosis (1, 19) and may be the secondary infection of pulmonary nocardiosis through hematogenous dissemination in immunosuppression patients (20). Primary cutaneous infections tends more common in immunocompetent patient due to the open injury (3). This patient firstly presented respiratory symptoms 10 days prior the admission and then developed skin swelling and pain, when was 7 days before the admission. This patient had taken corticosteroid drugs and tacrolimus for 5 months and he had not suffered open injury. Thus, we considered pulmonary nocardiosis as primary infection for this patient. It would be perfect if the intermuscular abscesses specimen was performed with mNGS. However, the cost of mNGS was a burden to this patient. Moreover, we considered the intermuscular abscesses were the result of primary pulmonary nocardiosis through hematogenous dissemination. Therefore, the necessity of intermuscular abscesses for mNGS could be limited.

The traditional diagnostic method of nocardiosis is culture. A culture of *Nocardia* usually takes 2–7 days, which may delay establishing the diagnosis. Moreover, most patients with nocardiosis may receive antibiotic treatment before samples collection, reducing microbial culture sensitivity (21). In 2005, 16S rRNA–based polymerase chain reaction (PCR) assay was reported to detect *Nocardia* on clinical samples, with high sensitivity and specificity (22), whereas the method requires the pre-considerations of *Nocardia* infection by clinicians. Metagenomic next-generation sequencing (mNGS) is a new approach with characteristics of high sensitivity, rapid detection and less affected by prior antibiotics usage (23, 24). This is an unbiased method and can theoretically detect all kinds of pathogens, which is suitable for difficult and atypical infectious diseases (24). The former study reported mean time to diagnosis was 42 days, which may lead to poor clinical prognosis especially in immunosuppression patients (14). In this case, mNGS only took 2 days to detect pathogens and significantly reduced hospital days. A recent study revealed that 14 samples were identified to be *Nocardia* spp. positive by mNGS, whereas only five of them obtained positive results of culture (25). This patient's negative blood culture may due to the influence of prior antibiotics usage.

mNGS also has its limitation. The expensive cost is a burden to some patients, which was one of the reasons that the intermuscular abscesses specimen was not performed with mNGS in this case. Moreover, the time of sample collection may influence the sensitivity. A former study indicated the false negative of mNGS in *Angiostrongylus cantonensis* detection, which maybe as the result of the improper sampling time during the life cycle of parasites (26). For pulmonary infection, sample types may also influence the sensitivity (27).

Treatment of nocardiosis is individualized (28). Sulfonamides were initially used as nocardiosis treatment (29). The most common treatment for *Nocardia* nowadays is trimethoprim-sulfamethoxazole (TMP-SMX) (5, 29). Linezolid and carbapenem are also used in nocardiosis (7, 30). In this case, previously carbapenem use did not control the infection, whereas treatment of TMP-SMX and linezolid provided symptomatic improvement.

Pseudomonas aeruginosa was also detected by mNGS in BALF. Carbapenem was applied at local hospital but without response. It could be deduced that the *Pseudomonas aeruginosa* of the patient responded less sensitive to carbapenem. There was another possibility that *Pseudomonas aeruginosa* was hospital-acquired infections in ICU of our hospital. Before the detection of *Pseudomonas aeruginosa,* the therapy of linezolid, piperacillin/tazobactam and TMP-SMX greatly relieved the patient's symptoms. Actually, linezolid and piperacillin/tazobactam also had clinical efficiency with *Pseudomonas aeruginosa* (31). Therefore, the former treatment was not changed and then patient completely recovered. *Pseudomonas aeruginosa* in this case may be more sensitive to piperacillin/tazobactam, or a combination of these drugs.

CONCLUSIONS

mNGS identified *Nocardia farcinica* of the patient and the appliance of accurate treatment made him complete recovery. mNGS, a new approach with high sensitivity and rapid detection, could assist to identify clinical infection disease pathogens, especially in culture-negative infections. We look forward that mNGS can provide more support for clinical diagnosis and treatment.

DATA AVAILABILITY STATEMENT

MNGS was performed at Illumina NextSeq 550 platform in Gene Hospital of Henan Province. Metagenomics whole genome shotgun sequences have been uploaded to EMBL. SRA accession: PRJEB43515.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board of the First Affiliated Hospital of Zhengzhou University. The ethics approval number is 2019-KY-330. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MJ, FL, and JL analyzed and interpreted patient data. XD, HY, and JD performed the experiments. MJ and FL analyzed the genomics data. MJ and XD wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was equally funded and supported by Chinese National Science and Technology Major Project 2018ZX10305410 and Beijing Health Alliance Charitable Foundation TB704016.

REFERENCES

1. Mcneil MM, Brown JM. The medically important aerobic actinomycetes: epidemiology and microbiology. *Clin Microbiol Rev.* (1994) 7:357–417. doi: 10.1128/CMR.7.3.357
2. Conville PS, Witebsky FG. The complexity of nocardia taxonomy: implications for the clinical microbiology laboratory. *Clin Microbiol Newslett.* (2010) 32:119–25. doi: 10.1016/j.clinmicnews.2010.07.004
3. Lerner PI. Nocardiosis. *Clin Infect Dis.* (1996) 22:891–905. doi: 10.1093/clinids/22.6.891
4. Rathish BZP. *Nocardia*. (2020). Available online at: https://www.ncbi.nlm.nih.gov/books/NBK560872/ (accessed September 29, 2020).
5. Wilson J. Nocardiosis: updates and clinical overview. *Mayo Clin Proc.* (2012) 87:403–7. doi: 10.1016/j.mayocp.2011.11.016
6. Brown-Elliott B, Brown J, Conville P, Wallace R. Clinical and laboratory features of the *Nocardia* spp. based on current molecular taxonomy. *Clin Microbiol Rev.* (2006) 19:259–82. doi: 10.1128/CMR.19.2.259-282.2006
7. Welsh O, Vera-Cabrera L, Salinas-Carmona M. Current treatment for nocardia infections. *Exp Opin Pharmacother.* (2013) 14:2387–98. doi: 10.1517/14656566.2013.842553
8. Han D, Li Z, Li R, Tan P, Zhang R, Li J. mNGS in clinical microbiology laboratories: on the road to maturity. *Crit Rev Microbiol.* (2019) 45:668–85. doi: 10.1080/1040841X.2019.1681933
9. Long PF. A retrospective study of *Nocardia* infections associated with the acquired immune deficiency syndrome (AIDS). *Infection*. (1994) 22:362–4. doi: 10.1007/BF01715551
10. Dorman SE, Guide SV, Conville PS, Decarlo ES, Malech HL, Gallin JI, et al. Nocardia infection in chronic granulomatous disease. *Clin Infect Dis.* (2002) 35:390–4. doi: 10.1086/341416
11. Jonsson S, Wallace RJ Jr, Hull SI, Musher DM. Recurrent *Nocardia* pneumonia in an adult with chronic granulomatous disease. *Am Rev Respir Dis.* (1986) 133:932–4.
12. Lebeaux D, Morelon E, Suarez F, Lanternier F, Scemla A, Frange P, et al. Nocardiosis in transplant recipients. *Eur J Clin Microbiol Infect Dis.* (2014) 33:689–702. doi: 10.1007/s10096-013-2015-5
13. Chouciño C, Goodman SA, Greer JP, Stein RS, Wolff SN, Dummer JS. Nocardial infections in bone marrow transplant recipients. *Clin Infect Dis.* (1996) 23:1012–9. doi: 10.1093/clinids/23.5.1012
14. Martínez Tomás R, Menéndez Villanueva R, Reyes Calzada S, Santos Durantez M, Vallés Tarazona JM, Modesto Alapont M, et al. Pulmonary nocardiosis: risk factors and outcomes. *Respirology.* (2007) 12:394–400. doi: 10.1111/j.1440-1843.2007.01078.x
15. Beaman BL, Burnside J, Edwards B, Causey W. Nocardial infections in the United States, 1972-1974. *J Infect Dis.* (1976) 134:286–9. doi: 10.1093/infdis/134.3.286
16. Saubolle MA, Sussland D. Nocardiosis: review of clinical and laboratory experience. *J Clin Microbiol.* (2003) 41:4497–501. doi: 10.1128/JCM.41.10.4497-4501.2003
17. Feigin DS. Nocardiosis of the lung: chest radiographic findings in 21 cases. *Radiology.* (1986) 159:9–14. doi: 10.1148/radiology.159.1.3952335
18. Anagnostou T, Arvanitis M, Kourkoumpetis TK, Desalermos A, Carneiro HA, Mylonakis E. Nocardiosis of the central nervous system: experience from a general hospital and review of 84 cases from the literature. *Medicine.* (2014) 93:19–32. doi: 10.1097/MD.0000000000000012
19. Lopes JO, Bassanesi MC, Alves SH, Salla A, Benevenga JP, Castro MS. Cutaneous *Nocardia* asteroides infection of nontraumatic origin. *Rev Inst Med Trop São Paulo.* (1994) 36:403–8. doi: 10.1590/S0036-46651994000500003
20. Kontoyiannis DP, Ruoff K, Hooper DC. Nocardia bacteremia. Report of 4 cases and review of the literature. *Medicine.* (1998) 77:255–67. doi: 10.1097/00005792-199807000-00004
21. Rouzaud C, Rodriguez-Nava V, Catherinot E, Méchaï F, Bergeron E, Farfour E, et al. Clinical assessment of a *Nocardia* PCR-based assay for diagnosis of nocardiosis. *J Clin Microbiol.* (2018) 56:e00002–18. doi: 10.1128/JCM.00002-18
22. Couble A, Rodríguez-Nava V, De Montclos MP, Boiron P, Laurent F. Direct detection of *Nocardia* spp. in clinical samples by a rapid molecular method. *J Clin Microbiol.* (2005) 43:1921–4. doi: 10.1128/JCM.43.4.1921-1924.2005
23. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis.* (2018) 67:S231–40. doi: 10.1093/cid/ciy693
24. Goldberg B, Sichtig H, Geyer C, Ledeboer N, Weinstock GM. Making the leap from research laboratory to clinic: challenges and opportunities for next-generation sequencing in infectious disease diagnostics. *mBio.* (2015) 6:e01888. doi: 10.1128/mBio.01888-15
25. Weng S-S, Zhang H-Y, Ai J-W, Gao Y, Liu Y-Y, Xu B, et al. Rapid detection of *Nocardia* by next-generation sequencing. *Front Cell Infect Microbiol.* (2020) 10:13. doi: 10.3389/fcimb.2020.00013
26. Feng L, Zhang A, Que J, Zhou H, Wang H, Guan Y, et al. The metagenomic next-generation sequencing in diagnosing central nervous system angiostrongyliasis: a case report. *BMC Infect Dis.* (2020) 20:691. doi: 10.1186/s12879-020-05410-y
27. Wang Q, Wu B, Yang D, Yang C, Jin Z, Cao J, et al. Optimal specimen type for accurate diagnosis of infectious peripheral pulmonary lesions by mNGS. *BMC Pulm Med.* (2020) 20:268. doi: 10.1186/s12890-020-01298-1
28. Muñoz J, Mirelis B, Aragón LM, Gutiérrez N, Sánchez F, Español M, et al. Clinical and microbiological features of nocardiosis 1997-2003. *J Med Microbiol.* (2007) 56:545–50. doi: 10.1099/jmm.0.46774-0
29. Mcneil MM, Brown JM, Hutwagner LC, Schiff TA. Evaluation of therapy for *Nocardia* asteroides complex infections. *Infect Dis Clin Pract.* (1995) 4, 287–92. doi: 10.1097/00019048-199507000-00011
30. Moylett EH, Pacheco SE, Brown-Elliott BA, Perry TR, Buescher ES, Birmingham MC, et al. Clinical experience with linezolid for the treatment of nocardia infection. *Clin Infect Dis.* (2003) 36:313–8. doi: 10.1086/345907
31. Driscoll JA, Brody SL, Kollef MH. The epidemiology, pathogenesis and treatment of pseudomonas aeruginosa infections. *Drugs.* (2007) 67:351–68. doi: 10.2165/00003495-200767030-00003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Jiao, Deng, Yang, Dong, Lv and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 11 June 2021
doi: 10.3389/fmed.2021.643473

Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of *Legionella pneumophila* Pneumonia in a Patient After Umbilical Cord Blood Stem Cell Transplantation

Yangyan Wang[1], *Yuanyuan Dai*[2], *Huaiwei Lu*[2], *Wenjiao Chang*[2], *Fan Ma*[1], *Ziran Wang*[3], *Zhican Liu*[4] *and Xiaoling Ma*[1,2*]

[1] Department of Clinical Laboratory, Affiliated Provincial Hospital of Anhui Medical University, Hefei, China, [2] Department of Clinical Laboratory, First Affiliated Hospital of University of Science and Technology of China, Hefei, China, [3] Department of Clinical Laboratory, Peking Union Medical College Hospital, Beijing, China, [4] Nanjing Medical laboratory, Beijing Genomics Institute, Nanjing, China

OPEN ACCESS

Edited by:
George Weinstock,
Jackson Laboratory for Genomic Medicine, United States

Reviewed by:
Alberto Cagigi,
Karolinska Institutet (KI), Sweden
Beiwen Zheng,
Zhejiang University, China

***Correspondence:**
Xiaoling Ma
maxiaoling@USTC.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 21 January 2021
Accepted: 30 April 2021
Published: 11 June 2021

Citation:
Wang Y, Dai Y, Lu H, Chang W, Ma F, Wang Z, Liu Z and Ma X (2021) Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of Legionella pneumophila Pneumonia in a Patient After Umbilical Cord Blood Stem Cell Transplantation. Front. Med. 8:643473. doi: 10.3389/fmed.2021.643473

We report a case of hospital-acquired *Legionella* pneumonia that was detected by metagenomic next-generation sequencing (mNGS) of blood from a 7-year-old girl after umbilical cord blood stem cell transplantation (UCBT) with myelodysplastic syndrome. UCBT is traditionally associated with an increased risk of infection, particularly during the first 3 months after transplantation. Controlling interstitial pneumonia and severe infection is the key to reducing patient mortality from infection. *Legionella pneumophila* can cause a mild cough to rapidly fatal pneumonia. After mNGS confirmed that the pathogen was *L. pneumophila*, azithromycin, cefoperazone sulbactam, and posaconazole were used for treatment, and the patient's temperature decreased and remained normal. The details of this case highlight the benefits of the timely use of metagenomic NGS to identify pathogens for the survival of immunocompromised patients.

Keywords: metagenomic next-generation sequencing, *Legionella pneumophila*, myelodysplastic syndrome, umbilical cord blood stem cell transplantation, hospital acquired

INTRODUCTION

Legionella, which is widely found in the natural environment, including in water sources and soil, includes 58 species and 3 subspecies (1). More than 90% of legionellosis is caused by *L. pneumophila*, of which Lp1 is the most common strain (1–4). Mortality from *Legionella* depends on the severity of the disease, the suitability of the initial antimicrobial treatment, the location where the *Legionella* infection was contracted, and host factors (2, 5). Immunocompromised patients are the most susceptible hosts. *Legionella* is a commonly misdiagnosed pathogen that causes severe hospital-acquired pneumonia, and multiple studies have shown it to be both underdiagnosed and undertreated (2). But mNGS technology based on high-throughput sequencing has been widely studied for use as a non-targeted and broad-spectrum pathogen screening technology for identifying clinically moderate and severe infections (6, 7). Here, we report the first case of an umbilical-cord blood stem cell patient with pulmonary *L. pneumophila* infection who was diagnosed by mNGS before the blood culture results were obtained.

CASE DESCRIPTION

A 7-year-old girl with refractory anemia with excess blasts and type 2 myelodysplastic syndrome was admitted to the hematology department in March 2019, and blood analysis showed that the number of primordial blood cells had reached 19%. The patient was treated with allogeneic cord blood stem cell transplantation under electrocardiographic monitoring; 15 days after transplantation, the patient's neutrophils exceeded 0.5 $\times$ 10^9/L, and thus she was transferred to the general ward for further treatment.

Thirty-nine days after transplantation, the patient developed a high fever, and her blood neutrophil count and inflammation indicators increased (**Figure 1**). Therefore, hematologists treated the patient with imipenem, cilastatin, amikacin, linezolid, and liposomal amphotericin B. After 1 week of treatment, the patient's body temperature had not returned to normal, and CT showed double pneumonia, in which the upper lobe of the left lung was consolidated (**Figure 2A**). We sent the patient's blood samples for mNGS, and 8,112 raw reads belonged to *L. pneumophila* (**Figure 3A**). The anti-infection regimen was changed to azithromycin combined with cefoperazone, sulbactam, and posaconazole on May 10, 2019 (**Figure 1**). We then tested the urine for *Legionella* antigen and sent the bronchoalveolar lavage fluid (BALF) for Gram staining, mNGS, and culture on *Legionella* MWY selective agar. The phagocytosis of gram-negative bacilli by leukocytes in BALF was observed under a microscope (**Figures 4A,B**). The number of raw reads of *L. pneumophila* in BALF was 1,960 (**Figure 3B**). Six days later, several off-white colonies were visible on the MWY

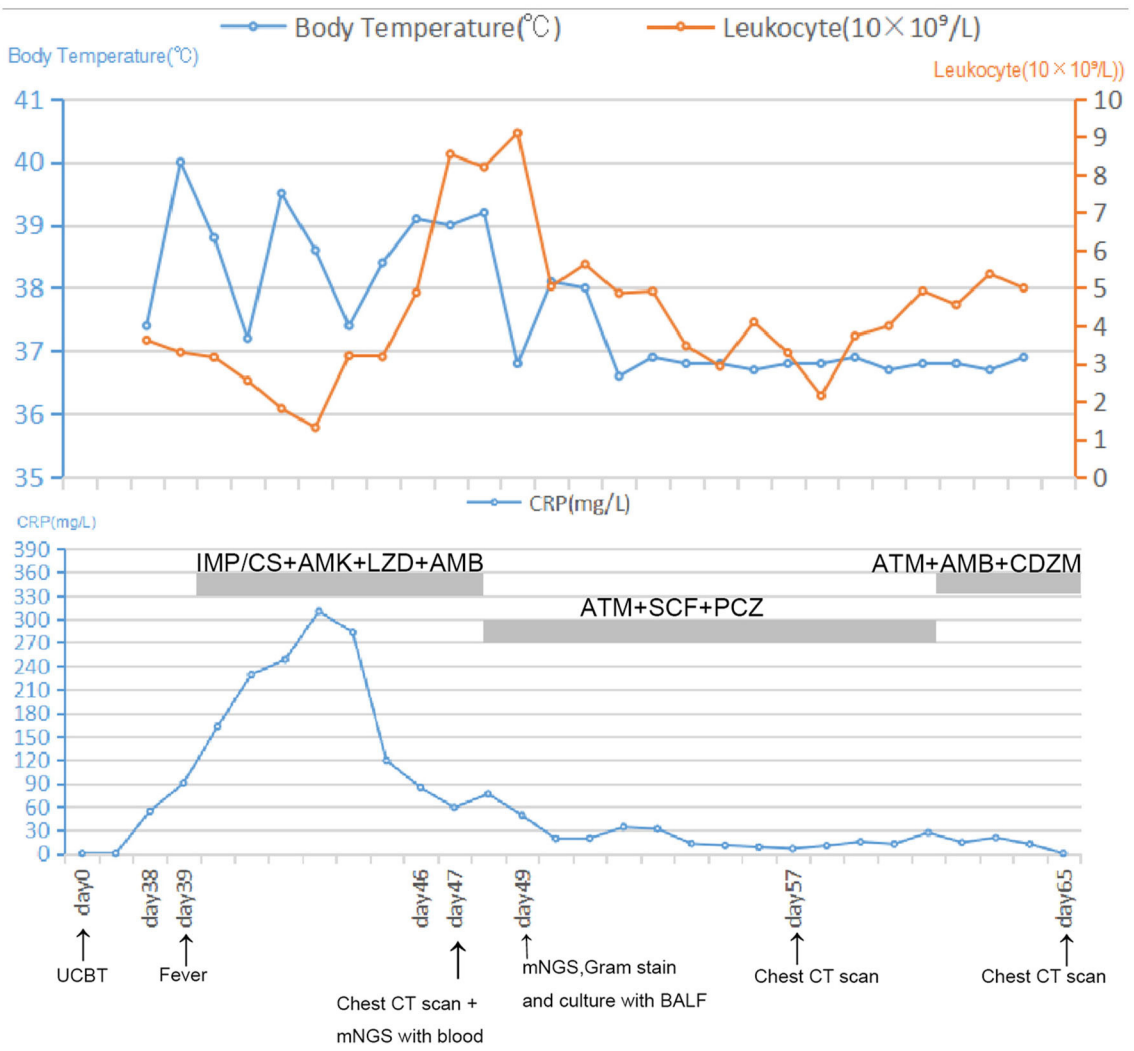


FIGURE 1 | Timeline with relevant data from the episode of care; body temperature curves, leukocyte counts, and CRP. Major events are indicated with arrows. Yellow line shows the leukocyte counts in peripheral blood. Blue line on the top shows body-temperature values. Blue line on the bottom shows CRP values. Horizontal thick gray lines show the medications administered: IMP/CS, Imipenem and cilastatin sodium; AMK, Amikacin; LZD, Linezolid; AMB, Amphotericin B; ATM, Azithromycin; SCF, Sulbactam and cefoperazone sodium; PCZ, Posaconazole; CDZM, Cefodizime.

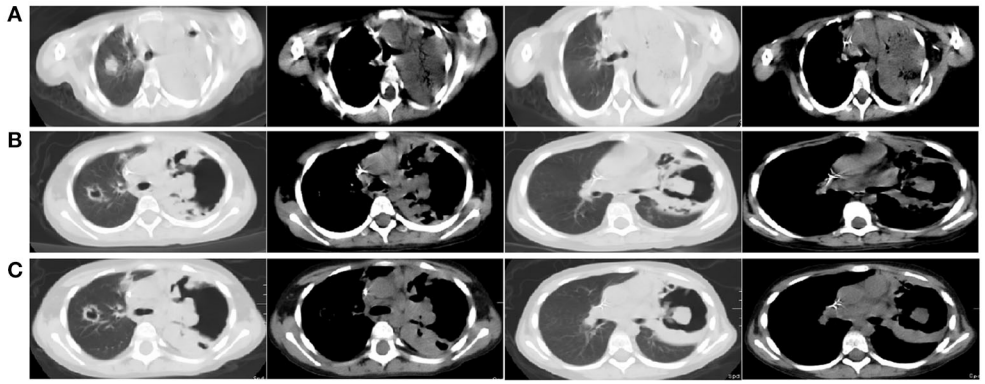


FIGURE 2 | (A) Chest CT scan showing double pneumonia: the upper lobe of the left lung was consolidated; **(B)** Chest CT scan showing inflammation in both lungs after 10 days of treatment with azithromycin; **(C)** Chest CT scan revealed that multiple lung lesions were absorbed 18 days later.

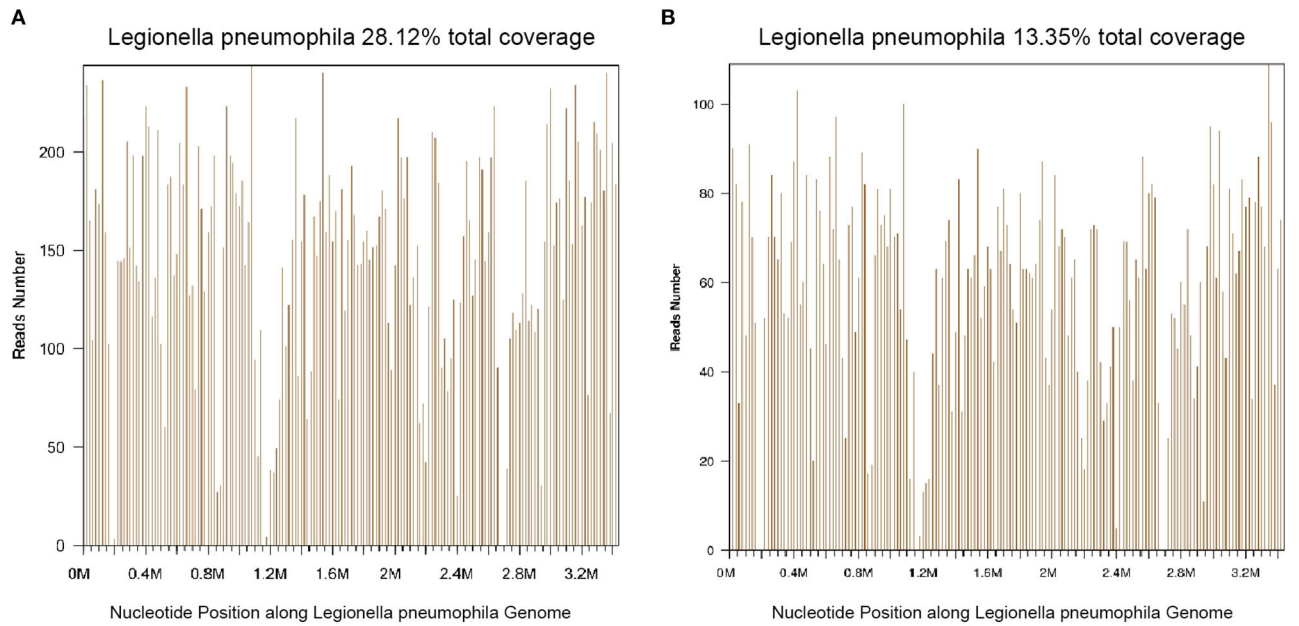


FIGURE 3 | Diagnosis of *Legionella pneumophila* infection using mNGS. **(A)** The majority of reads mapped to the *L. pneumophila* genome with coverage of 28.12%. **(B)** The majority of reads mapped to the *L. pneumophila* genome with coverage of 13.35%.

medium (**Figure 4C**). After Gram staining of the colonies, a large number of gram-negative bacilli were observed under microscopy (**Figure 4D**). The off-white colonies grown on the MWY medium were subsequently identified as *L. pneumophila* using time-of-flight mass spectrometry. The patient's body temperature returned to normal after 1 day of treatment with azithromycin and remained normal for 1 week (**Figure 1**), but 10 days after initiating azithromycin treatment, a CT scan still showed inflammation in both lungs (**Figure 2B**). A CT scan revealed that multiple lung lesions were absorbed 18 days later (**Figure 2C**). Finally, the patient's general condition improved after follow-up treatment, and she was discharged from hospital on June 3.

DISCUSSION

The most susceptible hosts of hospital-acquired *Legionella* pneumonia are immunocompromised patients, including organ transplant recipients (8) and cancer patients (9), as well as those receiving glucocorticoid therapy. Multiple studies have shown *Legionella* infections to be underdiagnosed and undertreated (1, 10, 11). In this case report, the patient was fortunate to be treated in time and was discharged from the hospital in good health.

There are many kinds of *Legionella* detection techniques. The immunological methods are limited by the patient's immune status and cross-reactions with other pathogens (12), and at present, this technology can only detect *L. pneumophila*

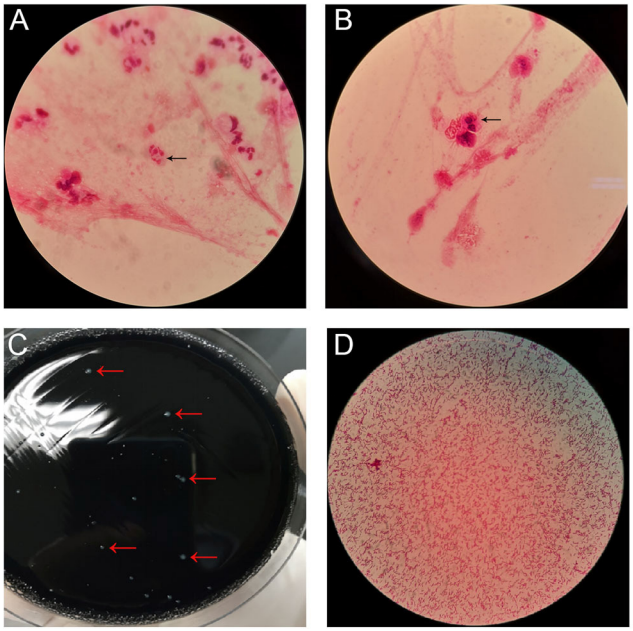


FIGURE 4 | (A,B) Gram stain of bronchoalveolar lavage fluid (BALF) with arrows indicating *Legionella pneumophila*, magnification ×1,000. **(C)** *L. pneumophila* colonies on MWY selective agar (red arrows) from BALF. **(D)** Gram stain of *L. pneumophila* colonies (magnification ×1,000).

serogroups 1, 3, and 6 (13). For immunocompromised patients, it is important to identify the infectious agent as early as possible in the evolution of the disease. The culture of *Legionella* species from respiratory specimens is the gold standard for the diagnosis of *Legionella* pneumonia (14); however, a *Legionella* culture requires more than 3 days and a medium containing L-cysteine (15, 16). When the patient is in the acute infection phase, samples should be quickly transported to the laboratory for culture, preferably before initiating antimicrobial therapy. The diagnosis of Legionnaires' disease is confirmed by means of culture in only 5% of cases (1). The widely used time-of-flight mass spectrometry detection method can only be used to identify bacterial species after the colonies are cultivated by traditional methods. Thus, the turn-around time is too long for critically ill patients. Obviously, the culture results in this report were obtained very much later than the mNGS results. Other techniques for detecting *Legionella* nucleic acids include PCR (1), isothermal amplification (17), probe hybridization (18), and second-generation sequencing.

This is the first report describing the use of mNGS to detect hospital-acquired *Legionella* pneumonia in a patient after umbilical cord blood transplantation. NGS has unique advantages for the detection of pathogens that are difficult to cultivate (19), especially for cases without target pathogens (20, 21). In addition, mNGS is extremely suitable for detecting unknown pathogens (22, 23), rare pathogens (24, 25), and between-species transmitted pathogens (26, 27). Of course, there are also shortcomings, such as high testing costs, analytical sensitivity, a complex laboratory workflow, and susceptibility to contamination (28). In this case, the mNGS provided directions for choosing traditional diagnosis methods, and the cultured colonies and immunological results were consistent with the mNGS. Although mNGS is relatively expensive, the patient's family considered it worthwhile to identify the pathogen as early as possible to reduce the patient's symptoms. We believe that, with the reductions in sequencing costs and the continuous improvements in medical standards in China, mNGS-based pathogenic diagnosis can be increasingly used to greatly improve the pertinence and timeliness of clinical pathogenic treatment.

DATA AVAILABILITY STATEMENT

The datasets for this study can be found in the NCBI https://www.ncbi.nlm.nih.gov/bioproject/PRJNA722154 under accession number PRJNA722154.

ETHICS STATEMENT

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YW and YD designed the study. HL, WC, and ZW helped collect data. ZL performed the statistical analysis. FM and XM revised the manuscript, which was written by YW. All authors read and approved the final manuscript.

FUNDING

This paper was supported by National Natural Science Foundation of China (Grant Number: 81972001).

ACKNOWLEDGMENTS

We thank Suzanne Leech, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

REFERENCES

1. Cunha BA, Burillo A, Bouza E. Legionnaires' disease. *Lancet.* (2016) 387:376–85. doi: 10.1016/S0140-6736(15)60078-2
2. Chahin A, Opal SM. Severe pneumonia caused by legionella pneumophila: differential diagnosis and therapeutic considerations. *Infect Dis Clin North Am.* (2017) 31:111–21. doi: 10.1016/j.idc.2016.10.009
3. Yu VL, Plouffe JF, Pastoris MC, Stout JE, Schousboe M, Widmer A, et al. Distribution of Legionella species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. *J Infect Dis.* (2002) 186:127–8. doi: 10.1086/341087
4. Beaute J, Plachouras D, Sandin S, Giesecke J, Sparen P. Healthcare-associated legionnaires' disease, Europe, 2008–2017. *Emerg Infect Dis.* (2020) 26:2309–18. doi: 10.3201/eid2610.181889

5. Heath CH, Grove DI, Looke DF. Delay in appropriate therapy of Legionella pneumonia associated with increased mortality. *Eur J Clin Microbiol Infect Dis.* (1996) 15:286–90. doi: 10.1007/BF01695659
6. Grumaz S, Stevens P, Grumaz C, Decker SO, Weigand MA, Hofer S, et al. Next-generation sequencing diagnostics of bacteremia in septic patients. *Genome Med.* (2016) 8:73. doi: 10.1186/s13073-016-0326-8
7. Xie Y, Du J, Jin W, Teng X, Cheng R, Huang P, et al. Next generation sequencing for diagnosis of severe pneumonia: China, 2010–2018. *J Infect.* (2019) 78:158–69. doi: 10.1016/j.jinf.2018.09.004
8. Servais S, Hannon M, Peffault De Latour R, Socie G, Beguin Y Reconstitution of adaptive immunity after umbilical cord blood transplantation: impact on infectious complications. *Stem Cell Investig.* (2017) 4:40. doi: 10.21037/sci.2017.05.03
9. Ehret KM, Chamberlain AT, Berkelman RL, Fridkin SK Preventing hospital-acquired Legionnaires' disease: A snapshot of clinical practices and water management approaches in US acute-care hospitals. *Infect Control Hosp Epidemiol.* (2018) 39:1470–2. doi: 10.1017/ice.2018.240
10. Spiegelman J, Pedutem T, Francisco MJ. Legionnaires' disease cases at a large community hospital-common and underdiagnosed. *Int J Environ Res Public Health.* (2020) 17:332. doi: 10.3390/ijerph17010332
11. Sreenath K, Dey AB, Kabra SK, Thakur B, Guleria R, Chaudhry R. Legionella pneumophila in Patients with Pneumonia at a Referral Hospital, New Delhi, India, 2015–2020. *Am J Trop Med Hyg.* (2020) 104:854–60. doi: 10.4269/ajtmh.20-0653
12. Edelstein PH, Meyer RD, Finegold SM. Laboratory diagnosis of Legionnaires' disease. *Am Rev Respir Dis.* (1980) 121:317–27.
13. Elverdal PL, Svarrer CW, Jorgensen CS, Skovsted IC, Uldum SA. Development and validation of ELISA for detection of antibodies to Legionella pneumophila serogroup 1, 3 and 6 in human sera. *J Microbiol Methods.* (2011) 86:298–303. doi: 10.1016/j.mimet.2011.05.022
14. Fields BS, Benson RF, Besser RE. Legionella and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev.* (2002) 15:506–26. doi: 10.1128/CMR.15.3.506-526.2002
15. Fard SY, Nomanpour B, Fatolahzadeh B, Mobarez AM, Darban-Sarokhalil D, Fooladi AA, et al. Hospital acquired pneumonia: comparison of culture and real-time PCR assays for detection of Legionella pneumophila from respiratory specimens at Tehran hospitals. *Acta Microbiol Immunol Hung.* (2012) 59:355–65. doi: 10.1556/amicr.59.2012.3.6
16. Mayock R, Skale B, Kohler RB. Legionella pneumophila pericarditis proved by culture of pericardial fluid. *Am J Med.* (1983) 75:534–6. doi: 10.1016/0002-9343(83)90362-5
17. Olabarria G, Eletxigerra U, Rodriguez I, Bilbao A, Berganza J, Merino S. Highly sensitive and fast Legionella spp. in situ detection based on a loop mediated isothermal amplification technique combined to an electrochemical transduction system. *Talanta.* (2020) 217:121061. doi: 10.1016/j.talanta.2020.121061
18. Nuthong B, Wilailuckana C, Tavichakorntrakool R, Boonsiri P, Daduang S, Bunyaraksyotin G, et al. One step for Legionella pneumophila detection in environmental samples by DNA-gold nanoparticle probe. *J Appl Microbiol.* (2018) 125:1534–40. doi: 10.1111/jam.14047
19. Kolb M, Lazarevic V, Emonet S, Calmy A, Girard M, Gaia N, et al. Next-generation sequencing for the diagnosis of challenging culture-negative endocarditis. *Front Med.* (2019) 6:203. doi: 10.3389/fmed.2019.00203
20. Lin Y, Wang BX, Zhang NN, Zhang L, Gao ZB, Tian J, et al. Metagenomic analysis identified stenotrophomonas maltophilia pneumonia in an infant suffering from unexplained very severe pneumonia. *Front Pediatr.* (2019) 7:380. doi: 10.3389/fped.2019.00380
21. Xiao N, Gai W, Hu WG, Li JX, Zhang Y, Zhao XY. Next-generation-sequencing technology used for the detection of Mycoplasma hominis in renal cyst fluid: a case report. *Infect Drug Resist.* (2019) 12:1073–9. doi: 10.2147/IDR.S198678
22. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature.* (2020) 579:265–9. doi: 10.1038/s41586-020-2008-3
23. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* (2020) 579:270–3. doi: 10.1038/s41586-020-2012-7
24. Wilson MR, Shanbhag NM, Reid MJ, Singhal NS, Gelfand JM, Sample HA, et al. Diagnosing balamuthia mandrillaris encephalitis with metagenomic deep sequencing. *Ann Neurol.* (2015) 78:722–30. doi: 10.1002/ana.24499
25. Du B, Tao Y, Ma J, Weng X, Gong Y, Lin Y, et al. Identification of sparganosis based on next-generation sequencing. *Infect Genet Evol.* (2018) 66:256–61. doi: 10.1016/j.meegid.2018.10.005
26. Harrison EM, Paterson GK, Holden MT, Larsen J, Stegger M, Larsen AR, et al. Whole genome sequencing identifies zoonotic transmission of MRSA isolates with the novel mecA homologue mecC. *EMBO Mol Med.* (2013) 5:509–15. doi: 10.1002/emmm.201202413
27. Ai JW, Zhang HC, Cui P, Xu B, Gao Y, Cheng Q, et al. Dynamic and direct pathogen load surveillance to monitor disease progression and therapeutic efficacy in central nervous system infection using a novel semi-quantitive sequencing platform. *J Infect.* (2018) 76:307–10. doi: 10.1016/j.jinf.2017.11.002
28. Greninger AL The challenge of diagnostic metagenomics. *Expert Rev Mol Diagn.* (2018) 18:605–15. doi: 10.1080/14737159.2018.1487292

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Wang, Dai, Lu, Chang, Ma, Wang, Liu and Ma. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

MINI REVIEW
published: 30 June 2021
doi: 10.3389/fmed.2021.686512

Early Detection of *Legionella pneumophila* and *Aspergillus* by mNGS in a Critically Ill Patient With *Legionella* Pneumonia After Extracorporeal Membrane Oxygenation Treatment: Case Report and Literature Review

Ruiming Yue[1,2], *Xiaoxiao Wu*[1,2], *Tianlong Li*[1,2], *Li Chang*[1,2], *Xiaobo Huang*[1,2*†] and *Lingai Pan*[1,2*†]

[1] Department of Critical Care Medicine, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, China, [2] Chinese Academy of Sciences Sichuan Translational Medicine Research Hospital, Chengdu, China

Legionella pneumophila can cause pneumonia, leading to severe acute respiratory distress syndrome (ARDS). Because of its harsh growth requirements, limited detection methods, and non-specific clinical manifestations, diagnosing *Legionella* pneumonia remains still challenging. Metagenomic next-generation sequencing (mNGS) technology has increased the rate of detection of *Legionella*. This study describes a patient who rapidly progressed to severe ARDS during the early stage of infection and was treated with extracorporeal membrane oxygenation (ECMO). Although his bronchoalveolar lavage fluid (BALF) was negative for infection and his serum was negative for anti-*Legionella* antibody, mNGS of his BALF and blood showed only the presence of *Legionella pneumophila* (blood mNGS reads 229, BALF reads 656). After antibiotic treatment and weaning from ECMO, however, he developed a secondary *Aspergillus* and *Klebsiella pneumoniae* infection as shown by mNGS. Mechanical ventilation and antibiotic treatment were effective. A search of PubMed showed few reports of secondary *Aspergillus* infections after *Legionella* infection. Severe pneumonia caused by any type of pathogenic bacteria may be followed by *Aspergillus* infection, sometimes during extremely early stages of infection. Patients with severe pneumonia caused by *Legionella* infection should undergo early screening for secondary infections using methods such as mNGS, enabling early and precise treatment, thereby simplifying the use of antibiotics and improving patient prognosis.

Keywords: *Legionella*, invasive pulmonary aspergillosis, mNGS, ECMO, diagnosis

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Beiwen Zheng,
Zhejiang University, China
Xiawei Jiang,
Zhejiang Chinese Medical University, China
Wanhong Yin,
Sichuan University, China

***Correspondence:**
Xiaobo Huang
drhuangxb@163.com
Lingai Pan
panlingai2004@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *27 March 2021*
Accepted: *31 May 2021*
Published: *30 June 2021*

Citation:
Yue R, Wu X, Li T, Chang L, Huang X and Pan L (2021) Early Detection of Legionella pneumophila and Aspergillus by mNGS in a Critically Ill Patient With Legionella Pneumonia After Extracorporeal Membrane Oxygenation Treatment: Case Report and Literature Review. Front. Med. 8:686512. doi: 10.3389/fmed.2021.686512

INTRODUCTION

Legionella pneumophila is a common atypical pathogen that can cause pneumonia, and is thought to be responsible for about 2–15% of patients with community-acquired pneumonia who require hospitalization (1, 2). *Legionella* pneumonia can rapidly lead to the development of acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) (3).

Legionella bacteria often grow and multiply in water. Patients with *Legionella* pneumonia often have a non-specific epidemiological history (e.g., bathing, travel history, and cruises) and clinical symptoms, and laboratory tests lack specificity. These patients can present with slow pulse, gastrointestinal symptoms, and/or muscle pain, as well as with involvement of the kidneys and/or nervous system (4).

Traditional methods of culturing *Legionella* are often time-consuming, and cultures are easily contaminated. Moreover, harsh culture conditions are required, further decreasing the positivity rate of traditional cultures (5). Clinically, *Legionella* is often diagnosed by the presence of anti-*Legionella* antibodies.

Most patients, however, develop anti-*Legionella* antibodies around 3 weeks after the onset of the disease, resulting in delayed diagnosis (4). The emergence of metagenomic next-generation sequencing (mNGS) has markedly improved the rate of early diagnosis of *Legionella* pneumonia. Although extracorporeal membrane oxygenation (ECMO) treatment has increased the survival rate of patients infected with *Legionella* bacteria, nosocomial infections and secondary fungal infections are common and serious complications of ECMO treatment. *Aspergillus* infection has been reported after ECMO treatment, especially secondary pulmonary *Aspergillus* infection in patients with severe influenza or COVID-19, and in immunosuppressed patients (6–10). Few reports, however, have described patients with *Legionella* pneumonia co-infected with invasive pulmonary aspergillosis (IPA). The present report describes a patient with early IPA and sensitive *Klebsiella pneumoniae* secondary to *Legionella pneumophila* serogroup one infection after ECMO treatment. To our knowledge, this report is the first to describe a patient with severe *Legionella* pneumonia treated by venous-venous ECMO (VV-ECMO) who developed a secondary IPA infection after the first stage of improvement. This study showed that mNGS technology can improve the early detection and treatment of *Legionella* pneumonia and secondary Aspergillus infection.

CASE PRESENTATION

A 46-year-old man was admitted to the intensive care unit (ICU) on February 4, 2021, after experiencing fever (highest temperature 39.0°C), accompanied by chills and diarrhea with yellow watery stool, for 2 days and dyspnea for 1 day. A chest computed tomography (CT) scan showed multiple subpleural infectious foci and interstitial lesions in his left lower lobe. He had traveled to Aba Prefecture 1 week earlier and had a smoking history of about 20 pack-years. An earlier CT scan suggested pulmonary bullae. His hypoxia symptoms progressed rapidly, and he was started on high-flow oxygen treatment, along with the antibiotics imipenem, moxifloxacin, and oseltamivir. The patient, however, quickly progressed to ARDS. Because mechanical ventilation with a 100% oxygen supply could not maintain saturation (IPPV, VT 480 ml; FIO2, 100%; PEEP, 14 cmH_2O; f, 20/min; saturation, 70%), he was transferred to the ICU for ECMO treatment. Laboratory examination showed a white blood cell (WBC) count of 6.99×10^9/L, a neutrophil rate of 91.7%, an aspartate transaminase concentration of 137.0 U/L, an alanine aminotransferase concentration of 25.0 U/L, a creatinine concentration of 414.0 μmol/L, a C-reactive protein (CRP) concentration of 259.39 mg/L, a procalcitonin (PCT) concentration of >200 ng/ml, a CD4+ lymphocyte count of 196/μl, and a CD8+ lymphocyte count of 27/μl. He was negative for β-(1,3)-glucan (BD), galactomannan, and influenza A and B. Chest CT showed multiple subpleural infectious lesions in the right lung, and interstitial changes in the lingual segment of the left upper lobe and left lower lobe (**Figure 1A**). The patient was treated with VV-ECMO at a flow rate of 3.8 L/min, a rotation speed of 7,400 rpm, and an airflow rate of 3.5 L/min. Later his blood and BALF mNGS showed only *Legionella pneumophila* infection (blood reads 229, BALF reads 656), and his antibiotic treatment was changed to moxifloxacin, azithromycin, and piperacillin-tazobactam sodium. Because of his acute kidney injury, ECMO was combined with continuous renal replacement therapy (CRRT). The patient's condition gradually improved, and he was weaned from ECMO on February 12 and from mechanical ventilation on February 13. A sputum smear showed no bacteria or fungi, sputum and blood cultures were negative, and he was negative for anti-*Legionella* antibodies. A comprehensive examination showed no evidence of immune system disease or tumor disease in this patient. A chest CT taken on February 19 showed significant improvement, and he was discharged to a local hospital for rehabilitation on February 20, 17 days after admission (**Figure 1B**). A blood test at that time showed a WBC count of 8.9×10^9/L, a total neutrophil count of 6.42×10^9/L, a neutrophil percentage of 72.2%, and a PCT concentration of 4.97 ng/ml.

Four days after returning to the local hospital, the patient again presented with dyspnea, accompanied by chills and fever. Saturation could not be maintained by non-invasive positive pressure ventilation (NPPV). The patient was again intubated, and a large amount of bloody sputum was aspirated from his airways. The patient was re-admitted to the ICU, and a CT scan on February 24 showed scattered speckled shadows and reticular blurred shadows, with partial consolidation, consistent with infectious interstitial lesions (**Figure 1C**). Blood gas analysis showed pH 7.228, PO_2 7.3 kpa, PCO_2 9.64 Kpa, Lac 1.9 mmol/L, K^+ 6.0 mmol/L, and 90% saturation (FIO_2, 60%). The patient was started on treatment with meropenem plus moxifloxacin. He was positive for anti-*Legionella* IgM antibody, and his PCT was >200 ng/mL, but fungal BD and GM tests were negative. mNGS of bronchoalveolar lavage fluid (BALF) on February 27 revealed *Aspergillus fumigatus* and *Klebsiella pneumoniae*, with reads of 80 and 2,325, respectively (**Figure 2**). A culture of BALF in our hospital also suggested *K. pneumonia*. CT imaging showed bronchial wall thickening with local bronchiectasis and interstitial changes, and bronchoscopy showed airway

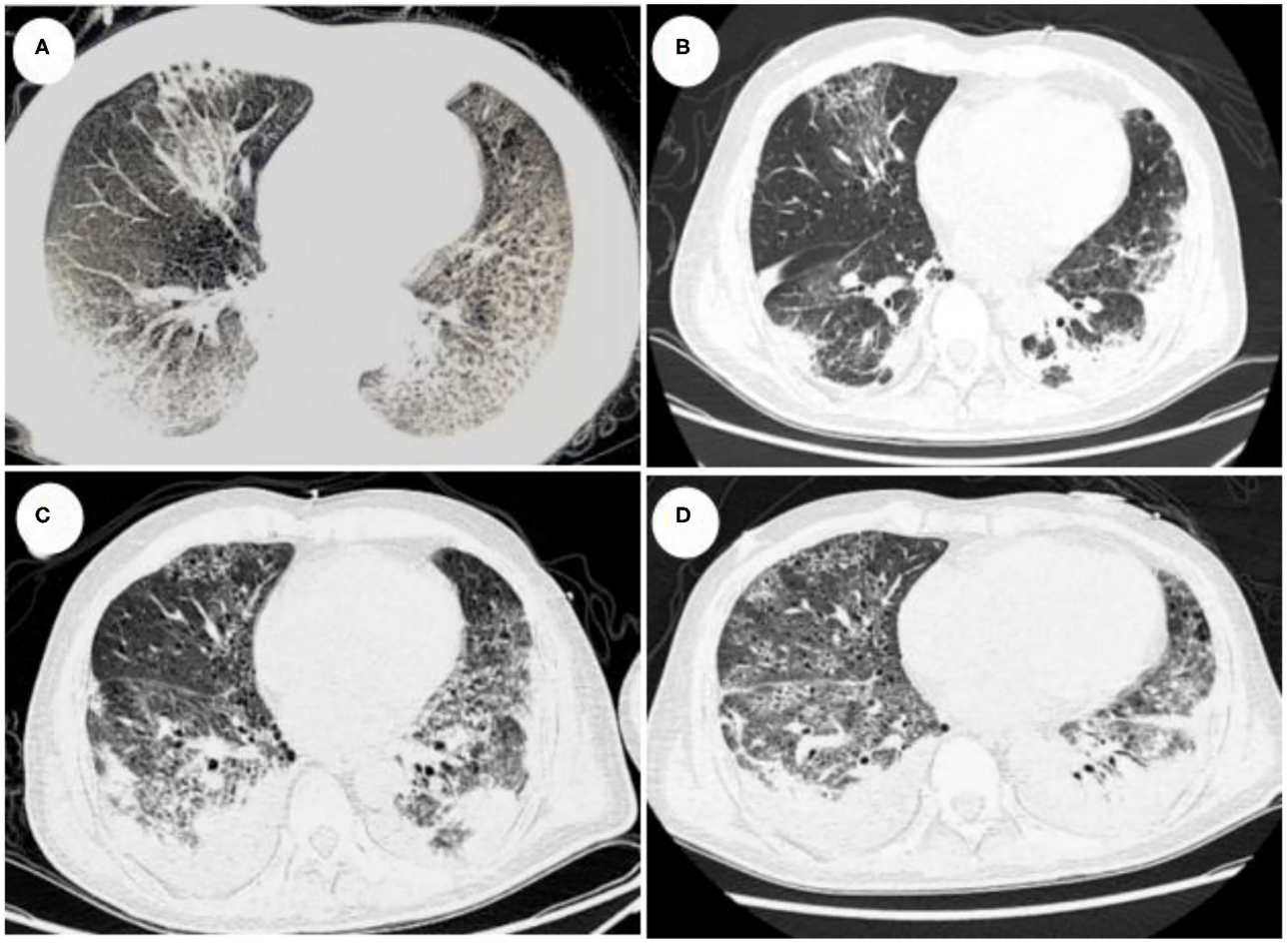


FIGURE 1 | (A) CT scan taken on February 5 (day 2), showing multiple infectious foci and interstitial lesions in the right lung, the lingual segment of the upper lobe of the left lung, and the subpleura of the lower lobe of the left lung. **(B)** CT scan taken on February 19 (day 16), 7 days after the patient was weaned from ECMO, showing scattered patchy shadows and meshwork blurred shadows in both lungs, as well as some consolidation, indicative of significant improvements. **(C)** CT scan taken on February 24 (day 21), after the patient again presented with dyspnea and was re-admitted to the ICU. The CT scan showed patches in lobular segments of both lungs, honeycomb-like changes, partial consolidation, and lesion expansion. **(D)** CT scan taken on March 01 (day 26), showing patchy, gridded, cellulite-like changes, with bronchiectasis in multiple lobes of the lungs.

erosion and congestion, resulting in a diagnosis of IPA and *K. pneumoniae* infection. Lung CT scan taken on March 1 indicated that the infection was still progressing (**Figure 1D**). After 16 days of mechanical ventilation, the patient was successfully weaned and transferred from the ICU on March 14. The treatment process is shown in **Figure 3**.

DISCUSSION

Legionella is a genus of aerobic gram-negative bacillus widely distributed in artificial cold water, natural water sources, moist soil, and hospital hot water systems. *Legionella* can be divided into 58 species with more than 70 different serotypes. *Legionella* pneumonia, which is mostly caused by *Legionella pneumophila* type 1, can progress rapidly to ARDS. The clinical diagnosis of *Legionella* is difficult, which may delay its treatment. At present, *Legionella* is diagnosed clinically by culturing by detecting antibodies in serum and antigens in urine, and by molecular detection techniques such as PCR; however, positivity rates are low (11). The patient described in this report, who had traveled elsewhere within 2 weeks prior to diagnosis, experienced an acute onset in the community and rapidly progressed to ARDS, combined with diarrhea and kidney damage. All these symptoms strongly suggested *Legionella* infection. Serum samples at the time of admission were negative for anti-*Legionella* antibody, and sputum cultures were negative. mNGS assays of BALF indicated *Legionella pneumophila*. *Legionella* grows in particular conditions, the positive culture rate is low, and more than 50% of early-stage patients have no sputum (4). Although urinary antigen tests are widely performed in clinical practice, these tests are sensitive only to *Legionella pneumophila* type 1. Moreover, urinary antigen tests were unavailable in our hospital. Metagenomic sequencing can significantly improve the early diagnosis rate of infectious diseases (12). Imaging studies

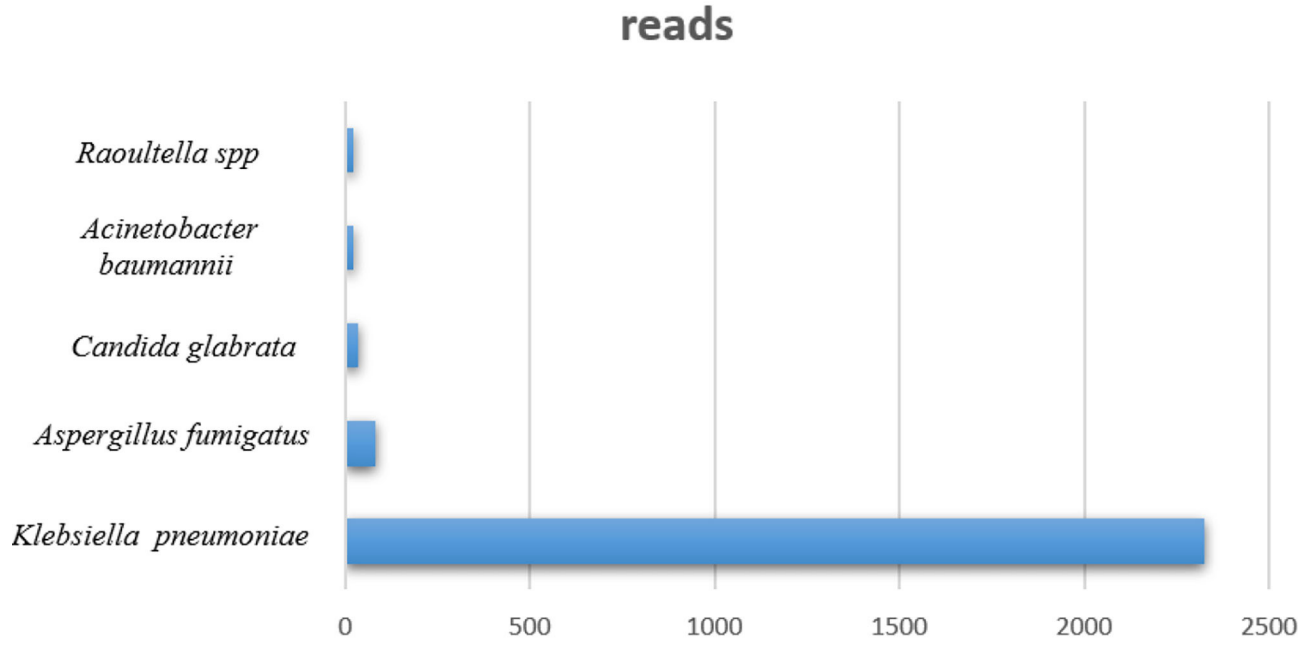


FIGURE 2 | Detection of pathogens by mNGS.

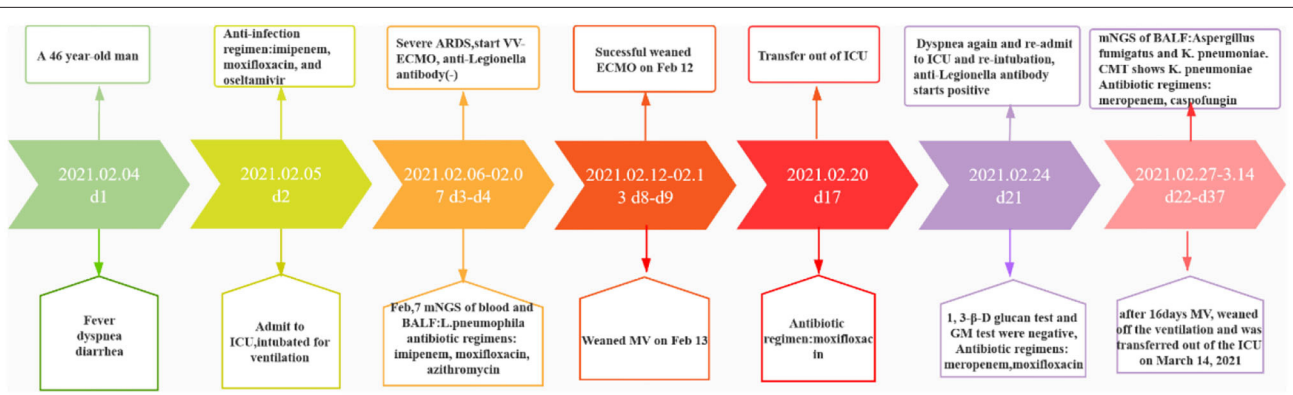


FIGURE 3 | Course of treatment of this patient over time.

of patients with clusters of onset, ineffective initial empirical treatment, severe community-acquired pneumonia, and travel history within 2 weeks before onset suggest that patients with multilobed lung lesions and bilateral pleural effusion should be routinely screened for *Legionella*.

Fluoroquinolones or macrolides are regarded as first line treatments for patients with *Legionella* pneumonia choice, with combination treatments recommended for critically ill or immunocompromised patients (13, 14). ECMO support has dramatically improved the survival rate of patients with severe *Legionella* pneumonia. For example, the survival rate of patients with *Legionella* pneumonia treated with ECMO was found to be 70% (15). Moreover, a single-center observational study of 112 ECMO-treated patients found that 12 of these patients had *Legionella* pneumonia, with a survival rate of 85.7% (16). The patient described in this report was weaned off ECMO after 6 days and recovered.

However, 12 days after ECMO withdrawal, the patient again presented with dyspnea and coughed up considerable bloody sputum. BALF culture indicated the presence of *K. pneumoniae*, and mNGS indicated *K. pneumoniae* and *A. fumigatus*. IPA occurs frequently in severely immunosuppressed patients, including organ transplant recipients, HIV-infected patients, patients with long-term or severe neutropenia, patients with tumors, and patients with long-term use of glucocorticoids. IPA, however, is a less well-recognized complication in patients without traditional risk factors. More recent reports have described patients with non-neutropenia-related IPA, including

TABLE 1 | Patients diagnosed with invasive pulmonary aspergillosis associated with *Legionella* pneumophila.

References	Underlying diseases	Immune status	Time from onset to diagnosis	Method of IPA diagnosis	Antifungal therapy	Outcome
Jiva et al. (21)	Asthma	Prednisone 40 mg/day, 3 weeks	11 days	Autopsy	Amphotericin B	Death
Vergne et al. (23)	Smoking history	Normal	6 days	Anatomo-pathological examination	Amphotericin B	Death
Guillouzouic et al. (24)	Broad passive nicotinism	Prednisone 100 mg/day, 1 month	Not mentioned	*A. fumigatus* isolation from BALF	Caspofungin and voriconazole	Death
Saijo et al. (25)	Mild alcoholic liver injury	Normal	8 days	*A. fumigatus* isolation from BALF	Micafungin and voriconazole	Death
Coulon et al. (22)	Hypertension, type 2, diabetes, Smoking history	Prednisone 50 mg/day, 9 days	12 days	*A. fumigatus* isolation from BALF	Amphotericin B	Death
Shorten et al. (26)	Leukemia	Neutropenia	16 days	*A. fumigatus* isolation from peritoneal fluid	Voriconazole	Death
Present patient	Smoking history	Normal	3 days	mNGS	Caspofungin	Recovery

patients with end-stage chronic obstructive pulmonary disease (COPD) and Child-Pugh C cirrhosis, and patients receiving immunosuppressive therapy (17). Patients with severe influenza admitted to the ICU due to respiratory failure are also at risk for IPA, a condition called influenza-related pulmonary aspergillosis (18). Accidental IPA has been observed in about 1% of critically ill patients who undergo lung biopsy after death. Factors associated with IPA in critically ill, non-immunocompromised patients include COPD, ARDS, liver failure, and organ dysfunction, as well as treatment with glucocorticoids or broad-spectrum antibiotics (19).

The diagnosis of IPA remains challenging in non-immunosuppressed patients. The criteria for IPA caused by neutropenia may be inapplicable to non-neutropenic patients. mNGS can be used to assist in the diagnosis of IPA. For example, a comparison of mNGS with traditional microbial diagnostic methods for diagnosing *Aspergillus* found that mNGS had an accuracy of 78.3%, owing chiefly to the difficulty of extracting nucleic acids from molds, but a specificity as high as 97.5% (20). A search of PubMed identified only six case reports describing patients with secondary invasive pulmonary *Aspergillus* infection after *Legionella* infection (21–26) (**Table 1**). Of these six patients, one was diagnosed by autopsy, one by histopathology, and four by microbiological isolation and culture. The earliest diagnosis time was 6 days, but this patient eventually died. To our knowledge, this was the first patient with early diagnosis of *Legionella* and IPA through mNGS who was successfully treated with ECMO. *Legionella* infection has been reported to be a potential risk factor for IPA infection (14). The risk factors of IPA in this patient may have included admission to the ICU, the use of broad-spectrum antibiotics, and the placement of the ECMO circuit. The incidence of VV-ECMO complicated by IPA was 7%, the median duration of ECMO treatment before Aspergillus isolation was 5 days, and immunosuppression and influenza virus infection may be risk factors (10). Physicians should therefore be highly vigilant for the occurrence of IPA in patients with influenza virus infection, immunodeficiency, and *Legionella* infection, especially those receiving ECMO support. These patients should undergo early fungal screening to avoid poor outcomes.

CONCLUSIONS

The present study described a patient with severe *Legionella* pneumonia, diagnosed early by mNGS, who was found to have secondary IPA infection by mNGS. The patient was quickly diagnosed and successfully treated with ECMO and antibiotics. *Legionella* may enhance infection by invasive aspergillus. Patients infected with *Legionella* should undergo early complete screening of secondary infection using mNGS technology.

AUTHOR CONTRIBUTIONS

XH and LP were involved in the conception and design of the work. RY wrote the first draft of the manuscript and revised it. LP revised the manuscript. TL, XW, and LC were responsible for the management of this patient, for collecting the clinical data, and for reviewing the literature throughout this study. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We would like to thank all our hospital members for their efforts. They are Ping Zhou, Fuxun Yang, Lei Deng from Department of Critical Care Medicine, Sichuan Provincial People's Hospital.

REFERENCES

1. Muder RR, Yu VL, Fang GD. Community-acquired Legionnaires' disease. *Semin Respir Infect.* (1989) 4:32–9.
2. Viasus D, Di Yacovo S, Garcia-Vidal C, Verdaguer R, Manresa F, Dorca J, et al. Community-acquired *Legionella pneumophila* pneumonia: a single-center experience with 214 hospitalized sporadic cases over 15 years. *Medicine.* (2013) 92:51–60. doi: 10.1097/MD.0b013e31827f6104
3. Chahin A, Opal SM. Severe pneumonia caused by *Legionella pneumophila*: differential diagnosis and therapeutic considerations. *Infect Dis Clin North Am.* (2017) 31:111–21. doi: 10.1016/j.idc.2016.10.009
4. Cunha BA, Burillo A, Bouza E. Legionnaires' disease. *Lancet*. (2016) 387:376–85. doi: 10.1016/S0140-6736(15)60078-2
5. Mercante JW, Winchell JM. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. *Clin Microbiol Rev.* (2015) 28:95–133. doi: 10.1128/CMR.00029-14
6. Armstrong-James D, Youngs J, Bicanic T, Abdolrasouli A, Denning D, Johnson E, et al. Confronting and mitigating the risk of covid-19 associated pulmonary aspergillosis. *Eur Respir J.* (2020) 56:2002554. doi: 10.1183/13993003.02554-2020
7. Huang LN, Xiong SY, Huang X, Zhan QY. The clinical characteristics of severe influenza virus pneumonia complicated with invasive pulmonary aspergillosis: a retrospective study of 15 cases. *Zhonghua Jie He He Hu Xi Za Zhi*. (2020) 43:437–43. doi: 10.3760/cma.j.cn112147-20200109-00013
8. Parcell BJ, Kumar B, Johnson EM, Fardon TC. Invasive pulmonary aspergillosis post extracorporeal membrane oxygenation support and literature review. *Med Mycol Case Rep.* (2014) 4:12–5. doi: 10.1016/j.mmcr.2014.01.003
9. Schauwvlieghe A, Rijnders B, Nele P, Verwijs R, Vanderbeke L, Tienen C, et al. Invasive aspergillosis in patients admitted to the intensive care unit with severe influenza: a retrospective cohort study. *Lancet Respir Med.* (2018) 6:782–92. doi: 10.1016/S2213-2600(18)30274-1
10. Rodriguez-goncer I, Thomas S, Foden P, Richardson MD, Ashworth A, Barker J. Invasive pulmonary aspergillosis is associated with adverse clinical outcomes in critically ill patients receiving veno-venous extracorporeal membrane oxygenation. *Eur J Clin Microbiol Infect Dis.* (2018) 37:1251–7. doi: 10.1007/s10096-018-3241-7
11. Almudena B, Luisa PM, Emilio B. Microbiology and epidemiology of Legionnaire's disease. *Infect Dis Clin North Am.* (2017) 31:7–27. doi: 10.1016/j.idc.2016.10.002
12. Simner PJ, Miller S, Carroll KC. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. *Clin Infect Dis.* (2018) 66:778–88. doi: 10.1093/cid/cix881
13. Zhai J. Guidelines for the diagnosis and treatment of community-acquired pneumonia in adults (Practice Edition·2018). *Chin J General Practitioners.* (2019) 18:127–33.
14. Jia X, Ren H, Nie X, Li Y, Li J, Qin T. Antibiotic resistance and azithromycin resistance mechanism of *Legionella pneumophila* serogroup 1 in China. *Antimicrob Agents Chemother*. (2019) 63:e00768-19. doi: 10.1128/AAC.00768-19
15. Naqvi A, Kapoor S, Pradhan M, Dicpinigaitis PV. Outcomes of severe Legionella pneumonia requiring extracorporeal membrane oxygenation (ECMO). *J Crit Care.* (2021) 61:103–6. doi: 10.1016/j.jcrc.2020.10.017
16. Roncon-Albuquerque R Jr, Vilares-Morgado R, van der Heijden GJ, Ferreira-Coimbra J, Mergulhão P, Paiva JA. Outcome and management of refractory respiratory failure with timely extracorporeal membrane oxygenation: single-center experience with Legionella pneumonia. *J Intensive Care Med.* (2019) 34:344–50. doi: 10.1177/0885066617700121
17. Russo A, Tiseo G, Falcone M, Menichetti F. Pulmonary aspergillosis: an evolving challenge for diagnosis and treatment. *Infect Dis Ther.* (2020) 9:511–24. doi: 10.1007/s40121-020-00315-4
18. Verweij PE, Rijnders BJA, Brüggemann RJM, Azoulay E, Bassetti M, Blot S, et al. Review of influenza-associated pulmonary aspergillosis in ICU patients and proposal for a case definition: an expert opinion. *Intensive Care Med.* (2020) 46:1524–35. doi: 10.1007/s00134-020-06091-6
19. Tudesq JJ, Peyrony O, Lemiale V, Azoulay E. Invasive pulmonary aspergillosis in nonimmunocompromised hosts. *Semin Respir Crit Care Med.* (2019) 40:540–7. doi: 10.1055/s-0039-1696968
20. Peng JM, Du B, Qin HY, Wang Q, Shi Y. Metagenomic next-generation sequencing for the diagnosis of suspected pneumonia in immunocompromised patients. *J Infect.* (2021) 82:22-7. doi: 10.1016/j.jinf.2021.01.029
21. Jiva TM, Kallay MC, Marin MG, Poe RH. Simultaneous legionellosis and invasive aspergillosis in an immunocompetent patient newly treated with corticosteroids. *Chest.* (1993) 104:1929–31. doi: 10.1378/chest.104.6.1929
22. Coulon P, Cordier C, Saint-Léger P, Lambiotte F, Loridant S, Mazars E. Invasive pulmonary aspergillosis in an ICU patient with Legionnaires' disease: a diagnostic challenge. *J Mycol Med.* (2020) 30:100985. doi: 10.1016/j.mycmed.2020.100985
23. Vergne S, Lathuile D, Bénosa B, Fragnier F, Destrac S, Avedan M, et al. severe Legionnaire's disease revealing an invasive aspergillosis in an apparently immunocompetent patient. *Press Med.* (2003) 32:1604–6.
24. Guillouzouic A, Bemer P, Gay-Andrieu F, Bretonnière C, Lepelletier D, Mahé PJ, et al. Fatal coinfection with *Legionella pneumophila* serogroup 8 and aspergillus fumigatus. *Diagn Microbiol Infect Dis.* (2008) 60:193–5. doi: 10.1016/j.diagmicrobio.2007.09.005
25. Saijo T, Izumikawa K, Takazono T, Kosai K, Kurihara S, Nakamura S, et al. A case of *Legionella pneumophila* pneumonia followed by invasive aspergillosis. *Jpn J Infect Dis.* (2008) 61:379–81.
26. Shorten RJ, Norman J, Sweeney LC. An unusual presentation of Legionella pneumonia in a returning traveller. *BMJ Case Rep.* (2019) 12:e230130. doi: 10.1136/bcr-2019-230130

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Yue, Wu, Li, Chang, Huang and Pan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 01 July 2021
doi: 10.3389/fmed.2021.675041

Diagnosis of *Mycobacterium tuberculosis* Septic Shock in Patients With Anti-synthetase Syndrome Based on Next-Generation Sequencing: A Case Report and Literature Review

Limin Sun[1†], Ziyue Yang[1†], Fei Yang[1], Zhenhua Wang[1], Hongqiang Li[1], Huifen Wang[2,3] and Tongwen Sun[1]*

[1] General Intensive Care Unit, Zhengzhou Key Laboratory of Sepsis, Henan Key Laboratory of Critical Care Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Gene Hospital of Henan Province, Precision Medicine Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Daxian Wu,
First Affiliated Hospital of Nanchang University, China
Armelia Sari Widyarman,
Trisakti University, Indonesia

***Correspondence:**
Tongwen Sun
suntongwen@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 02 March 2021
Accepted: 03 June 2021
Published: 01 July 2021

Citation:
Sun L, Yang Z, Yang F, Wang Z, Li H, Wang H and Sun T (2021) Diagnosis of Mycobacterium tuberculosis Septic Shock in Patients With Anti-synthetase Syndrome Based on Next-Generation Sequencing: A Case Report and Literature Review. Front. Med. 8:675041. doi: 10.3389/fmed.2021.675041

A 51-year-old woman was transferred to the intensive care unit with such symptoms as fever, swollen left knee joint, pain and hypotension. After preliminary evaluation, she was diagnosed as suffering acute suppurative arthritis and septic shock. Then, she was rescued and prescribed to receive treatment with broad-spectrum antibiotics. However, there was no source of infection identified except for the knee joint. The bacterial and fungal cultures of blood samples and articular effusion were shown to be negative, while the results obtained from the next-generation sequencing of blood and articular effusion revealed that *Mycobacterium tuberculosis* was positive. The patient was then put on five combinations of anti-tuberculosis therapeutic treatment. Nevertheless, despite the active anti-tuberculosis treatment put in place, her general condition still deteriorated progressively. As the level of her bilirubin continued to rise, further treatment was affected, which prompted the change made to the anti-tuberculosis treatment program. Her clinical condition continued to deteriorate, which led to the development of unstable vital signs and the multiple organ dysfunction syndrome. In spite of our best efforts to save her life, the patient still ended up with death.

Keywords: *Mycobacterium tuberculosis*, sepsis, septic shock, intensive care unit, bloodstream infection

INTRODUCTION

Anti-synthetase syndrome (ASS) is referred to as a clinical classification of idiopathic inflammatory myopathy, which is mainly treated with hormones and immunosuppressants (1). The disease requires long cycle and low immunity during treatment, which makes it easy for infection to cause various complications (2). For clinicians, it is very difficult to distinguish between different types of pathogens that could cause infection, and most of them are acute and critical. Therefore, it is essential to identify what type the pathogens fall into and carry out early targeted anti-infective treatment (3). At present, there is a low positive rate of bacterial culture commonly used in clinical

practice, and it requires a large amount of time, which hinders clinicians from carrying out targeted anti-infective treatment in a timely manner (4). As a novel diagnostic method, next-generation sequencing (NGS) technology can be applied to provide etiological basis at the genetic level and enable the identification of pathogens within 24 h (5). In addition, it can support the effort to make etiological diagnosis of difficult infection, for the early start of anti-infective treatment, which demonstrates significant clinical value. In this study, a dermatomyositis patient is reported who got admitted to the intensive care unit because of various clinical symptoms including knee joint redness, swelling, heat and pain, high fever, septic shock and multiple organ failure. As suggested by NGS, the pathogen was *Mycobacterium tuberculosis*. The late diagnosis rendered the prognosis of the patient's poor.

CASE PRESENTATION

A 51-year-old woman with a one-month history of redness and swollen left knee joint was transferred to our general intensive care unit (ICU) due to persistent high fever (up to 39.5°C) and sudden hypotension. After being diagnosed with dermatomyositis 2 years ago, she continued medication treatment on prednisone tablets with a 25 mg daily dose and cyclosporine capsule with a 75 mg dose twice per day. Last month, the results of blood culture and bacterial culture of joint cavity pus as obtained from other medical institutions were shown to be negative, with no special microbiological examination performed.

When the patient visited the rheumatology department for appointment with a doctor, she coughed up sputum intermittently and her body temperature reached up to 40°C. Physical examination revealed that the left knee was red and swollen with positive floating patellar test, along with red rash in the V area of face and neck. The right dark yellow pleural effusion displayed exudate and negative bacterial culture. Vancomycin was thus prescribed to reduce infection, and 20 mg of methylprednisolone was applied twice a day. Four days later, she was transferred to ICU due to hypotension (the detailed clinical course is shown in **Figure 1**). The patient exhibited low blood pressure and high heart rate, which was accompanied by elevated procalcitonin and multiple organ failure. Broad-spectrum anti-infective therapy (imipenem/cilastatin + linezolid) was initiated within the first hour of highly suspected septic shock. ECG suggested cardiac infarction and high myocardial enzyme index. In order to rule out the occurrence of acute cardiovascular event, no infarction was detected immediately after coronary angiography. Given the EF value of 30% for the patient, septic heart disease was suspected. At the same time, the patient suffered acute liver injury, acute renal injury and thrombocytopenia.

After the patient was transferred to ICU, joint cavity pus was also taken for culture in addition to blood culture samples (ultrasound: **Figures 2A,B**, MRI: **Figures 2C,D**). Considering that the patient had negative culture multiple times in the previous month, the NGS examination was conducted for joint cavity pus and blood samples at the same time. Subsequently, NGS results indicated that the pathogen was "*M. tuberculosis*," with acid-fast bacilli unexpectedly visible in articular cavity effusion. After an inquiry made about her medical history, it was known that the patient had a history of contact with pulmonary tuberculosis, and such symptoms as night sweating, coughing and expectoration were manifested since around the last month. Thus, the septic shock associated with *M. tuberculosis* was suspected. Five combinations of anti-tuberculosis drugs (HRZEO: Isoniazid, Rifampicin, Pyrazinamide, Ethambutol, Ofloxacin) were prescribed, while imipenem/cilastatin was downgraded to piperacillin/tazobactam and vitamin B6 was prescribed as a supplement. The bronchoalveolar lavage fluid was found negative for acid-fast staining. The T-SPOT results of the patients remained negative. The T lymphocyte count of the patient was shown to be extremely low. After about 10 days of anti-tuberculosis treatment, the body temperature improved, the ST segment of ECG decreased, myocardial enzymes returned to normal, shock was alleviated, and the count of platelets increased. Unfortunately, liver function deteriorated for the patient gradually. Five days after the adjustment made to anti-tuberculosis drugs, the patients showed such tuberculosis poisoning symptoms as high fever and shock, norepinephrine of 1.2 ug/kg/kg·min, which was accompanied by a decline in the count of platelet again. Afterwards, both liver function and cardiac function further deteriorated. Re-examination of blood NGS remained suggestive of *M. tuberculosis*. Our advice against further treatment was rejected by her family members.

DISCUSSION

In this study, a rare case of septic shock caused by the tuberculosis of the knee joint is reported. Sepsis puts a huge strain on global health care, and the severity of septicemia can affect the prognosis. For sepsis patients, the in-hospital mortality rate could exceed 10%, while the in-hospital mortality rate for the patients with septic shock and their serum lactate levels higher than 2 mmol/L could reach above 40% (3, 6–8). Additionally, there have been a large number of studies substantiating that the tuberculosis sepsis patients needing ICU treatment are at increased risk of in-hospital mortality (9–11). In one study, it was demonstrated that the previous medication on any antibiotics can cause delay to the diagnosis of *M. tuberculosis* (12). The latest evidence has been obtained to suggest that the delayed diagnosis of sepsis and late administration of antibiotics can increase the risk of mortality (13–16). In previous reports, the manifestation of *M. tuberculosis* septic shock was suggested as similar to that of bacterial septic shock, with early and appropriate antimicrobial treatment seemingly effective in reducing the risk of mortality (17). However, it is believed in this study that given the current routine bacterial culture of blood and other tissue samples, it is often difficult to identify early-stage tuberculosis septic shock in the absence of typical pulmonary lesions (18). A late start of anti-tuberculosis therapy will lead to the poor clinical outcomes (17). In this respect, NGS can provide more compelling evidence required for clinical diagnosis and treatment (19). Our patients showed a tendency to have their vital signs improved after NGS

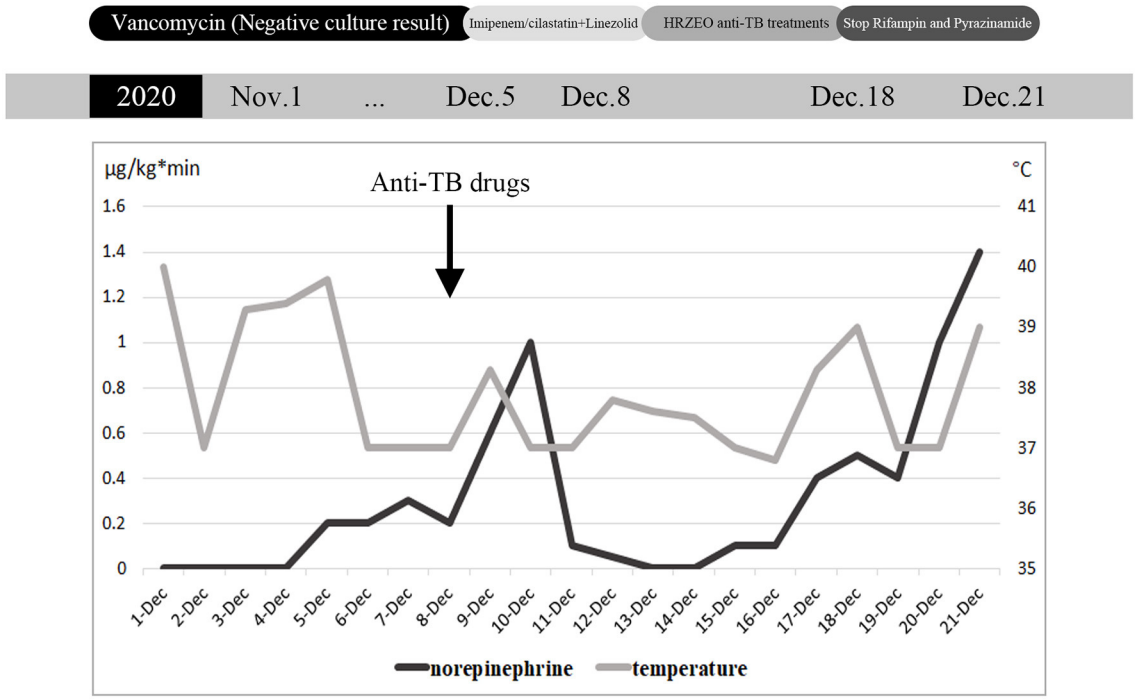


FIGURE 1 | Daily maximum body temperature and maximum norepinephrine pump speed during hospitalization, and antibiotic adjustment process.

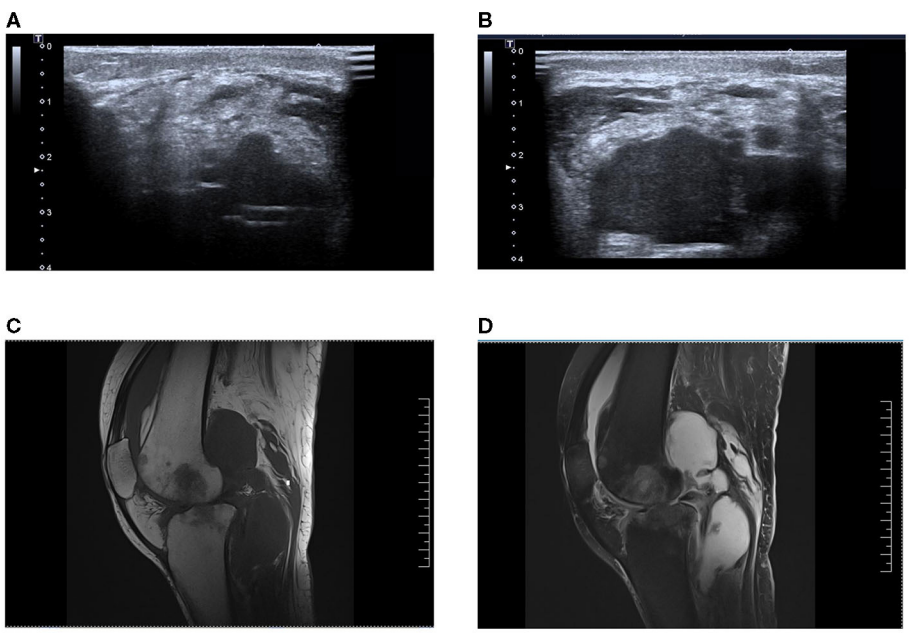


FIGURE 2 | (A,B) Bedside ultrasonographic examination of knee joint cavity effusion in this patient. **(C,D)** Magnetic resonance imaging of the patient's knee cavity effusion, T1 image and T2 image, respectively.

reported *M. tuberculosis* bacteremia and the timely use of anti-tuberculosis drugs, indicating the effectiveness of treatment. Due to late diagnosis and various complications, however, it failed to achieve a satisfactory outcome.

Though tuberculosis progressed slowly in most cases, it can be manifested in the form of disseminated tuberculosis with visible systemic symptoms. Tuberculosis sepsis shock (TBSS) is frequent to occur in those adults with low immunity, and the incidence is lower in children than in adults (20). TBSS can lead to such similar symptoms as fever, shortness of breath and multiple organ dysfunction to sepsis (17), especially in the patients with low immunity. In some studies, it has been suggested that TBSS can cause hyponatremia and anemia (9, 21–23). In this case, not only did the aforementioned situation occur following *M. tuberculosis* bacteremia, it also triggered various similar symptoms to G-bacilli bloodstream infection, for example, thrombocytopenia, elevated procalcitonin, persistent high fever and septic cardiomyopathy. Allowing for this, it is speculated in our study that TBSS may make it more difficult for clinicians to make accurate diagnosis in time than previously thought. A timely identification of pathogens and an early targeted use of antibiotics can be effective in improving prognosis for patients. However, active tuberculosis has long been the challenge facing clinical diagnosis and treatment. Due to atypical clinical manifestations and extrapulmonary tuberculosis infection, it is more difficult to make accurate diagnosis.

Given the microbiological characteristics of *M. tuberculosis*, the traditional etiological examination is disadvantaged by low sensitivity and long cycle, which cannot meet the urgent clinical needs for rapid etiological diagnosis of active tuberculosis (18). For quite long, the diagnosis of tuberculosis has been reliant on traditional acid-fast staining smears and the culture techniques of *M. tuberculosis*. Although culture remains the gold standard for the diagnosis of tuberculosis, it can not meet the requirements of rapid clinical diagnosis. In recent years, there have been a variety of new diagnostic techniques and methods for tuberculosis proposed. The emergence of molecular diagnosis technology in recent years achieves an important breakthrough, as represented by Xpert MTB/RIF detection kit (24, 25). WHO strongly recommends Xpert MTB/RIF for the preliminary screening of suspected drug-resistant tuberculosis among both adults and children. Besides, for such extrapulmonary specimens as cerebrospinal fluid, lymph nodes and other tissues derived from the patients suspected of extrapulmonary tuberculosis. Since its specificity exceeds 95%, it is applicable to the diagnostic test intended for tuberculosis. However, the sensitivity of both culture and Xpert MTB/RIF remains very low for the diagnosis of extrapulmonary tuberculosis. As a new laboratory method, next-generation sequencing (NGS) has been reported to be applied to assist the diagnosis of various pathogens, and in active pulmonary tuberculosis (19, 26). Plenty of studies have confirmed that NGS is quick to detect *M. tuberculosis* complex in various samples, with its sensitivity and specificity similar to Xpert MTB/RIF (5). The process of NGS detection mainly includes two parts: experimental operation (wet experiment) and bioinformatics analysis (dry experiment). Wet experiment mainly involves the following four steps: sample pre-processing, nucleic acid extraction, library construction and computer sequencing. Bioinformatics analysis mainly involves the following steps: data quality control, human sequence removal, microbial species comparison identification, drug resistance gene and virulence gene analysis. In recent years, the cost of NGS has been reduced to some extent, thus making it affordable for most families of ordinary patients. Compared with the huge amount of medical costs, actively defining the etiological basis and targeted medication can reduce the cost of treatment more significantly. Also, for patients with sepsis, besides *M. tuberculosis*, NGS can also be applied to screen other suspected pathogens fast, which is more beneficial to patients.

The clinical symptoms of tuberculosis exhibited by the patients with HIV-induced immunosuppression are different from those of immune hosts (27). Those infected with HIV are at increased risk to develop extrapulmonary tuberculosis compared to HIV-negative people. Those with less CD4 are more likely to develop extrapulmonary tuberculosis (28). The patients infected with tuberculosis and showing a small number of CD4 can show atypical chest imaging findings, and even normal chest imaging findings (29). In the relevant literature, it has been shown that in the case of immune disorders and destruction of the immune system, the T lymphocyte subset count is extremely low and the false negative rate of T-SPOT is fairly high, thus increasing the difficulty in making clinical diagnosis of *M. tuberculosis* infection. These atypical chest imaging examinations combined with atypical clinical manifestations could hinder the effort on early administration of anti-tuberculosis drugs, thus resulting in the poor prognosis of TBSS. This patient with anti-synthase syndrome was receiving immunosuppressant and hormone therapy for a long time. Consequently, the CD4 count was extremely low, as was the level of immunity. In case of infection with tuberculosis, the clinical signs of atypical extrapulmonary tuberculosis similar to those of patients with HIV infection would be manifested.

TBSS is extremely rare and there are only sporadic cases reported to date (9). The Centers for Disease Control and Prevention reported a decline in TB diagnosis. The rarity of TBSS makes this series of cases novel. According to our and previous cases, it can be found out that the case fatality rate of TBSS remains very high, with late diagnosis and delayed treatment being often contrary to the general epidemiological expectations of sepsis. The epidemic characteristics of tuberculosis vary in different countries and regions (2), so that each institution is supposed to determine its tuberculosis risk level according to the geographical location, the incidence of tuberculosis and the temporal trend of drug resistance. NGS is highly sensitive and rapid in the diagnosis of infectious pathogens, for which it is considered to be an important auxiliary diagnostic method for rare pathogens (5). Also, for those critically ill patients or the patients with unidentified pathogens, the effect of empirical treatment is not as satisfactory as expected, NGS can help identify the pathogen fast, for targeted anti-infective treatment to be carried out, which is of much significance to clinical treatment.

CONCLUSION

Though the epidemic characteristics of tuberculosis vary by region, the high mortality rate and high missed diagnosis

rate of TBSS remain common. Early diagnosis and early anti-tuberculosis treatment can be effective in improving the mortality of TBSS patients. Anti-tuberculosis treatment can be carried out directly in those high prevalence areas or for the patients with typical clinical manifestations. In this study, it is suggested that the application of NGS in sepsis patients can not only help clinicians identify the pathogen rapidly, but also screen rare pathogens, which is conducive to further reducing the mortality caused by sepsis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study has been approved by the Scientific Research and Clinical Trial Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Code 2019-KY-330). The patients/participants provided their written informed consent to participate in this study. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LS and ZY were responsible for managing the patient. ZY collected data. ZW, HL, and TS revised the article. All authors have read and approved the final manuscript and contributed substantially to the work presented in this article.

FUNDING

This study was supported by the United Fund of National Natural Science Foundation of China (Grant No. U2004110), Leading Talents Fund in Science and Technology Innovation in Henan Province (Grant No. 194200510017), the 51282 Project Leaders of Scientific and Technological Innovative Talents from Health and Family Planning Commission in Henan Province (2016-32), and Science and Technology people-benefit project of Zhengzhou (2019KJHM0001).

REFERENCES

1. Mehta P, Agarwal V, Gupta L. High early mortality in idiopathic inflammatory myopathies: results from the inception cohort at a tertiary care center in northern India. *Rheumatology.* (2021). doi: 10.1093/rheumatology/keab001
2. Muhammed H, Gupta L, Zanwar AA, Misra DP, Lawrence A, Agarwal V, et al. Infections are leading cause of in-hospital mortality in indian patients with inflammatory myopathy. *J Clin Rheumatol.* (2019) 27:114–9. doi: 10.1097/RHU.0000000000001214
3. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA.* (2016) 315:801–10. doi: 10.1001/jama.2016.0287
4. Cheng MP, Stenstrom R, Paquette K, Stabler SN, Akhter M, Davidson AC, et al. Blood culture results before and after antimicrobial administration in patients with severe manifestations of sepsis: a diagnostic study. *Ann Intern Med.* (2019) 171:547–54. doi: 10.7326/M19-1696
5. Huang ZD, Zhang ZJ, Yang B, Li WB, Zhang CJ, Fang XY,et al. Pathogenic detection by metagenomic next-generation sequencing in osteoarticular infections. *Front Cell Infect Microbiol.* (2020) 10:471. doi: 10.3389/fcimb.2020.00471
6. Gaieski DF, Edwards JM, Kallan MJ, Carr BG: Benchmarking the incidence and mortality of severe sepsis in the United States. *Crit Care Med.* (2013) 41:1167–74. doi: 10.1097/CCM.0b013e31827c09f8
7. Vincent JL, Opal SM, Marshall JC, Tracey KJ. Sepsis definitions: time for change. *Lancet.* (2013) 381:774–5. doi: 10.1016/S0140-6736(12)61815-7
8. Kaukonen KM, Bailey M, Pilcher D, Cooper DJ, Bellomo R. Systemic inflammatory response syndrome criteria in defining severe sepsis. *N Engl J Med.* (2015) 372:1629–38. doi: 10.1056/NEJMoa1415236
9. Mishra R, Patel HK, Singasani R, Vakde T. Tuberculosis septic shock, an elusive pathophysiology and hurdles in management: a case report and review of literature. *World J Crit Care Med.* (2019) 8:72–81. doi: 10.5492/wjccm.v8.i5.72
10. Silva DR, Menegotto DM, Schulz LF, Gazzana MB, Dalcin PT. Mortality among patients with tuberculosis requiring intensive care: a retrospective cohort study. *BMC Infect Dis.* (2010) 10:54. doi: 10.1186/1471-2334-10-54
11. Duro RP, Figueiredo Dias P, Ferreira AA, Xerinda SM, Lima Alves C, Sarmento AC, et al. Severe tuberculosis requiring intensive care: a descriptive analysis. *Crit Care Res Pract.* (2017) 2017:9535463. doi: 10.1155/2017/9535463
12. Wang M, Fitzgerald JM, Richardson K, Marra CA, Cook VJ, Hajek J, et al. Is the delay in diagnosis of pulmonary tuberculosis related to exposure to fluoroquinolones or any antibiotic? *Int J Tuberc Lung Dis.* (2011) 15:1062–8. doi: 10.5588/ijtld.10.0734
13. Taylor SP, Anderson WE, Beam K, Taylor B, Ellerman J, Kowalkowski MA. The association between antibiotic delay intervals and hospital mortality among patients treated in the emergency department for suspected sepsis. *Crit Care Med.* (2021) 49:741–7. doi: 10.1097/CCM.0000000000004863
14. Bassetti M, Rello J, Blasi F, Goossens H, Sotgiu G, Tavoschi L, et al. Systematic review of the impact of appropriate versus inappropriate initial antibiotic therapy on outcomes of patients with severe bacterial infections. *Int J Antimicrob Agents.* (2020) 56:106184. doi: 10.1016/j.ijantimicag.2020.106184
15. Gregorowicz AJ, Costello PG, Gajdosik DA, Purakal J, Pettit NN, Bastow S, et al. Effect of IV push antibiotic administration on antibiotic therapy delays in sepsis. *Crit Care Med.* (2020) 48:1175–9. doi: 10.1097/CCM.0000000000004430
16. Zasowski EJ, Bassetti M, Blasi F, Goossens H, Rello J, Sotgiu G, et al. A systematic review of the effect of delayed appropriate antibiotic treatment on the outcomes of patients with severe bacterial infections. *Chest.* (2020) 158:929–38. doi: 10.1016/j.chest.2020.03.087
17. Kethireddy S, Light RB, Mirzanejad Y, Maki D, Arabi Y, Lapinsky S, et al. *Mycobacterium tuberculosis* septic shock. *Chest.* (2013) 144:474–82. doi: 10.1378/chest.12-1286
18. Barr DA, Kerkhoff AD, Schutz C, Ward AM, Davies GR, Wilkinson RJ, et al. HIV-associated *mycobacterium tuberculosis* bloodstream infection is underdiagnosed by single blood culture. *J Clin Microbiol.* (2018) 56:e01914–17. doi: 10.1128/JCM.01914-17
19. Smith C, Halse TA, Shea J, Modestil H, Fowler RC, Musser KA, et al. Assessing nanopore sequencing for clinical diagnostics: a comparison of next-generation sequencing (NGS) methods for *Mycobacterium tuberculosis*. *J Clin Microbiol.* (2020) 59:e00583–20. doi: 10.1128/JCM.00583-20
20. Pavlinac PB, Lokken EM, Walson JL, Richardson BA, Crump JA, John-Stewart GC. *Mycobacterium tuberculosis* bacteremia in adults and children: a systematic review and meta-analysis. *Int J Tuberc Lung Dis.* (2016) 20:895–902. doi: 10.5588/ijtld.15.0773
21. Luies L, Du Preez I. The echo of pulmonary tuberculosis: mechanisms of clinical symptoms and other disease-induced systemic complications. *Clin Microbiol Rev.* (2020) 33. doi: 10.1128/CMR.00036-20

22. Sims EA, Welt LG, Orloff J, Needham JW. Asymptomatic hyponatremia in pulmonary tuberculosis. *J Clin Investig.* (1950) 29:846–47. doi: 10.1172/JCI102396
23. Shalhoub RJ, Antoniou LD. The mechanism of hyponatremia in pulmonary tuberculosis. *Ann Intern Med.* (1969) 70:943–62. doi: 10.7326/0003-4819-70-5-943
24. Theron G, Peter J, van Zyl-Smit R, Mishra H, Streicher E, Murray S, et al. Evaluation of the Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in a high HIV prevalence setting. *Am J Respir Crit Care Med.* (2011) 184:132–40. doi: 10.1164/rccm.201101-0056OC
25. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev.* (2014) 2014:Cd009593. doi: 10.1002/14651858.CD009593.pub3
26. Shi CL, Han P, Tang PJ, Chen MM, Ye ZJ, Wu MY, et al. Clinical metagenomic sequencing for diagnosis of pulmonary tuberculosis. *J Infect.* (2020) 81:567–74. doi: 10.1016/j.jinf.2020.08.004
27. Barr DA, Lewis JM, Feasey N, Schutz C, Kerkhoff AD, Jacob ST, et al. *Mycobacterium tuberculosis* bloodstream infection prevalence, diagnosis, and mortality risk in seriously ill adults with HIV: a systematic review and meta-analysis of individual patient data. *Lancet Infect Dis.* (2020) 20:742–52. doi: 10.1016/S1473-3099(19)30695-4
28. Xu W, Snell LM, Guo M, Boukhaled G, Macleod BL, Li M, et al. Early innate and adaptive immune perturbations determine long-term severity of chronic virus and *Mycobacterium tuberculosis* coinfection. *Immunity.* (2021) 54:526–41.e7. doi: 10.1016/j.immuni.2021.01.003
29. Kumar N, Bhargava SK, Agrawal CS, George K, Karki P, Baral D. Chest radiographs and their reliability in the diagnosis of tuberculosis. *JNMA J Nepal Med Assoc.* (2005) 44:138–42. doi: 10.31729/jnma.447

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Sun, Yang, Yang, Wang, Li, Wang and Sun. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 12 July 2021
doi: 10.3389/fmed.2021.687984

Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of Disseminated Tuberculosis of an Immunocompetent Patient

Yuanting Ye[1,2], *Naibin Yang*[3], *Jingying Zhou*[4], *Guoqing Qian*[3*] and *Jinguo Chu*[2*]

[1] School of Medicine, Ningbo University, Ningbo, China, [2] Department of General Practice, Ningbo First Hospital, Ningbo, China, [3] Department of Infectious Disease, Ningbo First Hospital, Ningbo, China, [4] Department of Hematology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, China

Disseminated tuberculosis (TB) is a rare disease and mainly occurs in immunodeficient patients. It is marked by hematogenous or lymphatic dissemination of *Mycobacterium tuberculosis*, causing tuberculous infection involving any organ system. Here, we report a case of disseminated TB involving lung, liver, spine, mediastinum, and prostate in an immunocompetent man. The present patient found a hepatic mass without any symptom during health examination. In the next 2 years, further examinations revealed multiple lesions in the lung, mediastinum, spine, and prostate. Imaging examinations, such as contrast-enhanced abdominal CT, F-18 FDG-PET/CT, and radionuclide bone scan, suggested the diagnosis of malignancy or metastatic tumor. Furthermore, histopathological results of the biopsies of the hepatic mass, mediastinal mass, and prostatic mass demonstrated granulomatous inflammation. Therefore, metagenomic next-generation sequencing (mNGS) was utilized to confirm the diagnosis. *Mycobacterium tuberculosis* complex was simultaneously detected in the spinal surgical resection specimens and bronchoalveolar lavage fluid (BALF), indicating the diagnosis of disseminated TB. mNGS is an emerging molecular diagnostic technology, and its application in disseminated TB has been rarely reported. We highlight that disseminated TB should be considered even in an immunocompetent patient, and mNGS can be performed when the diagnosis is difficult.

Keywords: disseminated tuberculosis, metagenomic next-generation sequencing, immunocompetence, extrapulmonary tuberculosis, diagnosis

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Eirini Christaki,
University of Cyprus, Cyprus
Reza Yazdani,
Tehran University of Medical Sciences, Iran

***Correspondence:**
Jinguo Chu
chujg@126.com
Guoqing Qian
bill.qian@outlook.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *30 March 2021*
Accepted: *14 June 2021*
Published: *12 July 2021*

Citation:
Ye Y, Yang N, Zhou J, Qian G and Chu J (2021) Case Report: Metagenomic Next-Generation Sequencing in Diagnosis of Disseminated Tuberculosis of an Immunocompetent Patient. Front. Med. 8:687984. doi: 10.3389/fmed.2021.687984

INTRODUCTION

Tuberculosis (TB) is a widespread infectious disease caused by *Mycobacterium tuberculosis* (MTB), which can involve any organ system and mostly the lungs (1, 2). Extrapulmonary infection of MTB was also reported in recent decades, including lymph glands, pleura, bones, joints, urogenital tract, and central nervous system (CNS) (3–7). Besides, disseminated TB is a rare form accounting for about 1–5% of all TB cases and defined as tuberculous infection involving two or more non-adjacent body sites via hematogenous or lymphatic spread of MTB from the primary lesion (8). The dissemination mainly occurs in patients with risk factors including HIV immunodeficiency,

long-term use of immunosuppressants, poorly controlled diabetes, hematologic diseases, and alcohol abuse (9). However, it is less likely to appear in immunocompetent individuals.

Metagenomic next-generation sequencing (mNGS) is a new molecular diagnostic technology. mNGS application in TB of single organ has been reported, such as pulmonary TB, osteoarticular TB, and tuberculous meningitis, but rarely in disseminated TB until now (10–12). Here, we report a case of disseminated TB with a hepatic mass as the first manifestation. The patient failed to be diagnosed despite several biopsies of liver mass, mediastinal mass, and prostatic nodules. With the application of mNGS, the patient was finally diagnosed with disseminated TB.

CASE PRESENTATION

On December 14, 2017, a 51-year-old man visited our General Internal Medicine department for finding a hepatic mass without any discomfort during health examination. The entire process of diagnosis and treatment is briefly depicted in **Figure 1**. The patient claimed no history of hepatitis or TB and no family history of liver cancer. Physical examination was unremarkable. Detailed laboratory examination data are presented in **Table 1**. Contrast-enhanced abdominal computed tomography (CT) showed a hypodense lesion with mild to moderate enhancement in the right hepatic lobe near hepatic hilar, which indicated the possibility of cholangiocellular carcinoma; in addition, a soft tissue density lesion with heterogeneous enhancement in the left posterior mediastinum suggested the possibility of neurogenic tumor (**Figures 1A,B**).

In the following 2 years, he had visited several hospitals and had underwent a huge number of blood tests, CT scans, and magnetic resonance imaging (MRI) examinations. Mantoux test was positive (24 × 22 mm). The results of T-SPOT.TB tests elevated progressively from 14 to 84 pg/ml. Repeated sputum smear examinations for acid-fast bacilli (AFB) were all negative. Chest CT displayed multiple small lung nodules and a left posterior mediastinal mass. 18F-fluorodeoxyglucose positron emission tomography (F-18 FDG-PET)/CT showed intense accumulation of FDG in the right hepatic lobe, right abdomen, lower left lung, left hilar, and lower mediastinal lymph nodes, suggesting the possibility of malignancy. MRI showed multiple cystic nodules in the prostate. Histopathological examinations of specimens obtained by fine-needle aspiration biopsy from the liver mass, mediastinal mass, and prostatic nodules suggested granulomatous inflammation, and special stains including acid-fast staining, Grocott's methenamine silver (GMS), Periodic acid-Schiff (PAS), and gram were all negative. The diagnosis remained unclear.

On December 18, 2019, the patient was admitted to our hospital again with backache for 50 days. MRI of thoracic spine demonstrated multiple bone destructions at T3/T4 and T7/T8 with surrounding soft tissue lesions, suggesting spinal TB with cold abscesses (**Figures 1C–E**). Radionuclide bone scan demonstrated that the metabolism of vertebral bodies of T4 and T8 was abnormal, and metastatic bone tumors were considered (**Figure 1F**). He underwent a thoracic spinal operation. Considering the possibility of TB, the surgical specimens were subjected to both mNGS and histopathological examination. The process of mNGS is provided in the **Supplementary Material**. The mNGS revealed 25 total reads of MTB complex (**Table 2**). Significantly, because of the high internal homology and relatively insufficient coverage, the species within the MTB complex was not determined (13). Meanwhile, histopathological examination showed caseous necrosis with a significant number of inflammatory cells and multinucleate giant cells (**Figure 1G**). Therefore, the patient was diagnosed with spinal TB and immediately treated with anti-TB drugs including isoniazid, rifampicin, ethambutol, and pyrazinamide.

On March 1, 2020, the patient presented to our hospital for the third time due to coughing for a week. His bronchoalveolar lavage fluid (BALF) was obtained by fiberoptic bronchoscopy for mNGS and Xpert MTB/RIF assay. The process of Xpert MTB/RIF is provided in the **Supplementary Material**. MTB complex was successfully detected by mNGS. In addition, the result of Xpert MTB/RIF was positive for MTB gene and negative for rifampicin resistance. Based on the available evidence, it was eventually considered that the patient suffered from disseminated TB with systemic multi-organ involvement, including the lung, spine, mediastinum, liver, and prostate. To enhance the treatment effect, moxifloxacin was added to the original anti-TB treatment regimen.

According to the national laws and regulations, the patient was subsequently referred to the TB designated hospital for systematic treatment because of intrapulmonary TB. Through telephone follow-up, we learned that pyrazinamide was discontinued because of hyperuricemia shortly after the referral, and then the patient received anti-TB treatment with isoniazid, rifampicin, ethambutol, levofloxacin, and linezolid for 1 year. Diammonium glycyrrhizinate, which protects the liver, was continuous in the course of anti-TB therapy. The anti-TB drug regimen was well-tolerated and achieved a remarkable effect. At the latest follow-up, the patient reported significant reduction in the size of the lesions on imaging tests, reconfirming the diagnosis of TB, and started receiving anti-TB treatment with isoniazid, rifampicin, and ethambutol. He thanked us for our contribution to the diagnosis.

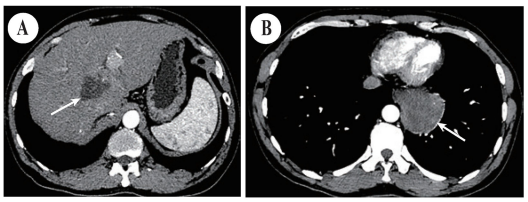
DISCUSSION

The clinical presentation of disseminated TB can vary greatly according to the involved organ system. It can present with atypical extrapulmonary symptoms as the first manifestation, often leading to a diagnostic dilemma. In our case, the patient was immunocompetent and came to our hospital due to a mass in the liver without any typical symptoms. For most clinicians, malignancy and infectious diseases are primary considerations among the broad differential diagnosis. Their identification needs evidence from additional tests.

December 14, 2017

The patient visited our outpatient department for finding a hepatic mass during health examination.

Contrast-enhanced abdominal CT: panel A and B



The patient refused further examination and visited other hospitals.

External hospital examinations:

F-18 FDG-PET/CT: intense accumulation of FDG in the right hepatic lobe, right abdomen, lower left lung, left hilar, and lower mediastinal lymph nodes

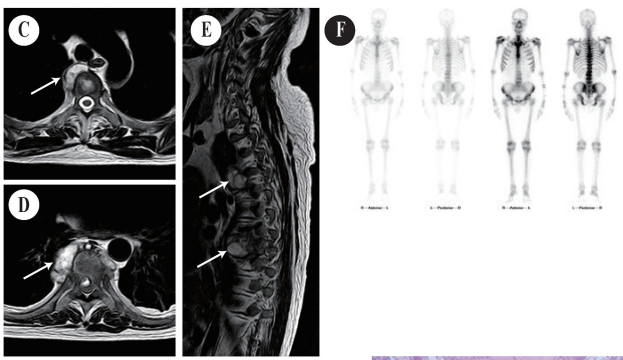
Liver biopsy: granulomatous inflammation
Biopsy of mediastinal mass: granulomatous inflammation
Prostate biopsy: granulomatous inflammation

December 18, 2019

The patient was admitted to our hospital with backache for 50 days.

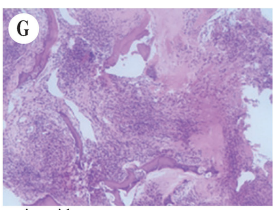
MRI of thoracic spine: panel C-E

Radionuclide bone scan: panel F



Thoracic spinal operation

mNGS (surgical specimens): MTB complex
Histopathological examination (surgical specimens): panel G



anti-TB drugs: isoniazid, rifampicin, ethambutol, and pyrazinamide

March 1, 2020

The patient presented to our hospital due to coughing for a week.

mNGS (BALF): MTB complex
Xpert MTB/RIF (BALF): MTB gene (+), rifampicin resistance gene (-)

Anti-TB drugs: isoniazid, rifampicin, ethambutol, pyrazinamide, and moxifloxacin

March 6, 2020

The patient was referred to the TB designated hospital.

Anti-TB drugs: isoniazid, rifampicin, ethambutol, levofloxacin, and linezolid

Continued follow-up:

The patient did not report any discomfort symptoms.

March 2021

The latest follow-up:

The patient reported significant reduction in the size of the lesions on imaging tests.

Anti-TB drugs: isoniazid, rifampicin, and ethambutol

FIGURE 1 | Timeline of the diagnosis and treatment process. **(A)** Contrast-enhanced abdominal CT revealed a hypodense lesion (white arrow) with mild to moderate enhancement in the right hepatic lobe near hepatic hilar. **(B)** Contrast-enhanced abdominal CT revealed a soft tissue density lesion (white arrow) with heterogeneous

(Continued)

FIGURE 1 | enhancement in the left posterior mediastinum. **(C)** MRI of thoracic spine revealed a soft tissue lesion (white arrow) at T3/T4. **(D)** MRI of thoracic spine showed a soft tissue lesion (white arrow) at T7/T8. **(E)** MRI of thoracic spine displayed multiple bone destructions (white arrows) at T3/T4 and T7/T8 with surrounding soft tissue lesions. **(F)** Radionuclide bone scan showed that the metabolism of vertebral bodies of T4 and T8 was abnormal. **(G)** Histopathological examination of the lesion at T7/T8 showed caseous necrosis with a significant number of inflammatory cells and multinucleate giant cells. CT, computed tomography; F-18 FDG-PET, 18F-fluorodeoxyglucose positron emission tomography; MRI, magnetic resonance imaging; T, thoracic vertebra; BALF, bronchoalveolar lavage fluid.

TABLE 1 | Laboratory examination results of the patient.

Items	Values	Reference range
White blood cell count ($\times 10^9$/L)	6.19	3.50–9.50
Neutrophil count ($\times 10^9$/L)	3.9	1.8–6.3
Lymphocyte count ($\times 10^9$/L)	1.2	1.1–3.2
Monocyte count ($\times 10^9$/L)	0.9	0.1–0.6
Red blood cell count ($\times 10^{12}$/L)	4.02	4.3–5.8
Hemoglobin (g/L)	129.0	130.0–175.0
Platelet count ($\times 10^9$/L)	273.0	125.0–350.0
Erythrocyte sedimentation rate (mm/h)	10	<21
Alanine aminotransferase (U/L)	33	9–50
Aspartate aminotransferase (U/L)	27	15–40
γ-Glutamyl transpeptidase (U/L)	252	10–60
Alkaline phosphatase (U/L)	183	45–125
Total bilirubin (μmol/L)	12.9	3.4–20.5
Direct bilirubin (μmol/L)	3.8	0.0–6.84
Indirect bilirubin (μmol/L)	9.1	1.7–13.7
Carcinoembryonic antigen (ng/ml)	1.48	0.00–5.00
Alpha-fetoprotein (ng/ml)	3.11	0.00–9.00
Hepatitis B surface antigen (IU/ml)	0	0.00–0.05
Hepatitis B surface antibody (mIU/ml)	18.16	0.00–10.00
Hepatitis C virus antibody	Negative	Negative
Human immunodeficiency virus antibody	Negative	Negative
Treponema pallidum antibody	Negative	Negative

TABLE 2 | Result of mNGS of the spinal surgical resection specimens.

Genus			Species	
Name	**Sequence number**	**Relative abundance (%)**	**Name**	**Sequence number**
Mycobacterium tuberculosis complex	25	1.0	/	/
Staphylococcus	38	3.9	*S. haemolyticus*	23
Moraxella	13	1.2	*M. osloensis*	13
Propionibacterium	7	0.5	*P. acnes*	3

WHO reported that nearly 30% TB cases failed to be diagnosed (14). The hepatic TB are largely non-specific and exhibit an extensive overlap with more common primary or metastatic liver carcinoma under image findings (15). Initially, this case was misdiagnosed as hepatic carcinoma according to the contrast-enhanced abdominal CT scan. Recently, F-18 FDG-PET/CT has emerged as an effective tool for the diagnosis and evaluation of malignant tumors. However, it is difficult for F-18 FDG-PET/CT to distinguish between inflammatory and malignant lesions because of the strong accumulation of F-18 FDG in both tissues (16). In our case, the F-18 FDG-PET/CT report was more favorable of malignancy vs. inflammation. Regrettably, due to the influence of the above imaging results or the lack of attention by clinicians to infectious diseases, the specimens from three biopsies were just collected for histopathological examinations, but no pathogenic detections, which led to diagnostic delay and worse clinical course.

At present, the dominating methods of diagnosing disseminated TB are still pathogen culture, AFB smear, nucleic acid amplification test (NAAT), and histopathological examination (17). MTB culture is currently considered as the gold standard of diagnosis, but low sensitivity and long culture time make it cannot meet the need of rapid clinical diagnosis (18). AFB smear, as a simple and fast tool, is unsatisfying due to lower sensitivity and specificity (19, 20). Just like the biopsies in our case, histopathological examination sometimes only shows granulomas without typical caseous necrosis, which is non-specific for the diagnosis of TB. PCR is a valuable diagnostic technique, but it always requires presupposing specific pathogens, which sometimes cannot detect rare pathogens and mixed infections (21). Additionally, Xpert MTB/RIF, a new automated molecular test, has been endorsed by WHO for the initial detection of TB and rifampicin resistance (14). Nevertheless, the sensitivity of Xpert MTB/RIF varies greatly with the type of extrapulmonary samples (22, 23). Significantly, mNGS exhibits better diagnostic performance than Xpert MTB/RIF in detecting MTB among various samples. Mutual combination can stimulate further elevation of the performance (13, 24).

mNGS, an unbiased culture-independent high-throughput sequencing technology, has shown a considerable clinical application prospect in diagnosis of infectious diseases over the past few years. In our case, mNGS finally unmasked TB after 2 years of delayed diagnosis and misdiagnosis. Notably, several prospective studies have indicated that mNGS shows an excellent diagnostic performance in suspected TB patients with 62–87.5% sensitivity for intrapulmonary samples, 47.4–60% sensitivity for extrapulmonary samples, and almost 100% specificity in various samples (13, 24, 25). In contrast to some traditional microbiological tests designed to specifically detect just one or a limited spectrum of known pathogens at a time, mNGS can simultaneously detect thousands of pathogens, including bacteria, fungi, viruses, and even parasites, without requiring any prior knowledge (26). Additionally, the turnaround time of mNGS, usually 2–3 days, is shorter than several weeks of traditional MTB culture, which might

benefit clinicians in making rapid diagnosis and guiding precise antimicrobial therapy. On the other hand, mNGS can obtain all nucleotide sequence information in clinical samples, including nucleotide sequences from human, contaminant during operations, harmless parasitic microbes, and true pathogens, thus can create challenges in data processing and interpretation of results (27). MTB, as one of the intracellular bacteria, is characterized by releasing fewer nucleic acids into extracellular environment, resulting in a small number of sequences detected, which may also lead to the difficulty of detecting MTB by mNGS (28). The detection of resistance genes and the strain discrimination of the MTB complex are hard to achieve clinically because they highly depend on genome coverage, and combining with targeted PCR might be a good choice (13). The combination of mNGS and Xpert MTB/RIF shows great potential in detecting MTB, which is superior to traditional pathogenic detection methods. In our case, MTB was reconfirmed by fiberoptic bronchoscopy combining with mNGS and Xpert MTB/RIF, meeting the requirement for etiology diagnosis and proving the clinical value of the combined application. Thus, mNGS alone or in combination with Xpert MTB/RIF provides clinicians with a reliable TB diagnosis method.

In conclusion, we emphasize that disseminated TB should also be considered in an immunocompetent patient even when imaging results favor malignant lesions based on the experience from this case. It is essential for clinicians to perform pathogenic examinations in time when suspicious specimens are encountered, and mNGS can be used as a valuable complementary means for TB diagnosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patient provided his written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YY and NY analyzed and interpreted the clinical data. YY, NY, and JZ drafted the manuscript. GQ and JC revised the manuscript. All authors have read and approved the final manuscript.

FUNDING

This research was supported by Key Program of Natural Science Foundation of Ningbo under Grant No. 202003N4019.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.687984/full#supplementary-material

REFERENCES

1. Click ES, Moonan PK, Winston CA, Cowan LS, Oeltmann JE. Relationship between *Mycobacterium tuberculosis* phylogenetic lineage and clinical site of tuberculosis. *Clin Infect Dis.* (2012) 54:211–9. doi: 10.1093/cid/cir788
2. Suárez I, Fünger SM, Kröger S, Rademacher J, Fätkenheuer G, Rybniker J. The diagnosis and treatment of tuberculosis. *Deutsches Aerzteblatt Online.* (2019) 116:729–35. doi: 10.3238/arztebl.2019.0729
3. Fujikawa T, Senoo A. Recurrent erythema nodosum as a warning of tuberculous lymphadenitis. *Mayo Clin Proc.* (2019) 94:174–5. doi: 10.1016/j.mayocp.2018.11.018
4. Nishizawa S, Tobino K. Asymptomatic tuberculous pleurisy mimicking mesothelioma. *Am J Respir Crit Care Med.* (2020) 201:1149. doi: 10.1164/rccm.201906-1223IM
5. Mahjoub S, Lahmar AA, Zaraa M, Belhaj G, Abdelkafi M, Mbarek M. Extensive lytic tuberculosis of the humeral head. *Joint Bone Spine.* (2018) 85:767. doi: 10.1016/j.jbspin.2018.04.005
6. Ran P, Liang X, Zhang Y, Sun P, Dong A, FDG. PET/CT in a case of bilateral tuberculous epididymo-orchitis. *Clin Nucl Med.* (2019) 44:757–60. doi: 10.1097/RLU.0000000000002606
7. Machida A, Amano E, Otsu S, Akagawa S. Corticosteroid-dependent tuberculous meningitis: a case report. *J Neurol Sci.* (2019) 396:232–4. doi: 10.1016/j.jns.2018.11.021
8. Suarez I, Maria Funger S, Jung N, Lehmann C, Reimer RP, Mehrkens D, et al. Severe disseminated tuberculosis in HIV-negative refugees. *Lancet Infect Dis.* (2019) 19:e352–9. doi: 10.1016/S1473-3099(19)30162-8
9. Crump JA, Reller LB. Two decades of disseminated tuberculosis at a university medical center: the expanding role of mycobacterial blood culture. *Clin Infect Dis.* (2003) 37:1037–43. doi: 10.1086/378273
10. Shi CL, Han P, Tang PJ, Chen MM, Ye ZJ, Wu MY, et al. Clinical metagenomic sequencing for diagnosis of pulmonary tuberculosis. *J Infect.* (2020) 81:567–74. doi: 10.1016/j.jinf.2020.08.004
11. Zhao M, Tang K, Liu F, Zhou W, Fan J, Yan G, et al. Metagenomic next-generation sequencing improves diagnosis of osteoarticular infections from abscess specimens: a multicenter retrospective study. *Front Microbiol.* (2020) 11:2034. doi: 10.3389/fmicb.2020.02034
12. Yan L, Sun W, Lu Z, Fan L. Metagenomic Next-Generation Sequencing (mNGS) in cerebrospinal fluid for rapid diagnosis of Tuberculosis meningitis in HIV-negative population. *Int J Infect Dis.* (2020) 96:270–5. doi: 10.1016/j.ijid.2020.04.048
13. Zhou X, Wu H, Ruan Q, Jiang N, Chen X, Shen Y, et al. Clinical evaluation of diagnosis efficacy of active *Mycobacterium tuberculosis* complex infection via metagenomic next-generation sequencing of direct clinical samples. *Front Cell Infect Microbiol.* (2019) 9:351. doi: 10.3389/fcimb.2019.00351
14. WHO. *Global Tuberculosis Report 2020*. WHO (2020). Available online at: https://www.who.int/publications-detail-redirect/9789240013131 (accessed October 15, 2020).
15. Kakkar C, Polnaya AM, Koteshwara P, Smiti S, Rajagopal KV, Arora A. Hepatic tuberculosis: a multimodality imaging review. *Insights Imaging.* (2015) 6:647–58. doi: 10.1007/s13244-015-0440-y
16. Li YJ, Zhang Y, Gao S, Bai RJ. Systemic disseminated tuberculosis mimicking malignancy on F-18 FDG PET-CT. *Clin Nucl Med.* (2008) 33:49–51. doi: 10.1097/RLU.0b013e31815c5004
17. Lewinsohn DM, Leonard MK, LoBue PA, Cohn DL, Daley CL, Desmond E, et al. Official American thoracic society/infectious diseases society of America/centers for disease control and prevention clinical practice guidelines: diagnosis of tuberculosis in adults and children. *Clin Infect Dis.* (2017) 64:111–5. doi: 10.1093/cid/ciw778

18. Tyrrell FC, Budnick GE, Elliott T, Gillim-Ross L, Hildred MV, Mahlmeister P, et al. Probability of negative mycobacterium tuberculosis complex cultures based on time to detection of positive cultures: a multicenter evaluation of commercial-broth-based culture systems. *J Clin Microbiol.* (2012) 50:3275–82. doi: 10.1128/JCM.01225-12
19. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis.* (2006) 6:570–81. doi: 10.1016/S1473-3099(06)70578-3
20. Ninet B, Rohner P, Metral C, Auckenthaler R. Assessment of use of the COBAS AMPLICOR system with BACTEC 12B cultures for rapid detection of frequently identified mycobacteria. *J Clin Microbiol.* (1999) 37:782–4. doi: 10.1128/JCM.37.3.782-784.1999
21. Huang Z, Zhang C, Hu D, Shi K, Li W, Zhang C, et al. Diagnosis of osteoarticular tuberculosis via metagenomic next-generation sequencing: a case report. *Exp Ther Med.* (2019) 18:1184–8. doi: 10.3892/etm.2019.7655
22. Tadesse M, Abebe G, Bekele A, Bezabih M, Yilma D, Apers L, et al. Xpert MTB/RIF assay for the diagnosis of extrapulmonary tuberculosis: a diagnostic evaluation study. *Clin Microbiol Infect.* (2019) 25:1000–5. doi: 10.1016/j.cmi.2018.12.018
23. Denkinger CM, Schumacher SG, Boehme CC, Dendukuri N, Pai M, Steingart KR. Xpert MTB/RIF assay for the diagnosis of extrapulmonary tuberculosis: a systematic review and meta-analysis. *Eur Respir J.* (2014) 44:435–46. doi: 10.1183/09031936.00007814
24. Chen P, Sun W, He Y. Comparison of metagenomic next-generation sequencing technology, culture and GeneXpert MTB/RIF assay in the diagnosis of tuberculosis. *J Thorac Dis.* (2020) 12:4014–24. doi: 10.21037/jtd-20-1232
25. Sun W, Lu Z, Yan L. Clinical efficacy of metagenomic next-generation sequencing for rapid detection of *Mycobacterium tuberculosis* in smear-negative extrapulmonary specimens in a high tuberculosis burden area. *Int J Infect Dis.* (2021) 103:91–6. doi: 10.1016/j.ijid.2020.11.165
26. Simner PJ, Miller S, Carroll KC. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. *Clin Infect Dis.* (2018) 66:778–88. doi: 10.1093/cid/cix881
27. Zhu YM, Ai JW, Xu B, Cui P, Cheng Q, Wu H, et al. Rapid and precise diagnosis of disseminated tmarneffei infection assisted by high-throughput sequencing of multifarious specimens in a HIV-negative patient: a case report. *BMC Infect Dis.* (2018) 18:379. doi: 10.1186/s12879-018-3276-5
28. Zhang HC Ai JW, Cui P, Zhu YM, Hong-Long W, Li YJ, et al. Incremental value of metagenomic next generation sequencing for the diagnosis of suspected focal infection in adults. *J Infect.* (2019) 79:419–25. doi: 10.1016/j.jinf.2019.08.012

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Ye, Yang, Zhou, Qian and Chu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 26 July 2021
doi: 10.3389/fmed.2021.679755

Metagenomic Next-Generation Sequencing Can Clinch Diagnosis of Non-Tuberculous Mycobacterial Infections: A Case Report

He Zhu[1], *Min Zhu*[2], *Jia-Hui Lei*[1], *Ya-Li Xiao*[1] *and Li-Min Zhao*[1,2]*

[1] *Department of Respiratory and Critical Care Medicine, People's Hospital of Zhengzhou University, Henan Provincial People's Hospital, Zhengzhou, China,* [2] *Department of Respiratory and Critical Care Medicine, Henan Provincial People's Hospital, Zhengzhou, China*

Non-tuberculou *Mycobacteria* (NTM) is ubiquitous in the environment and is conditional pathogen. Due to NTM and *Mycobacterium tuberculosis* belong to the genus *Mycobacterium*, their pathogenic mechanisms and clinical manifestations are similar. Therefore, NTM can cause tuberculosis-like lesions and lead to misdiagnosis. Early diagnosis and treatment greatly improve prognosis. However, traditional pathogenic microorganism detection has limitations, and it is difficult to accurately identify strains in clinical practice. Here, we report a 65-year-old man with NTM who presented with recurrent fever and cough. Computed tomography of the chest revealed a lung infection. The previous improper diagnosis and treatment did not improve his condition. With the aid of metagenomic next-generation sequencing, the pathogen was identified as *Mycobacterium avium complex*. Subsequently, he received accurate treatment and made significant improvements in clinical and radiology.

Keywords: non-tuberculous mycobacteria, metagenomic next-generation sequencing, *mycobacterium avium complex*, diagnosis, case report

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Robson Leão,
Rio de Janeiro State University, Brazil
Chun Chang,
Peking University Third Hospital, China

***Correspondence:**
Li-Min Zhao
zlm9898@126.com

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *12 March 2021*
Accepted: *30 June 2021*
Published: *26 July 2021*

Citation:
Zhu H, Zhu M, Lei J-H, Xiao Y-L and Zhao L-M (2021) Metagenomic Next-Generation Sequencing Can Clinch Diagnosis of Non-Tuberculous Mycobacterial Infections: A Case Report. Front. Med. 8:679755. doi: 10.3389/fmed.2021.679755

INTRODUCTION

Non-tuberculous *Mycobacteria* (NTM) refers to a general term for a large group of mycobacteria other than *Mycobacterium tuberculosis complex* and *Mycobacterium leprae*. According to statistics, more than 190 species of NTM strains and 14 subspecies have been discovered so far, of which only a few are pathogenic to humans and belong to conditional pathogens (1, 2). Among pathogenic bacteria, the *Mycobacterium avium complex* (*M. avium complex,* MAC) is the mycobacterium with the most new species or subspecies (3). NTM disease means that the human body is infected with NTM and causes the disease of related tissues and organs, most commonly in the lungs. In recent years, as the infection rate of NTM has increased year by year, NTM pulmonary disease (NTM-PD) has become a common clinical disease, accounting for about 70–80% in the United States, while China currently has no specific information data in this area (4, 5). Because the clinical characteristics of the disease are similar to those of tuberculosis, it often leads to misdiagnosis and missed diagnosis, which delays treatment and seriously threatens human life and health.

NTM-PD often occurs in patients with original underlying diseases, especially chronic respiratory diseases, such as chronic obstructive pulmonary disease, bronchiectasis, and cystic pulmonary fibrosis. Due to the weakened autoimmune function, NTM infection cannot be

effectively controlled, and eventually it progresses to NTM-PD (6, 7). The main clinical symptoms of NTM-PD are fever, cough and sputum. Due to the atypical clinical manifestations, it often needs to be differentiated from other diseases of the respiratory system. In the case of sterile species identification results, it can be misdiagnosed as *Mycobacterium tuberculosis* infection for a long time. Here, we report a case in which a patient's alveolar lavage fluid sample was identified by metagenomic next-generation sequencing (mNGS) and the final diagnosis was MAC lung disease.

CASE PRESENTATION

We report a 65-year-old man who attended teaching hospital at Henan with a 10-day history of fever, intermittent cough, expectoration and wheezing. Five days prior to admission, the patient had been treated with anti-infective drugs at his local hospital (such as rifampicin, ethambutol, clarithromycin, piperacillin sodium and tazobactam sodium,and levofloxacin), but the therapeutic effect was not good. He had a past medical history of chronic obstructive pulmonary disease (COPD). He lived in the rural Henan province, China, and has no recent travel history out of the province.

Physical examination showed that his underarm temperature was 38 °C, pulse was 108 beats/min, breathing was 25 beats/min, and the patient had a barrel-shaped chest, partial collapse of the upper left anterior thorax, clear voice during percussion of both lungs, regular breathing, and scattered dry and wet rales on auscultation of the lungs.

The laboratory studies revealed elevated erythrocyte sedimentation rate (69 mm/h), C-reactive protein (61.77 mg/L), rheumatoid factor quantification (74.7I U/mL), procalcitonin (0.06 ng/mL), blood glucose (7 mmol/L), cancer antigen 125 (35.21 U/mL), neuron specific enolase (20.56 ng/mL) and D-dimer (1.41 μg/mL). While the percent of lymphocytes (19%), red blood cell (3.47 × 109/L), hemoglobin (106 g/L), serum potassium (3.32 mmol/L), and albumin (27.1 g/L) were reduced. Other indicators, including GM test, T-SPOT.TB, Xpert MTB (Sputum) and antistreptococcal "o" quantification were negative.

Coughed up sputum smear examination: no bacteria and fungi were found in the microscopic examination, and acid-fast positive bacilli (+) were found in the microscopic examination. It was necessary to further distinguish *Mycobacterium tuberculosis* and non-tuberculous *Mycobacteria*.

Chest computed tomography scan indicated multiple flaky, strand-like and light mist-like high-density shadows in both lung fields. Visible effusion in the left pleural cavity (**Figure 1**). Hence, he was preliminarily diagnosed with lung infection and the nature of the upper right lung space to be investigated. We decided to treat the patient with intravenous biapenem for empirical anti-infective and oral rifabutin, ethambutol hydrochloride tablets and clarithromycin sustained-release tablets for anti-tuberculosis treatment, but his condition did not improve.

On day 3 of the admission, a bronchoscopy was performed, and the apical bronchus of the right upper lobe was blindly examined. Pathological results showed granulomatous inflammation with necrosis, special staining results showed acid-fast (-), molecular pathological results showed *Mycobacterium tuberculosis* DNA determination (TB-DNA) (PCR) (-) (**Figure 2**). Bronchoalveolar lavage fluid (BALF) was collected for mNGS and *M. avium complex* was detected (**Table 1**). Combined with clinical symptoms, laboratory test results and characteristics of MAC, the patient was diagnosed with MAC lung disease. Discontinued biapenem and switched to intravenous amikacin and oral compound sulfa tablets; and continued oral rifabutin, ethambutol hydrochloride tablets and clarithromycin. After 7 days of treatment, the patient's fever, cough, expectoration and wheezing had gradually improved. At the follow-up 3 months later, the patient's above symptoms had been completely relieved.

DISCUSSION

MAC disease is a zoonotic infectious disease caused by MAC infection. According to statistics, MAC is the main strain on all continents of the world (5, 8, 9). In China, the more common infections for NTM-PD are *M. kansasii* and *M. intercelleulare* (10). MAC infection can invade various tissues and organs, including lungs, bone marrow, and lymph nodes. It may also cause diseases of the synovium, bursa, tendon sheath, joint, bone and bone marrow (4, 5). Due to its pathological changes, bacteriological characteristics and antigenic components are

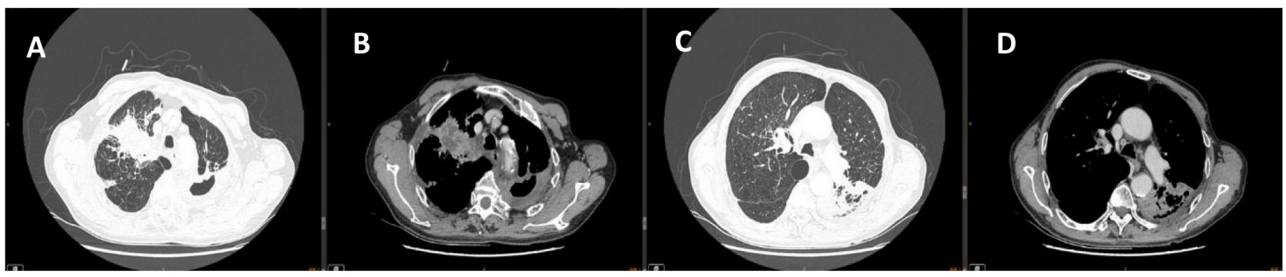


FIGURE 1 | Chest CT of **(A,C)** lung window images showed multiple flaky, strand-like and light mist-like high-density shadows in both lung fields. Chest CT of **(B,D)** corresponding mediastinum window images showed Spot-like calcification, thickened bronchial walls of both lungs, narrowed lumen, and left pleural effusion.

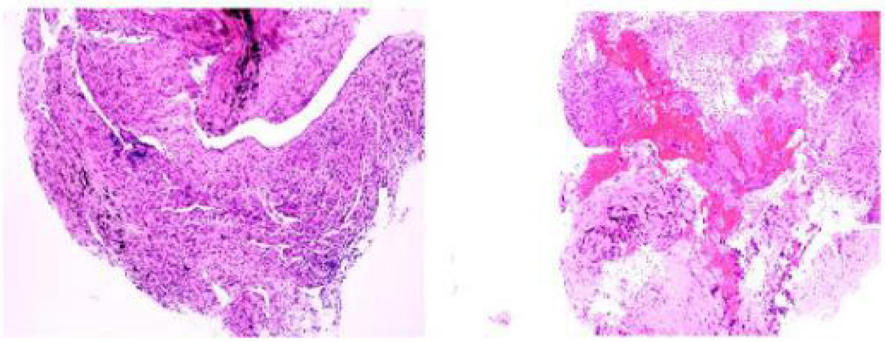


FIGURE 2 | The microscope showed no acid fast bacilli and fungi.

TABLE 1 | Results of mNGS in bronchoalveolar lavage fluid of patient.

	Genus		Species		
Type	**Name**	**Sequence number**	**Name**	**Sequence number**	**Relative abundance**
G+	*Mycobacterium*	23	*Mycobacterium avium complex*	2	0.005%

similar to *Mycobacterium tuberculosis*, sputum acid-fast staining can be positive. If the bacteria is not further identified, it is easy to be misdiagnosed as *Mycobacterium tuberculosis* infection for a long time, but traditional pathogenic microorganism detection technology has limitations such as low positive rate and long detection cycle, and it is difficult to accurately identify strains, which easily lead to clinical misdiagnosis and seriously affect the prognosis of patients. Especially for patients with positive acid-fast staining of respiratory tract specimens and the anti-tuberculosis treatment is not effective, it is necessary to be highly vigilant against NTM infection. In this case, sputum smear microscopy alone cannot distinguish *Mycobacterium tuberculosis* from NTM. In order to further determine the pathogen, the mNGS detection technology covering the pathogen is used to accurately detect the MAC infection. Combined with the patient's past history of COPD, clinical imaging features and mNGS test results, the final diagnosis was MAC lung disease. This case shows that mNGS has the advantages of high sensitivity and high specificity in the diagnosis and treatment of NTM-PD.

The diagnosis of NTM-PD requires the combination of respiratory system and systemic clinical symptoms, lung imaging findings, and NTM isolation and culture. NTM isolation and culture are the key to diagnosing NTM-PD and distinguishing tuberculosis (11). Based on mNGS does not rely on traditional microbial culture, no specific primers are required, and all DNA/RNA genome information in the sample can be determined in a single run. FEDRIZZI (12) used mNGS to sequence 47 types of NTM strains, including 11 rapidly-growing Mycobacterium strains and 36 slow-growing Mycobacterium strains. At the same time, 41 previously undescribed NTM species were reconstructed and analyzed and expanded the understanding of NTM. Therefore, mNGS has played a huge role in the identification of NTM-PD bacteria, genotyping, clinical diagnosis and treatment, and improvement of patient prognosis.

In the past, due to insufficient knowledge of non-tuberculous mycobacterial infections and limited diagnostic techniques, patients were misdiagnosed as tuberculosis infections, resulting in poor treatment effects and severely affecting the prognosis of patients. In this case, the patient's condition had been repeated for more than 40 years, and eventually progressed to NTM-PD, due to the inability to effectively control the NTM infection. For different NTM infections, the type of medication and the course of treatment are also different (13, 14). Compared with pulmonary infections caused by *Mycobacterium tuberculosis*, NTM-PD has a longer treatment time and a more complicated drug treatment plan. After NTM is tested and identified by mNGS technology, individualized drug treatment can be carried out. Therefore, mNGS detection technology can quickly and accurately detect the types of pathogenic bacteria infected by patients, so as to provide help for clinicians to diagnose and treat patients.

CONCLUSION

mNGS is a new type of pathogenic diagnosis technology, which has shown great potential in the identification of pathogenic pathogens of infectious diseases, and has the highest resolution of strain identification. With the increasing popularity and cost reduction of mNGS, it will play an increasingly important role in the diagnosis of NTM diseases. Finding and curing the source of infection in time and reducing contact with patients with NTM are the best measures to prevent NTM infection. Therefore, it is recommended to use mNGS to help clinicians improve the accuracy of identifying *Mycobacterium tuberculosis* and NTM.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HZ, J-HL, and Y-LX collected and analyzed patient data. HZ and MZ wrote the manuscript. L-MZ provided supervision. All authors approved the final version. Written consent for publication was obtained from the patient.

ACKNOWLEDGMENTS

We thank all colleagues who are engaged in medical work in the Department of Respiratory and Critical Care Medicine of Henan Provincial People's Hospital.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.679755/full#supplementary-material

REFERENCES

1. Daley CL, Iaccarino JM, Lange C, Cambau E, Wallace Jr RJ, Andrejak C, et al. Treatment of nontuberculous mycobacterial pulmonary disease: an official ATS/ERS/ESCMID/IDSA clinical practice guideline. *Eur Respir.* (2020) 56:200053. doi: 10.1183/13993003.00535-2020
2. Haworth CS, Banks J, Capstick T, Fisher AJ, Gorsuch T, Laurenson IF, et al. British thoracic society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). *Thorax.* (2017) 72(Suppl. 2): ii1–ii64. doi: 10.1136/thoraxjnl-2017-210929
3. Van Ingen J, Turenne CY, Tortoli E, Wallace Jr RJ, Brown-Elliott BA. A definition of the mycobacterium avium complex for taxonomical and clinical purposes, a review. *Int J Syst Evol Microbiol.* (2018) 68:3666–77. doi: 10.1099/ijsem.0.003026
4. Tang JS, Li L, Yan XF, Wu SM. Guidelines for the diagnosis and treatment of non-tuberculous mycobacterial diseases (2020 edition). *Chin J Tubercul Res Dis.* (2020) 11:918–46. doi: 10.3760/cma.j.cn112147-20200508-00570
5. Smith GS, Ghio AJ, Stout JE, Messier KP, Hudgens EE, Murphy MS, et al. Epidemiology of nontuberculous mycobacteria isolations among central North Carolina residents, 2006-2010. *J Infect.* (2016) 72:678–86. doi: 10.1016/j.jinf.2016.03.008
6. Orme IM, Ordway D. Host response to nontuberculous mycobacterial infections of current clinical importance. *Infect Immun.* (2014) 82:3516–22. doi: 10.1128/IAI.01606-13
7. Honda JR, Knight V, Chan ED. Pathogenesis and risk factors for nontuberculous mycobacterial lung disease. *Clin Chest Med.* (2015) 36:1–11. doi: 10.1016/j.ccm.2014.10.001
8. Prevots DR, Marras TK. Epidemiology of human pulmonary infection with nontuberculous mycobacteria: a review. *Clin Chest Med.* (2015) 36:13–34. doi: 10.1016/j.ccm.2014.10.002
9. Kwon BS, Lee JH, Koh Y, Kim WS, Song JW, Oh YM, et al. The natural history of non-cavitary nodular bronchiectatic mycobacterium avium complex lung disease. *Respir Med.* (2019) 150:45–50. doi: 10.1016/j.rmed.2019.02.007
10. Wu J, Zhang Y, Li J, Lin S, Wang L, Jiang Y, et al. Increase in nontuberculous mycobacteria isolated in Shanghai, China: results from a population-based study. *PLoS ONE.* (2014) 9:e109736. doi: 10.1371/journal.pone.0109736
11. Saifi M, Jabbarzadeh E, Bahrmand AR, Karimi A, Pourazar S, Fateh A, et al. HSP65- PRA identification of non-tuberculosis mycobacteria from 4892 samples suspicious for mycobacterial infections. *Clin Microbiol Infect*. (2013) 1:723–8. doi: 10.1111/j.1469-0691.2012.04005.x
12. Fedrizzi T, Meehan CJ, Grottola A, Giacobazzi E, Serpini GF, Tagliazucchi S, et al. Genomic characterization of nontuberculous mycobacteria. *Sci Rep*. (2017) 7:45258. doi: 10.1038/srep45258
13. Boyle DP, Zembower TR, Reddy S, Qi C. Comparison of clinical features, virulence, and relapse among mycobacterium avium complex species. *Am J Respir Crit Care Med.* (2015) 191:1310–7. doi: 10.1164/rccm.201501-0067OC
14. Falkinham JO 3rd. Current epidemiologic trends of the nontuberculous mycobacteria (NTM). *Curr Environ Health Rep*. (2016) 3:161–7. doi: 10.1007/s40572-016-0086-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Zhu, Zhu, Lei, Xiao and Zhao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Public Health

CASE REPORT
published: 13 August 2021
doi: 10.3389/fpubh.2021.663093

Case Report: *Tropheryma whipplei* Hide in an AIDS Patient With *Pneumocystis* Pneumonia

*Jun Yan, Binhai Zhang, Zhongdong Zhang, Jinchuan Shi, Shourong Liu, Jianjiang Qi and Mengyan Wang**

Department II of Infectious Diseases, Xixi Hospital of Hangzhou, Zhejiang University, Hangzhou, China

Introduction: *Pneumocystis* pneumonia (PCP) is one of the most common opportunistic infections in HIV-infected patients. However, coinfection with *Tropheryma whipplei* is infrequent in AIDS patients with PCP.

Case Presentation: We report a 28-year-old male AIDS patient coinfected with *T. whipplei* and *Pneumocystis jirovecii* diagnosed in the bronchoalveolar lavage. After sulfamethoxazole–trimethoprim and meropenem treatment, the patient showed clinical improvement in 2 weeks.

Conclusion: Clinicians need to be alert to the occurrence of *T. whipplei* infection in AIDS patients with PCP and timely diagnosis and antibacterial treatments are essential. This case may help clinicians for timely diagnosis of the coinfection of *T. whipplei* and *P. jirovecii* in AIDS patients.

Keywords: *Tropheryma whipplei*, *Pneumocystis* pneumonia, HIV, case report, NGS

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UCONN Health, United States

Reviewed by:
Yaxsier De Armas,
Pedro Kouri National Institute of Tropical Medicine (IPK), Cuba
Citra Fragrantia Theodorea,
University of Indonesia, Indonesia

****Correspondence:***
Mengyan Wang
21618054@zju.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *02 February 2021*
Accepted: *19 July 2021*
Published: *13 August 2021*

Citation:
Yan J, Zhang B, Zhang Z, Shi J, Liu S, Qi J and Wang M (2021) Case Report: Tropheryma whipplei Hide in an AIDS Patient With Pneumocystis Pneumonia. Front. Public Health 9:663093. doi: 10.3389/fpubh.2021.663093

INTRODUCTION

Pneumocystis pneumonia (PCP) is one of the most common opportunistic infections in HIV-infected patients with high morbidity and mortality. The in-hospital mortality rate has been decreasing since the implementation of combination antiretroviral therapies and chemoprophylaxis. However, the reported mortality rate for PCP still ranged from 12.8 to 33.1% after antiretroviral therapies and chemoprophylaxis (1, 2). *Tropheryma whipplei* pneumonia is very infrequent and easy to be overlooked, especially in AIDS-PCP patients. *T. whipplei* can cause endocarditis, encephalitis, and other acute infections. *T. whipplei* was first detected in Whipple disease which was considered as a metabolic disease in the 20th century (3). In this study, we first report a case of *T. whipplei* coinfection with *Pneumocystis jirovecii* in an AIDS patient.

CASE PRESENTATION

A 28-year-old male patient had a dry cough, chest tightness after exercise, and shortness of breath for 1 week. He took oral cephalosporin for 3 days without any efficiency, and his symptoms got worse. On admission, leukoplakia in the mouth, little wet rales at the bottom of both lungs, and multiple swollen lymph nodes were found on clinical examination. His temperature was 37.6°C. His blood test results showed that the white blood cell count was $6.52*10^{9/L}$, with an elevation in neutrophil ratio of 72.7%, high level of C-reactive protein (CRP) at 166 mg/L and serum amyloid protein A at 130 mg/L, low level of oxygen partial pressure at 87.5 mmHg and albumin

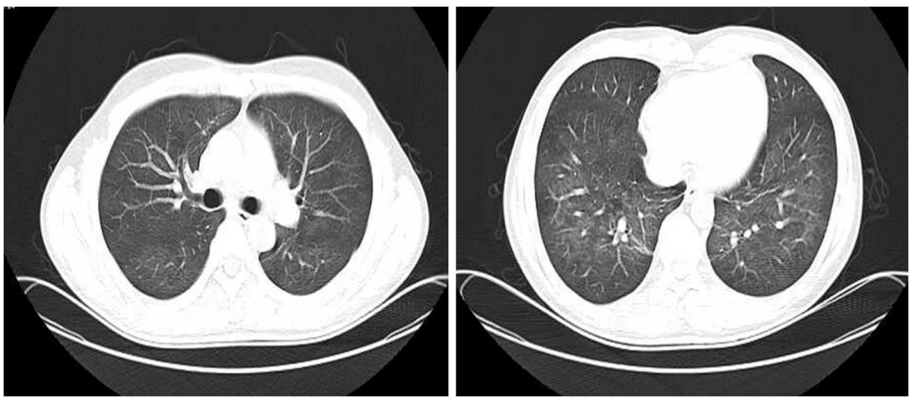


FIGURE 1 | CT of the lung revealed multifocal bilateral ground-glass opacities on admission.

at 30.3 g/L. Blood culture, antibody tests of *Legionella pneumophila*, *Streptococcus pneumoniae,* and *Mycoplasma pneumoniae*, and PCR-based detection of SARS-CoV-2 were negative. Cryptococcal capsular antigen, 1-3-β-D glucan detection, aspergillus galactomannan detection, and interferon-gamma release assays were negative. The viral load of HIV-RNA was 84,100 IU/ml. $CD4^+$ T-cell count was 11 cells/μl. CT of the lung revealed multifocal bilateral ground-glass opacities (**Figure 1**). According to the clinical diagnosis of PCP, the patient was treated with four pills of sulfamethoxazole–trimethoprim (sulfamethoxazole 1.2 g, trimethoprim 240 mg) three times a day.

After treatment for 2 days (on day 3), the symptoms of the patient were more severe, and his temperature got to 39.9°C. We performed a bronchoscopy on the patient, and it showed bronchial mucosa hyperemia and bronchial inflammatory changes. Nucleated cell counts of bronchoalveolar lavage (BAL) were $20 * 10^{6/L}$. Cryptococcal antigen, 1-3-β-D glucan detection, and culture of BAL were negative. Hexamine silver staining of *P. jiroveci* and PCR-based detection of cytomegalovirus and *Mycobacterium tuberculosis* were also negative. In addition, next-generation sequencing technologies (NGS) of BAL were carried out, the BAL was sent to a laboratory, and DNA was extracted for whole genomic sequencing, purified, and sonicated to a size of 100–150 bp. The DNA libraries were constructed followed by end repair, joint connection, no bias PCR amplification, and sequenced using the MGlseq-2000 platform after quality control. High-quality sequencing data were generated by removing low-quality reads. After removing human sequences, the remaining sequencing data were aligned to the bacterial, viral, fungal, and protozoan databases.

After treatment for 5 days (on day 6), CT of the lung revealed that it was worse than before (**Figure 2**). Results of NGS reported 205,658 unique reads of *P. jirovecii*, with coverage of identified genes 1.70% and 115 unique reads of *T. whipplei*, with coverage of identified genes 1.00% in BAL. The *P. jirovecii*-specific SYBR Green quantitative real-time PCR (qPCR) assay targeting a 301 bp fragment using a CFX96 Real-Time System was performed. The forward primer (pH207 5-ACAAATCGGACTAGGATAT AGCTGGT-3) and the reverse primer pAZ102-E were used to detect the mtLSUrRNA gene (4), and the copy number of *P. jiroveci* was 4,787.36 copies/ml. For specific *T. whipplei* qPCR, the specimen was tested by using Twhi3F (5-TTGTGTATT TGGTATTAGATGAAACAG-3), Twhi3R (5-CCCTACAATATG AAACAGCCTTTG-3) primer pair, and the specific TaqMan probe Twhi3 (6-FAM-GGGATAGAGCAGGAGGTGTCTGTC TGG-TAMRA). When the specimen was positive in this assay, the result was confirmed by a second qPCR by using Twhi2F (5-TGAGGATGTATCTGTGTATGGGACA-3) andTwhi2R (5-TCCTGTTACAAGCAGTACAAAACAAA-3) primer set, and the Twhi2 probe (6-FAM-GAGAGATGGGGTGCAGGACAG GG-TAMRA) (5). The copy number of *T. whipplei* was 11,717.3 copies/ml. Considering the progressed condition of the patient, meropenem 1 g q8h and caspofungin 70 mg per day were added for treating the infections of *T. whipplei* and *P. jirovecii*. Methylprednisolone 40 mg BID was added due to the severe symptoms. On day 12, there was a clinical improvement, and CT of the lungs showed obviously better than before (**Figure 3**), and the patient was discharged on day 16.

DISCUSSION AND CONCLUSIONS

A previous study had found that *T. whipplei* could easily colonize in the lungs of HIV individuals by analyzing bacterial gene sequences in BAL (6). An AIDS patient with *T. whipplei* pneumonia had been reported before, and Stein *et al.* suggested that *T. whipplei* should be considered in the differential diagnosis of pneumonia in AIDS patients (7). In this study, we reported the coinfection of *T. whipplei* and *P. jirovecii* pneumonia, which frequently had similar symptoms of fever, cough, and dyspnea (8–10), and a previous case-control study revealed that *P. jirovecii* was frequently associated with the *T. whipplei* pneumonia (11).

As we all know, lung infections by opportunistic or virulent pathogens are the major reasons that cause high mortality in AIDS patients. However, it is difficult to diagnose

Abbreviations: AIDS, Acquired immune deficiency syndrome; PCP, Pneumocystis pneumonia; BLAF, Bronchoalveolar lavage fluid; NGS, next-generation sequencing; CRP, C-reactive protein; PCR, polymerase chain reaction.

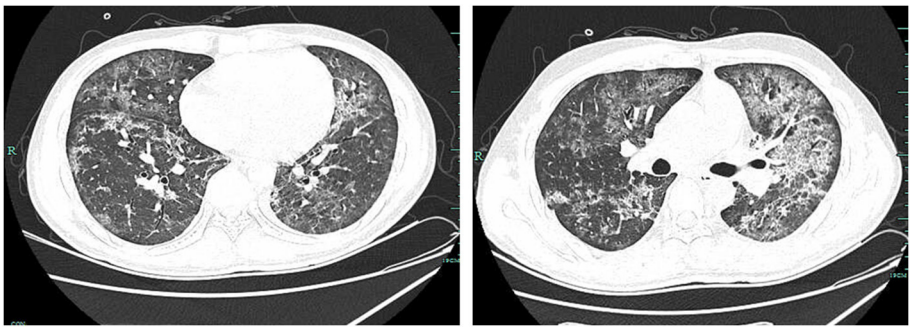


FIGURE 2 | CT of the lung revealed that multiple infections occurred in bilateral lungs, and they were scattered with diffuse patchy, ground glass-like, and high density of shadows on day 6.

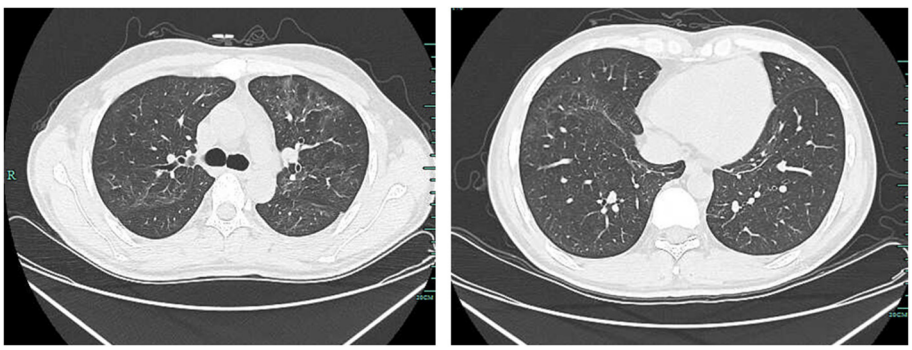


FIGURE 3 | CT of the lung showed that multiple infections occurred in bilateral lungs, and they were better, slightly diffuse, and with a ground glass-like high density of shadows on day 12.

specific pathogens of lung infection. Due to the difficulty of culture, the diagnosis methods of *P. jirovecii* included direct cytochemical staining, immunofluorescent staining with monoclonal antibodies, and molecular methods such as PCR, with no ideal specificity and sensitivity (12). Similar to *P. jirovecii*, the culture of *T. whipplei* is difficult, which may not be suitable for the clinic. Therefore, PCR, a more specific and sensitive technique than the other methods, has become a preferred technique for diagnosing *T. whipplei* infection (13). In this report, we detected the presence of *P. jirovecii* and *T. whipplei* by NGS using BAL. NGS technology has been booming since 2014 for pathogenic testing (14). Nowadays, NGS has been widely applied to clinics.

Antibiotics including penicillin, streptomycin, tetracycline, ceftriaxone, meropenem, co-trimoxazole, doxycycline, and hydroxychloroquine have been used for the treatment of *T. whipplei* infection (13). In this case, oral sulfamethoxazole-trimethoprim treatment seems to be slow to work in the first few days. Adding intravenous meropenem for 1 week helped the patient to clinically improve.

Pneumocystis pneumonia is one of the common opportunistic infections in HIV-infected patients. This case highlights the occultation of acute *T. whipplei* pneumonia in an AIDS patient with PCP. Clinicians need to be alert to the occurrence of *T. whipplei* infection and timely diagnosis and antibacterial treatments are essential.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Xixi Hospital of Hangzhou. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MW, JY, and BZ were involved in the clinical management of this patient. JY, JS, BZ, and ZZ collected the data of the patient. SL and

JQ contributed to manuscript preparation. MW wrote the article. All authors read and approved the final manuscript.

FUNDING

This work was funded by Key projects of the Ministry of Science and Technology (No. 2018YFC1705701) and the Technology Plan of Medicine Health Science in Zhejiang Province (No. 2019KY533). The funding body had no role in the design of the study and collection, analysis, interpretation of data, or preparation of the manuscript.

ACKNOWLEDGMENTS

The authors thank the staff in the HIV/AIDS ward of the Xixi Hospital of Hangzhou.

REFERENCES

1. Schmidt JJ, Lueck C, Ziesing S, Stoll M, Haller H, Gottlieb J, et al. Clinical course, treatment and outcome of Pneumocystis pneumonia in immunocompromised adults: a retrospective analysis over 17 years. *Crit Care.* (2018) 22:307. doi: 10.1186/s13054-018-2221-8
2. Xiao J, Gao G, Li Y, Zhang W, Tian Y, Huang Y, et al. Spectrums of opportunistic infections and malignancies in HIV-infected patients in tertiary care hospital, China. *PLoS ONE.* (2013) 8:e75915. doi: 10.1371/journal.pone.0075915
3. Raoult D. From whipple disease to Tropheryma whipplei infection. *Clin Infect Dis.* (2019) 68:1098–9. doi: 10.1093/cid/ciy668
4. Wang M, Dai X, Huang Y, Sun J, Dong X, Guo Y, et al. The presence of Pneumocystis jirovecii DNA in plasma is associated with a higher mortality rate in patients with AIDS-associated Pneumocystis pneumonia. *Med Mycol.* (2018). doi: 10.1093/mmy/myy110
5. Fenollar F, Laouira S, Lepidi H, Rolain JM, Raoult D. Value of Tropheryma whipplei quantitative polymerase chain reaction assay for the diagnosis of Whipple disease: usefulness of saliva and stool specimens for first-line screening. *Clin Infect Dis.* (2008) 47:659–67. doi: 10.1086/590559
6. Lozupone C, Cota-Gomez A, Palmer BE, Linderman DJ, Charlson ES, Sodergren E, et al. Widespread colonization of the lung by Tropheryma whipplei in HIV infection. *Am J Respir Crit Care Med.* (2013) 187:1110–7. doi: 10.1164/rccm.201211-2145OC
7. Stein A, Doutchi M, Fenollar F, Raoult D. Tropheryma whipplei pneumonia in a patient with HIV-2 infection. *Am J Respir Crit Care Med.* (2013) 188:1036–7. doi: 10.1164/rccm.201304-0692LE
8. Fenollar F, Ponge T, La Scola B, Lagier JC, Lefebvre M, Raoult D. First isolation of Tropheryma whipplei from bronchoalveolar fluid and clinical implications. *J Infect.* (2012) 65:275–8. doi: 10.1016/j.jinf.2011.11.026
9. Bassene H, Mediannikov O, Socolovschi C, Ratmanov P, Keita AK, Sokhna C, et al. Tropheryma whipplei as a Cause of Epidemic Fever, Senegal, 2010-2012. *Emerg Infect Dis.* (2016) 22:1229–334. doi: 10.3201/eid2207.150441
10. Masur H, Brooks JT, Benson CA, Holmes KK, Pau AK, Kaplan JE. Prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: Updated Guidelines from the Centers for Disease Control and Prevention, National Institutes of Health, and HIV Medicine Association of the Infectious Diseases Society of America. *Clin Infect Dis.* (2014) 58:1308–11. doi: 10.1093/cid/ciu094
11. Lagier JC, Papazian L, Fenollar F, Edouard S, Melenotte C, Laroumagne S, et al. Tropheryma whipplei DNA in bronchoalveolar lavage samples: a case control study. *Clin Microbiol Infect.* (2016) 22:875–9. doi: 10.1016/j.cmi.2016.07.010
12. Esteves F, Lee CH, de Sousa B, Badura R, Seringa M, Fernandes C, et al. (1-3)-beta-D-glucan in association with lactate dehydrogenase as biomarkers of Pneumocystis pneumonia (PcP) in HIV-infected patients. *Eur J Clin Microbiol Infect Dis.* (2014) 33:1173–80. doi: 10.1007/s10096-014-2054-6
13. Dolmans RA, Boel CH, Lacle MM, Kusters JG. Clinical manifestations, treatment, and diagnosis of Tropheryma whipplei infections. *Clin Microbiol Rev.* (2017) 30:529–55. doi: 10.1128/CMR.00033-16
14. Wilson MR, Naccache SN, Samayoa E, Biagtan M, Bashir H, Yu G, et al. Actionable diagnosis of neuroleptospirosis by next-generation sequencing. *N Engl J Med.* (2014) 370:2408–17. doi: 10.1056/NEJMoa1401268

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Yan, Zhang, Zhang, Shi, Liu, Qi and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 10 September 2021
doi: 10.3389/fcimb.2021.674235

Homologous *Escherichia coli* Identified in Cerebrospinal Fluid and Bloodstream

Qingmiao Shi[1,2†], *Jun Zhang*[3†], *Jinghui Wang*[4], *Lijuan Du*[4], *Zhaoyang Shi*[4], *Min Xu*[5*] and *Yonggang Luo*[4*]

[1] Gene Hospital of Henan Province, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Department of Intensive Care Unit, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5] Department of Clinical Laboratory, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
George Weinstock,
Jackson Laboratory for Genomic Medicine, United States

Reviewed by:
Rafael Franco-Cendejas,
National Institute of Rehabilitation Luis Guillermo Ibarra Ibarra, Mexico
Lingyun Guo,
Capital Medical University, China

***Correspondence:**
Min Xu
xumin3568@sina.com
Yonggang Luo
Luoyg_514@126.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 01 March 2021
Accepted: 11 August 2021
Published: 10 September 2021

Citation:
Shi Q, Zhang J, Wang J, Du L, Shi Z, Xu M and Luo Y (2021) Homologous Escherichia coli Identified in Cerebrospinal Fluid and Bloodstream. Front. Cell. Infect. Microbiol. 11:674235. doi: 10.3389/fcimb.2021.674235

Background: *Escherichia coli* is an opportunistic bacterium that causes a wide range of diseases, such as bloodstream infection and central nervous system infection. The traditional culture-based method to detect *E. coli* usually takes more than 2 days. The object of this study is to explore the value of metagenomic next-generation sequencing (mNGS) in identifying *E. coli* from human cerebrospinal fluid. In addition, we investigated the infection source of *E. coli* through whole genome sequencing and phylogenetic analysis.

Methods: We combined a clinical example to analyze the function of mNGS in pathogen detection from cerebrospinal fluid. NextSeq 550Dx platform was applied for mNGS. Next, whole genome sequencing was performed to obtain the genomic characterization of *E. coli*. Furthermore, we screened 20 *E. coli* strains from the National Center for Biotechnology Information and conducted a phylogenetic analysis.

Results: A middle-aged patient who attended our hospital was diagnosed with craniopharyngioma and received surgery. The patient had recurrent fever and persistent lethargy after surgery. Cerebrospinal fluid culture firstly failed to grow the bacteria. Next the cerebrospinal fluid sample was detected by mNGS and the sequence readings of *E. coli* were identified. Later, *E. coli* was reported *via* the second cerebrospinal fluid culture, certifying the result of mNGS. Moreover, we also cultured carbapenem-resistant *E. coli* from the patient's bloodstream. Through whole genome sequencing and phylogenetic analysis, we found that the *E. coli* isolated from cerebrospinal fluid and the bloodstream was 100% homologous, indicating the *E. coli* central nervous system infection was originated from the bloodstream.

Conclusion: Metagenomic next-generation sequencing is a valuable tool to identify the pathogens from cerebrospinal fluid, and seeking the infection source is of great significance in clinical diagnosis and treatment. Furthermore, carbapenem-resistant *E. coli* is a serious problem as the cause of bloodstream infection and central nervous

system infection, and effective and adequate measures to prevent and control the present circumstance are urgent.

Keywords: *Escherichia coli*, whole genome sequencing, phylogenetic analysis, homology, craniopharyngioma, metagenomic next-generation sequencing

INTRODUCTION

Escherichia coli is a common gram-negative bacterium that can be a harmless inhabitant of the gastrointestinal tract in humans (Croxen et al., 2013). These symbiotic *E. coli* strains seldom contribute to illness. However, once the individual's immune dysfunction or gut barrier is destroyed, it is also pathogenic and can result in many diseases, including diarrheal and extraintestinal diseases such as bloodstream infection, urinary tract infection, and central nervous system (CNS) infection (Kaper et al., 2004). The traditional diagnosis for pathogenic *E. coli* has depended on culture-based techniques (Peña-Gonzalez et al., 2019), but it usually takes more than 2 days. With the development of molecular diagnostic tools such as metagenomic next-generation sequencing (mNGS), it provides a handy sensitivity method to rapidly detect the pathogens (Li et al., 2021).

Craniopharyngioma is a rare benign epithelial tumor of the CNS derived from residual cells of Rathke's pouch, which includes two different histopathological type, adamantinomatous and papillary craniopharyngioma (Müller, 2014). It constitutes about 2–5% of primary intracranial tumors with an overall morbidity of 0.5–2 cases per million (Erfurth et al., 2013). Approximately 60% of all cases emerge in children, and the second peak of onset is the age of 40 to 44 years old. The invasion, adhesion, and compression of key structures around the sellar region lead to high mortality of craniopharyngioma even while receiving treatment (Karavitaki et al., 2005; Zoicas and Schöfl, 2012). The most common postoperative complications are involved in neuroendocrine dysfunction, visual deficits, and hypothalamic metabolic disorder. Although intracranial infection is seldom reported after surgery, it's a serious problem and shouldn't be ignored by clinicians.

Our study herein reported a febrile and lethargic patient who had undergone excision surgery of adamantinomatous craniopharyngioma. We identified *E. coli* sequence readings from the patient's cerebrospinal fluid (CSF) by mNGS when the traditional CSF culture first failed to grow a pathogen. Furthermore, we confirmed that the *E. coli* intracranial infection was sourced from bloodstream through whole genome sequencing and phylogenetic analysis, thus providing a modest recommendation for clinical management **(Figure 1)**.

MATERIALS AND METHODS

Patient and Samples

Our study involved a middle-aged patient who was diagnosed with craniopharyngioma and received surgery at our hospital.

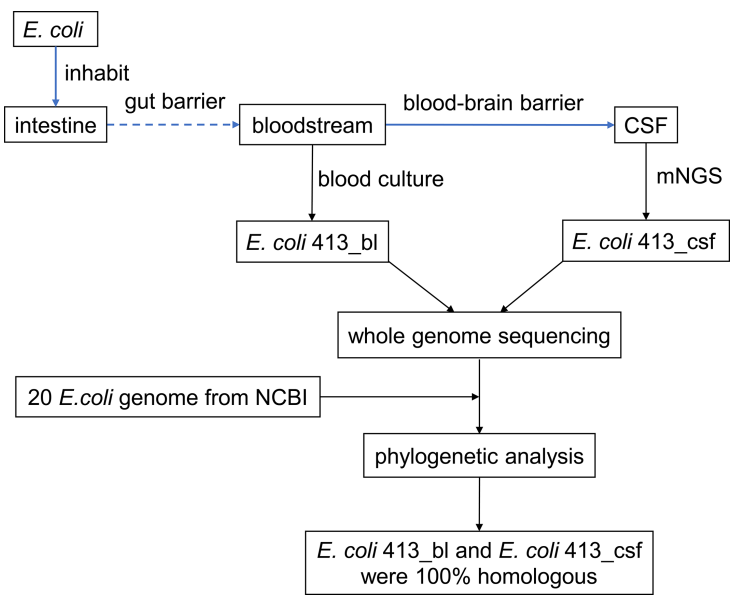


FIGURE 1 | Study design and flow diagram. CSF, cerebrospinal fluid; mNGS, metagenomic next-generation sequencing; NCBI, National Center for Biotechnology Information.

Because of recurrent fever and persistent lethargy after surgery, the patient's CSF sample was collected after lumbar puncture by an experienced intensive care unit doctor, and the *E. coli* sequence readings were detected from CSF by mNGS. In addition, two suits of blood cultures were taken for the patient; one suit was from peripheral vein and another from central venous line. Each set included an aerobic bottle and an anaerobic bottle. And all the four bottles cultured carbapenem-resistant *E. coli*. The antimicrobial susceptibility test was detected by Kirby-Bauer and broth dilution method.

Metagenomic Next-Generation Sequencing

After CSF sample preprocessing, the microbial genomic DNA extraction was performed by TIANamp Micro DNA Kit (TIANGEN Biotech, Beijing) according to the manufacturer's instruction. Next, library construction was carried out, including genomic DNA fragmented, end-repair, 3' adenylated, adapters ligation, PCR amplifying, and purified products. The qualified single-stranded circular DNA library was sequenced on NextSeq 550Dx platform (illumina, San Diego, USA) with a single-end 75bp sequencing strategy. We obtained the sequencing depth (**Supplementary 1**) using the complete genome of *Escherichia coli* str. K-12 substr. MG1655 (NCBI Reference Sequence: NC_000913.3) as genome reference sequence. Finally, we conducted a bioinformatics analysis and determined the mNGS-based pathogenic microorganism. The result revealed that mNGS identified 32,296 sequence readings of *E. coli* in the CSF sample.

Whole Genome Sequencing

Next, the *E. coli* strains isolated from CSF and the bloodstream were sequenced by whole genome sequencing, respectively. Firstly, the genomic DNA of *E. coli* was extracted by using MagPure DNA Kit (Cat. No. MD5118-05F, Magen, China). Secondly, we determined the concentration and purity of DNA through Qubit fluorometer and Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Thirdly, library construction and whole genome sequencing was performed on the MGISEQ-2000 platform at BGI (Shenzhen, China). The antibiotic-resistant genes were annotated using CARD (Comprehensive Antibiotic Resistance Database Version 3.1.2).

Screening of *E. coli* Strains

Twenty *E. coli* strains with genome information were screened out from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). The 20 *E. coli* strains meet the following criteria: (1) the hosts were homo sapiens or human; (2) the isolation source was blood, CSF, or feces; (3) the collection date was in the last 10 years, except that the strain CE10 isolated from CSF was collected in the 1970s. The genome assembly and annotation data of the 20 *E. coli* strains were downloaded from Genbank (https://ftp.ncbi.nlm.nih.gov/genomes/genbank/) for a genomic phylogenetic analysis.

Phylogenetic Analysis

Roary, a pipeline that structures large-scale pan genomes at a high speed (Page et al., 2015), was used to construct the pan genome and core genome of 22 *E. coli* strains. Next, we adopted ClonalFrameML to analyze and detect recombination in bacterial whole genomes (Didelot and Wilson, 2015). Single nucleotide polymorphisms distance matrix with snp-dists was calculated. Furthermore, we performed phylogenetic analysis and a phylogenetic tree of 22 *E. coli* strains with branch lengths was reconstructed to display the evolutionary relationships.

RESULTS

Case Presentation

A middle-aged patient who complained of blurred vision with headache for three years was admitted to the neurosurgery department on July 23, 2020. The patient had a two-year history of type II diabetes and received oral hypoglycemic drugs regularly. Laboratory tests showed the fasting plasma glucose of 6.76 mmol/L, with glycated hemoglobin of 7.10%. Other tests were within normal range including blood routine examination, blood biochemistry, coagulation function, and thyroid function. Head magnetic resonance imaging scanning indicated a suprasellar space-occupying lesion (**Figure 2A**), which was suspected as craniopharyngioma. After a thorough evaluation, on August 3, the patient was administered the resection of the lesion and repair of CSF rhinorrhea by endoscopic endonasal transsphenoidal approach, which was confirmed to be adamantinomatous craniopharyngioma by histopathological examination of a surgical specimen afterwards (**Figure 2B**).

Despite using cefotiam (1g, q12h) as prophylactic treatment for surgery, the patient developed fever and lethargy the following day post-surgery. Reexamination of blood routine reported raised white blood cell of 13.49×10^9/L (reference range $3.9–9.5\times10^9$/L) and percentage of neutrophils of 92.1% (reference range 40–75%). The hemoglobin content 106.0 g/L (reference range 115–150 g/L) and percentage of lymphocyte 6.2% (reference range 20–50%) were reduced. Lumbar puncture was performed and CSF tests revealed elevated glucose 15.29 mmol/L (reference range 2.5–4.4 mmol/L), total protein 5982.00 mg/L (reference range 100–300 mg/L), and count of white blood cells 192.00×10^6/L. Continuous monitoring of the CSF indicated no sign of CNS infection. Bacterial culture and gram stains of CSF reported negative results, respectively. The patient was given empiric anti-infective treatment with latamoxef sodium (1.5g, q12h) and vancomycin (1g, q12h) intravenously. Six days later, the patient's condition was improved.

On August 18, the patient occurred CSF rhinorrhea. Repair surgery of CSF rhinorrhea was performed by endoscopic endonasal transsphenoidal approach immediately. However, the patient developed fever and lethargy again the day after surgery, which had high possibility of intracranial infection considering that the characteristics of CSF was bloody and cloudy. Vancomycin (1g, q12h) and cefoperazone-sulbactam (3g, q12h) were given for anti-infection intravenously, but the fever didn't reduce. To make matters worse, the patient emerged in diabetic ketoacidosis and was transmitted to intensive care unit urgently. CSF was bloody and cloudy with 0.77 mmol/L of glucose, and the glucose ratio was 0.06. The count of white blood cells in CSF was increased to 187385.00×10^6/

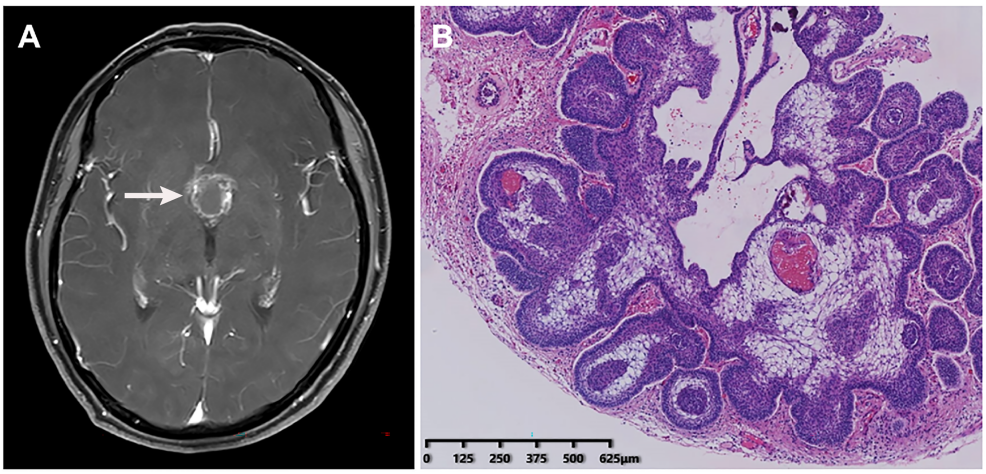


FIGURE 2 | Radiological imaging and histopathological examination of the patient. **(A)** Head magnetic resonance imaging showed a suprasellar space-occupying lesion. **(B)** Histopathological examination of a surgical specimen indicated adamantinomatous craniopharyngioma.

L and the total protein was 4098.5 mg/L. But the bacterial culture of CSF was negative as before. In order to further seek the pathogen, the CSF was tested by mNGS and 32,296 sequence readings of *E. coli* was identified in CSF. Next, *E. coli* was also reported *via* the second cerebrospinal fluid culture, certifying the result of mNGS. After consultation with the pharmacy department, meropenem (2g, q8h), tigecycline (100mg, q12h), and linezolid (0.6g, q12h) were applied intravenously for this patient. To our surprise, carbapenem-resistant *E. coli* was cultured in the patient's bloodstream later. The minimum inhibitory concentration (MIC) was measured by broth dilution method. The antimicrobial susceptibility test showed a multi-drug resistance of *E. coli* to doxycycline (MIC ≥ 16 ug/ml), piperacillin-tazobactam (MIC ≥ 128 ug/ml), cefoperazone/sulbactam (MIC ≥ 64 ug/ml), ceftazidime (MIC ≥ 64 ug/ml), cefepime (MIC ≥ 32 ug/ml), aztreonam (MIC ≥ 64 ug/ml), meropenem (MIC ≥ 16 ug/ml), imipenem (MIC ≥ 16 ug/ml), levofloxacin (MIC ≥ 8 ug/ml), and so on.

Questioning the medical history of the patient, we came to know that the patient had symptoms of diarrhea after the surgery for craniopharyngioma. Thus, we inferred that the gut microbiota translocations occurred in the state of surgical stress. The *E. coli* residing in the intestine became opportunistic bacterium and translocated into the blood, resulting in bloodstream infection. Furthermore, the *E. coli* in the bloodstream moved through the blood-brain barrier and entered the CSF, causing CNS infection. To prove the conjecture, whole genome sequencing analysis of *E. coli* strains isolated from CSF sample and bloodstream sample were performed. After phylogenetic analysis, the results suggested that the *E. coli* from CSF was 100% homologous with the *E. coli* from the blood. It indicated that the intracranial *E. coli* infection of the patient was not caused by the invasive surgical procedure but originated from the bloodstream. So far, the cause of the patient's intracranial infection can be clarified.

According to combined drug susceptibility test, the anti-infective regimen was adjusted to intravenous administration of ceftazidime-avibactam (2.5g, q8h, ivgtt) and aztreonam (2g, q6h, ivgtt), along with intrathecal injection of polymyxin B (5WIU). Considering the neurotoxicity of polymyxin B, its use was discontinued after seven times intrathecal injections. Through 20 days of anti-infection treatment, the fever of this patient was controlled and her condition was getting better. CSF was transparent with white blood cells count of 53×10^6/L, glucose of 5.49 mmol/L, and total protein of 1319.0 mg/L. Next the antibiotics were discontinued, the patient remained in the hospital under observation for a week, and then was discharged. Two weeks later, we followed up with the family of the patient. Unfortunately, we learned that the patient has expired outside hospital due to the uncontrolled infection caused by newly developed and untreated CSF leak. The schematic of the patient's clinical course was showed in **Figure 3**.

The *E. coli* Strains Identified in Our Study

From the CSF sample, we detected 32,296 sequence readings of *E. coli* genome by mNGS and this strain *E. coli* was named 413_csf. From the blood culture sample, we identified carbapenem-resistant *E. coli*, which was named 413_bl. Through whole genome sequencing, it showed that the *E. coli* 413_bl and 413_csf were homologous. The antibiotics-resistant genes data of whole genome sequencing was presented in **Supplementary 2**. In addition, the carbapenem resistance arose from β-lactam ring hydrolysis by dedicated carbapenems enzymes and the carbapenems come from ambler class B metallo-β-lactamases.

Genomic Characteristics

We filtered out 20 *E. coli* strains with whole genome sequencing information from the National Center for Biotechnology Information. The genomic characteristics of 20 *E. coli* strains were presented in **Table 1**. Fourteen *E. coli* strains were isolated from the blood of homo sapiens; one strain was from CSF and five strains were from feces. The genome size and protein-coding sequences for 20 *E. coli* strains ranged from 4.82 Mb to 5.71 Mb

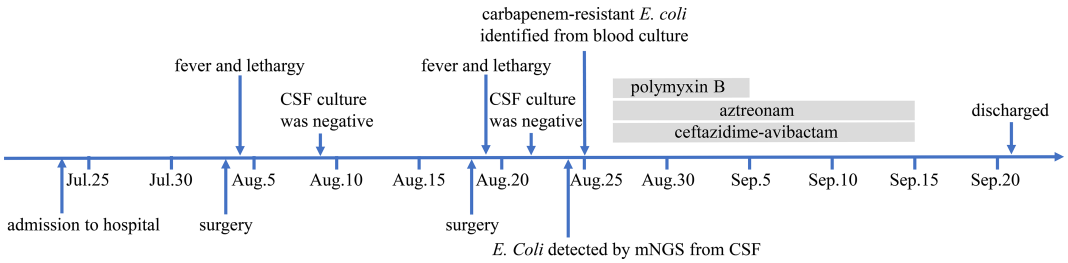


FIGURE 3 | The schematic of the patient's clinical course. CSF, cerebrospinal fluid; mNGS, metagenomic next-generation sequencing.

and 4006 to 5179 genes. The G+C contents of the 20 *E. coli* genome were between 50.39% and 51.21%.

Genomic Recombination Analysis

We used Roary to create a pan-genome and core-genome of 22 strains and got 2654 core genes (99%≤strains ≤ 100%) and 14,854 pan genes (0%≤strains ≤ 100%). Then we identified recombination with ClonalFrameML and got a pairwise single nucleotide polymorphisms distance matrix with snp-dists (**Supplementary 3**) from a FASTA sequence alignment after we masked the recombination regions. We found that the snp-dists between *E. coli* 413_bl and 413_csf was 0, which provided evidence that the two *E. coli* strains were highly homologous.

Phylogenetic Analysis for *E. coli*

We performed phylogenetic analysis for *E. coli* and conducted a phylogenetic tree based on the whole genome sequencing data of 22 *E. coli* strains (**Figure 4**). It showed that *E. coli* 413_bl and 413_csf exist in the same clade with another five strains, including *E. coli* GZEC065, SJ7, 2EC1, NCCP15955, RM-103-MS, indicating that *E. coli* 413_bl and 413_csf have close genetic relationship with them. Combining the result that the snp-dists between *E. coli* 413_bl and 413_csf was 0, which further confirmed the 100% homologousness of them. *E. coli* GZEC065 was isolated from the blood of homo sapiens. *E. coli* SJ7, 2EC1, NCCP15955, and RM-103-MS were isolated from the feces of homo sapiens. Thus, we inferred that the *E. coli* 413_bl in our case may be from the intestine, and the *E. coli* 413_bl in the bloodstream entered the cerebrospinal fluid through the blood-brain barrier, causing intracranial infection. The only strain isolated from CSF, *E. coli* CE10, had a relatively far connection with *E. coli* 413_csf. We infer that that's because *E. coli* CE10 was collected from newborn with bacterial meningitis in 1970s, which is ancient and not sourced from the intestine (Lu et al., 2011).

DISCUSSION

In this study, we identified *E. coli* from cerebrospinal fluid by mNGS under circumstance of initial traditional culture-negative, to assist

TABLE 1 | Genome statistical information of the 20 *Escherichia coli* strains selected from NCBI.

Strain	Assembly.NO	Genome Size (Mb)	CDS	G+C (mol%)	Country	Isolation source	Host disease	Collection date
188B	GCA_014898615.1	5.13381	4096	51.21	Turkey	Blood	Sepsis	2018
188	GCA_014898635.1	5.1333	4006	51.11	Turkey	Blood	Sepsis	2018
2D	GCA_013425955.1	5.3633	4930	50.61	China	Blood	Bloodstream infection	2019
190693	GCA_011022275.1	5.15195	4714	50.60	UK	Blood	Bacteremia	2016
p11A	GCA_011330935.1	5.54584	5166	50.76	USA	Blood	Bacteremia	2016
p4A	GCA_011331215.1	5.2555	4861	50.70	USA	Blood	Bacteremia	2016
GZEC065	GCA_010092405.1	5.10792	4610	50.75	China	Blood	NA	2017
69	GCA_007107705.1	5.41794	5028	50.71	USA	Blood	Bloodstream infection	2014
95	GCA_007107905.1	5.06629	4662	50.72	USA	Blood	Bloodstream infection	2013
131	GCA_007108085.1	5.21412	4804	50.79	USA	Blood	Bloodstream infection	2012
963	GCA_007108305.1	5.25381	4756	50.70	USA	Blood	Bloodstream infection	2012
strain Z247	GCA_002142715.1	5.40007	4972	50.39	China	Blood	Bloodstream infection	2015
Z1002	GCA_002142675.1	5.62852	5152	50.52	China	Blood	Bloodstream infection	2014
SF-468	GCA_001280345.1	5.35864	5012	50.61	USA	Blood	Bloodstream infection	2010
CE10	GCA_000227625.1	5.37873	4911	50.58	USA	CSF	Neonatal meningitis	1970
RM-103-MS	GCA_014217095.1	5.70703	4353	50.50	Bangladesh	Feces	Healthy individual	2017
E686	GCA_011045295.1	5.16231	4516	50.60	Korea	Feces	NA	2019
2EC1	GCA_009905115.1	4.86859	4383	50.90	Singapore	Feces	Healthy individual	2019
SJ7	GCA_008761535.3	5.57204	5179	50.66	USA	Feces	Bloody diarrhea	2015
NCCP 15955	GCA_009873375.1	4.82062	4266	50.60	China	Feces	Diarrhea	2014

CDS, coding sequences; CSF, cerebrospinal fluid; NA, not available.

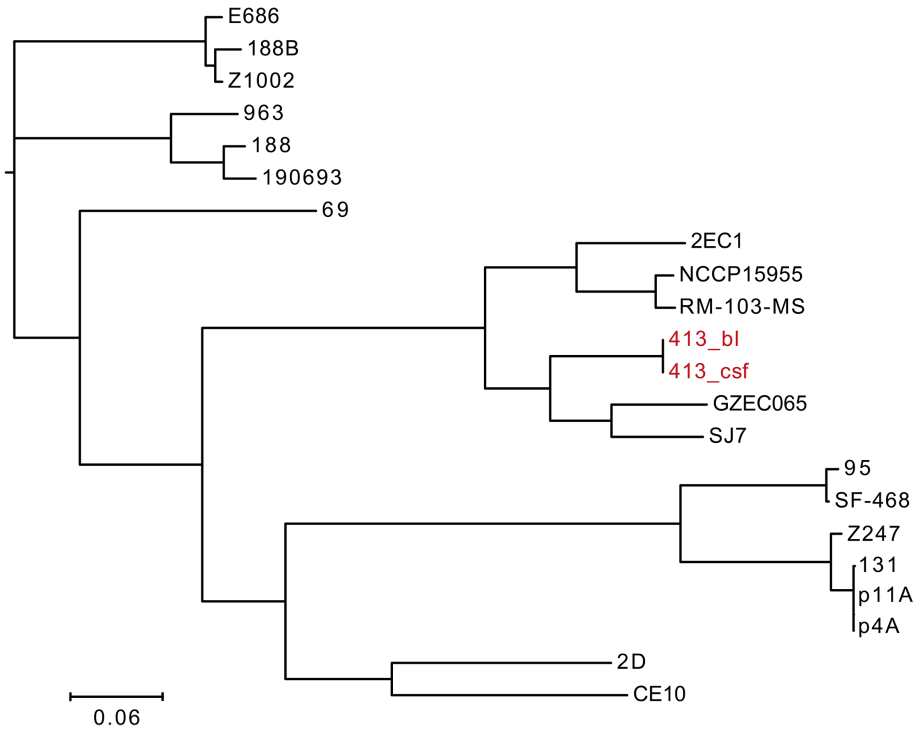


FIGURE 4 | Phylogenetic tree constructed using whole genomes of 22 *E. coli* strains, including *E. coli* 413_bl strains isolated from bloodstream, *E. coli* 413_csf isolated from cerebrospinal fluid, and 20 *E. coli* strains screened from the National Center for Biotechnology Information.

the clinicians in detecting infectious pathogen rapidly. And the second cerebrospinal fluid culture reported *E. coli*, further indicating the precision of mNGS. It's reported that the positive rate of cerebrospinal fluid culture in meningitis is only between 5.4% and 24.3% in developing countries like China (Li et al., 2014; Zhang et al., 2020). In addition, the prophylactic use of inappropriate broad-spectrum antibiotics reduces the sensitivity of pathogens detection by conventional culture, causing challenges in diagnosing the pathogens (Paul et al., 2010).

Accumulating evidence have proved that mNGS is an unbiased and comprehensive diagnostic strategy and is less disturbed by preliminary antibiotics exposure (Miao et al., 2018). A prospective multicenter study revealed that the positive rate of mNGS in cerebrospinal fluid infection was 57.0% (Xing et al., 2020). Moreover, it's found that the patient—as in our study—with CSF WBC $>300\times10^6$/L, CSF protein > 500 mg/L, or glucose ratio ≤ 0.3 had a notably higher mNGS detection rate (Miao et al., 2018). Another retrospective observational study focused on CSF samples from 99 pediatric bacterial meningitis patients found that mNGS appeared with higher sensitivity (68.7%) than culture (55.6%), and the top four detected pathogens were *Streptococcus pneumoniae*, *Streptococcus Agalactiae*, *Staphylococcus aureus*, and *Escherichia coli* (Guo et al., 2019). In a word, it demonstrated that mNGS had preferable diagnosis efficiency to culture in CNS infection.

Postoperative CNS infection remains a severe question requiring instant attention in patients undergoing neurosurgery. A previous research in America found that 0.8% (14/1587) patients after cranial operations were complicated with intracranial infection, and the morbidity of bacterial meningitis was 0.3% (4/1587). In addition, this research observed no associations between cerebrospinal fluid leakage, diabetes mellitus, and CNS infection (McClelland and Hall, 2007). However, a retrospective study in China revealed that the risk of meningitis after major craniotomy was increased by the presence of diabetes mellitus and lumbar drainage (Chen et al., 2014). In our study, the patient had undergone surgery of craniopharyngioma resection by endoscopic endonasal transsphenoidal approach, with type 2 diabetes, cerebrospinal fluid leakage, and lumbar cistern drainage. No research reported the postoperative CNS infection associated risk factors of neurosurgical endoscopic endonasal transsphenoidal approach. Thus, it's meaningful to further explore this topic to provide appropriate recommendations for clinicians around reducing the complications after neurosurgical procedures.

In addition to the identification of *E. coli* in the CSF, we also cultured *E. coli* in the bloodstream. To explore the homology of the two isolated *E. coli* strains, whole genome sequencing was performed. After phylogenetic analysis, we confirmed that the two *E. coli* strains posed 100% homologous—meaning they are the same strain, which helped clinicians inferring the source of *E. coli* in CSF. Because the patient occurred CNS infection post-surgery, we couldn't exclude that the patient was infected during the surgery if without evidence. However, according to the results of whole genome sequencing, we concluded that the *E. coli* in CSF was originated from the bloodstream. In a way, it provides a reliable basis for the clinicians to clarify the causation

to the patient. And the *E. coli* in bloodstream was carbapenem-resistant *via* drug susceptibility test, thus contributing to formulating a precise antibiotic treatment.

E. coli is a major pathogen that causes bloodstream infection. In Oxfordshire, UK, about half of *E. coli* bloodstream infections were associated with urinary tract infection and three quarters of bloodstream infection with *E. coli* were community-onset (Vihta et al., 2018). With the increase of antibiotic resistance, the scope of therapy choices is shrinking (Harris et al., 2018). Our study identified carbapenem-resistant *E. coli* from bloodstream infection in the hospital, which could be related to diarrhea. According to the statistics, carbapenem-resistant *Enterobacteriaceae* has been a major global public health problem (Xu et al., 2015). Patients with carbapenem-resistant *Enterobacteriaceae* infection usually have a history of prolonged exposure to health care facilities, which is consistent with long-term and diversified antibiotic consumption. Antibiotics available to treat carbapenem-resistant *Enterobacteriaceae* are limited, and combination therapy with multiple antibiotics has been shown to reduce mortality. In our study, anti-infective treatment was used for 20 days in combination with ceftazidime-avibactam and aztreonam, and the fever of the patient was relieved.

LIMITATIONS

One pivotal limitation of our study was a lack of pathogen identification of the patient's stool, meaning that our inferences about the relation of *E. coli* bloodstream infection and diarrhea need to be confirmed. Second, it's important to administer early postoperative enteral nutrition to maintain the gastrointestinal homeostasis and the stability of gut microbiota. Third, we should strengthen the management of cerebrospinal fluid leakage in patients, trying to avoid the occurrence of it.

CONCLUSION

In summary, our study demonstrated the usefulness of mNGS in improved identification of neurosurgical postoperative CNS infection. Actively tracing the infection source is of great significance to clinical diagnosis and treatment. In addition, carbapenem-resistant *E. coli* is a serious problem as the cause of bloodstream infection and CNS infection, and effective and adequate measures to prevent and control the present circumstance are urgent.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb). The names of the repository/repositories and accession number(s) can be found below: https://db.cngb.org/, CNP0002005.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-330). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL and MX designed the study. QS collected the data and performed the data analyses. JZ provided medication guidance. JW, LD, and ZS involved in patient management and provided clinical information. MX performed laboratory investigations. QS and JZ wrote the original manuscript. YL and MX revised and supervised the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was equally funded and supported by Chinese National Science and Technology Major Project 2018ZX10305410, Henan Province Science and Technology Research Project grant 192102310054 and 212102310321, Fund of the First Affiliated Hospital of Zhengzhou University 2018020136.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.674235/full#supplementary-material

REFERENCES

Chen, C., Zhang, B., Yu, S., Sun, F., Ruan, Q., Zhang, W., et al. (2014). The Incidence and Risk Factors of Meningitis After Major Craniotomy in China: A Retrospective Cohort Study. *PloS One* 9 (7), e101961. doi: 10.1371/journal.pone.0101961

Croxen, M., Law, R., Scholz, R., Keeney, K., Wlodarska, M., and Finlay, B. (2013). Recent Advances in Understanding Enteric Pathogenic Escherichia Coli. *Clin. Microbiol. Rev.* 26 (4), 822–880. doi: 10.1128/cmr.00022-13

Didelot, X., and Wilson, D. (2015). ClonalFrameML: Efficient Inference of Recombination in Whole Bacterial Genomes. *PloS Comput. Biol.* 11 (2), e1004041. doi: 10.1371/journal.pcbi.1004041

Erfurth, E., Holmer, H., and Fjalldal, S. (2013). Mortality and Morbidity in Adult Craniopharyngioma. *Pituitary* 16 (1), 46–55. doi: 10.1007/s11102-012-0428-2

Guo, L., Li, Y., Liu, L., Wu, H., Zhou, J., Zhang, Y., et al. (2019). Detection of Pediatric Bacterial Meningitis Pathogens From Cerebrospinal Fluid by Next-Generation Sequencing Technology. *J. Infect.* 78 (4), 323–337. doi: 10.1016/j.jinf.2018.12.001

Harris, P., Tambyah, P., Lye, D., Mo, Y., Lee, T., Yilmaz, M., et al. (2018). Effect of Piperacillin-Tazobactam *vs* Meropenem on 30-Day Mortality for Patients With E Coli or Klebsiella Pneumoniae Bloodstream Infection and Ceftriaxone Resistance: A Randomized Clinical Trial. *JAMA* 320 (10), 984–994. doi: 10.1001/jama.2018.12163

Kaper, J., Nataro, J., and Mobley, H. (2004). Pathogenic Escherichia Coli. *Nat. Rev. Microbiol.* 2 (2), 123–140. doi: 10.1038/nrmicro818

Karavitaki, N., Brufani, C., Warner, J., Adams, C., Richards, P., Ansorge, O., et al. (2005). Craniopharyngiomas in Children and Adults: Systematic Analysis of 121 Cases With Long-Term Follow-Up. *Clin. Endocrinol.* 62 (4), 397–409. doi: 10.1111/j.1365-2265.2005.02231.x

Li, N., Cai, Q., Miao, Q., Song, Z., Fang, Y., and Hu, B. (2021). High-Throughput Metagenomics for Identification of Pathogens in the Clinical Settings. *Small. Methods* 5 (1), 2000792. doi: 10.1002/smtd.202000792

Li, Y., Yin, Z., Shao, Z., Li, M., Liang, X., Sandhu, H., et al. (2014). Population-Based Surveillance for Bacterial Meningitis in China, September 2006-December 2009. *Emerging. Infect. Dis.* 20 (1), 61–69. doi: 10.3201/eid2001.120375

Lu, S., Zhang, X., Zhu, Y., Kim, K., Yang, J., and Jin, Q. (2011). Complete Genome Sequence of the Neonatal-Meningitis-Associated Escherichia Coli Strain CE10. *J. Bacteriol.* 193 (24), 7005. doi: 10.1128/jb.06284-11

McClelland, S., and Hall, W. (2007). Postoperative Central Nervous System Infection: Incidence and Associated Factors in 2111 Neurosurgical Procedures. *Clin. Infect. Dis.* 45 (1), 55–59. doi: 10.1086/518580

Miao, Q., Ma, Y., Wang, Q., Pan, J., Zhang, Y., Jin, W., et al. (2018). Microbiological Diagnostic Performance of Metagenomic Next-Generation Sequencing When Applied to Clinical Practice. *Clin. Infect. Dis.* 67, S231–S240. doi: 10.1093/cid/ciy693

Müller, H. (2014). Craniopharyngioma. *Endocrine. Rev.* 35 (3), 513–543. doi: 10.1210/er.2013-1115

Page, A., Cummins, C., Hunt, M., Wong, V., Reuter, S., Holden, M., et al. (2015). Roary: Rapid Large-Scale Prokaryote Pan Genome Analysis. *Bioinf. (Oxford. England).* 31 (22), 3691–3693. doi: 10.1093/bioinformatics/btv421

Paul, M., Shani, V., Muchtar, E., Kariv, G., Robenshtok, E., and Leibovici, L. (2010). Systematic Review and Meta-Analysis of the Efficacy of Appropriate Empiric Antibiotic Therapy for Sepsis. *Antimicrob. Agents Chemother.* 54 (11), 4851–4863. doi: 10.1128/aac.00627-10

Peña-Gonzalez, A., Soto-Girón, M., Smith, S., Sistrunk, J., Montero, L., Páez, M., et al. (2019). Metagenomic Signatures of Gut Infections Caused by Different Escherichia coli Pathotypes. *Appl. Environ. Microbiol.* 85 (24), e01820–19. doi: 10.1128/aem.01820-19

Vihta, K., Stoesser, N., Llewelyn, M., Quan, T., Davies, T., Fawcett, N., et al. (2018). Trends Over Time in Escherichia Coli Bloodstream Infections, Urinary Tract Infections, and Antibiotic Susceptibilities in Oxfordshire, U-2016: A Study of Electronic Health Records. *Lancet Infect. Dis.* 18 (10), 1138–1149. doi: 10.1016/s1473-3099(18)30353-0

Xing, X., Zhang, J., Ma, Y., He, M., Yao, G., Wang, W., et al. (2020). Metagenomic Next-Generation Sequencing for Diagnosis of Infectious Encephalitis and Meningitis: A Large, Prospective Case Series of 213 Patients. *Front. Cell. Infect. Microbiol.* 10, 88. doi: 10.3389/fcimb.2020.00088

Xu, Y., Gu, B., Huang, M., Liu, H., Xu, T., Xia, W., et al. (2015). Epidemiology of Carbapenem Resistant Enterobacteriaceae (CRE) During 2000-2012 in Asia. *J. Thoracic. Dis.* 7 (3), 376–385. doi: 10.3978/j.issn.2072-1439.2014.12.33

Zhang, Y., Cui, P., Zhang, H., Wu, H., Ye, M., Zhu, Y., et al. (2020). Clinical Application and Evaluation of Metagenomic Next-Generation Sequencing in Suspected Adult Central Nervous System Infection. *J. Trans. Med.* 18 (1), 199. doi: 10.1186/s12967-020-02360-6

Zoicas, F., and Schöfl, C. (2012). Craniopharyngioma in Adults. *Front. Endocrinol.* 3, 46. doi: 10.3389/fendo.2012.00046

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Shi, Zhang, Wang, Du, Shi, Xu and Luo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 14 September 2021
doi: 10.3389/fcimb.2021.720132

Metagenomic Next Generation Sequencing in the Detection of Pathogens in Cerebrospinal Fluid of Patients After Alternative Donor Transplantation: A Feasibility Analysis

Binglei Zhang[1,2,3,4], Jian Zhou[2], Ruirui Gui[2], Zhen Li[2], Yingling Zu[2], Juan Wang[2], Fengkuan Yu[2], Yanli Zhang[2], Huifang Zhao[2], Zhenyu Ji[3*†] and Yongping Song[2*†]*

[1] School of Basic Medical Sciences, Zhengzhou University, Zhengzhou, China, [2] Department of Hematology, Affiliated Cancer Hospital of Zhengzhou University and Henan Cancer Hospital, Zhengzhou, China, [3] Henan Academy of Medical and Pharmaceutical Sciences, Zhengzhou University, Zhengzhou, China, [4] Academy of Medical Sciences, Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Xiao Xu Ma,
First Affiliated Hospital of Zhengzhou University, China
Xuejie Jiang,
Southern Medical University, China

***Correspondence:**
Jian Zhou
zhoujiandoctor@163.com
Zhenyu Ji
jizhenyu@zzu.edu.cn
Yongping Song
songyongping001@126.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 03 June 2021
Accepted: 20 August 2021
Published: 14 September 2021

Citation:
Zhang B, Zhou J, Gui R, Li Z, Zu Y, Wang J, Yu F, Zhang Y, Zhao H, Ji Z and Song Y (2021) Metagenomic Next Generation Sequencing in the Detection of Pathogens in Cerebrospinal Fluid of Patients After Alternative Donor Transplantation: A Feasibility Analysis. Front. Cell. Infect. Microbiol. 11:720132. doi: 10.3389/fcimb.2021.720132

Central nervous system (CNS) complications can occur in 9%–15% of patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The clinical manifestations of the CNS complications are non-specific, with most of them being disturbances of consciousness, convulsions, headaches, fever, and epilepsy, making it difficult to infer the cause of the complications based on clinical manifestations. We retrospectively analyzed the sensitivity and feasibility of metagenomic next generation sequencing (mNGS) in the diagnosis of CNS infections after allo-HSCT. Lumbar punctures were performed on 20 patients with CNS symptoms after receiving alternative donor HSCT(AD-HSCT) at the Affiliated Cancer Hospital of Zhengzhou University from February 2019 to December 2020, and their cerebrospinal fluid (CSF) was collected. The mNGS technique was used to detect pathogens in the CSF. Routine CSF testing, biochemical analyses, G experiments, GM experiments, ink staining, acid-fast staining, and bacterial cultures were carried out, and quantitative PCR (qPCR) tests were used to detect cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK polyomavirus (BKPyV), and human alphaherpesvirus (HHV). A total of 29 tests were performed with 21 of them being positive. Of the five negative patients, three were diagnosed with a posterior reversible encephalopathy syndrome, one as having transplantation-associated thrombotic microangiopathy, and one with transient seizure caused by hypertension. Fifteen patients tested positive, of which four had single infections and eleven had mixed infections. Five cases of fungal infections, six cases of bacterial infections, and 13 cases of viral infections were detected. Among the 13 cases of viral infections, ten cases were CMV(HHV-5); three were BKPyV; two were Torque teno virus (TTV); Two were HHV-1,two were EBV(HHV4), and one each

of HpyV5 and HHV-6B. Thirteen patients tested positive for virus while the qPCR detection method of 6 identical specimens were below the minimum detection limit ($<1\times10^3$ U/ml). The mNGS technique is highly sensitive, and it can be used to diagnose CNS infections after allo-HSCT.

Keywords: metagenomics next generation sequencing (mNGS), alternative donor, hematopoietic stem cell transplantation, cerebrospinal fluid, pathogen

1 INTRODUCTION

Central nervous system (CNS) complications can occur in 9%–15% of patients after allogeneic hematopoietic stem cell transplantations (allo-HSCT) (Schmidt-Hieber et al., 2016; Das et al., 2020) with the common causes being mainly the posterior reversible encephalopathy syndrome (PRES), transplant-related thrombotic microangiopathies (TA-TMA), CNS graft *versus* host disease (CNS-GVHD), CNS infiltration of malignant disease, CNS infections and non-specific neurological symptoms (Chaudhary et al., 2017; Maffini et al., 2017; Balaguer-Rosello et al., 2019). Furthermore, the clinical manifestations of CNS complications are non-specific, with most of them being disturbances of consciousness, convulsions, headaches, fever, and epilepsy. While it is difficult to infer the cause of the complications based on clinical manifestations. At present, CNS infections are still the main cause of CNS complications after transplantation, with an incidence rate of up to 15% (Schmidt-Hieber et al., 2016). Moreover, the incidence of allo-HSCT is significantly higher than that of autologous HSCT, with the fatality rate also being high (Chaudhary et al., 2017). This is a risk factor for poor prognosis in patients after allo-HSCT (Dowling et al., 2018). For CNS infections after transplantation, traditional detection methods cannot meet clinical needs by offering a quick and accurate means to diagnose the infections and the types of infectious pathogens. Therefore, rapid and accurate detection methods for pathogenic microorganisms have become the focus of current research.

As an emerging detection method, metagenomic next generation sequencing (mNGS) was first recognized for its success in the diagnosis of CNS infectious diseases. The sensitivity of mNGS was more than 90% in untreated patients and 66.67% in patients receiving empirical treatment with a specificity as high as 100%. Since the test results are less affected by antibiotics and other drugs, they can also be used to evaluate the effects of treatment and the monitoring of diseases (Zhang et al., 2019; Zhang et al., 2020). Furthermore, there have been many reports of successful cases that have used the mNGS method in the diagnosis of CNS infections (Wilson et al., 2014; Wilson et al., 2018). Research has shown that the mNGS method also has a high diagnostic value in children with unexplained encephalitis (Haston et al., 2020). In addition, multi-center prospective clinical trials have confirmed its application value in clinical diagnoses (Xing et al., 2020). However, there are few studies on the use of the mNGS method in CNS infections after allo-HSCT. Therefore, we analyzed the sensitivity and feasibility of the mNGS method in the diagnosis of CNS infections after allo-HSCT to provide a reference for the diagnosis and guidance for treatment.

Abbreviations: CNS, central nervous system; allo-HSCT, allogeneic hematopoietic stem cell transplantation; TA-TMA, transplant-related thrombotic microangiopathies; GVHD, graft versus host disease; OS, overall survival; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HHV, Human alphaherpesvirus; TTV, Torque teno virus; HpyV5, Human polyomavirus 5; SAA, severe aplastic anemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; TBI, total-body irradiation; FLU, fludarabine; BU, busulfan; Ara-c, cytarabine; ALG, antilymphocyte globulin; ATG, antithymocyte globulin; VP16, etoposide; Mel melphalan; CsA, cyclosporine A; MMF, mycophenolate mofetil; MNC, mononuclear cells; PRES, posterior reversible encephalopathy syndrome.

2 METHODS

2.1 General Information

The study protocol was approved by the Ethics Committee of the Affiliated Cancer Hospital of Zhengzhou University, and the study was carried out in accordance with the Declaration of Helsinki. We retrospectively analyzed the data of 20 patients with CNS symptoms who received AD-HSCT at the Affiliated Cancer Hospital of Zhengzhou University between February 2019 and December 2020. The clinical data of the patients were recorded, and the related contraindications excluded. Lumbar punctures were performed under aseptic conditions, and CSF specimens were collected, stored at a low temperature, and tested within six hours. The mNGS method was used to detect pathogens in the CSF. Routine CSF testing, biochemical analyses, G experiments (There is no fungal infection below 70 pg/ml, 70-95 pg/ml is the observation period, continuous testing is given, and deep fungal infection is suspected if it is greater than 95pg/ml), GM experiments(Less than 0.65 ug/L is negative, greater than 0.85 ug/L is positive), ink staining, acid-fast staining, and bacterial cultures were performed, and quantitative PCR (qPCR) was used to detect the cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK polyomavirus (BKPyV), and human alphaherpesvirus (HHV). At the same time, neuroimaging examinations (such as MRI and CT) and other means were used to assist the diagnosis. All the patients provided informed consent for their inclusion in the study. For the children, their parents and guardians provided informed consent.

2.2 Methods and Process of mNGS

2.2.1 Sample Processing and Sequencing

2mL CSF was inactived at 80°C for 10 minutes immediately after collection. 1.5mL microcentrifuge tube with 0.8mL sample and 2 g

0.5 mm glass beads were attached to a horizontal plat form on a vortex mixer and agitated vigorously at 3000 RPM for 15 minutes, all Sample was then centrifuged at 12000 RPM for 1 minute, 0.6 mL sample was separated into a new 2.0mL microcentrifuge tube and DNA was extracted using the Humoral microbial DNA Kit (PMD101, Nanjing Practice Medicine Diagnostics Co., Ltd) according to the manufacturer's recommendation.

According to the protocol of the BGISEQ-200 sequencing platform, the DNA library was constructed through DNA fragmentation, end-repair, adapter-ligation, and PCR amplification. The constructed library was qualified by Agilent 2100 (Agilent Technologies, USA) and Qubit 4.0 (Thermo Fisher, USA). The qualified double-strand DNA library was transformed into a single-stranded circular DNA library through DNA-denaturation and circularization. DNA nanoballs (DNBs) were generated from single-stranded circular DNA using rolling circle amplification (RCA). The DNBs were qualified using Qubit 4.0. Qualified DNBs were loaded into the flow cell and sequenced (50 bp, single-end) on the BGISEQ-200 platform.

2.2.2 Bioinformatic Analysis

High-quality sequencing data were generated by removing low-quality and short (length<35 bp) reads using fastp software (Chen et al., 2018), followed by computational subtraction of human host sequences mapped to the human reference genome (hg38) using STAR alignment (Dobin et al., 2013). After the removal of low complexity and duplicated reads using PRINSEQ algorithms (Schmieder and Edwards, 2011), the remained data were classified by simultaneously aligning to in-house microbial genome databases, consisting of viruses, bacteria, fungi, and parasites, which were mainly downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/) using Kraken2 software (Wood et al., 2019). The sequencing data list was analyzed in terms of species-specific read number (SSRN), reads per million (RPM) and genome coverage (%).

2.2.3 Threshold Criteria for Interpretation of Metagenomic Analysis

The microbial list obtained from the above analysis process was compared with an in-house background database, which contains microorganisms appearing in more than 50% samples in the laboratory in the past three months. The suspected background microorganisms were removed from the microbial list.

For different types of microbes, the thresholds were set as follows: Extracellular bacteria/Fungus (excluding *Cryptococcus*)/Parasites: SSRN≥30 (RPM≥1.5), ranked among the top 10 for bacteria, fungi, or parasites. Organisms detected in the negative control group or that were present in≥25% of samples from the previous 30 days were excluded but only if the detected SSRN was≥10-fold than that in the negative control group or other organisms. Additionally, organisms present in≥75% of samples from the previous 30 days were excluded. (1) Intracellular Bacteria (excluding *Mycobacterium tuberculosis* and *Brucella*)/Cryptococcus: SSRN≥10 (RPM≥0.5), ranked among the top 10 for bacteria or fungi. Pathogens detected in the negative control group or that were present in≥25% of samples from the previous 30 days were excluded but only if the detected SSRN was≥10-fold than that in the negative control group or other organisms. (2) Virus/*Brucella*: SSRN≥3 (RPM≥0.15), Pathogens detected in the negative control group were excluded but only if the detected SSRN was≥10-fold than that in the negative control group. (3) *Mycobacterium tuberculosis*: SSRN≥1 (RPM≥0.05) (Xing et al., 2020).

3 RESULTS

3.1 Characteristics of the Patients

Among the 20 patients, ten were male and 10 were female. The median age was 12 years (range, 3 – 56 years), seven had severe aplastic anemia (SAA), seven acute lymphoblastic leukemia (ALL), and four acute myeloid leukemia (AML), including one case of chronic myeloid leukemia (CML) with acute myeloid degeneration and two were myelodysplastic syndrome (MDS). Ten cases accepted unrelated donor transplantation, and ten haploidentical transplantation. There were 12 cases with positive genes mutation/fusion genes and eight with complex chromosomal karyotypes. All the patients underwent peripheral blood stem cell transplantation and high-resolution HLA testing. Three cases were HLA10/10 compatible, six were HLA9/10 compatible, four were HLA8/10 compatible, one was HLA6/10 compatible, and the HLA5/10 matched six cases. (**Table 1** and **Supplementary Table 1**).

3.2 Conditioning Regimens and GVHD Prophylaxis

In this study, the conditioning regimens included total-body irradiation (TBI) + fludarabine (FLU) + busulfan (BU) + cytarabine (Ara-c) + antilymphocyte globulin (ALG) in five cases, TBI + FLU + Cyclophosphamide (CTX) + antithymocyte globulin (ATG) in five cases, FLU + BU + Ara-c + ALG in four cases, TBI + FLU + BU + Ara-C+ etoposide (VP16) in three cases, FLU + CTX + ATG + melphalan (Mel) in one case, FLU+BU+Mel in one case and TBI+FLU+CTX+ATG+Mel in one case. For the aGVHD prophylaxis, 13 patients received CTX + ALG + cyclosporine A (CsA) + mycophenolate mofetil (MMF) + ruxolitinib, and seven patients received CTX + ALG + CsA + MMF + ruxolitinib + cord blood pluripotent stem cells (**Table 1** and **Supplementary Table 1**). Patients received 20 mg/kg CTX for the unrelated donor transplantation and 40 mg/kg for the haploidentical transplantation, at +3 d and +4 d. At -4 d to -1 d they received ALG, 12.0 mg/(kg · d) for the unrelated donor transplantation; at +8 d, 5 mg/(kg · d) for the haploidentical transplantation, and at +5 d, CsA and MMF were used. The initial dose of the CsA was 2 mg/kg.d for the adults and 2.5 mg/kg.d for the children. The dose was adjusted according to the concentrations of CsA. For the haploidentical transplant patients, the dose was reduced six months after the transplantation and stopped after 9–10 months. For the unrelated donor transplantation, the dose was reduced six months after the transplantation and stopped after 6–8 months. The plasma concentrations of CsA were assessed every three days and

TABLE 1 | Characteristics of patients (N = 20).

Characteristics	N = 20 (*n*, %)
Gender (*n*, %)	
Male	10 (50)
Female	10 (50)
Primary disease (*n*, %)	
SAA	7 (35)
ALL	7 (35)
AML	4 (20)
MDS	2 (10)
Genes mutation/fusion genes	
Yes	12 (60)
No	8 (40)
Source of donors (*n*, %)	
Unrelated donors	10 (50)
Haploidentical donors	10 (50)
Conditioning regimens (*n*, %)	
TBI+FLU+BU+ Ara-c+ ALG	5 (25)
TBI + FLU + CTX+ ATG	5 (25)
FLU + BU + Ara-c + ALG	4 (20)
TBI + FLU + BU + Ara-C+VP16	3 (15)
FLU + CTX + ATG + Mel	1 (5)
TBI+FLU+CTX+ATG+Mel	1 (5)
FLU+BU+Mel	1 (5)
aGVHD prophylaxis (*n*, %)	
PTCY + ALG + CsA + MMF + ruxolitinib	13 (65)
PTCY + ALG + CsA + MMF + ruxolitinib + CBPSC	7 (35)
The median age (year)	12 (3–56)
The median number of reinfused MNCs ($\times 10^8$/kg)	16.49 (5.67– 46.61)
The median number of reinfused CD34+ cells($\times 10^6$/kg)	6.41 (2.13–15.7)
The median time for the neutrophil engraftment(d)	12 (10–15)
The median time for the platelet engraftment(d)	12 (7–15)

SAA, Severe aplastic anemia; AML Acute myeloid leukemia; ALL, Acute lymphocytic leukemia; MDS, Myelodysplastic syndrome; TBI, total-body irradiation; FLU, Fludarabine; BU, busulfan; Ara-c, Cytarabine; ALG, Antilymphocyte globulin; ATG, Antithymocyte globulin; VP16, Etoposide; Mel, Melphalan; GVHD, graft-versus-host disease; PTCY, posttransplant cyclophosphamide; CsA, cyclosporine A; MMF, mycophenolate mofetil; MNC, mononuclear cells; CBPSC, cord blood pluripotent stem cells.

maintained at 150–250 ng/mL. The dosage of MMF was usually 500 mg twice a day, halved at four weeks after transplantation, and then stopped at six weeks. The concentrations of MMF were not assessed in all the patients. Ruxolitinib 5 mg/d and 0.07–0.1 mg/kg·d were administered to the adults and children, respectively, from the day of neutrophil engraftment to 100 days post-transplantation.

3.3 Hematopoietic Stem Cell Infusion and Engraftment

The median number of reinfused mononuclear cells (MNCs) and CD34+ cells were 16.49 (5.67– 46.61) $\times 10^8$/kg and 6.41 (2.13–15.7) $\times$ 10^6/kg, respectively. The median time for the neutrophil and platelet engraftment were day 12 (10–15) and day 12 (7–15), respectively. (**Table 1** and **Supplementary Table 1**).

3.4 Symptoms of Central Nervous System and Laboratory Examination Results

The median time between the onset of the neurological symptoms and the transplantation was 64 (22–320) days, and 20 patients achieved hematopoietic reconstitution when they developed CNS symptoms. The symptoms of CNS included one case with a respiratory status change, three with fever, four with headache, 13 with mental status change, and 14 with convulsion. A total of 29 tests were performed with 21 of them being positive. Of the five negative patients, three cases were diagnosed as PRES (**Figure 1**), one as a TA-TMA (**Figure 2**), and one was considered as a transient seizure caused by hypertension. fifteen patients had infections, of which four were single infections and eleven mixed infections. Five cases of fungal infections, six cases of bacterial infections, and 13 cases of viral infections were detected (**Figure 3**). Among the 13 cases of viral infections, ten cases were CMV, three were BKPyV, and two were Torque teno virus(TTV), two were HHV-1, two were EBV and with one each of HpyV5 and HHV-6B. While all 13 of the patients had positive virus test results, six identical specimens tested negative for the virus using qPCR. Among the 15 patients with CNS infections, one had simultaneous central nervous system leukemia (**Figure 4**) and two had PRES. The results of the MRI, mNGS, bacterial cultures, acid-fast staining, and G experiments/GM experiments are shown in **Table 2**, **Supplementary Table 3** and **Figures 1–4**. All patients were tested by CSF smear, and no erythrocytes were detected. Peripheral blood contamination can be ruled out.

3.5 Other Transplant-Related Complications

Among the 20 patients, eight had aGVHD, of which five were grade II-IV aGVHD, two had cGVHD, 14 had pulmonary infections, nine had intestinal infections, seven had bacteremia, nine had hemorrhagic cystitis, seven had EBV infections, and 14 had CMV infections. (**Supplementary Table 2**). All those comorbidities were administered symptomatic and supportive treatment.

3.6 Treatment and Prognosis

According to the pathogen test results, drug sensitivities, and types of resistance, treatment was administered based on the Guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Medical Oncology (DGHO) (Schmidt-Hieber et al., 2016) and multi-center research experience (Lin and Liu, 2013; Gao et al., 2016). The neurological symptoms of ten patients improved after treatment, and the treatment effect was not obvious in 10 patients. As of July 2021, the median follow-up time after transplantation was 7.5 (2.1–24.5) months, and the median survival time of 15 patients with CNS infections was 30 (7–152) days. Twelve patients died, and the main cause of death was a nervous system infection in 10 patients, and severe pneumonia and organ failure in two patients. Eight patients survived. The survival rate of patients without CNS infection was 80% (4/5), and the survival rate of patients with CNS infection was only 26.7% (4/15).

4 DISCUSSION

CNS infection was an important factor affecting patient survival. The overall mortality rates after development of encephalitis were for the most common viruses as follows: 67% for HHV-6,

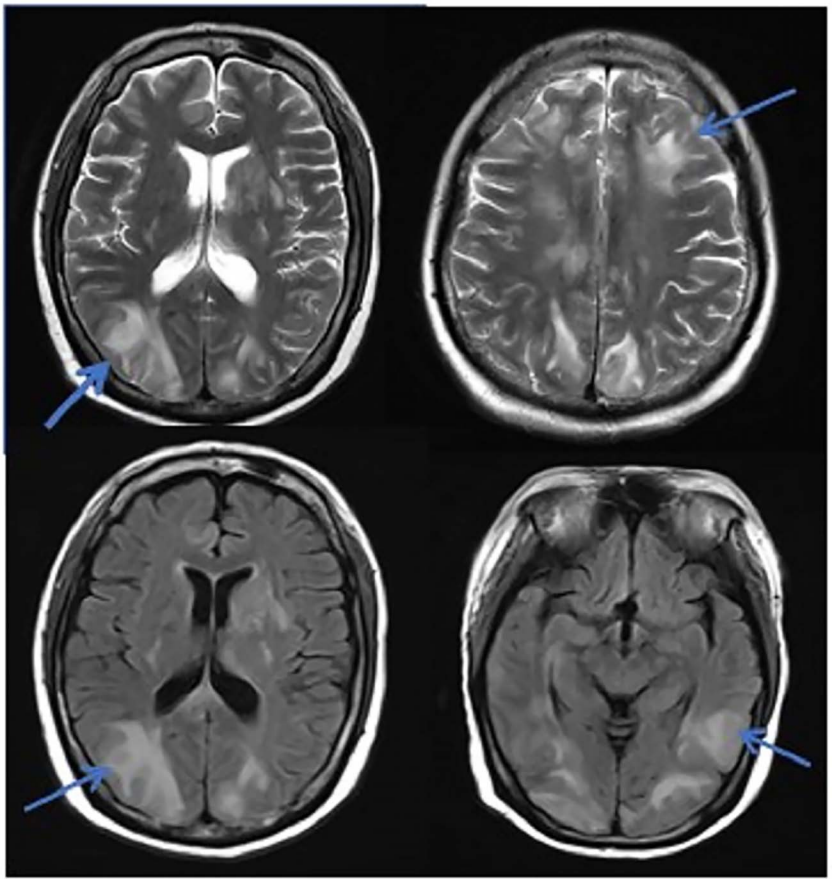


FIGURE 1 | MRI in patients with PRES. The patient diagnosed with PRES showed a patchy low-intensity T1WI on the left occipital lobe, a high-intensity shadow on the T2WI and water pressure image, a high-intensity shadow in the DWI sequence part, unclear borders, and a shallow fission near the sulci.

83% for EBV, 80% for more than one detected virus (Schmidt-Hieber et al., 2011). Allo-HSCT patients are a high-risk group for CNS infections, with a total incidence of up to 15%, and most of them occurring within six months after transplantation (Pruitt et al., 2013). Studies have shown that 30% of patients with CNS complications after allo-HSCT had infections, of which 13% were bacterial, 10% fungal, and 7% viral (Colombo et al., 2017). The toxicities of pretreatment and GVHD preventive drugs can lead to a low immunity in patients after allo-HSCT and an increased risk of CNS infections. Once an infection occurs, it can lead to varying degrees of encephalitis, meningitis, and brain abscesses. Because the patients' immune function is low, the early clinical symptoms are not obvious and are masked easily by other symptoms enabling the disease to progress rapidly. If the patient cannot be diagnosed and treated in time, the fatality rate is high (Schmidt-Hieber et al., 2016). In an autopsy study, more than 90% of the patients who died after allo-HSCT had neurological abnormalities (Weber et al., 2008). However, since allo-HSCT patients usually have multiple complications after transplantation, the symptoms of CNS lack specificity, making CNS infection an independent risk factor affecting the survival of allo-HSCT patients (Balaguer et al., 2017; Chaudhary et al., 2017). Hanajiri retrospectively analyzed the clinical data of 353 cases of CNS infections in allo-HSCT recipients. The median overall survival (OS) of patients after the CNS infections was 107 days, while the OS of patients with CNS infections was significantly lower than that of patients without CNS infections (Hanajiri et al., 2017). In our study, the survival rate of patients without CNS infection was 80%, but the survival rate of patients with CNS infection was only 26.7%. Therefore, the early, rapid, and precise diagnosis of CNS infections and pathogen types together with the implementation of timely, targeted treatment is essential for the improvement, survival, and prognosis of allo-HSCT patients.

Many methods such as G/GM experiments, ink staining, acid-fast staining, bacterial cultures, and quantitative PCR have been used for the diagnosis of CNS infections; However, they all have limitations. Although the conventional pathogen culture is the gold standard, it takes a long time and the rate of positivity is low. The G/GM experiments can only be used with low specificity and sensitivity for the preliminary approximate discrimination of fungi and the test results are usually affected

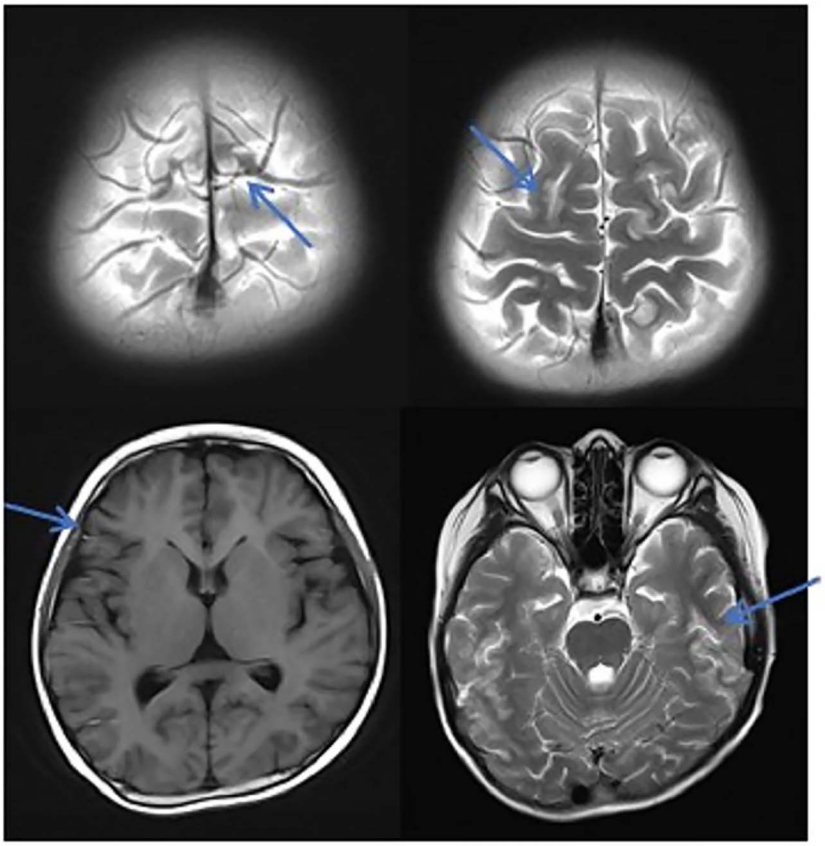


FIGURE 2 | MRI in patients with TA-TMA. Patients with TA-TMA showed bilateral frontal, parietal, and occipital sulcus edges with a sheet-like low signal on the T1WI, a slightly high signal on the T2WI, a FLAIR high signal, unclear borders, a bilateral parietal dotted DWI high signal, and unclear borders.

by many factors. False positive and false negative results can occur, and the threshold for CSF detection is not clear. While acid-fast staining and ink staining can be used only for the detection of Mycobacterium tuberculosis and Cryptococcus, respectively, their sensitivities are low. Furthermore, although qPCR technology is widely used, its sensitivity is also low. There are difficulties in detecting viruses with a copy number of less than 10^3 copies(U/ml), it is difficult to detect the early infections in time, and these methods can only detect specific pathogens, such as EBV, CMV, BKPyV, and adenovirus. At the same time, the number of specimens needed for detection is large, and it is difficult to obtain a sufficient amount of CSF in clinical practice. Therefore, traditional detection methods cannot detect pathogens early and accurately, and disease progression is often serious when discovered, which seriously affects the prognosis of CNS patients after allo-HSCT. The emergence of mNGS technology has solved these problems. There is no need to isolate and cultivate pathogens, and it can analyze multiple pathogens simultaneously. It has the advantages of a high detection sensitivity, short cycles, and a wide application range (Brown et al., 2018). In addition, the pathogen detection rate of mNGS is significantly better than that of traditional detection methods such as qPCR (Carpenter et al., 2019; Fei et al., 2020). mNGS's unbiased approach broadens viral infection diagnosis, theoretically detecting "all" viral nucleotide sequences or viral infections present. Viral primary infections and reactivations are common complications after allo-HSCT and are associated with significant morbidity and mortality (Zanella et al., 2021). In our study, there were 13 viral infections, and most patients were mixed infections, the overall mortality of CNS infections after transplantation was 73.3%(11/15). The mortality was lower than previous research results (80%) (Schmidt-Hieber et al., 2011). Therefore, early accurate diagnosis and timely effective treatment are essential to improve the survival and prognosis of patients with CNS infection after transplantation.

In this study, due to the specificity of the patients' primary disease treatment and the allo-HSCT, there were many reasons for the CNS symptoms of the patients. Approximately 61.1% of the CNS complications after allo-HSCT occurred within 100 days, such as PRES, GVHD, TA-TMA, and infections (Ke et al., 2019). The early clinical manifestations in these patients were not specific. Therefore, the diagnosis of the etiology of allo-HSCT

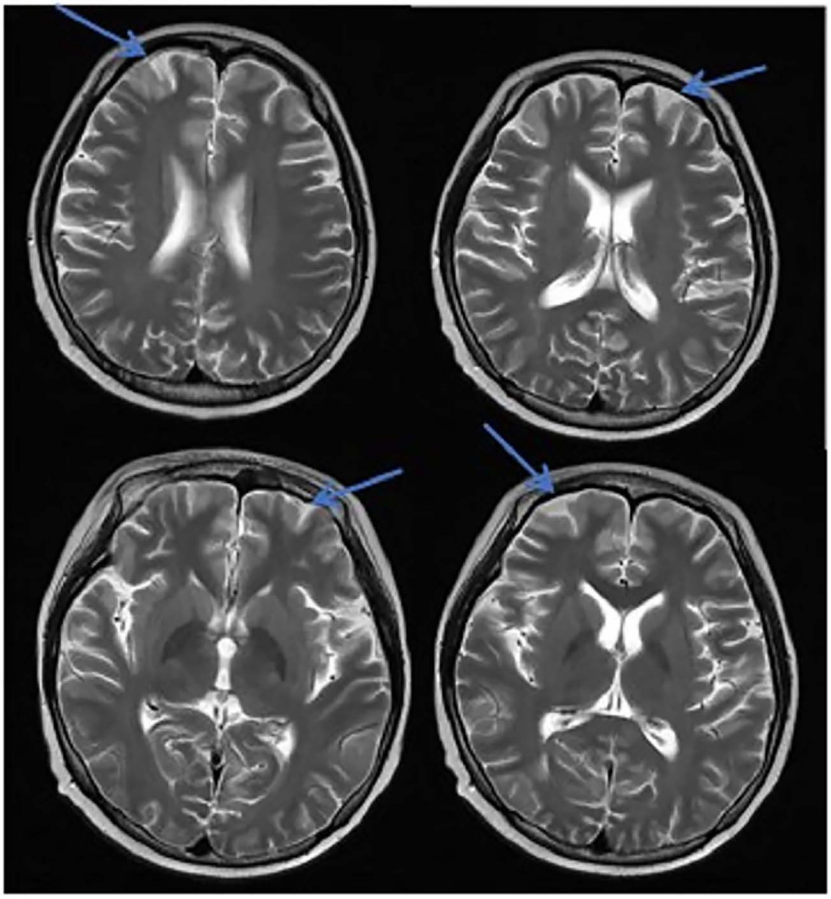


FIGURE 3 | MRI in patients with CNS infection. The CNS infection showed flair hyperintensity in the cerebral cortex, but no clear display on the T2WI, T1WI, DWI, and no enhancement after enhancement.

with CNS symptoms requires the use of comprehensive auxiliary examination methods, especially in patients with CNS symptoms in the early stage. The MRI is the most commonly used detection method for CNS diseases. A variety of neurological diseases can be diagnosed initially through their MRI manifestations, while it needs to be combined with other detection methods for differential diagnosis. Research has shown that the MRI can be used as the diagnostic gold standard for PRES (Picchi et al., 2019).The MRI of the patient diagnosed with PRES in this study showed a patchy low-intensity T1WI on the left occipital lobe, a high-intensity shadow on the T2WI and water pressure image, a high-intensity shadow in the DWI sequence part, unclear borders, and a shallow fission near the sulci (**Figure 1**). The MRI in patient with TA-TMA showed bilateral frontal, parietal, and occipital sulcus edges with a sheet-like low signal on T1WI, a slightly high signal on T2WI, a FLAIR high signal, unclear borders, bilateral parietal dotted DWI high signal, and unclear borders (**Figure 2**). The primary central nervous system infiltration MRI showed a lesion with mixed signals on the T1WI and nodules with mixed signals on the T2WI and T2WI flair. A DWI spot-like hyperintensity was observed in the lesion, and an enhancement was observed after the enhancement (**Figure 4**). However, the MRI of the CNS infections showed flair hyperintensities in the cerebral cortexes, but no clear display on the T2WI, T1WI, DWI, and no enhancement after the enhancement (**Figure 3**). However, the results of MRI have limitations in the diagnosis of infections, and they are not specific, making it difficult to confirm the existence of infections and the types of infectious pathogens. Detection methods, such as the mNGS, CSF smear and bacterial culture, are needed to assist in the diagnoses.

There were no typical or specific changes in the MRI after CNS infections. In addition, due to the immunodeficiencies of the allo-HSCT patients, MRI imaging after CNS infections is even less specific. Even if the infection can be confirmed by an MRI, only the lesion of the infections can be observed, and the type of pathogen type causing the infections cannot be determined (Chaudhary et al., 2017). On the other hand, the mNGS method has a high positive rate for pathogen detection and strong reproducibility. The smallest unit of detection was

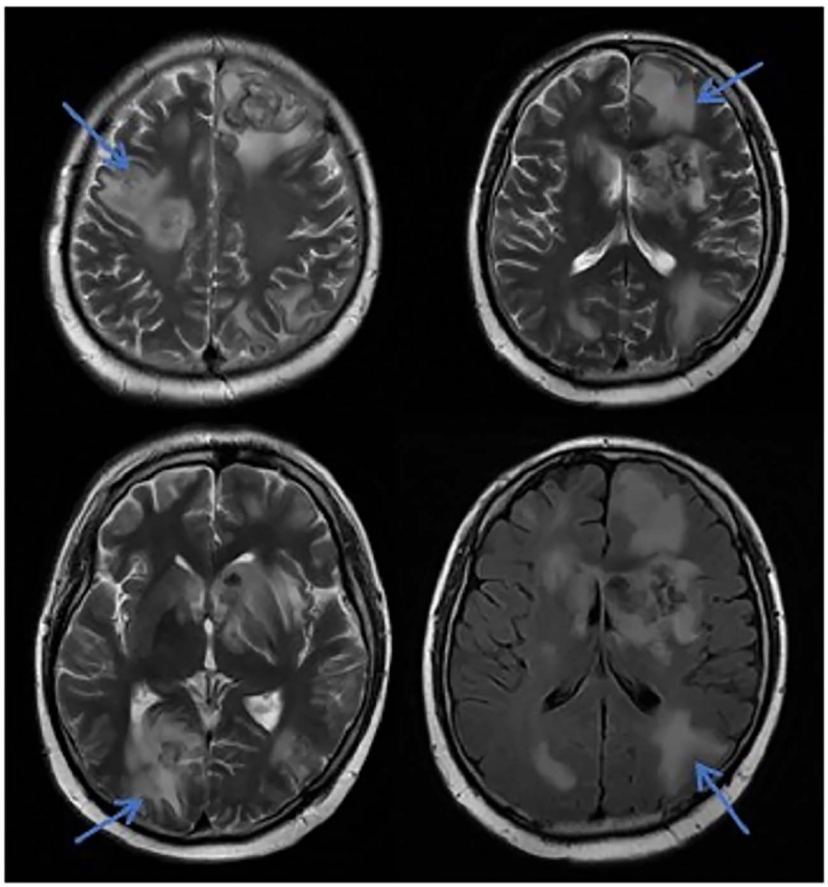


FIGURE 4 | MRI in patients with CNS leukemia and infection. Primary central nervous system infiltration MRI indicated that the lesion showed mixed signals on the T1WI and nodules with mixed signals on the T2WI and T2WI flair. A DWI spot-like hyperintensity was observed in the lesion, and the enhancement.

one read. In particular, for the detection of virus infection and the sensitivity was significantly better than that of a traditional qPCR. In this study, among the 13 patients who tested positive for the virus, six identical specimens tested negative($<10^3$U/ml) for the virus using qPCR. For patients with fungal infections, the test results of the G/GM experiment were imprecise. The mNGS method can be used to perform untargeted and undifferentiated detections of specimens. It can not only target and identify specific microorganisms, but also detect different pathogens at the same time in one sequence, discover the sequences of known and unknown pathogens, and prioritize them according to the types of pathogens (Wilson et al., 2018). However, there are problems. Due to the high sensitivity, false-positive results may occur, and it is impossible to distinguish whether the detected pathogen is a pathogenic or parasitic bacterium. Therefore, it is not suitable for specimens that are easily contaminated by parasites, such as sputum and pharynx swabs. Therefore, attention must be paid to aseptic operations in the process of specimen collection, transportation, and storage. The CSF specimens used in this study were free of parasitic contamination. When obtaining cerebrospinal fluid during a lumbar puncture, it is necessary to pay attention to the disinfection of the local skin; CSF smear was tested to avoid the cerebrospinal fluid being mixed with blood, leading to false positive results. Simultaneous mNGS testing of the CSF and peripheral blood can be used to overcome this. However, this may increase the financial burden on patients. Overall, the application of the mNGS method has greatly improved the detection rate of patients with CNS infections after transplantation (Boers et al., 2019). An early and efficient diagnosis not only enables patients to receive timely and targeted treatment, to buy time for patients treatment, but is also essential to improve the prognosis of patients and to increase the survival rate of patients with CNS infections after transplantation (Schmidt-Hieber et al., 2020).

The mNGS method detects pathogens quickly, has a high sensitivity, and a wide detection range for microorganisms. It can assist in the early diagnosis of CNS infections after allo-HSCT and provide a basis for the timely selection of effective anti-infective drugs. However, clinicians still need to be cautious

TABLE 2 | Symptoms of CNS, imaging examination and prognosis (N = 20).

Case	Symptoms	Time from the onset of CNS symptom post-transplantation/d	mNGS			Brain MRI	Prognosis
			Virus	Bacteria	Fungus		
1	Fever, convulsion	107	+	+	+	Abnormal signal in frontal parietal lobe	Death
2	Convulsion	68	+	+	–	Abnormal signals in the cerebral cortex	Death
3	Convulsion, mental status change	51	–	–	–	Bilateral parietal abnormal signal	Survive
4	Breathing and mental status change	240	+	–	–	Subacute cerebral infarction	Death
5	Convulsion, headache	180	+	–	–	Abnormal signals in both cerebral hemispheres	Death
6	Convulsion	59	–	+	+	Bilateral frontal and parietal abnormal signal	Death
7	Mental status change	41	+	–	–	Bilateral parietal abnormal signal	Death
8	Convulsion	187	–	–	+	Abnormal signal in left basal ganglia	Death
9	Convulsion, mental status change	29	–	–	–	Abnormal signal of left frontal plate block	Survive
10	Fever, convulsion, mental status change	22	+	+	+	Abnormal signals in the center of the bilateral semioval and lateral ventricle Formation of bilateral frontal subdural effusion	Death
11	Headache, Convulsion, mental status change	49	+	+	+	Left pituitary nodule	Survive
12	Mental status change	32	+	–	–	No data	Death
13	Fever, mental status change	215	+	–	–	No data	Death
14	Convulsion	97	–	–	–	Small vessel thrombosis	Death
15	Convulsion	181	–	–	–	Abnormal white matter signal in the right frontal lobe	Survive
16	Headache, mental status change	60	+	–	–	Extensive subcortical white matter lesions; subacute cerebral infarction	Death
17	Convulsion, mental status change	189	–	–	–	Bilateral frontal and parietal abnormal signal	Survive
18	Headache, mental status change	152	+	–	–	No data	Survive
19	Convulsion, mental status change	320	+	+	–	The sulci and the cistern were widened and deepened	Survive
20	Convulsion, mental status change	55	+	–	–	Multiple abnormal signals in the brain	Survive

+, Yes; -, No.

when diagnosing CNS infections. They need to use a combination of clinical symptoms, imaging examinations, bacteriological cultures, mNGS techniques, and other auxiliary testing methods. They also need to adhere strictly to the principles of sterility during specimen collection, transportation, and testing. Simultaneously, to obtain more accurate test results, blood samples and CSF smear should be tested to exclude contamination and false positive results. In addition, large samples, multi-center, prospective studies are needed to confirm the application standards and timing of the use of the mNGS method in patients with CNS symptoms after hematopoietic stem cell transplantation.

DATA AVAILABILITY STATEMENT

All the data generated or analyzed during this study are included in this published article. Genome sequences had been uploaded into NCBI (BioProject ID: PRJNA756408).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Affiliated Cancer Hospital of Zhengzhou University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BZ, JZ, and YS designed the study. RG, ZL, YZu, JW, and HZ implemented this research. BZ, YZh, and ZJ collected medical records. BZ, JZ, and YS drafted the manuscript. All the authors participated in the revision of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported, in part, by the Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, China.

ACKNOWLEDGMENTS

We would like to thank all the patients for their cooperation and the Department of Hematology, the Affiliated Cancer Hospital of Zhengzhou University. Thanks to Nanjing Practice Medicine Diagnostics. Co., Ltd and Beijing Genskey Co., Ltd, China for providing sequencing technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.720132/full#supplementary-material

REFERENCES

Balaguer, R. A., Bataller, L., Lorenzo, I., Jarque, I., Salavert, M., Gonzalez, E., et al. (2017). Infections of the Central Nervous System After Unrelated Donor Umbilical Cord Blood Transplantation or Human Leukocyte Antigen-Matched Sibling Transplantation. *Biol. Blood Marrow Transplant.* 23 (1), 134–139. doi: 10.1016/j.bbmt.2016.10.005

Balaguer-Rosello, A., Bataller, L., Pinana, J. L., Montoro, J., Lorenzo, I., Villalba, A., et al. (2019). Noninfectious Neurologic Complications After Allogeneic Hematopoietic Stem Cell Transplantation. *Biol. Blood Marrow Transplant.* 25 (9), 1818–1824. doi: 10.1016/j.bbmt.2019.05.024

Boers, S. A., Jansen, R., and Hays, J. P. (2019). Understanding and Overcoming the Pitfalls and Biases of Next-Generation Sequencing (Ngs) Methods for Use in the Routine Clinical Microbiological Diagnostic Laboratory. *Eur. J. Clin. Microbiol. Infect. Dis.* 38 (6), 1059–1070. doi: 10.1007/s10096-019-03520-3

Brown, J. R., Bharucha, T., and Breuer, J. (2018). Encephalitis Diagnosis Using Metagenomics: Application of Next Generation Sequencing for Undiagnosed Cases. *J. Infect.* 76 (3), 225–240. doi: 10.1016/j.jinf.2017.12.014

Carpenter, M. L., Tan, S. K., Watson, T., Bacher, R., Nagesh, V., Watts, A., et al. (2019). Metagenomic Next-Generation Sequencing for Identification and Quantitation of Transplant-Related DNA Viruses. *J. Clin. Microbiol.* 57 (12). doi: 10.1128/JCM.01113-19

Chaudhary, R. K., Dhakal, P., Aryal, A., and Bhatt, V. R. (2017). Central Nervous System Complications After Allogeneic Hematopoietic Stem Cell Transplantation. *Future Oncol.* 13 (25), 2297–2312. doi: 10.2217/fon-2017-0274

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). Fastp: An Ultra-Fast All-in-One Fastq Preprocessor. *Bioinformatics* 34 (17), i884–i890. doi: 10.1093/bioinformatics/bty560

Colombo, A. A., Marchioni, E., Diamanti, L., Di Matteo, A. M., Baldanti, F., Furione, M., et al. (2017). Neurological Complications Involving the Central Nervous System After Allogeneic Hematopoietic Stem Cell Transplantation During a Period of Evolution in Transplant Modalities: A Cohort Analysis. *Transplantation* 101 (3), 616–623. doi: 10.1097/TP.0000000000001257

Das, J., Gill, A., Lo, C., Chan-Lam, N., Price, S., Wharton, S. B., et al. (2020). A Case of Multiple Sclerosis-Like Relapsing Remitting Encephalomyelitis Following Allogeneic Hematopoietic Stem Cell Transplantation and a Review of the Published Literature. *Front. Immunol.* 11:668. doi: 10.3389/fimmu.2020.00668

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). Star: Ultrafast Universal RNA-Seq Aligner. *Bioinformatics* 29 (1), 15–21. doi: 10.1093/bioinformatics/bts635

Dowling, M. R., Li, S., Dey, B. R., McAfee, S. L., Hock, H. R., Spitzer, T. R., et al. (2018). Neurologic Complications After Allogeneic Hematopoietic Stem Cell Transplantation: Risk Factors and Impact. *Bone Marrow Transplant.* 53 (2), 199–206. doi: 10.1038/bmt.2017.239

Fei, X., Li, C., Zhang, Y., Zhang, H., Liu, X., Ji, X., et al. (2020). Next-Generation Sequencing of Cerebrospinal Fluid for the Diagnosis of Neurocysticercosis. *Clin. Neurol. Neurosurg.* 193:105752. doi: 10.1016/j.clineuro.2020.105752

Gao, L., Sun, Y., Meng, F., Han, M., Huang, H., Wu, D., et al. (2016). Antifungal Prophylaxis of Patients Undergoing Allogenetic Hematopoietic Stem Cell Transplantation in China: A Multicenter Prospective Observational Study. *J. Hematol. Oncol.* 9 (1), 97. doi: 10.1186/s13045-016-0305-y

Hanajiri, R., Kobayashi, T., Yoshioka, K., Watanabe, D., Watakabe, K., Murata, Y., et al. (2017). Central Nervous System Infection Following Allogeneic Hematopoietic Stem Cell Transplantation. *Hematol. Oncol. Stem Cell Ther.* 10 (1), 22–28. doi: 10.1016/j.hemonc.2016.08.008

Haston, J. C., Rostad, C. A., Jerris, R. C., Milla, S. S., McCracken, C., Pratt, C., et al. (2020). Prospective Cohort Study of Next-Generation Sequencing as a Diagnostic Modality for Unexplained Encephalitis in Children. *J. Pediatr. Infect. Dis. Soc* 9 (3), 326–333. doi: 10.1093/jpids/piz032

Ke, P., Bao, X., Qiu, H., Zhuang, J., Hu, X., Wu, X., et al. (2019). Central Nervous System Complications Caused by 3-4 Grade Agvhd in Adult Patients Occurred in Hla-Mismatched Recipients Majorly After Allogeneic Hematopoietic Stem Cell Transplantation. *Bone Marrow Transplant.* 54 (7), 1155–1157. doi: 10.1038/s41409-019-0443-2

Lin, R., and Liu, Q. (2013). Diagnosis and Treatment of Viral Diseases in Recipients of Allogeneic Hematopoietic Stem Cell Transplantation. *J. Hematol. Oncol.* 6:94. doi: 10.1186/1756-8722-6-94

Maffini, E., Festuccia, M., Brunello, L., Boccadoro, M., Giaccone, L., and Bruno, B. (2017). Neurologic Complications After Allogeneic Hematopoietic Stem Cell Transplantation. *Biol. Blood Marrow Transplant.* 23 (3), 388–397. doi: 10.1016/j.bbmt.2016.12.632

Picchi, E., Di Giuliano, F., Marziali, S., Minosse, S., Ferrazzoli, V., Da, R. V., et al. (2019). Radiological Findings of Posterior Reversible Encephalopathy Syndrome in Transplanted Children Previous Affected by Hemoglobinopathy: A Neuroimaging Retrospective Analysis. *Eur. J. Radiol. Open* 6, 144–151. doi: 10.1016/j.ejro.2019.03.001

Pruitt, A. A., Graus, F., and Rosenfeld, M. R. (2013). Neurological Complications of Transplantation: Part I: Hematopoietic Cell Transplantation. *Neurohospitalist* 3 (1), 24–38. doi: 10.1177/1941874412455338

Schmidt-Hieber, M., Engelhard, D., Ullmann, A., Ljungman, P., Maertens, J., Martino, R., et al. (2020). Central Nervous System Disorders After Hematopoietic Stem Cell Transplantation: A Prospective Study of the Infectious Diseases Working Party of Ebmt. *J. Neurol.* 267 (2), 430–439. doi: 10.1007/s00415-019-09578-5

Schmidt-Hieber, M., Schwender, J., Heinz, W. J., Zabelina, T., Kuhl, J. S., Mousset, S., et al. (2011). Viral Encephalitis After Allogeneic Stem Cell Transplantation: A Rare Complication With Distinct Characteristics of Different Causative Agents. *Haematologica* 96 (1), 142–149. doi: 10.3324/haematol.2010.029876

Schmidt-Hieber, M., Silling, G., Schalk, E., Heinz, W., Panse, J., Penack, O., et al. (2016). Cns Infections in Patients With Hematological Disorders (Including Allogeneic Stem-Cell Transplantation)-Guidelines of the Infectious Diseases Working Party (Agiho) of the German Society of Hematology and Medical Oncology (Dgho). *Ann. Oncol.* 27 (7), 1207–1225. doi: 10.1093/annonc/mdw155

Schmieder, R., and Edwards, R. (2011). Quality Control and Preprocessing of Metagenomic Datasets. *Bioinformatics* 27 (6), 863–864. doi: 10.1093/bioinformatics/btr026

Weber, C., Schaper, J., Tibussek, D., Adams, O., Mackenzie, C. R., Dilloo, D., et al. (2008). Diagnostic and Therapeutic Implications of Neurological Complications Following Paediatric Haematopoietic Stem Cell Transplantation. *Bone Marrow Transplant.* 41 (3), 253–259. doi: 10.1038/sj.bmt.1705905

Wilson, M. R., Naccache, S. N., Samayoa, E., Biagtan, M., Bashir, H., Yu, G., et al. (2014). Actionable Diagnosis of Neuroleptospirosis by Next-Generation Sequencing. *N Engl. J. Med.* 370 (25), 2408–2417. doi: 10.1056/NEJMoa1401268

Wilson, M. R., O'Donovan, B. D., Gelfand, J. M., Sample, H. A., Chow, F. C., Betjemann, J. P., et al. (2018). Chronic Meningitis Investigated via Metagenomic Next-Generation Sequencing. *JAMA Neurol.* 75 (8), 947–955. doi: 10.1001/jamaneurol.2018.0463

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved Metagenomic Analysis With Kraken 2. *Genome Biol.* 20 (1), 257. doi: 10.1186/s13059-019-1891-0

Xing, X. W., Zhang, J. T., Ma, Y. B., He, M. W., Yao, G. E., Wang, W., et al. (2020). Metagenomic Next-Generation Sequencing for Diagnosis of Infectious Encephalitis and Meningitis: A Large, Prospective Case Series of 213 Patients. *Front. Cell Infect. Microbiol.* 10:88. doi: 10.3389/fcimb.2020.00088

Zanella, M. C., Cordey, S., Laubscher, F., Docquier, M., Vieille, G., Van Delden, C., et al. (2021). Unmasking Viral Sequences by Metagenomic Next-Generation Sequencing in Adult Human Blood Samples During Steroid-Refractory/Dependent Graft-*Versus*-Host Disease. *Microbiome* 9 (1), 28. doi: 10.1186/s40168-020-00953-3

Zhang, Y., Cui, P., Zhang, H. C., Wu, H. L., Ye, M. Z., Zhu, Y. M., et al. (2020). Clinical Application and Evaluation of Metagenomic Next-Generation Sequencing in Suspected Adult Central Nervous System Infection. *J. Transl. Med.* 18 (1), 199. doi: 10.1186/s12967-020-02360-6

Zhang, X. X., Guo, L. Y., Liu, L. L., Shen, A., Feng, W. Y., Huang, W. H., et al. (2019). The Diagnostic Value of Metagenomic Next-Generation Sequencing for Identifying Streptococcus Pneumoniae in Paediatric Bacterial Meningitis. *BMC Infect. Dis.* 19 (1), 495. doi: 10.1186/s12879-019-4132-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Zhang, Zhou, Gui, Li, Zu, Wang, Yu, Zhang, Zhao, Ji and Song. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 23 September 2021
doi: 10.3389/fmed.2021.682758

Case Report: Diagnostic Value of Metagenomics Next Generation Sequencing in Intracranial Infection Caused by Mucor

YuChen Liu[1,2], Jun Zhang[3†], Bing Han[4†], LiJuan Du[4], ZhaoYang Shi[4], ChunCheng Wang[4], Min Xu[4] and YongGang Luo[5*]*

[1] Department of Gynecology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Gene Hospital of Henan Province, Precision Medicine Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Department of Intensive Care Unit, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5] Department of Clinical Laboratory, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UCONN Health, United States

Reviewed by:
Beiwen Zheng,
Zhejiang University, China
Parisa Badiee,
Shiraz University of Medical Sciences, Iran
Teclegiorgis Gebremariam,
Lundquist Institute for Biomedical Innovation, United States

***Correspondence:**
YongGang Luo
Luoyg_514@126.com
Min Xu
xumin3568@sina.com

[†]These authors share first authorship

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 19 March 2021
Accepted: 30 August 2021
Published: 23 September 2021

Citation:
Liu YC, Zhang J, Han B, Du LJ, Shi ZY, Wang CC, Xu M and Luo YG (2021) Case Report: Diagnostic Value of Metagenomics Next Generation Sequencing in Intracranial Infection Caused by Mucor. Front. Med. 8:682758. doi: 10.3389/fmed.2021.682758

mNGS(metagenomics Next Generation Sequencing), as a novel culture-independent approach, demonstrated the capability of rapid, sensitive, and accurate pathogen identification. At present, there have been many case reports about the use of mNGS to assist in the diagnosis of bacterial, fungal, viral and parasitic infections and to guide clinicians to determine appropriate treatment. However, the clinical understanding of this technique is not comprehensive, and the experience of using it is relatively limited. We reported a 53-year-old man who was admitted to hospital with a high fever and headache. His inflammatory biomarkers were markedly elevated. Based on the clinical presentation, He was initially diagnosed as having an intracranial infection of unknown etiology and received empirical antibiotics and systemic supportive treatment. But these did not relieve his symptoms. Both the blood and CSF specimens were examined using traditional culture, serological testing, and mNGS. Traditional culture and serological testing produced negative results, while the mNGS revealed the presence of a potential pathogen, *mucor*, in the CSF specimen. Then targeted antifungal treatment was selected quickly and his temperature gradually returned to normal. Thus, we report the case in which mNGS was an auxiliary method to diagnose *mucormycosis*, and discuss this case in combination with relevant literature, in order to improve the clinical cognition of this technology.

Keywords: *mucormycosis*, *Lichtheimia ramosa*, metagenomics Next Generation Sequencing, precision treatment, intracranial infection

INTRODUCTION

Mucormycosis, also known as *zygomycosis*, is a highly invasive conditional fungal infection. It has low incidence, about 1.2 per 1 million people per year (1) and high mortality about 87% (2). Clinically, *mucormycosis* can be divided into many types, among which the rhino-orbito-cerebral (ROCM) is the most common type, accounting for about 75% of reported cases (3), that is, the fungus in the nasal cavity, nasal sinus mucosa accompanied by orbital and intracranial invasion (4).

The early symptoms of the disease are not typical, and it is difficult to be detected by traditional etiological detection methods, so the clinical diagnosis still faces great challenges and delayed treatment significantly affects patient prognosis (5). Therefore, it is crucial to rapidly and accurately identify the pathogens of *mucor*, and then optimize medical therapy. Clinically most patients with *mycosis* are treated empirically without a clear diagnosis. Given the risk of death due to delayed treatment, there is an urgent clinical need for a technology that can quickly and accurately identify pathogens.

Metagenomics Next Generation Sequencing (mNGS) is a new genomics-based pathogen detection technology, which has the advantages of high detection rate and fast detection speed. Thus, we report the case of intracranial infection caused by *mucor*, in which traditional culture was negative but mNGS provided a positive finding.

CASE RESCRIPTION

A 53-year-old man presented to our hospital with a 7-day history of fever and severe headache and a 1-day history of left extension of the tongue and slurred speech.11 days before arriving at our hospital, the patient developed periodontal pain and maxillofacial swelling without obvious inducement and received antibiotics in the local hospital. However, his symptoms were not significantly relieved, neither did his body temperature drop.

On admission, vital signs were shown as: body temperature was 37.2°C, respiration was 20 beats per minute, heart rate was 84 beats per minute, blood pressure was 158/81 mmHg. Physical examination revealed a 1 cm lesion near the gum of the patient's upper incisor; the right side of the light reflection disappeared and the left side is dull; the left nasolabial groove is shallow and the patient has a left extension of the tongue. Blood routine examination (BRE) showed that white blood cells were 27.62 × 109/L (neutrophil accounted for 89.1%). The procalcitonin (PCT) level was elevated to 0.120 ng/mL, C-reactive protein (CRP) reached 178.30 mg/L. Magnetic resonance imaging (MRI) showed a lesion in the brainstem. Hence, based on previous clinical experience, the patient was diagnosed preliminarily with brainstem encephalitis and purulent gingivitis. An initial treatment of meropenem (2g/8h iv) and linezolid (0.6g/12 h iv) had been empirically administrated. At the same time, enteral nutrition, reducing intracranial pressure, anti-heart failure, organ function protection, analgesia and other symptomatic treatment were given. But there was no significant relief of his symptoms.

In order to improve the etiological examination, both his peripheral blood and CSF specimens were examined to investigate possible etiology using traditional culture and mNGS. The CSF presented a colorless and transparent appearance, intracranial pressure was 600 mmH_20;cytology showed 46 × 106 leukocyte/L, glucose: 4.05mmol/L, chloride: 125.3 mmol/L, lactic acid: 2.90 mmol/L and there was no evidence of *fungal*, or *acid-fast bacilli* infection. Serological testing was performed, including 1,3-β-D glucan test (G test), galactomannan antigen test (GM test) and bacterial smear test; however they were all negative. Computerized tomography (CT) showed paranasal sinusitis and bilateral hypertrophic inferior turbinate. Consider the possibility of infection by a particular pathogen, empirical antifungal and antiviral therapy of ganciclovir intravenous (0.5g/12h iv) and voriconazole (400mg/12h iv) was given.

The fourth day after admission, the patient was unconscious and his temperature level reached 39.6°C. Black eschar lesions and bloody secretion are seen in the external auditory canal (**Figure 1**). Based on this, pathogen culture was performed on external auditory canal secretions. Laboratory tests to show CRP level to 340.50 mg/L. To exclude pulmonary infection, fiberoptic bronchoscopy was performed, and the results showed no significant abnormalities. MRI showed multiple intracranial soft tissue infections, sinus infections, soft tissue thickening of the posterior pharyngeal wall, suppurative otitis media, and gingival infection. Therefore, we detected nasal secretions by pathogen culture. Secretions of external auditory canal and nasal, peripheral blood and cerebrospinal fluid specimens were negative. However, within 48 h, mNGS revealed a total of 35 single-end reads in the genomic DNA of the CSF specimen, of which 4 reads were *Lichtheimia ramosa* (**Table 1**), and its relative abundance is 50%. Because the test results are consistent with the clinical manifestations, we adjusted the treatment regimen to amphotericin B liposomes intravenous (500 mg/24h iv). After a discussion with the otolaryngology department, we recommended surgical treatment for patient, but was rejected by the patient's family.

On the eighth day of admission, after four days of conservative treatment, the patient's temperature was back to normal (37.2°C) but he suffered developed brainstem failure. We use high doses of vasoactive drugs and still have difficulty maintaining his blood pressure. After communicating with the patient's family, they requested to be discharged.

DISCUSSION

Here, we reported a case in which mNGS helped clinicians rapidly and accurately identifying *mucor* in a patient with intracranial infection. This patient was admitted to hospital due to high fever and headache. Traditional culture and serological testing did not determine possible infection etiology, while mNGS identified *mucor* in the CSF specimen. Combined with his clinical characteristics, his diagnosis was confirmed to be a ROCM.

Route of Infection

This disease generally seen in people with malignant blood diseases, diabetes, organ transplantation and long term use of antibiotics, hormones, immunosuppressant. But a recent epidemiological study found that 19.16% of cases of *mucormycosis* occurred in patients with "no underlying disease" (6). This patient was a healthy adult before the onset of the disease and did not have any of the above risk factors. However, before the onset of the disease, he had received intravenous infusion treatment in a local primary hospital for "toothache and maxillofacial swelling". Therefore, we suspect that the disease

FIGURE 1 | Black eschar lesions of the patient.

TABLE 1 | The results of mNGS.

Genus			Species			
Type	**Name**	**Sequence number**	**Name**	**Sequence number**	**Realtive abundance**	**Attention**
G-	*Lichtheimia*	4	*Lichtheimia ramosa*	2	50.0%	High

may be caused by the retrograde infection of pathogens along the bloodstream after tooth extraction.

Clinical Manifestation

Early symptoms of ROCM are atypical and include runny nose, stuffy nose, headache, fever, facial swelling, vision loss, and mental distress. With the progression of the disease, persistent high fever, eye movement disorders, intracranial infection, and consciousness disorders may occur (7, 8). The typical presentation is sinusitis with painless black eschar formation in the face, nasal cavity or palate, but it is only seen in a few patients and suggests a poor prognosis. In this case, the patient presented only toothache and maxillofacial swelling at first, then fever and headache appeared 1 week later. With the progress of the disease, the patient presented brainstem involvement such as extending tongue to the left and speaking unclearly and black eschars were also observed around the eyes and the external auditory canal. Soon after, he progressed to brainstem failure. Consistent with literature reports, the onset of typical lesions is late and associated with poor prognosis.

Survival Situation

The overall survival rate for ROCM is about 85% (5), and the main factors affecting the survival rate include the time of diagnosis, type of diagnosis, treatment methods (9). Yohai pointed out that if the delay between diagnosis and treatment of mucormycosis exceeds 6 days, survival rates will be significantly reduced (5). Anand analyzed 224 cases of mucormycosis reported since 1943, of which 123 (55%) had a 67% reduction in fatality due to early diagnosis and early surgical treatment (10). Therefore, the timing of diagnosis may directly affect patient survival. Delayed treatment, such as the appearance of intracranial involvement, results in a significantly reduced prognosis.

Diagnostic Method

However, the diagnosis of fungal infection of central nervous system is difficult, which requires comprehensive analysis of history, epidemiology, basic diseases, clinical manifestations, imaging manifestations and laboratory examination results (11, 12). The commonly used clinical diagnostic methods include the following:

Routine and biochemical examinations of CSF: It analyzes possible pathogens by measuring the patient's intracranial pressure and the levels of cells, proteins, glucose and chloride in the CSF. However, the specificity of this method is poor, and sometimes false negative results may occur.

Etiological examination of CSF: It involves microscopic examination and culture. However, due to the influence of clinical use of antibiotics, the detection rate is low, and the requirements for specimen collection and transportation are strict, and the accuracy of the diagnosis of bacteria with similar phenotypes and biochemical characteristics is poor. For mucormycosis, its growth rate is slow, so the culture cycle is long, which is not conducive to early diagnosis (13–15).

Blood tests: It includes blood smear, pathogen culture, serum antigen and antibody detection, etc. Its diagnostic significance is equivalent to that of CSF.

Immunological examination: Common methods include galactomannan antigen test (GM test), (1, 3) – β-D – dextran test (G test), antibody test, etc. The principle is to identify the pathogen by measuring the cell wall or antigen of the fungus, but there are some defects, such as high false positive rate, difficult to distinguish the past infection from the current infection. Because

the cell wall of mucor contains little or almost no (1, 3) – β-D – glucan, the result of this test was negative in our patient.

Imaging examination: Imaging examination is helpful for the detection of mucosal edema and thickening, extraocular muscle and soft tissue swelling, bone destruction, brain parenchymal lesions, intracranial vascular occlusion and other conditions in the affected areas before the appearance of clinical symptoms, and can also be used to judge the therapeutic effect and determine surgical methods. Patient's CT showed paranasal sinusitis and bilateral hypertrophic inferior turbinate and his MRI showed multiple intracranial soft tissue infections, suppurative otitis media, and gingival infection. All of his imaging findings are not typical and have poor clinical suggestive effect.

Molecular biological examination: The commonly used clinical molecular diagnostic method is PCR, which is more sensitive than pathogen culture (16–18) and has a shorter detection time (19, 20). However, false negative results may occur when the DNA content is low, and false positive results may occur when the DNA of other fungi is interfered. Due to the lack of uniform standards and clinical validation (21), the application of this technique in patients with mucormycosis is rarely reported (22, 23), and is still under study.

Histopathological examination: Pathological results obtained by puncture of intracranial lesions can indicate the type of pathogenic bacteria, and mycelium found by local tissue biopsy is the gold standard for the diagnosis of mycormycosis (24–26). However, this procedure is invasive and is poorly tolerated by patients.

In a study of suspected central nervous system infections in adults, only 25% of cases resulted in a clear etiological diagnosis (27). Failure to obtain clear etiological evidence will affect the implementation of precise antimicrobial therapy, lead to unnecessary use of antibiotics, increase the risk of bacterial resistance, and increase the medical cost. In our case, In the early stage of the disease, there was no obvious suggestive evidence, and the traditional etiological test was negative. However, because the therapeutic effect of broad spectrum antibiotics was not good, we timely took the cerebrospinal fluid and sent it to mNGS for detection, and the result detected *Lichtheimia ramosa*, which confirmed the diagnosis of ROCM in combination with clinical manifestations. In conclusion, mNGS can be used as an effective complementary means for etiological diagnosis when traditional methods cannot be used in time (28).

Clinical Application of mNGS

In a retrospective study of organ transplant recipients, Palacios identified a new virus using mNGS techniques, which were subsequently validated by pathogen culture, PCR, immunohistochemistry and serological analysis (29). This study is the first to show that mNGS can be used as a new tool to identify novel pathogens.

mNGS is a rapid diagnostic method which has a wide application prospect. Unlike PCR, mNGS does not require primers or specific amplification and is less affected by the use of antibiotics and the body's immune status (30). It can directly sequence the base sequences in the samples (31) and obtain the genome sequences of a variety of pathogens that may be infected in the samples by one-time detection, which is characterized by rapid and high accuracy (32). Therefore, it can be used to cultivate pathogens that are difficult to find or have a relatively low culture rate or a long period of time (33), predict infection in advance by detecting microecology (34), predict drug sensitivity (31), interpret the diversity and abundance of pathogen population, and explore the relationship between pathogen, environment and host. At the same time, mNGS can be considered as an alternative non-invasive testing tool for the detection of infections that cannot tolerate invasive testing.

Application Status

Currently, mNGS can detect more than 6,350 bacteria, 1,798 viruses, 10,664 fungi and 234 parasites (30, 35–37). In recent years, researchers have used mNGS technology to provide diagnostic basis for human herpesvirus, pseudorabies virus, cysticercosis, toxoplasma gondii and other infections (38), and it can guide the treatment effectively. In addition, when mNGS detects related pathogens, the CSF culture program can be adjusted to improve the positive rate of related pathogens culture. What's more, reliable negative results also play a role in clinical diagnosis and treatment, helping to reduce the concern of clinicians about active infection.

Applications in the Central Nervous System

A growing number of studies in recent years have shown that mNGS play a key role in pathogen recognition of central nervous system infections (28, 39, 40). When the clinical symptoms are atypical, the traditional etiological detection methods may omit some pathogens that have not been considered, but mNGS technology can make a supplement to reduce the rate of missed diagnosis. This technology also plays an important role in the identification of difficult-to-diagnose infections, such as fever of unknown origin, repeated negative clinical tests, or unknown pathogens. A growing number of cases and studies are demonstrating the great potential of mNGs, which are changing the way clinicians think about treating infections caused by rare pathogens, from traditional medicine to genome-based diagnostics.

Li et al. evaluated the diagnostic efficacy of mNGS in central nervous system infections and found that the sensitivity of mNGS was 61.74% and the specificity was nearly 100%, the area under the ROC curve was 0.81 (41), which indicate that mNGS has a strong diagnostic ability in infectious diseases of the central nervous system. Compared with CSF culture, mNGS can indicate central nervous system infection with a higher accuracy and a shorter time (about 36 hours), which provides a new diagnostic clue for patients with immunodeficiency or severe diseases that are difficult to diagnose.

Diagnosis and Treatment Experience

In this case, the early medication was not targeted and did not successfully prevent the progression of the disease. It was not until the mNGS in the CSF indicated the infection of *Lichtheimia ramosa* that the patient was finally diagnosed. However, due to a variety of factors such as late diagnosis, intracranial lesions indicated by MRI and large necrotic area which makes it difficult

for drugs to penetrate, the overall prognosis of patients is poor. It suggests that the following issues should be paid attention to in the process of clinical diagnosis and treatment: (1) The susceptible people should consider this disease when they have symptoms such as fever, headache, swelling of maxillofacial region and nasal congestion and should carry on the etiology examination and the tissue biopsy pathology examination in time to achieve early diagnosis and treatment. (2) The discovery of mucor's characteristic by biopsy is the gold standard for diagnosis of this disease, but medication can be taken as soon as possible according to clinical experience, so as not to delay diagnosis and treatment. (3) For patients with intracranial infection with atypical symptoms and rapid disease progression, the pathogen must be identified as soon as possible, and mNGS has a certain auxiliary role in diagnosis.

In conclusion, specific pathogens should be highly suspected based on the patient's underlying disease history, clinical manifestations, and ineffective use of conventional antibiotics. However, due to the diversity of pathogens, the limitations of traditional detection methods, and the uneven judgment of clinicians on target pathogens, many infectious diseases can not be identified in clinical practice. Traditional etiological detection methods are time-consuming and have low sensitivity, which cannot meet the needs of early diagnosis and treatment of this kind of disease. Sensitive, rapid, accurate and simple diagnostic techniques are needed. In recent years, the emerging mNGS technology has helped to improve the level of diagnosis, and has played a role in the detection of a variety of pathogens. While the technique is currently used only as a second-line option in cases where testing for other pathogens is inconclusive, but with the rapid advances in genomics and sequencing, mNGS will be more and more widely used in the traceability, detection, typing and drug resistance evaluation of infectious diseases, and will develop rapidly in the direction of fast and economic development.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YCL, JZ, and YGL analyzed and interpreted patient data. BH, LJD, and ZYS performed the experiments. YCL, CCW, and MX analyzed the genomics data. YCL and BH wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was equally funded and supported by Henan Province Science and Technology Project Grant 192102310054. Fund of the First Affiliated Hospital of Zhengzhou University 2018020136.

REFERENCES

1. Cornely O A, Arikan-Akdagli S, Dannaoui E, Groll A H, Lagrou K, Chakrabarti A, et al. ESCMID and ECMM joint clinical guidelines for the diagnosis and management of mucormycosis 2013. *Clin Microbiol Infect.* (2014) 20:5–26. doi: 10.1111/1469-0691.12371
2. Roden M M, Zaoutis T E, Buchanan W L, Knudsen T A, Sarkisova T A, Schaufele R L, et al. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. *Clin Infect Dis.* (2005) 41:634–53. doi: 10.1086/432579
3. Hong G, Xiaojun C, Weihua W, Haibo C, Jingwen J. The clinical and pathological features of rhinocerebral mucormycosis. *Chin J Internal Med.* (2004) 43:686–9. doi: 10.3760/j.issn:0578-1426.2004.09.015
4. Akoz T, Civelek B, Akan M. Rhinocerebral mucormycosis: report of two cases. *Ann Plast Surg.* (1999) 43:309–12. doi: 10.1097/00000637-199909000-00016
5. Yohai R A, Bullock JD, Aziz A A, Markert R J. Survival factors in rhino-orbital-cerebral mucormycosis. *Surv Ophthalmol.* (1994) 39:3–22. doi: 10.1016/S0039-6257(05)80041-4
6. Cano P, Horseman M A, Surani S. Rhinocerebral mucormycosis complicated by bacterial brain abscess. *Am J Med Sci.* (2010) 340:507–10. doi: 10.1097/MAJ.0b013e3181f0aad1
7. Bakshi S S. Rhino-orbital mucormycosis in a patient with diabetes. *Lancet Diabetes Endocrinol.* (2017) 5:234. doi: 10.1016/S2213-8587(15)00522-7
8. Chen Y X, He Y X, Zhou H, Wang M, Su S O. Rapidly progressive rhino-orbito-cerebral mucormycosis in a patient with type 2 diabetes: a case report. *Exp Ther Med.* (2017) 13:1054–6. doi: 10.3892/etm.2017.4074
9. Chamilos G, Lewis R E, Kontoyiannis D P. Delaying amphotericin B-based frontline therapy significantly increases mortality among patients with hematologic malignancy who have zygomycosis. *Clin Infect Dis.* (2008) 47:503–9. doi: 10.1086/590004
10. Anand V K, Alemar G, Jr. Griswold JA. Intracranial complications of mucormycosis: an experimental model and clinical review. *Laryngoscope.* (1992) 102:656–62. doi: 10.1288/00005537-199206000-00011
11. Wilson M R, Shanbhag N M, Reid M J, Singhal N S, Gelfand J M, Sample H A, et al. Diagnosing balamuthia mandrillaris encephalitis with metagenomic deep sequencing. *Ann Neurol.* (2015) 78:722–30. doi: 10.1002/ana.24499
12. Wei C, Jun-Ji W, Ren-Zhi W, Zu-Yuan R. The diagnosis aand treatment of fungal infections in central nervous system. *Basic Clin Med.* (2012) 32:358–61.
13. Rockwood N, Wojno J, Ghebrekristos Y, Nicol M P, Meintjes G, Wilkinsona R J. Utility of second-generation line probe assay (Hain MTBDRplus) directly on 2-month sputum specimens for monitoring tuberculosis treatment response. *J Clin Microbiol.* (2017) 55:1508–15. doi: 10.1128/JCM.00025-17
14. Moodley B, Tempia S, Frean J A. Comparison of quantitative real-time PCR and direct immunofluorescence for the detection of Pneumocystis jirovecii. *PLoS ONE.* (2017) 12:e0180589. doi: 10.1371/journal.pone.0180589
15. Mingqiang Z. Advances of diagnosis of invasive fungal disease in children. *J Pediatric Pharm.* (2020) 26:51–4. doi: 10.13407/j.cnki.jpp.1672-108X.2020.03.016
16. Zhijuan L, Xiaolin H, Zhenhai W, Aicui L. Extraction of Bacterial Genomic DNA from Cerebrospinal Fluid of Bacterial Meningitis Patients and Identification of 16S rDNA. *Acta Med Univ Sci Technol Huazhong.* (2013) 42:314–6. doi: 10.3870/j.issn.1672-0741.2013.03.016

17. Rajesh N T, Dutta S, Prasad R, Narang A. Effect of delay in analysis on neonatal cerebrospinal fluid parameters. *Arch Dis Childh Fetal Neonatal Ed.* (2010) 95:F25–F9. doi: 10.1136/adc.2008.150292
18. Sarookhani M R, Ayazi P, Alizadeh S, Foroughi F, Sahmani A, Adineh M. Comparison of 16S rDNA-PCR amplification and culture of cerebrospinal fluid for diagnosis of bacterial meningitis. *Iran J Pediatr.* (2010) 20:471–5. doi: 10.2139/ssrn.2611957
19. Esparcia O, Montemayor M, Ginovart G, Pomar V, Soriano G, Pericas R, et al. Diagnostic accuracy of a 16S ribosomal DNA gene-based molecular technique (RT-PCR, microarray, and sequencing) for bacterial meningitis, early-onset neonatal sepsis, and spontaneous bacterial peritonitis. *Diagn Microbiol Infect Dis.* (2011) 69:153–60. doi: 10.1016/j.diagmicrobio.2010.10.022
20. Kumar A, Asthana M, Gupta P, Yadav S, Sharma D, Singh K N, et al. 16SrRNA sequencing of dye decolorizing bacteria isolated from soil. *Bioinformation.* (2015) 11:1–5. doi: 10.6026/97320630011001
21. Groll A H, Castagnola E, Cesaro S, Dalle J H, Engelhard D, Hope W, et al. Fourth European Conference on Infections in Leukaemia (ECIL-4): guidelines for diagnosis, prevention, and treatment of invasive fungal diseases in paediatric patients with cancer or allogeneic haemopoietic stem-cell transplantation. *Lancet Oncol.* (2014) 15:e327–40. doi: 10.1016/S1470-2045(14)70017-8
22. Kasai M, Harrington S M, Francesconi A, Petraitis V, Petraitiene R, Beveridge M G, et al. Detection of a molecular biomarker for zygomycetes by quantitative PCR assays of plasma, bronchoalveolar lavage, and lung tissue in a rabbit model of experimental pulmonary zygomycosis. *J Clin Microbiol.* (2008) 46:3690–702. doi: 10.1128/JCM.00917-08
23. Rickerts V, Just-Nubling G, Konrad F, Kern J, Lambrecht E, Bohme A, et al. Diagnosis of invasive aspergillosis and mucormycosis in immunocompromised patients by seminested PCR assay of tissue samples. *Eur J Clin Microbiol Infect Dis.* (2006) 25:8–13. doi: 10.1007/s10096-005-0078-7
24. Limei W, Bin D, Yanqiao W, Jun L, A. report of chronic rhinocerebralmucormycosis misdiagnosed as tubercular meningitis. *Chin J Otorhinolaryngol Head Neck Surg.* (2017) 52:608–10. doi: 10.3760/cma.j.issn.1673-0860.2017.08.010
25. Wende L, Yunjun L, Aipin W, Lihua C, Qun W, Ruxiang X. Isolated cerebral mucormycosis(a report of 1 case and review of literature). *J Clin Neurosurg.* (2013) 10:371–2. doi: 10.3969/j.issn.1672-7770.2013.06.019
26. Mbarek C, Zribi S, Khamassi K, Hariga I, Ouni H, Ben Amor M, et al. Rhinocerebral mucormycosis: five cases and a literature review. *B-ENT.* (2011) 7:189–93. doi: 10.1159/000323276
27. Khatib U, Van De Beek D, Lees J A, Brouwer M C. Adults with suspected central nervous system infection: A prospective study of diagnostic accuracy. *J Infect.* (2017) 74:1–9. doi: 10.1016/j.jinf.2016.09.007
28. Wilson M R, Naccache S N, Samayoa E, Biagtan M, Bashir H, Yu G, et al. Actionable diagnosis of neuroleptospirosis by next-generation sequencing. *N Engl J Med.* (2014) 370:2408–17. doi: 10.1056/NEJMoa1401268
29. Palacios G, Druce J, Du L, Tran T, Birch C, Briese T, et al. A new arenavirus in a cluster of fatal transplant-associated diseases. *N Engl J Med.* (2008) 358:991–8. doi: 10.1056/NEJMoa073785
30. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis.* (2018) 67:S231–S40. doi: 10.1093/cid/ciy693
31. Su M, Satola S W, Read T D. Genome-based prediction of bacterial antibiotic resistance. *J Clin Microbiol.* (2019). 57: doi: 10.1128/JCM.01405-18
32. Mchugh K E, Gersey M, Rhoads D D, Procop G W, Zhang Y, Booth C N, et al. Sensitivity of cerebrospinal fluid cytology for the diagnosis of cryptococcal infections: a 21-year single-institution retrospective review. *Am J Clin Pathol.* (2019) 151:198–204. doi: 10.1093/ajcp/aqy133
33. Ruppe E, Lazarevic V, Girard M, Mouton W, Ferry T, Laurent F, et al. Clinical metagenomics of bone and joint infections: a proof of concept study. *Sci Rep.* (2017) 7:7718. doi: 10.1038/s41598-017-07546-5
34. Langelier C, Kalantar K L, Moazed F, Wilson M R, Crawford E D, Deiss T, et al. Integrating host response and unbiased microbe detection for lower respiratory tract infection diagnosis in critically ill adults. *Proc Natl Acad Sci U S A.* (2018) 115:E12353–E62. doi: 10.1073/pnas.1809700115
35. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. *Genome Med.* (2016) 8:51. doi: 10.1186/s13073-016-0307-y
36. Schlaberg R, Chiu C Y, Miller S, Procop G W, Weinstock G, Professional Practice C, et al. Validation of metagenomic next-generation sequencing tests for universal pathogen detection. *Arch Pathol Lab Med.* (2017) 141:776–86. doi: 10.5858/arpa.2016-0539-RA
37. Guo F, Kang L, Xu M, A. case of pediatric visceral leishmaniasis-related hemophagocytic lymphohistiocytosis diagnosed by mNGS. *Int J Infect Dis.* (2020) 97:27–9. doi: 10.1016/j.ijid.2020.05.056
38. Hu Z, Weng X, Xu C, Lin Y, Cheng C, Wei H, et al. Metagenomic next-generation sequencing as a diagnostic tool for toxoplasmic encephalitis. *Ann Clin Microbiol Antimicrob.* (2018) 17:45. doi: 10.1186/s12941-018-0298-1
39. Wilson M R, Suan D, Duggins A, Schubert R D, Khan L M, Sample H A, et al. A novel cause of chronic viral meningoencephalitis: cache valley virus. *Ann Neurol.* (2017) 82:105–14. doi: 10.1002/ana.24982
40. Hoffmann B, Tappe D, Hoper D, Herden C, Boldt A, Mawrin C, et al. A variegated squirrel bornavirus associated with fatal human encephalitis. *N Engl J Med.* (2015) 373:154–62. doi: 10.1056/NEJMoa1415627
41. Li X, Tan S, Han M, Ye M, Li S. Evaluation of metagenomic next-generation sequencing in the diagnosis of central nervous system infections. *Jilin Med J.* (2020) 41:1864–5.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Liu, Zhang, Han, Du, Shi, Wang, Xu and Luo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 04 October 2021
doi: 10.3389/fmed.2021.715519

Metagenomic Next-Generation Sequencing in the Diagnosis of HHV-1 Reactivation in a Critically Ill COVID-19 Patient: A Case Report

*Lei Shi[1†], Han Xia[2†], Matthew D. Moore[3], Chao Deng[1], Na Li[1], Hui Ren[1], Yunru Chen[1], Jinfeng Liu[1], Fenjing Du[1], Gezhi Zheng[1], Jing Li[1], Qunying Han[1], Wanhu Fan[1], Feng Ye[1], Shumei Lin[1], Zhengwen Liu[1], Hongjuan Liu[1], Yawen Wang[1], Jian Yang[1], Qingguang Liu[1], Yingren Zhao[1] and Tianyan Chen[1]**

[1] The First Affiliated Hospital of Xi'an Jiaotong University, Shaanxi, China, [2] Hugobiotech Beijing Co., Ltd., Beijing, China, [3] Department of Food Science, University of Massachusetts, Amherst, MA, United States

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Armelia Sari Widyarman,
Trisakti University, Indonesia
Kevin Dieckhaus,
UCONN Health, United States

***Correspondence:**
Tianyan Chen
chentianyan@xjtufh.edu.cn

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 27 May 2021
Accepted: 08 September 2021
Published: 04 October 2021

Citation:
Shi L, Xia H, Moore MD, Deng C, Li N, Ren H, Chen Y, Liu J, Du F, Zheng G, Li J, Han Q, Fan W, Ye F, Lin S, Liu Z, Liu H, Wang Y, Yang J, Liu Q, Zhao Y and Chen T (2021) Metagenomic Next-Generation Sequencing in the Diagnosis of HHV-1 Reactivation in a Critically Ill COVID-19 Patient: A Case Report. Front. Med. 8:715519. doi: 10.3389/fmed.2021.715519

Background: Secondary infections pose tremendous challenges in Coronavirus disease 2019 (COVID-19) treatment and are associated with higher mortality rates. Clinicians face of the challenge of diagnosing viral infections because of low sensitivity of available laboratory tests.

Case Presentation: A 66-year-old woman initially manifested fever and shortness of breath. She was diagnosed as critically ill with COVID-19 using quantitative reverse transcription PCR (RT-qPCR) and treated with antiviral therapy, ventilator and extracorporeal membrane oxygenation (ECMO). However, after the condition was relatively stabled for a few days, the patient deteriorated with fever, frequent cough, increased airway secretions, and increased exudative lesions in the lower right lung on chest X-rays, showing the possibility of a newly acquired infection, though sputum bacterial and fungal cultures and smears showed negative results. Using metagenomic next-generation sequencing (mNGS), we identified a reactivation of latent human herpes virus type 1 (HHV-1) in the respiratory tract, blood and gastrointestinal tract, resulting in a worsened clinical course in a critically ill COVID-19 patient on ECMO. Anti-HHV-1 therapy guided by these sequencing results effectively decreased HHV-1 levels, and improved the patient's clinical condition. After 49 days on ECMO and 67 days on the ventilator, the 66-year-old patient recovered and was discharged.

Conclusions: This case report demonstrates the potential value of mNGS for evidence-based treatment, and suggests that potential reactivation of latent viruses should be considered in critically ill COVID-19 patients.

Keywords: COVID-19, HHV-1, metagenomic next-generation sequencing (mNGS), diagnosis, case report

BACKGROUND

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the seventh known coronavirus that can infect humans after HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, and MERS-CoV. The Coronavirus disease 2019 (COVID-19) global pandemic caused by SARS-CoV-2 has affected 205,372,963 of the world's population, with 4,334,178 total deaths, as of August 13, 2021 (https://coronavirus.jhu.edu/map.html). The symptoms in the majority of patients infected with SARS-CoV-2 have been relatively mild, but 10–15% patients developed severe infections (1). Compared with patients with mild symptoms, the severe patients are older, with more comorbidities and lower lymphocyte counts (2, 3) and experience higher mortality. Secondary infections or co-infections, mainly by bacteria or viruses, occur in 10–30% of hospitalized COVID-19 patients (4), contributing to the high incidence of severe infection and mortality of COVID-19.

Human herpes virus type 1 (HHV-1) is a double stranded DNA virus that infects a large population worldwide. According to the conservative estimate, more than 90% of the human adult population has latently infected by HHV-1 globally (5–8). HHV-1 establishes latent infection within ganglial cells, of which the trigeminal ganglia are the most commonly affected. When the body's immunity weakens, HHV-1 can be reactivated and cause opportunistic infection. The mortality of influenza patients is increased with concomitant HHV-1 infection, but the pathogenesis remains unclear (9). Whether SARS-CoV-2 coinfected HSV may aggravate the condition of COVID-19 has not been reported yet. We present a case of COVID-19 infection, followed by HHV-1 reactivation that exacerbated the condition of COVID-19.

CASE PRESENTATION

A 66-year-old woman presented to the First Affiliated Hospital of Xi'an Jiaotong University on February 5, 2020, with a 4-day history of fever and shortness of breath. Apart from hypertension, the patient had no other underlying medical conditions. Laboratory tests were unremarkable except for a low lymphocyte count of 0.72×10^9 cells/L (reference range 1.1–3.2 $\times 10^9$ cells/L) and high C-reactive protein of 71 mg/L (reference range 0-10 mg/L). The respiratory rate was 30 breaths/min, and oxygen saturation was 83% on ambient air. Chest computed tomography (CT) showed multifocal bilateral ground glass opacities (GGO) with patchy consolidations, prominent in the peripheral distribution and predilected to the posterior and lower lobes (**Figure 1**). Given a history of having contact with a confirmed COVID-19 patient and characteristic changes on CT imaging, a nasopharyngeal swab (NP) specimen was sent for SARS-CoV-2 testing by quantitative reverse transcription PCR (RT-qPCR). The swab registered as positive for SARS-CoV-2 while antigen tests for influenza A and B registered negative.

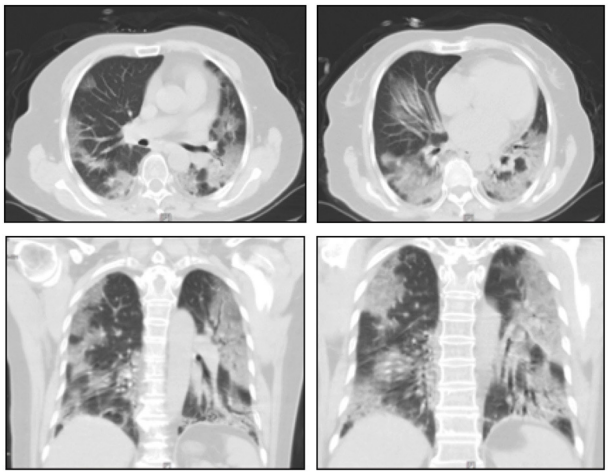


FIGURE 1 | Representative images of the thoracic CT scans. Chest computed tomograms showing multifocal bilateral ground glass opacities with patchy consolidations, prominent in the peripheral distribution on day 4 after symptom onset.

Upon admission, arbidol for antiviral therapy (0.2 g, oral, three times daily) and moxifloxacin for anti-bacterial therapy (400 mg, intravenous drips, once a day) were implemented to control patient's rapidly deteriorating condition. On Day 2 of hospitalization, the patient developed shortness of breath and chest tightness, and required supplemental oxygen *via* oxymask (oxygen flow at 10 L/min). Vitals included: respiratory rate 30–45 breaths per minute, oxygen saturation of 93–97% *via* pulse oximetry, and arterial blood gas PO_2 55 mmHg on mask oxygen at 10 L/min. Non-invasive ventilator-assisted ventilation was initiated.

On Day 3, the patient's chest tightness and shortness of breath persisted. Chest X-rays indicated a superimposed pneumonic process. Hypoxemia showed no significant improvement after increasing PEEP and oxygen concentration. Based on the Extracorporeal Life Support Organization guidelines, extracorporeal membrane oxygenation (ECMO) (V-V mode) was initiated on Day 5. During this period, the patient's vital signs remained stable and SARS-CoV-2 viral copy number in NP samples continued to decrease (**Figure 2**).

On Day 14, the patient developed a frequent productive cough, and pulse oxygen saturation dropped to 88%, accompanied by increased heart rate (117 beats per minute), breathing rate (42 breaths per minute), and a temperature of 37.8°C. A small amount of light blood fluid was suctioned out of the endotracheal tube. Chest X-rays showed increased exudative lesions in the lower right lung. The white blood cell count increased to 18.39×10^9 cells/L (reference range 3.5–9.5 $\times 10^9$ cells/L). Blood procalcitonin was negative, and sputum bacterial and fungal cultures and smears were negative, raising the possibility of a newly acquired infection, but with unknown etiology.

Abbreviations: COVID-19, Coronavirus disease 2019; HHV-1, Human herpes virus type 1; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; CT, Computed tomography; NP, nasopharyngeal swab; RT-qPCR, Quantitative reverse transcription PCR; ECMO, Extracorporeal membrane oxygenation; mNGS, Metagenomic next-generation sequencing.

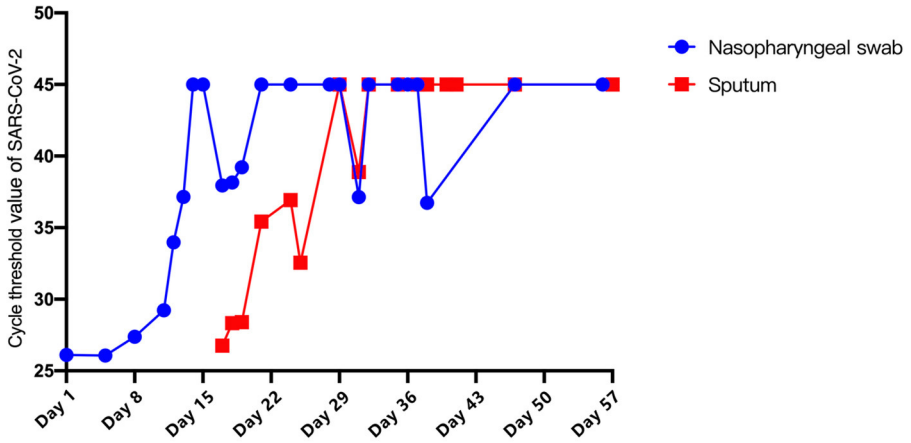


FIGURE 2 | Cycle threshold value of SARS-CoV-2. Blue: Nasopharyngeal swab. Red: Sputum.

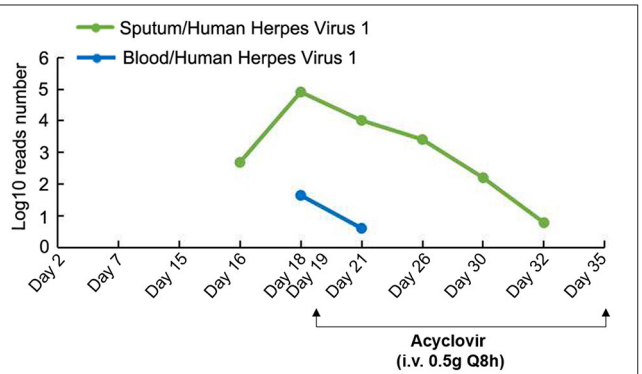


FIGURE 3 | mNGS detection and anti HHV-1 therapy. Acyclovir: 0.5 g intravenous every 8 h from Day 19 to Day 35.

To further identify the presence of any newly acquired respiratory pathogens, PACEseq metagenomic next-generation sequencing (mNGS) (Hugobiotech, Beijing) of sputum was performed on Day 16 for pan-pathogen detection. The mNGS report came out on Day 18 (48 h after inspection) and showed that in addition to SARS-CoV-2 (9,266 reads/million), HHV-1 (484 reads/million) was predominant in the sputum. Then, mNGS re-examinations of samples including sputum, blood, NP, lung lavage, and stomach fluid were performed immediately on Day 18. The results showed the amount of HHV-1 in sputum increased to 81,098 reads per million (RPM). HHV-1 was also detected in blood (44 RPM), NP (89,833 RPM), lung lavage (8 RPM) and stomach fluid (46 RPM), suggesting a latent reactivation of HHV-1 (**Figure 3**). Guided by these sequencing results, an anti HHV-1 agent, acyclovir (0.5 g, intravenous every 8 h), was initiated. After 14 days of anti-HHV-1 therapy, the number of HHV-1 reads decreased and reached to single digit level of RPM. The patient's clinical condition improved, with normal body temperature, heart rate (62 beats per minute), breathing rate (15 breaths per minute), oxygen saturation of 97–100%, and white blood cell count (8.97×10^9 cells/L). Chest X-ray showed the lesions in the lower right lung were no longer visible.

In subsequent days, the patient's clinical symptoms continued to improve, SARS-CoV-2 tests in sputum and NP maintained negative, and mNGS revealed no HHV-1 reads in sputum and blood. After 49 days on ECMO (February 9th-March 28th) and 67 days on the ventilator (February 6th-April 13th), the patient recovered and was finally discharged.

DISCUSSION AND CONCLUSIONS

To the best of our knowledge, this is the first report describing re-activation of latent HHV-1 in a critically ill COVID-19 patient with prolonged ECMO support. HHV-1 is a double stranded DNA virus of genus Simplexvirus, family Herpesviridae. HHV-1 commonly resides in sensory ganglia in latency. A variety of stimuli such as stress, fever and immune suppression can trigger lytic replication and result in production of infectious virus (10). In an immune competent patient, HHV-1 infection is mild and self-limiting. However, HHV-1 infection can be severe and prolonged in immunocompromised individuals without treatment, leading to significant morbidity and mortality (11). In this report, the lymphocyte count of the patient decreased significantly when admitted to the hospital for COVID-19 and during hospitalization. Lymphopenia is common in severe COVID-19 patients (12). ECMO therapy is also associated with a decrease of lymphocyte count and functionality (13). Interestingly, $CD8^+$ T cells play an essential role in preventing HHV-1 reactivation (14). The lower levels of lymphocytes in the reported patient might have permitted or promoted the reactivation of HHV-1 virus (15). Anti-HHV-1 therapy using acyclovir decreased HHV-1 load and alleviated the patient's clinical symptoms, indicating the significance of HHV-1 infection in the COVID-19 patient. We don't have direct evidence of HHV-1 pneumonia, but the clinical scenario (mNGS detection and the effect of anti HHV-1 treatment) is highly suggestive of causation.

Our report calls for awareness of latent virus reactivation in severe COVID-19 patients, particularly when infectious agents

are unknown. Identification of latent virus activation becomes a challenging task for the routine diagnostic tests if the causative agents are unknown. Our report utilized mNGS technology, which successfully detected HHV-1 DNA in multiple body sites, and was valuable in monitoring the dynamics of viral loads over treatment course.

mNGS is a new pan-pathogen detection technology, which can theoretically detect all known pathogens including bacteria, viruses, fungi, and parasites in one run, with unbiased results. Compared to the conventional diagnostic methods, mNGS has a higher sensitivity and specificity and costs less time. In this case report, mNGS successfully detected HHV-1 in different types of samples, including sputum, blood, NP, lung lavage, and stomach fluid within 2 days, indicating significant advantages in the diagnosis of pathogens, which contributes to a timely treatment to the patient.

In conclusion, we present a case of COVID-19 with HHV-1, followed by HHV-1 reactivation that exacerbated the condition of COVID-19. mNGS was used for the rapid clinical diagnosis of the pathogen, demonstrating the potential value of metagenomics for evidence-based treatment. This case report suggests that potential reactivation of latent viruses should be considered in critically ill COVID-19 patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The study was approved by the Ethics Review Committee of the First Affiliated Hospital of Xi'an Jiaotong University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LS, QL, YZ, and TC designed research. LS, HX, FD, GZ, JL, QH, WF, FY, SL, ZL, HL, YW, JY, and TC analyzed data. LS, HX, MM, CD, NL, HR, YC, JLiu, and TC wrote the paper. All authors have read and approved the manuscript.

FUNDING

This study was funded by the Key R&D plan of Shaanxi Province, China (number 2020ZDXM-SF-004). The funding body had no role in study design; collection, analysis, and interpretation of data; writing of the report; or the decision to submit the paper for publication.

ACKNOWLEDGMENTS

We thank all health care workers and show the greatest appreciation to all those involved in the treatment of disease. We also thank Dr. Yanjiao Zhou for language editing of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.715519/full#supplementary-material

REFERENCES

1. Lake MA. What we know so far: COVID-19 current clinical knowledge and research. *Clin Med.* (2020) 20:124-7. doi: 10.7861/clinmed.2019-coron
2. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. *JAMA.* (2020) 323:1061-9. doi: 10.1001/jama.2020.1585
3. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunologic features in severe and moderate coronavirus disease 2019. *J Clin Invest.* (2020) 130:2620-9. doi: 10.1172/JCI137244
4. Kim D, Quinn J, Pinsky B, Shah NH, Brown I. Rates of co-infection between SARS-CoV-2 and other respiratory pathogens. *JAMA.* (2020) 323:2085-6. doi: 10.1001/jama.2020.6266
5. Ayoub HH, Chemaitelly H, Abu-Raddad LJ. Characterizing the transitioning epidemiology of herpes simplex virus type 1 in the USA: model-based predictions. *BMC Med.* (2019) 17:57. doi: 10.1186/s12916-019-1285-x
6. Sukik L, Alyafei M, Harfouche M, Abu-Raddad LJ. Herpes simplex virus type 1 epidemiology in Latin America and the Caribbean: Systematic review and meta-analytics. *PLoS ONE.* (2019) 14:e0215487. doi: 10.1371/journal.pone.0215487
7. Khadr L, Harfouche M, Omori R, Schwarzer G, Chemaitelly H, Abu-Raddad LJ. The epidemiology of herpes simplex virus type 1 in Asia: systematic review, meta-analyses, and meta-regressions. *Clin Infect Dis.* (2019) 68:757-72. doi: 10.1093/cid/ciy562
8. Chaabane S, Harfouche M, Chemaitelly H, Schwarzer G, Abu-Raddad LJ. Herpes simplex virus type 1 epidemiology in the Middle East and North Africa: systematic review, meta-analyses, and meta-regressions. *Sci Rep.* (2019) 9:1136. doi: 10.1038/s41598-018-37833-8
9. Cantan B, Luyt CE, Martin-Loeches I. Influenza infections and emergent viral infections in intensive care unit. *Semin Respir Crit Care Med.* (2019) 40:488-97. doi: 10.1055/s-0039-1693497
10. Suzich JB, Cliffe AR. Strength in diversity: Understanding the pathways to herpes simplex virus reactivation. *Virology.* (2018) 522:81-91. doi: 10.1016/j.virol.2018.07.011
11. Herget GW, Riede UN, Schmitt-Graff A, Lubbert M, Neumann-Haefelin D, Kohler G. Generalized herpes simplex virus infection in an immunocompromised patient–report of a case and review of the literature. *Pathol Res Pract.* (2005) 201:123-9. doi: 10.1016/j.prp.2004.12.003
12. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. China medical treatment expert group for, clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
13. Bizzarro MJ, Conrad SA, Kaufman DA, Rycus P, Extracorporeal Life Support Organization Task Force on Infections. Infections acquired during extracorporeal membrane oxygenation in neonates, children, and adults. *Pediatr Crit Care Med.* (2011) 12:277-81. doi: 10.1097/PCC.0b013e3181e28894

14. Orr MT, Mathis MA, Lagunoff M, Sacks JA, Wilson CB. CD8 T cell control of HSV reactivation from latency is abrogated by viral inhibition of MHC class I. *Cell Host Microbe.* (2007) 2:172-80. doi: 10.1016/j.chom.2007.06.013
15. Wang F, Nie J, Wang H, Zhao Q, Xiong Y, Deng L, et al. Characteristics of peripheral lymphocyte subset alteration in COVID-19 pneumonia. *J Infect Dis.* (2020) 221:1762-9. doi: 10.1093/infdis/jiaa150

Conflict of Interest: HX is employed by Hugobiotech Beijing Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Shi, Xia, Moore, Deng, Li, Ren, Chen, Liu, Du, Zheng, Li, Han, Fan, Ye, Lin, Liu, Liu, Wang, Yang, Liu, Zhao and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

ORIGINAL RESEARCH
published: 06 October 2021
doi: 10.3389/fmed.2021.686247

A Retrospective Paired Comparison Between Untargeted Next Generation Sequencing and Conventional Microbiology Tests With Wisely Chosen Metagenomic Sequencing Positive Criteria

Hanyu Qin[1], Jinmin Peng[1], Ling Liu[2], Jing Wu[3], Lingai Pan[4,5], Xiaobo Huang[4,5], Man Huang[3], Haibo Qiu[2], Bin Du[1] and the China Critical Care Clinical Trials Group (CCCCTG)[1]*

[1] State Key Laboratory of Complex, Severe and Rare Disease, Medical ICU, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China, [2] Department of Critical Care Medicine, School of Medicine, Zhongda Hospital, Southeast University, Nanjing, China, [3] Department of Intensive Care Unit, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [4] Department of Critical Care Medicine, Sichuan Provincial Hospital, University of Electronic Science and Technology of China, Chengdu, China, [5] Sichuan Translational Medicine Research Hospital, Chinese Academy of Sciences, Chengdu, China

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Vania Aparecida Vicente Aparecida Vicente,
Federal University of Paraná, Brazil
Naveen Kumar Devanga Ragupathi,
The University of Sheffield,
United Kingdom

***Correspondence:**
Bin Du
dubin98@gmail.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 26 March 2021
Accepted: 25 August 2021
Published: 06 October 2021

Citation:
Qin H, Peng J, Liu L, Wu J, Pan L, Huang X, Huang M, Qiu H, Du B and the China Critical Care Clinical Trials Group (CCCCTG) (2021) A Retrospective Paired Comparison Between Untargeted Next Generation Sequencing and Conventional Microbiology Tests With Wisely Chosen Metagenomic Sequencing Positive Criteria. Front. Med. 8:686247. doi: 10.3389/fmed.2021.686247

Objectives: To evaluate the performance of metagenomic next generation sequencing (mNGS) using adequate criteria for the detection of pathogens in lower respiratory tract (LRT) samples with a paired comparison to conventional microbiology tests (CMT).

Methods: One hundred sixty-seven patients were reviewed from four different intensive care units (ICUs) in mainland China during 2018 with both mNGS and CMT results of LRT samples available. The reads per million ratio ($RPM_{sample}/RPM_{non-template-control}$ ratio) and standardized strictly mapped reads number (SDSMRN) were the two criteria chosen for identifying positive pathogens reported from mNGS. A McNemar test was used for a paired comparison analysis between mNGS and CMT.

Results: One hundred forty-nine cases were counted into the final analysis. The $RPMsample/RPM_{NTC}$ ratio criterion performed better with a higher accuracy for bacteria, fungi, and virus than SDSMRN criterion [bacteria ($RPMsample/RPM_{NTC}$ ratio vs. SDSMRN), 65.1 vs. 55.7%; fungi, 75.8 vs. 71.1%; DNA virus, 86.3 vs. 74.5%; RNA virus, 90.9 vs. 81.8%]. The mNGS was also superior in bacteria detection only if an SDSMRN $\geq$3 was used as a positive criterion with a paired comparison to culture [SDSMRN positive, 92/149 (61.7%); culture positive, 54/149 (36.2%); $p < 0.001$]; however, it was outperformed with significantly more fungi and DNA virus identification when choosing both criteria for positive outliers [fungi ($RPMsample/RPM_{NTC}$ ratio vs. SDSMRN vs. culture), 23.5 vs. 29.5 vs. 8.7%, $p < 0.001$; DNA virus ($RPMsample/RPM_{NTC}$ ratio vs. SDSMRN vs. PCR), 14.1 vs. 20.8 vs. 11.8%, $p < 0.05$].

Conclusions: Metagenomic next generation sequencing may contribute to revealing the LRT infection etiology in hospitalized groups of potential fungal infections and in situations with less access to the multiplex PCR of LRT samples from the laboratory by choosing a wise criterion like the RPMsample/RPM_{NTC} ratio.

Keywords: metagenomic sequencing, pathogen detection, lower respiratory tract infection, conventional microbiology tests, positive criteria

INTRODUCTION

Lower respiratory tract (LRT) infection is a common cause of intensive care unit (ICU) admission. The Management of Severe sepsis in Asia's Intensive Care unitS (MOSAICS) study revealed that among 1,285 severe sepsis patients admitted to Asian ICUs, 37.4% of the sources of infection were attributed to the lungs (1). Meanwhile, acute respiratory infection often leads to an unfavorable outcome, with in-hospital mortality increasing by 21.8% over a decade in one French region (2). Indeed, a point prevalence study of ICU infection, which recruited 1,150 centers, reported that less than two-thirds (5,259/8,135, 65%) of the ICU patients with probable or definite infections received at least one positive culture (3). Even combining cultures, targeted molecular methods and serology all together to routinely investigate lower respiratory tract (LRT) infection microbial etiology, Leven et al. found out that the potential pathogen detection rate was only 59% (1,844/3,104) in this study (4). Approximately 40% of the cases were causative agents undetermined by conventional microbiological tests (CMT).

Next generation sequencing, as a high-throughput technique, with a cost that was largely reduced since 2004 (5), is now widely used in clinical metagenomics, specifically for unbiased pathogen detection. In particular, metagenomic next generation sequencing (mNGS) allows for the novel findings and taxonomical classification of microorganisms, which could tremendously impact infectious disease diagnosing (6).

Large sample size studies evaluating mNGS diagnostic performance compared with CMTs either as a first-line test or the last resort in clinical scenarios were conducted. Parize et al. designed a prospective study with a cohort of 101 patients and a 30-day follow-up. They reported that untargeted next generation sequencing had a high negative predictive value of 98.4% (95% CI 95.3–100%) in bacteria and viruses, but with a restriction on the identification of fungi or parasites (7). Xing et al. claimed in his 213 case series that the mNGS detection rate was 57%, while in the laboratory of Chiu, mNGS succeeded in 32 central nervous system (CNS) infection diagnoses from 204 patients. The performance of mNGS remains controversial, and studies in LRT specimens were limited (8, 9).

A multicentered research was conducted with patients with suspected LRT infections, and both mNGS and CMT results reported from LRT samples, which aimed to interpret mNGS results in a fair way for common infectious agents.

METHODS

Study Design

One hundred sixty-seven patients admitted to four different ICUs in mainland China during 2018 were retrospectively studied. These cases were reviewed considering the following inclusion criteria: (i) patients with respiratory symptoms requiring oxygen therapy or any other organ support in the ICU, (ii) patients with radiology images (chest x-ray or CT scanning) showing lung infiltration, (iii) patients with a suspected infection causing the conditions described meeting criteria (i) and (ii), and (iv) patients with lower respiratory tract samples collected for both mNGS and CMT.

Data Collected From Medical Records

Demographic information, comorbidities, immune state, disease severity upon ICU admission, duration between ICU admission and attempt to sequence LRT specimen, ICU intervention, clinical outcome, and conventional microbiological test results were all collected from the medical record of each patient through the hospital information system. Disease severity was assessed using an Acute Physiology and Chronic Health Evaluation (APACHE) II score (10) and a Sequential Organ Failure Assessment (SOFA) score (11). A higher score indicates more severe clinical presentation. Furthermore, ICU intervention in this study was listed but not limited to vasopressor utilization, invasive or non-invasive mechanical ventilation, and continuous renal replacement therapy. Clinical outcome was illustrated as the length of stay in the ICU and ICU mortality.

Retrieving the LRT Sample and CMT Positive Agreement on Clinically Significant Microbes

Lower respiratory tract samples such as sputum, trachea aspirates, or bronchoalveolar lavage fluids were investigated in this study. The available CMTs of LRT samples (smear, culture, and multiplex PCR) in participating centers and significant pathogen consideration were consistent with previous publication (**Supplementary Tables 1**, **2**) (12). Microbes detected *via* CMT were identified as true pathogens rather than commensals or contaminants. Serum antigen test results were also reviewed, while serum antibody test results were excluded due to a failure to distinguish acute infection. More specifically, immunoglobulin G (IgG) antibodies reported in our cohort lacked track of a 4-fold rise while IgM antibody test results were not recommended for the infection diagnoses of adenovirus,

Mycoplasma pneumoniae, and *Legionella*/*Chlamydia* spp. (13, 14).

Library Construction, Sequencing, Bioinformatic Analysis, and Criteria for a Positive Result

After extraction, nucleic acid fragments underwent end repairing, adapter ligation, and amplification to construct the library, quality control of which was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). The qualified library was sequenced on the BGISEQ-100/50 platform. A non-template specimen was parallelly run for the purpose of contamination control (non-template control, NTC).

Raw sequencing data were filtered by removing low-quality reads, and clean reads were mapped to the human reference genome (hg19) to subtract the host portion by Burrows-Wheeler Alignment. The remaining reads were classified by simultaneously mapping to four microbial genome databases, composed of 4,152 whole genome sequences of viral taxa, 3,446 bacteria, 206 fungi, and 140 parasites associated with human disease (15).

The mapped reads number of each microbe in each respiratory sample was normalized in three ways (15, 16).

(i) Mapped reads abundance relative to other microbes in the same sample:

$$\text{Reads per million (RPM)} = \frac{\text{Mapped reads number(MRN)} * 10^6}{\text{Total sequencing reads}}$$

(ii) Mapped reads abundance relative to the same microbe at the species level in other samples of this study cohort using Z-score.

$$\text{Z} - \text{score} = \frac{\chi - \mu}{\sigma}$$

χ is the log10 transformation of RPM of one microbe. μ is the mean of $\log_{10}$RPM of the same microbe in this study cohort. σ is the SD of $\log_{10}$RPM of the same microbe in this study cohort.

(iii) Uniquely mapped reads in this study were described as stringently mapped reads number (SMRN) and normalized as SDSMRN.

$$\text{SDSMRN} = \frac{\text{SMRN}^*20^*10^6}{\text{Total sequencing reads}}$$

The positive criteria for the mNGS result were set as follows, which were consistent with literature reviews at the species level and those that found that microbes were evidenced of pathogenicity in the lungs (15–17). A value of "1" was adjusted to the RPM of the non-template control with no pathogenic bacteria, fungi, or viruses (18).

(i) RPMsample/RPM_{NTC} ratio $\geq$10, or SDSMRN $\geq$3 (mycobacteria excluded) for bacteria
(ii) RPMsample/RPM_{NTC} ratio $\geq$1, or SDSMRN $\geq$3 for fungi/DNA virus
(iii) RPMsample/RPM_{NTC} ratio $\geq$1, or SDSMRN $\geq$1 for RNA virus
(iv) SDSMRN $\geq$100 for parasites
(v) SDSMRN $\geq$3 for *Mycoplasma*/*Chlamydia* spp.
(vi) SDSMRN $\geq$1 (or SDSMRNG $\geq$1 at genus level) for *Mycobacterium tuberculosis* (MTB) complex
(vii) Reported *Nocardia* spp. by mNGS all considered positive

Statistical Analysis

A Mann–Whitney *U*-test was used for non-parametric data analysis. A 2 × 2 contingency table was drawn to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Data were reported as absolute values with 95% CI (7). The marginal frequencies of the 2 × 2 table were tested by a McNemar test. Significance was considered when $p < 0.05$. The SPSS 22.0 software was used.

RESULTS

Patient Characteristics and General Sequencing Information

One hundred forty-nine cases were enrolled into the final analysis since the total sequencing reads in the mNGS reports of 18 patients were missing, making both RPM and SDSMRN infeasible to calculate. Among these 149 patients [median age 59, interquartile range (IQR) from 47.5 to 70; 104 (69.8%) male], 75 (50.3%) were at immunocompromised states. Fifty (33.6%) were prescribed prolonged corticosteroids (defined as a minimum dose of 0.3 mg/kg/d of prednisone or an equivalent for 3 weeks at least) (12), constructing the therapy of autoimmune disease [37 (24.8%)] and post-transplantation [8 (5.4%)] or other. Twenty-one (14.9%) were receiving chemotherapy for cancer, leukemia, or lymphoma. The median APACHE II and sofa scores were 20 (IQR from 15 to 24) and 9 (IQR from 6 to 11), respectively. The median ICU stay was 12 days (IQR from 7 to 21.5 days), and ICU mortality was 51%. The LRT samples of patients were retrieved and sent for mNGS 1 day (IQR from 1 to 3 days) after ICU admission (**Table 1**).

In the metagenomic analysis of these 149 samples, 96 were tested by DNA sequencing (DNAseq), 35 were tested by RNA sequencing (RNAseq), and 18 were tested by DNAseq and RNAseq parallelly. No significant difference was found between the mean total yield of DNAseq and RNAseq (21,261,618 vs. 22,862,055 reads, $p = 0.49$) and for human proportion (97.1 vs. 96.8%, $p = 0.09$) (**Figures 1**, **2**).

Comparison of mNGS and CMT in Pathogen Detection

Positive mNGS Results Fulfilling RPM (Sample/NTC) Ratio Criterion

Bacteria were identified in 64 (64/149, 43%) cases by an RPMsample/RPM_{NTC} ratio $\geq$10, with the top 3 being *Acinetobacter baumannii* (30/149, 20.1%), *Pseudomonas aeruginosa* (14/149, 9.4%), and *Klebsiella pneumonia* (8/149, 5.4%). Of the 149 cases, 54 (54/149, 36.2%) were bacterial culture

TABLE 1 | Patient characteristics and clinical outcome.

Characteristic	Total patients (n = 149)
Age (year), median (IQR)	59 (47.5, 70)
Male, *n* (%)	104 (69.8%)
Disease severity	
APACHE II score, median (IQR)	20 (15, 24)
SOFA score, median (IQR)	9 (6, 11)
ICU intervention	
Vasopressor, *n* (%)	83 (55.7%)
Invasive mechanical ventilation, *n* (%)	122 (81.9%)
CRRT, *n* (%)	27 (18.1%)
Duration between mNGS test and admission (day)	1 (1, 3)
Clinical outcome	
ICU death, *n* (%)	76 (51.0%)
ICU LOS (day), median (IQR)	12 (7, 21.5)
Immunocompromised state	**75 (50.3%)**
Comorbidity	
Autoimmune disease	37 (24.8%)
Hematologic malignancy	16 (10.7%)
Cancer	5 (3.4%)
Solid organ transplantation	8 (5.4%)

IQR, interquartile range; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; ICU, intensive care unit; CRRT, continuous renal replacement therapy; LOS, length of stay.

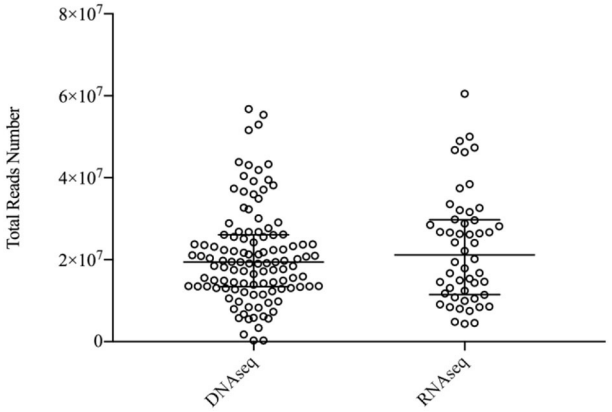


FIGURE 1 | Total sequencing reads of the reports of a total of 149 patients counted to the final analysis.

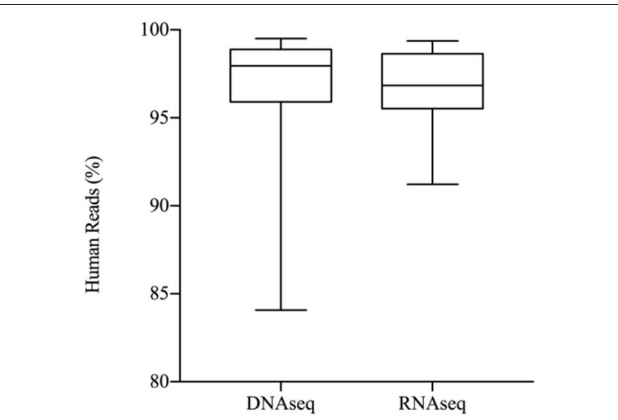


FIGURE 2 | Host background of sequencing data.

positive. The bacterium detection rate of mNGS seemed higher, but no significant difference was found in the paired comparison with culture by the McNemar test ($p = 0.212$) (**Figures 3**, **4**).

Thirty-five (35/149, 23.5%) cases met the criterion of having an RPMsample/RPM_{NTC} ratio $\geq$1 for fungi detection, while culture suggested less (13/149, 8.7%) fungal infection ($p < 0.001$). The detection rate of the culture itself for *Aspergillus* spp. was the same as that of mNGS if choosing RPM ratio criterion (11/149, 7.4%), while the probable diagnosis of invasive pulmonary aspergillosis (IPA) would be made in 46 (46/149, 30.9%) cases using comprehensive CMTs (either culture *Aspergillosis* spp. positive or serum/bronchoalveolar lavage fluid galactomannan positive). The mNGS was not superior to comprehensive CMTs for diagnosing IPA ($p < 0.001$) (**Figures 3**, **4**).

Pathogen Identified by SDSMRN Criterion and Comparison of Two Criteria

The mNGS detected 92 (92/149, 61.7%) bacterium cases with the fulfilment of SDSMRN $\geq$3. The SDSMRN criterion identified $\sim$1.5 times more bacterium cases than the RPM ratio criterion, also with a better bacterium identification rate than culture ($p < 0.001$). More fungal infections were suggested by SDSMRN $\geq$3 (44/149, 29.5%) than RPMsample/RPM_{NTC} ratio $\geq$1. The detection rate of mNGS for fungi was higher than culture with either criterion chosen in the paired analysis of 149 patients (SDSMRN vs. culture, $p < 0.001$; RPM ratio vs. culture, $p < 0.001$) (**Figure 4**).

Meanwhile, the SDSMRN criterion had a higher sensitivity but lower accuracy than the RPM ratio criterion for both bacteria [sensitivity (SDSMRN vs. RPM ratio), 61.1 vs. 74.1%; accuracy, 65.1 vs. 55.7%] and fungi [sensitivity (SDSMRN vs. RPM ratio), 53.8 vs. 46.2%; accuracy, 71.1 vs. 75.8%] identification. The negative predictive value of mNGS in the cohort was over 75% regardless of choosing either criterion [bacteria (SDSMRN vs. RPM ratio), 75.3 vs. 75.4%; fungi, 94.3 vs. 93.9%]. The accuracy of mNGS in detecting fungi was higher than bacteria, which was also criteria independent (**Table 2**).

The SDSMRN criterion identified 13 (13/149, 8.7%) aspergillosis positive and was also not superior to comprehensive CMTs ($p < 0.001$). The accuracy of the SDSMRN criterion in identifying *Aspergillus* spp. was the same as that of the RPM ratio criterion (89.3%, 95% CI 84.3–94.2%), but the SDSMRN criterion had a higher sensitivity (36.4%, 95% CI 12.4–68.4%) if comparing mNGS with culture (**Figure 3**, **Table 3**).

The SDSMRN criterion also suggested the possible infection of *M. tuberculosis* ($n = 2$), scedosporium ($n = 1$), metopneumovirus ($n = 1$), and *Chlamydia* spp. ($n = 1$), which would not be revealed by the RPM ratio criterion (**Figure 3**).

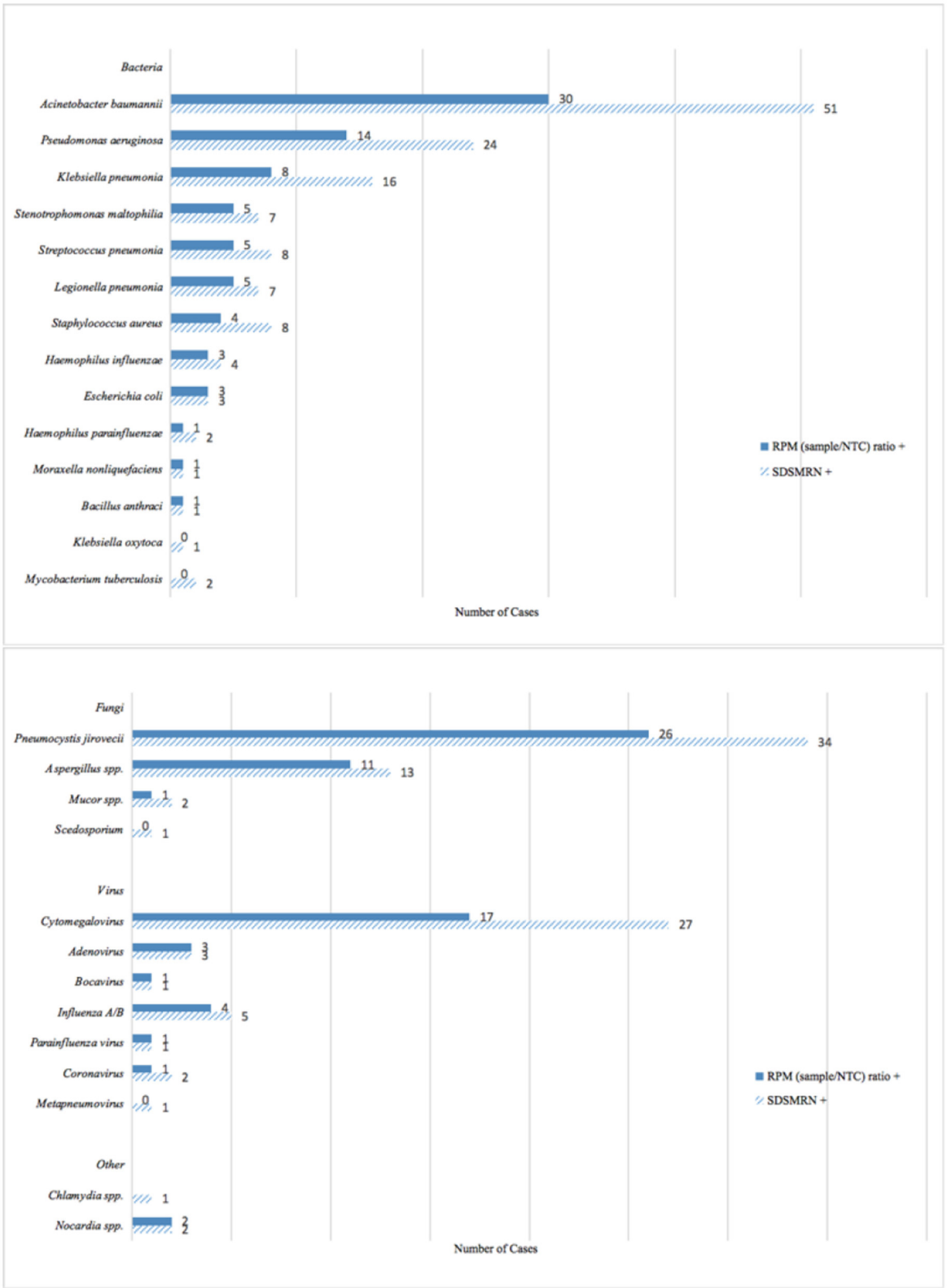


FIGURE 3 | Pathogen distribution according to metagenomic sequencing positive criteria of the reports of a total of 149 patients counted to the final analysis.

TABLE 2 | Performance of metagenomic sequencing for bacterium and fungus detection.

	Bacteria culture					Fungi culture				
	Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
RPM_{sample}/RPM_{NTC} ratio ≥ 10/1	61.1% (46.9–73.8%)	67.4% (56.9–76.4%)	51.6% (38.8–64.1%)	75.3% (64.5–83.7%)	65.1% (57.4–72.8%)	46.2% (20.4–73.9%)	78.7% (70.7–85.0%)	17.1% (7.2–34.3%)	93.9% (87.3–97.3%)	75.8% (69.0–82.7%)
SDSMRN ≥ 3	74.1% (60.1–84.6%)	45.3% (35.1–55.8%)	43.5% (33.3–54.2%)	75.4% (62.0–85.5%)	55.7% (47.7–63.7%)	53.8% (26.1–79.6%)	72.8% (64.4–79.9%)	15.9% (7.2–30.7%)	94.3% (87.5–97.7%)	71.1% (63.9–78.4%)

NPV, negative predictive value; PPV, positive predictive value.

TABLE 3 | Diagnostic performance of metagenomic sequencing for invasive pulmonary aspergillosis.

	***Aspergillus* spp. culture**					**Culture or Serum/BAL fluid galactomannan**				
	Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
RPM_{sample}/RPM_{NTC} ratio ≥ 1	27.3% (7.3–60.7%)	94.2% (88.5–97.3%)	27.3% (7.3–60.7%)	94.2% (88.5–97.3%)	89.3% (84.3–94.2%)	15.2% (6.8–29.5%)	96.1% (89.8–98.7%)	63.6% (31.6–87.6%)	71.7% (63.3–78.9%)	71.1% (63.3–78.9%)
SDSMRN ≥ 3	36.4% (12.4–68.4%)	93.5% (87.6–96.8%)	30.8% (10.4–61.1%)	94.9% (89.3–97.7%)	89.3% (84.3–94.2%)	17.4% (8.3–32.0%)	95.1% (88.5–98.2%)	61.5% (32.3–84.9%)	72.1% (63.6–79.2%)	71.1% (63.9–78.4%)

NPV, negative predictive value; PPV, positive predictive value.

TABLE 4 | Diagnostic performance of metagenomic sequencing for *Pneumocystis jirovecii* pneumonia.

	***Pneumocystis jirovecii* PCR**					**Gomori methenamine stain**				
	Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
RPM_{sample}/RPM_{NTC} ratio ≥ 1	80% (58.7–92.4%)	100% (87.0–100%)	100% (80.0–100%)	86.8% (71.1–95.1%)	91.4% (84.2–98.6%)	100% (56.1–100%)	74.5% (60.1–85.2%)	35% (16.3–59.1%)	100% (88.6–100%)	77.6% (66.9–88.3%)
SDSMRN ≥ 3	96% (77.7–99.8%)	97.0% (82.5–99.8%)	96% (77.7–99.8%)	97.0% (82.5–99.8%)	96.6% (91.9–100%)	100% (56.1–100%)	64.7% (50.0–77.2%)	28% (12.9–49.6%)	100% (87.0–100%)	69.0% (57.1–80.9%)

NPV, negative predictive value; PPV, positive predictive value.

TABLE 5 | Performance of metagenomic sequencing for virus detection.

	DNA virus PCR					**RNA virus PCR**				
	Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
RPM_{sample}/RPM_{NTC} ratio ≥ 1	100% (51.7–100%)	84.4% (69.9–93.0%)	46.2% (20.4–73.9%)	100% (88.6–100%)	86.3% (76.8–95.7%)	75% (21.9–98.7%)	94.4% (70.6–99.7%)	75% (21.9–98.7%)	94.4% (70.6–99.7%)	90.9% (78.9–100%)
SDSMRN ≥ 3/1	100% (51.7–100%)	71.1% (55.6–83.2%)	31.6% (13.6–56.5%)	100% (86.7–100%)	74.5% (62.5–86.5%)	75% (21.9–98.7%)	83.3% (57.7–95.6%)	50% (13.9–86.1%)	93.8% (67.7–99.7%)	81.8% (65.7–97.9%)

NPV, negative predictive value; PPV, positive predictive value.

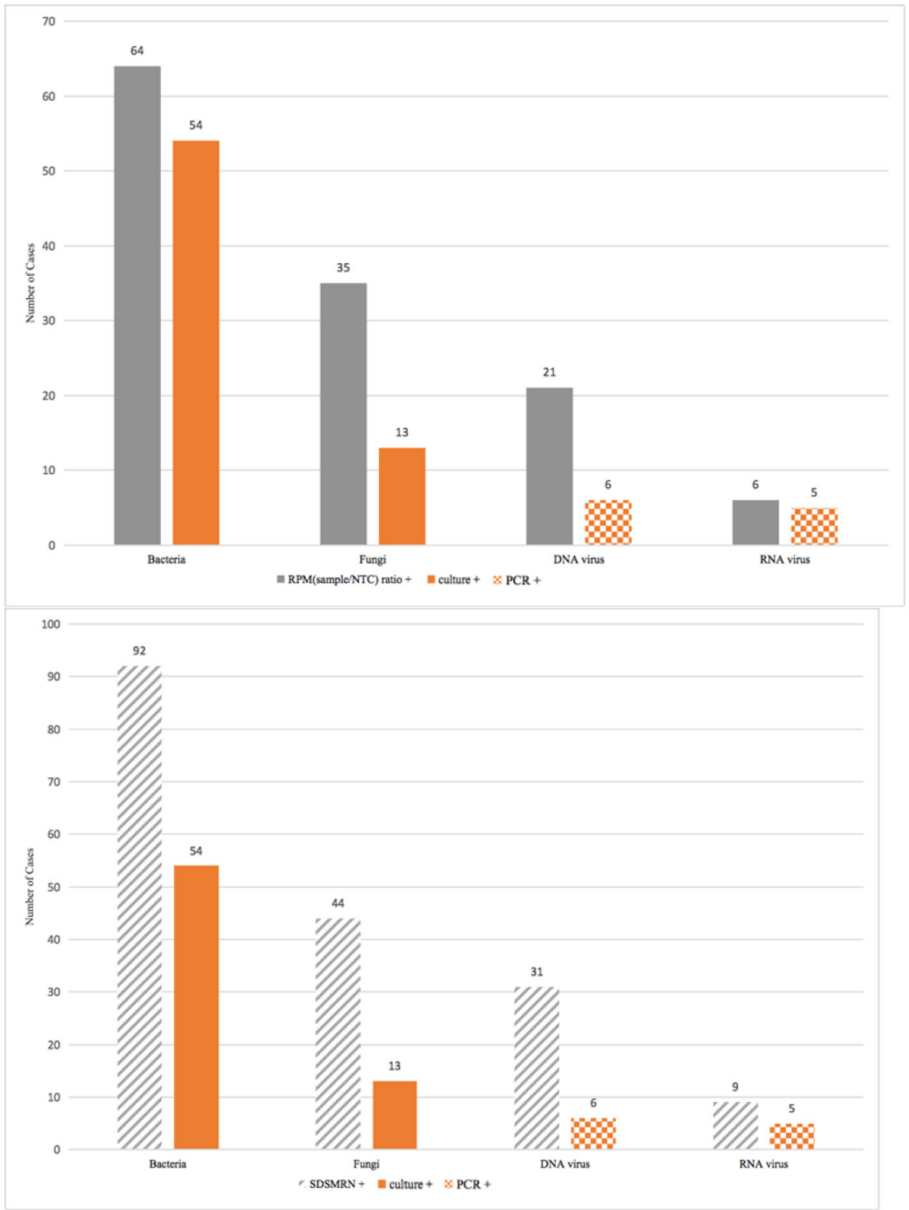


FIGURE 4 | Positive cases identified by metagenomic sequencing or conventional microbiology tests (culture/PCR) from a total of 149 patients counted to the final analysis.

Identification of Pathogens in Culture Negative Samples by mNGS

Streptococcus pneumonia (if using RPMsample/RPM_{NTC} ratio $\geq$10 criterion, $n = 5$; while using SDSMRN $\geq$3 criterion, $n = 8$), *Haemophilus influenzae*, *Legionella pneumonia,* and *Bacillus anthraci* were only detected by mNGS other than bacterium culture. Forty-eight (48/149, 32.2%) samples were sent for the *L. pneumonia* PCR, and only one (1/48, 2.1%) case tested positive. Of the 48 cases with a paired comparison between mNGS and Legionella PCR, both criteria also identified only one legionella positive case, which consisted of the PCR result (**Figure 3**).

Due to the inability of culturing *Pneumocystis jirovecii* (19), a gomori methenamine stain or PCR was used for the *P. jirovecii* pneumonia diagnosis. Fifty-eight (58/149, 38.9%) patients had both their gomori methenamine stain and PCR results reported, among which 25 (25/58, 43.1%) cases were *P. jirovecii* PCR positive, while the gomori methenamine stains only made 7 (7/58, 12.1%) *P. jirovecii* pneumonia confirmations. The RPMsample/RPM_{NTC} ratio $\geq$1 suggested 26 (26/149, 17.4%) *P. jirovecii* pneumonia cases with both sensitivity and accuracy [sensitivity (95% CI), 80% (58.7–92.4%); accuracy (95% CI), 91.4% (84.2–98.6%)] slightly lower than that of SDSMRN $\geq$3

[sensitivity (95% CI), 96% (77.7–99.8%); accuracy (95% CI), 96.6% (91.9–100%)], which identified 34 (34/149, 22.8%) *P. jirovecii* positive cases. When comparing mNGS and CMT in the paired 58 cases, mNGS performed no better than a simplex PCR but beat the gomori methenamine stain for *P. jirovecii* pneumonia diagnosis with either positive criterion chosen (RPM ratio vs. stain, $p < 0.001$; SDSMRN vs. stain, $p < 0.001$) (**Figure 3**, **Table 4**).

Viral etiology was revealed only in one-third (54/149, 36.2%) of the patients in this study when using a multiplex PCR. Viruses of the PCR interests were limited to cytomegalovirus (CMV), influenza A/B, adenovirus, rhinovirus, respiratory syncytial virus (RSV), metapneumovirus, and parainfluenza virus. Because the samples of 96 patients only underwent a metagenomic DNAseq analysis, from which RNA viruses would be impossibly discovered, the accuracy of mNGS was evaluated for DNA virus and RNA virus cases separately. Both an RPMsample/RPM_{NTC} ratio $\geq$1 (21/149, 14.1%) and an SDSMRN $\geq$3 (31/149, 20.8%) identified more DNA virus cases than the PCR (6/51, 11.8%). The mNGS was superior to the PCR detecting DNA viruses after a paired comparison between the two criteria and the multiplex PCR of the 51 cases (RPM ratio vs. PCR, $p = 0.007$; SDSMRN vs. PCR, $p < 0.001$). Six (6/53, 11.3%) RNA viral infections were suggested by the RPM ratio criterion and nine (9/53, 17.0%) by SDSMRN $\geq$1. The PCR revealed less cases (5/46, 10.9%) but without significant difference compared with mNGS (RPM ratio vs. PCR, $p = 0.5$; SDSMRN vs. PCR, $p = 0.313$) (**Figure 4**).

Metagenomic next generation sequencing presented a sensitivity of 100% (95% CI, 51.7–100%) for DNA viruses and a negative predictive value of over 90% for RNA viruses. The mNGS detection accuracy of RNA viruses [RPM ratio (95% CI), 90.9% (78.9–100%); SDSMRN (95% CI), 81.8% (65.7–97.9%)] was higher than that of DNA virus [RPM ratio (95% CI), 86.3% (76.8–95.7%); SDSMRN (95% CI), 74.5% (62.5–86.5%)]. Still, the RPM ration criterion performed better than the SDSMRN criterion in identifying virus cases, with higher accuracy (**Table 5**).

The bocavirus [$n = 1/1$ (RPM ratio positive/SDSMRN positive)] and coronavirus ($n = 1/2$) were the two novel viruses reported by mNGS outranging permits of the multiplex PCR in this cohort (**Figure 3**).

DISCUSSION

Microbiological etiology was only revealed in 12.7% of the hospitalized patients with pneumonia diagnoses in mainland China by CMTs (20). The mNGS was seen as a promising tool for its broad-spectrum and unbiased pathogen detection, with a sensitivity increase of 15% in diagnosing infection compared to culture (21). Our study first used the RPM ratio and SDSMRN as criteria for the positive identification of pathogens reported by mNGS in such a large-scale cohort of LRT infections. We also paired compared mNGS with the CMT of LRT samples in terms of their performance in detecting causative microbes. The RPM ratio criterion performed better, with a higher accuracy in identifying bacteria, fungi, and viruses, than the SDSMRN criterion in this cohort. The mNGS was only superior in bacteria detection if using SDSMRN $\geq$3 as the positive criterion with a paired comparison with culture but outperformed with significantly more fungi and DNA virus identification by choosing both criteria.

The mNGS test has been lacking a unified criterion for reporting clinically significant microbes due to varied sequencing instruments, the bioinformatic analysis pipeline, and the unstandardized productivity and quality control of each platform. Some studies defined mNGS positive as high-ranking microbes by sequencing abundance relative to other microbes in the same sample, probably with the purpose of balancing host background (21, 22). The human proportion of the total sequencing yield from an upper respiratory sample like an oropharyngeal swab [median (IQR), 5.1% (1.1–39.1%)] was clearly less than that of an LRT sample, which might result in more reads left for microbe mapping (23). However, a case series also proved absent microbes of the top 10 pathogens listed by mNGS in the validation run of nanopore sequencing with much longer read length, which also had a recognized 96.6% sensitivity for pathogen detection in LRT samples (24, 25). The evaluation of others for mNGS using absolute values as criteria like SMRN or unique reads number remained controversial since the difficulty of extracting nuclei acids varies from species to species and the total sequencing yield varies from sample to sample (26). It was believed that RPM could be an adequate criterion for mNGS because of its documented success in maximizing specificity for bacteria and sensitivity for fungi or viruses among immunocompromised children with pulmonary infections (18). A study by Zinter also used a combination with a Z-score $\geq$2 to set thresholds, which was, unfortunately, not applicable for this study. A Z-score $\geq$2 left eight mNGS-positive cases (8/149, 5%) in the study population (**Supplementary Figure 1**). The RPM to RPM (sample/NTC) ratio was also adjusted for the subtraction of contaminants to the platform. The RPM ratio criterion and the SDSMRN criterion, which was reported with a decent sensitivity (95%), were compared, detecting respiratory bacteria and fungi by Qian et al. (27).

The mNGS sensitivity for fungi detection was similar to a study by Li, which also made paired comparisons between mNGS and culture, while the mNGS of this study achieved higher specificity by wisely choosing a criterion with RPMsample/RPM_{NTC} ratio $\geq$1 (28). Consistent with the previous research results, mNGS also did not outperform comprehensive CMTs for IPA diagnosis but was superior to gomori methenamine stains for *P. jirovecii* pneumonia. By setting the clinical view as the final call of infection and comparing mNGS and simplex PCR of *P. jirovecii* with the diagnoses of the clinician, both the sensitivity and specificity of mNGS and the *P. jirovecii* PCR were very close from the previous study (12). In this cohort, with a paired analysis, no significant difference was found between the SDSMRN criterion and *P. jirovecii* PCR, but the *P. jirovecii* PCR showed its advantage over the RPM ratio criterion. Probably in the population with highly suspected *P. jirovecii* pneumonia, there was no need to use mNGS to look for a needle in a haystack (28). For viral detection, mNGS definitely exceeds the hypothesis of causative agents made by the clinician, which multiplex PCR is restricted to. However, novel emerging virus

identification requires the annotated microbe genome reference, long read, and depth sequencing methods (29).

Strengths

The majority of studies published by researchers evaluated the diagnostic performance of mNGS by setting up the final call for infection of clinicians as the golden standard. However, the mNGS performance in pathogen detection might have been disvalued if clinical impressions involved bias. By reviewing existing reports of both mNGS and CMTs from suspected severe pneumonia patients, this study utilized direct paired comparisons between mNGS and CMT to reveal whether these two methods were significantly different in causative pathogen detection. The study implied that mNGS performed better than fungi culture. Thus, clinicians who are faced with immunocompromised populations or who are working in less-developed areas without access to simplex fungus PCRs or antigen tests are suggested to order mNGS for LRT specimens.

It was also discovered that RPM could be widely used for mNGS results interpretation among clinicians and that the RPMsample/RPM_{NTC} ratio criterion outperformed regardless of pathogen categorizing.

Limitations

The research was clearly limited in several ways. Not all samples from this cohort had a PCR test of the suspected viral pathogen. The mNGS performance in detecting common viruses responsible for LRT infection was only evaluated in a small proportion of patients due to the need to compare with available PCR results. This could impact less in a future prospective study with all samples sent for both mNGS and PCR screens. Also, the criteria that were selected in identifying positive microbes reported by mNGS were documented ones from previous studies. It would be better to come up with a threshold based on the quantity of sequenced species under the circumstance that quantitative PCR was run to validate those in each sample. Lastly, effects on the clinical decision of mNGS results were not discussed, which would be better evaluated in a randomized controlled trial.

CONCLUSIONS

The RPM ratio criterion performed better with a higher accuracy for bacteria, fungi, and viruses than the SDSMRN criterion. By choosing positive criteria wisely, mNGS may contribute to revealing LRT infection etiology in hospitalized groups of potential fungal infections and in situations with less access to the multiplex PCR of LRT samples from the laboratory.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethic Committee of Peking Union Medical College Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HQin drafted the paper. JP, LL, JW, LP, XH, MH, HQiu, and BD contributed to the data collection. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Ministry of Science and Technology of China [Grant number: 2020YFC0841300].

ACKNOWLEDGMENTS

We thank Honglong Wu and Jiali Zhou for providing the sequencing data from the BGI platform.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.686247/full#supplementary-material

REFERENCES

1. Phua J, Koh Y, Du B, Tang Y-Q, Divatia JV, Tan CC, et al. Management of severe sepsis in patients admitted to Asian intensive care units: prospective cohort study. *BMJ Br Medical J.* (2011) 342:d3245. doi: 10.1136/bmj.d3245
2. Laporte L, Hermetet C, Jouan Y, Gaborit C, Rouve E, Shea KM, et al. Ten-year trends in intensive care admissions for respiratory infections in the elderly. *Ann Intensive Care.* (2018) 8:84. doi: 10.1186/s13613-018-0430-6
3. Vincent J-L, Sakr Y, Singer M, Martin-Loeches I, Machado FR, Marshall JC, et al. Prevalence and outcomes of infection among patients in intensive care units in 2017. *JAMA.* (2020) 323:1478–87. doi: 10.1001/jama.2020.2717
4. Ieven M, Coenen S, Loens K, Lammens C, Coenjaerts F, Vanderstraeten A, et al. Aetiology of lower respiratory tract infection in adults in primary care: a prospective study in 11 European countries. *Clin Microbiol Infec.* (2018) 24:1158–63. doi: 10.1016/j.cmi.2018.02.004
5. Chiu C, Miller S. Next-generation sequencing. In: Persing DH, et al. editor. *Molecular Microbiology: Diagnostic Princples and Practice*. 3rd ed. Washington, DC: ASM Press (2016), 68–79.
6. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathology Mech Dis.* (2018) 14:1–20. doi: 10.1146/annurev-pathmechdis-012418-012751
7. Parize P, Muth E, Richaud C, Gratigny M, Pilmis B, Lamamy A, et al. Untargeted next-generation sequencing-based first-line diagnosis of infection

in immunocompromised adults: a multicentre, blinded, prospective study. *Clin Microbiol Infec.* (2017) 23:574.e1–6. doi: 10.1016/j.cmi.2017.02.006
8. Xing X-W, Zhang J-T, Ma Y-B, He M-W, Yao G-E, Wang W, et al. Metagenomic next-generation sequencing for diagnosis of infectious encephalitis and meningitis: a large, prospective case series of 213 patients. *Front Cell Infect Mi.* (2020) 10:88. doi: 10.3389/fcimb.2020.00088
9. Wilson MR, Sample HA, Zorn KC, Arevalo S, Yu G, Neuhaus J, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. *New Engl J Med.* (2019) 380:2327–40. doi: 10.1056/NEJMoa1803396
10. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II. *Crit Care Med.* (1985) 13:818–29. doi: 10.1097/00003246-198510000-00009
11. Vincent J-L, Moreno R, Takala J, Willatts S, Mendonça AD, Bruining H, et al. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. *Intens Care Med.* (1996) 22:707–10. doi: 10.1007/s001340050156
12. Peng J-M, Du B, Qin H-Y, Wang Q, Shi Y. Metagenomic next-generation sequencing for the diagnosis of suspected pneumonia in immunocompromised patients. *J Infect.* (2021) 82:22–7. doi: 10.1016/j.jinf.2021.01.029
13. Miller JM, Binnicker MJ, Campbell S, Carroll KC, Chapin KC, Gilligan PH, et al. A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 2018 update by the infectious diseases society of America and the American society for microbiology. *Clin Infect Dis.* (2018) 67:e1–94. doi: 10.1093/cid/ciy381
14. Landry ML. Immunoglobulin M for acute infection: true or false? *Clin Vaccine Immunol.* (2016) 23:540–5. doi: 10.1128/CVI.00211-16
15. Wang H, Lu Z, Bao Y, Yang Y, Groot R de, Dai W, et al. Clinical diagnostic application of metagenomic next-generation sequencing in children with severe nonresponding pneumonia. *PLoS ONE.* (2020) 15:e0232610. doi: 10.1371/journal.pone.0232610
16. Zinter MS, Dvorak CC, Mayday MY, Iwanaga K, Ly NP, McGarry ME, et al. Pulmonary metagenomic sequencing suggests missed infections in immunocompromised children. *Clin Infect Dis.* (2018) 68:1847–55. doi: 10.1093/cid/ciy802
17. Langelier C, Zinter MS, Kalantar K, Yanik GA, Christenson S, O'Donovan B, et al. Metagenomic sequencing detects respiratory pathogens in hematopoietic cellular transplant patients. *Am J Resp Crit Care.* (2018) 197:524–8. doi: 10.1164/rccm.201706-1097LE
18. Simner PJ, Miller HB, Breitwieser FP, Monsalve GP, Pardo CA, Salzberg SL, et al. Development and optimization of metagenomic next-generation sequencing methods for cerebrospinal fluid diagnostics. *J Clin Microbiol.* (2018) 56:e00472–18. doi: 10.1128/JCM.00472-18
19. Liu Y, Fahle GA, Kovacs JA. Inability to culture Pneumocystis jirovecii. *Mbio.* (2018) 9:e00939–18. doi: 10.1128/mBio.00939-18
20. Chen L, Zhou F, Li H, Xing X, Han X, Wang Y, et al. Disease characteristics and management of hospitalised adolescents and adults with community-acquired pneumonia in China: a retrospective multicentre survey. *BMJ Open.* (2018) 8:e018709–9. doi: 10.1136/bmjopen-2017-018709
21. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis.* (2018) 67:S231–40. doi: 10.1093/cid/ciy693
22. Zhou X, Wu H, Ruan Q, Jiang N, Chen X, Shen Y, et al. Clinical evaluation of diagnosis efficacy of active mycobacterium tuberculosis complex infection via metagenomic next-generation sequencing of direct clinical samples. *Front Cell Infect Mi.* (2019) 9:351. doi: 10.3389/fcimb.2019.00351
23. Zou X, Tang G, Zhao X, Huang Y, Chen T, Lei M, et al. Simultaneous virus identification and characterization of severe unexplained pneumonia cases using a metagenomics sequencing technique. *Sci China Life Sci.* (2017) 60:279–86. doi: 10.1007/s11427-016-0244-8
24. Wang K, Li P, Lin Y, Chen H, Yang L, Li J, et al. Metagenomic diagnosis for a culture-negative sample from a patient with severe pneumonia by nanopore and next-generation sequencing. *Front Cell Infect Mi.* (2020) 10:182. doi: 10.3389/fcimb.2020.00182
25. Charalampous T, Kay GL, Richardson H, Aydin A, Baldan R, Jeanes C, et al. Nanopore metagenomics enables rapid clinical diagnosis of bacterial lower respiratory infection. *Nat Biotechnol.* (2019) 37:1–14. doi: 10.1038/s41587-019-0156-5
26. Li H, Gao H, Meng H, Wang Q, Li S, Chen H, et al. Detection of pulmonary infectious pathogens from lung biopsy tissues by metagenomic next-generation sequencing. *Front Cell Infect Mi.* (2018) 8:205. doi: 10.3389/fcimb.2018.00205
27. Qian Y-Y, Wang H-Y, Zhou Y, Zhang H-C, Zhu Y-M, Zhou X, et al. Improving pulmonary infection diagnosis with metagenomic next generation sequencing. *Front Cell Infect Mi.* (2021) 10:567615. doi: 10.3389/fcimb.2020.567615
28. Chiu CY. Viral pathogen discovery. *Curr Opin Microbiol.* (2013) 16:468–78. doi: 10.1016/j.mib.2013.05.001
29. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med.* (2020) 382:727–33. doi: 10.1056/NEJMoa2001017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Qin, Peng, Liu, Wu, Pan, Huang, Huang, Qiu, Du and the China Critical Care Clinical Trials Group (CCCCTG). This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 22 October 2021
doi: 10.3389/fmed.2021.675030

Case Report: Metagenomics Next-Generation Sequencing Can Be Performed for the Diagnosis of Disseminated Mucormycosis

Yi Sun[1,2], HuiLing Li[3], JiaJun Chen[4], ZhongHui Ma[2,5], Pin Han[1,2], YuChen Liu[1,2], Jing Wen[1,2], Fang Ren[1] and XiaoXu Ma[3]**

[1] Department of Gynecology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Gene Hospital of Henan Province, Precision Medicine Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Department of Respiratory, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] School of Public Health, Zhengzhou University, Zhengzhou, China, [5] Department of Stomatology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UCONN Health, United States

Reviewed by:
Yiyou Gu,
Lundquist Institute for Biomedical Innovation, United States
Adam Matson,
Connecticut Children's Medical Center, United States

***Correspondence:**
Fang Ren
renfang@foxmail.com
XiaoXu Ma
fccmaxx@zzu.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 02 March 2021
Accepted: 20 September 2021
Published: 22 October 2021

Citation:
Sun Y, Li H, Chen J, Ma Z, Han P, Liu Y, Wen J, Ren F and Ma X (2021) Case Report: Metagenomics Next-Generation Sequencing Can Be Performed for the Diagnosis of Disseminated Mucormycosis. Front. Med. 8:675030. doi: 10.3389/fmed.2021.675030

Mucormycosis is an infection caused by a group of filamentous molds with in the order Mucorales. In developing countries, most cases of mucormycosis occur in persons with poorly controlled diabetes mellitus or subjects with normal post-traumatic immune function. Mucormycosis exhibits a marked propensity for invading blood vessels. The mortality rate of invasive mucormycosis is very high (>30–50%), and 90% of mortality is related to disseminated diseases. We report a 62-year-old man with underlying diseases, such as diabetes and psoriatic arthritis, with a history of trauma before admission. Chest CT showed multiple cavities. Based on the suspected clinical manifestation of mucormycosis infection, the patient received a microbiological culture of bronchoalveolar lavage fluid, and metagenomics next generation sequencing (mNGS) was performed. The results suggested *Klebsiella pneumoniae* infection. However, *Rhizopus microsporus* strains were shown by the mNGS of transpulmonary puncture tissue. Therefore, we report a case in which rare pathogens are identified by mNGS.

Keywords: mucormycosis, diabetes, pulmonary cavity, alveolar lavage, metagenomics next generation

INTRODUCTION

Mucor is a rare and serious-conditioned pathogenic fungus, and can be found in the oral cavity and nasopharynx of normal people. However, in the presence of high-risk factors, such as diabetes, malignant tumors, agranulocytosis, AIDS, malnutrition, and the use of glucocorticoids (1), mucormycosis has become the third cause of invasive fungal infection after *Aspergillus* and *Candida* (2). The disease progresses rapidly, and the mortality is high after mucormycosis infection. However, its clinical manifestation is not specific, and results of laboratory tests are not specific either. Metagenomics next-generation sequencing (mNGS) refers to the direct extraction of nucleic acids of all microorganisms from clinical or environmental samples, construction of a metagenomic sequencing library, and sequencing. Through mNGS, some microbial species that cannot be cultured or difficult to be cultured are found in samples, and unknown and rare microbial species in complex samples are discovered (3). It has the advantages of high speed and high accuracy.

Herein, we report a case of *Rhizopus* infection with a variety of underlying diseases, such as diabetes and psoriatic arthritis. The clinical manifestations of the patient were not specific, and the underlying diseases were obvious. CT showed that the lung cavity was not specific. At first, *Klebsiella pneumoniae* was diagnosed and treated according to the results of bronchoalveolar lavage, but the effect was not satisfactory. Later, mNGS was performed to detect the lung tissue of the patient, and the presence of *R. microsporus* was quickly found, so the cause was finally determined.

CASE DESCRIPTION

We report a 62-year-old man with mucormycosis who suffered from chronic hepatitis B for 20 years. A month before admission, trauma caused chest pain, wounds on the right upper limb and right knee joint. Six days before admission, there was no obvious inducement for aggravation of joint pain and chest pain. Chest CT was performed locally and showed the shadow of the lungs. The local diagnosis was rheumatoid arthritis, type 2 diabetes, and hepatitis B. He received related treatment in a local hospital. However, his joint pain was not significantly relieved, while coughing and coughing dark red sputum occurred at the same time, so he went to the rheumatic immunology department of our hospital.

The vital signs were normal on admission. Physical examination showed that the posterior pharyngeal wall was red and swollen. The shoulder and knee joints were swollen. An irregular wound could be seen on the inside of the upper limb and knee joint. Relevant examinations and tests were improved after admission. Test results are shown in **Table 1**. Chest CT showed multiple cavities in the lower lobe of both lungs (**Figure 1**). Therefore, the patient was initially diagnosed with rheumatoid arthritis, pulmonary infection, and hepatitis B. He was given levofloxacin 0.5 g IV once daily (QD) for anti-infective treatment and metformin 50 mg orally (PO) two times daily to control blood sugar.

Three days after admission, the patient still had a severe cough with dark red sputum. Therefore, he was transferred to the respiratory department for treatment on the third day of admission. The physical examination showed that there was a small amount of wet rale in the left lower lung and a large ulcer in the right upper limb. On the first day after the transfer, a large amount of purulent secretions from the left main bronchus and a small amount of purulent secretions from the right main bronchus were observed by tracheoscopy. Bronchoalveolar lavage fluid (BALF) was collected and tested for microbial culture and mNGS. On the third day after transfer, the patient developed fever, and the highest temperature was 38.5°C, which returned to normal after physical cooling. The fever of the patient was thought to be due to infection. Microbial culture and mNGS of BALF showed *Klebsiella pneumoniae* infection (**Table 2**). On the fourth day after transfer, according to the results of drug sensitivity, the antibiotic was adjusted to piperacillin sodium 4.5 g IV every eight hours (Q8h). Low blood glucose was detected at this point, so he stopped using metformin. On the seventh day after transfer, a blood routine examination showed that white blood cells were 4.8×10^9/L and C-reactive protein (CRP) was 85.6 mg/L.

TABLE 1 | Test results after admission.

Inspection item	Actual value	Unit	Normal value
WBC	9.60	10^9/L	3.5–9.5
Neut%	84.0	%	40–75
Lymph%	6.50	%	20–50
PCT	0.40	ng/mL	Suggested antibiotics
CRP	104.83	mg/mL	104.83
(1–3)-β-D-glucan	<10.00	pg/mL	0–100
HumanGlactomannan	0.250	μg/mL	0–0.5
AspergillusGlactomannan	≤0.25	μg/mL	<0.85
Anti-CCP	1162.60	RU/mL	0–25
Anti-MCV	523.82	U/mL	0–30
RFIgG	1:100 (±)		(–)
RF IgM	196.4	IU/mL	0–30
AKA	(+)		(–)
APF	(+)		(–)

The 9th day after transfer to respiratory department recommended debridement treatment and sent it to mNGS. In the process of waiting for the results, the patient coughed intermittently with grayish-brown sputum. During this period, we continued the anti-infection treatment and maintained good blood glucose control, so we did not continue to use drugs for blood glucose. Finally, the result of mNGS submitted by debridement tissue indicated *R. microsporus* infection (**Table 2**). Combined with upper limb mNGS and imaging results, the possibility of lung complicated with *Mucor* infection is considered. Therefore, the amphotericin B antifungal therapy was added on the 11th day after transfer.

Thirteen days after being transferred to the respiratory department, the chest CT showed that the lung lesions were worse than before (**Figure 2**), and the empirical anti-infection treatment was poor. The diagnosis was further confirmed by CT-guided biopsy, and the punctured tissue was sent to pathology for mNGS detection. However, the patient suddenly developed massive hemoptysis on the day of the biopsy and died after the rescue was ineffective. The final pathology showed that a small amount of suppurative exudate with acute fibrinous tissue surrounding lung tissue causing pneumonia. The mNGS result returned as *R microsporus* infection (**Table 2**), which was consistent with the left upper limb wound test result. A brief summary of the patient's treatment is given (**Figure 3**).

DISCUSSION

The incidence of pulmonary mucormycosis reported in different countries is quite different. In developed countries such as those in Europe and America, the incidence rate is about 0.01/100,000–.2/100,000 (4–6), and the incidence rate in developing countries, such as India, is about 14/100,000 (7). Based on anatomic

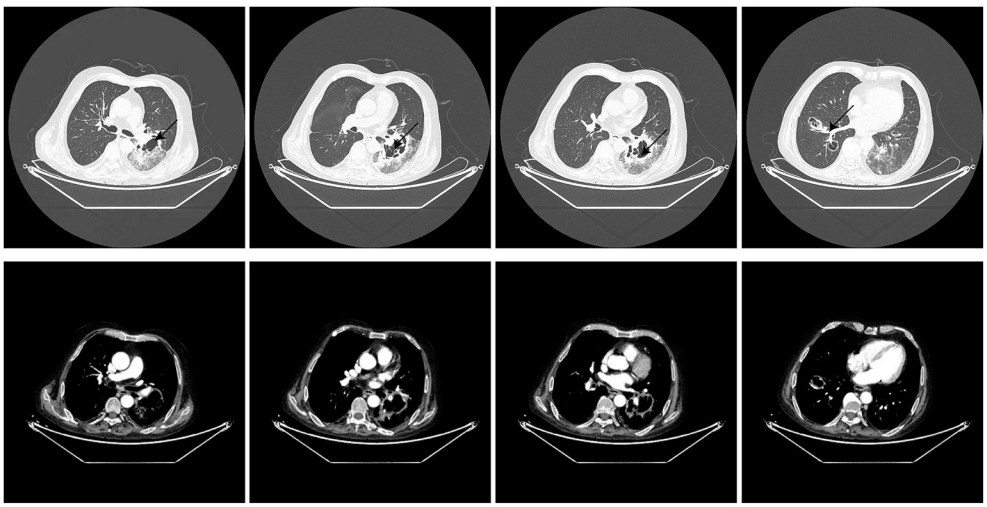


FIGURE 1 | Chest CT upon admission to the hospital.

TABLE 2 | Results of three metagenomics next-generation sequencing (mNGS).

Sampling tissue	Name	Sequence number	Name	Sequence number	Attention
BALF	Klebsiella	54,738	Klebsiella pneumoniae	41,019	High
Skin	Rhizopus	220	Rhizopus microsporus	197	High
Lung tissue	Rhizopus	56	Rhizopus microsporus	48	High

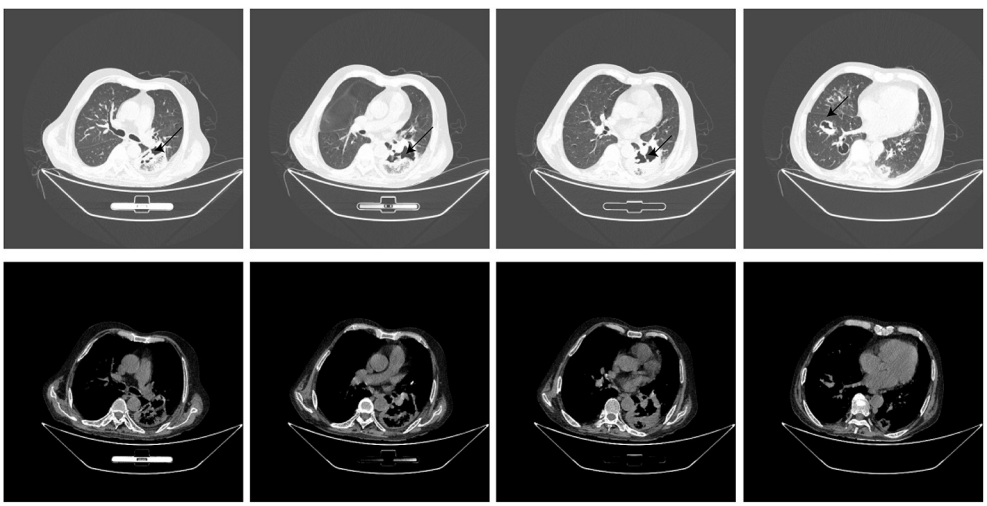


FIGURE 2 | Chest CT 10 days after transfer.

localization, mucormycosis can be classified as one of six forms: rhino-orbital-cerebral mucormycosis (ROCM), pulmonary, cutaneous, gastrointestinal (GI), disseminated, and mucormycosis of uncommon sites. Pulmonary mucormycosis is the second most common of the six types (8).

Patel et al. (9) reported that the mortality rate of mucormycosis, a serious fatal disease, was as high as 61%. Most patients have one or more risk factors, such as diabetes, silver scrap arthritis, and so on. In this case, the patient suffered from hepatitis B for 20 years and took an immunosuppressant regularly. At the same time, the patient also suffered from psoriasis arthritis, which was in a state of gradual aggravation in recent 1 year. More importantly, although the patient was diagnosed with type 2 diabetes after admission, according to the description of the patient, we suspected that he had been suffering from diabetes for many years. Therefore, the patient in this case also had the above several high-risk factors.

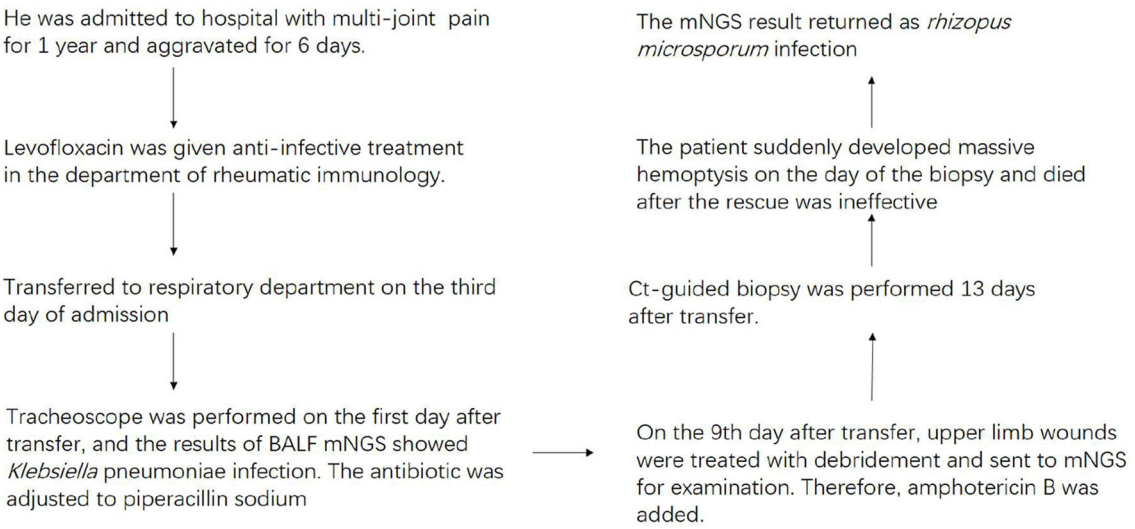


FIGURE 3 | Diagnosis and treatment of the patient.

In the process of diagnosis and treatment of the patient, we finally diagnosed him with pulmonary *Mucor* infection, *Klebsiella* pneumoniae infection, and skin *Mucor* infection. Although the pathogen was finally identified, the patient eventually died because of an untimely diagnosis. This is mainly because the mNGS test results of the alveolar lavage sent for inspection indicated *Klebsiella* pneumoniae infection. The most common pulmonary infections in diabetes are *Klebsiella* infection and *Mucor* infection (10–12). A typical CT of *Klebsiella pneumoniae* is characterized by large patches with blurred boundaries and locally protruding backward to form a typical "stalactite disease." Lesions caused by *Klebsiella pneumoniae* can form smooth cavities with smooth walls in a short period of time, and the diameter of cavities is rarely larger than 2 cm (13). Chest CT manifestations of pulmonary mucormycosis are non-specific. The common manifestations are multiple pulmonary nodules (>10), pleural effusion, pulmonary infarction, "anti-halo sign" (14). Eighty-seven percent of images of mucormycosis can appear "anti-halo sign" (15), but this sign is not characteristic.

Mucormycosis is an invasive fungal disease (10, 16). *Mucor* is characterized by its invasiveness of blood vessels, which leads to rapid and disseminated infection (8). It may be because of this characteristic of *Mucor* that the patient died of hemoptysis in a short time. Therefore, a timely diagnosis is extremely important for rapid therapeutic or surgical intervention and for maximizing the survival rate of patients. For *Mucor*, it has no specific serological index (17, 18). At present, the main methods for diagnosing mucormycosis are biopsy and microbial culture (19). However, microbial culture needs to provide appropriate culture conditions. It has the disadvantages of long culture time, complicated operation and low detection rate. More importantly, it even can delay the optimal treatment time. Routine histopathological examination is accurate but time-consuming.

In recent years, molecular biology techniques, mainly Polymerase Chain Reaction (PCR) and mNGS, have been widely used for the diagnosis of *Mucor*. PCR technology is developing rapidly. It has many outstanding advantages, such as specificity, simplicity, and so on (20). At the same time, it cannot be ignored that PCR also has certain limitations. For example, its specificity depends on the design of primers, and its sensitivity is prone to false-positive results after inspection contamination. However, the emergence of mNGS overcomes the shortcomings of the above methods. mNGS can detect almost all DNA or RNA information in a sample at the same time. It does not require pre-guessing or hypothesis about the type of pathogen causing the infection and overcomes the limitations of targeted diagnostic methods such as being time-consuming and labor-intensive, and slow detection process (21–24). However, mNGS also has some limitations. In the early stages of disease treatment, the mNGS of the early alveolar lavage fluid did not detect the *R. microsporus* infection. It may be because of the thick and hard hyphae of *Rhizopus*. It is difficult to lavage it from the lesion through alveolar lavage (25).

Mucor normally exists in environmental surroundings, which may cause the false positive diagnostic of mucormycosis. Therefore, we added a negative control in the test. And we have confirmed mucormycosis was exclusively detected in our samples mentioned in this manuscript and not detected in any other clinical samples and negative controls in the same run.

For the treatment of this patient, two points were very important. The control of blood sugar was the basis of the treatment of this patient, and we did it well. The second was the treatment of *Mucor*. For this patient, although we added amphotericin B in time after the skin tissue suggested *Mucor* infection, we did not give an adequate dose. The dosage of amphotericin B is 0.75–1 mg kg^{-1}·day^{-1}. The initial dose of amphotericin B liposome was 1–5 mg/day, and the dose of amphotericin B liposome was 3–5 mg kg^{-1}·day^{-1}. At present, posaconazole and esaconazole are also commonly used in clinics (26). The patient eventually died of massive hemoptysis, which

has something to do with our untimely treatment. Therefore, for the treatment of *Mucor*, we should be timely and accurate.

CONCLUSION

Mucormycosis has the characteristics of rapid progress, high mortality, and difficult diagnosis. Therefore, if *Mucor* infection is considered in a clinic, appropriate samples can be taken as soon as possible and submitted for mNGS for a definite diagnosis of pathogenic microorganisms and accurate treatment. The emergence of this extended-spectrum pathogen screening technology broadens the idea of traditional pathogen detection and provides a promising broad-spectrum screening method for solving difficult problems such as clinical unknown infection.

DATA AVAILABILITY STATEMENT

Metagenomics whole genome shotgun sequences have been uploaded to EMBL accession: PRJEB41159.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YS was involved in analyzing data, analyzing diagnosis and treatment, reading literature, and writing manuscripts. XM was involved in diagnosis and treatment, selecting imaging images, and directing the writing of manuscripts. FR directed the writing of the manuscript. HL and JC participate in the diagnosis and treatment of the disease and improve the cases. ZM, PH, YL, and JW help to obtain clinical data and analyze clinical data. All the authors have read and approved the final manuscript.

FUNDING

This research was equally funded and supported by Chinese National Science and Technology Major Project 2018ZX10305410, Henan Province Medical Science and Technique Project grant 2018020001, and Henan province postdoctoral research grant 001801005.

REFERENCES

1. Riley T, Muzny C, Swiatlo E, Legendre D. Breaking the mold: a review of mucormycosis and current pharmacological treatment options. *Ann Pharmacother.* (2016) 50:747–57. doi: 10.1177/1060028016655425
2. Eucker J, Sezer O, Graf B, Possinger K. Mucormycoses. *Mycoses.* (2001) 44:253–60. doi: 10.1111/j.1439-0507.2001.00656.x
3. Gu W, Miller S, Chiu C. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol.* (2019) 14:319–38. doi: 10.1146/annurev-pathmechdis-012418-012751
4. Bitar D, Van Cauteren D, Lanternier F, Dannaoui E, Che D, Dromer F, et al. Increasing incidence of zygomycosis (mucormycosis), France, 1997-2006. *Emerg Infect Dis.* (2009) 15:1395–401. doi: 10.3201/eid1509.090334
5. Ruhnke M, Groll A, Mayser P, Ullmann A, Mendling W, Hof H, et al. Estimated burden of fungal infections in Germany. *Mycoses.* (2015) 22–8. doi: 10.1111/myc.12392
6. Rees J, Pinner R, Hajjeh R, Brandt M, Reingold A. The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992-1993: results of population-based laboratory active surveillance. *Clin Infect Dis.* (1998) 27:1138–47. doi: 10.1093/clinids/27.5.1138
7. Chakrabarti A, Singh R. Mucormycosis in India: unique features. *Mycoses.* (2014) 57:85–90. doi: 10.1111/myc.12243
8. Muggeo P, Calore E, Decembrino N, Frenos S, De Leonardis F, Colombini A, et al. Invasive mucormycosis in children with cancer: a retrospective study from the Infection Working Group of Italian Pediatric Hematology Oncology Association. *Mycoses.* (2019) 62:165–70. doi: 10.1111/myc.12862
9. Patel A, Kaur H, Xess I, Michael J, Savio J, Rudramurthy S, et al. A multicentre observational study on the epidemiology, risk factors, management and outcomes of mucormycosis in India. *Clin Microbiol Infect.* (2020) 26:944.e9–15. doi: 10.1016/j.cmi.2019.11.021
10. Jeong W, Keighley C, Wolfe R, Lee W, Slavin M, Kong D, et al. The epidemiology and clinical manifestations of mucormycosis: a systematic review and meta-analysis of case reports. *Clin Microbiol Infect.* (2019) 25:26–34. doi: 10.1016/j.cmi.2018.07.011
11. Prakash H, Ghosh A, Rudramurthy S, Singh P, Xess I, Savio J, et al. A prospective multicenter study on mucormycosis in India: epidemiology, diagnosis, and treatment. *Med Mycol.* (2019) 57:395–402. doi: 10.1093/mmy/myy060
12. Soleimanpour S, Gupta A, Bakay M, Ferrari A, Groff D, Fadista J, et al. The diabetes susceptibility gene Clec16a regulates mitophagy. *Cell.* (2014) 157:1577–90. doi: 10.1016/j.cell.2014.05.016
13. Okada F, Ando Y, Honda K, Nakayama T, Kiyonaga M, Ono A, et al. Clinical and pulmonary thin-section CT findings in acute Klebsiella pneumoniae pneumonia. *Euro Radiol.* (2009) 19:809–15. doi: 10.1007/s00330-008-1233-1
14. Skiada A, Lass-Floerl C, Klimko N, Ibrahim A, Roilides E, Petrikkos G. Challenges in the diagnosis and treatment of mucormycosis. *Med Mycol.* (2018) 56:93–101. doi: 10.1093/mmy/myx101
15. Bourcier J, Heudes P, Morio F, Gastinne T, Chevallier P, Rialland-Battisti F, et al. Prevalence of the reversed halo sign in neutropenic patients compared with non-neutropenic patients: data from a single-centre study involving 27 patients with pulmonary mucormycosis (2003-2016). *Mycoses.* (2017) 60:526–33. doi: 10.1111/myc.12624
16. Chitasombat M, Niparuck P. Deferiprone as adjunctive treatment for patients with invasive mucormycosis: a retrospective case series. *Infect Dis Rep.* (2018) 10:7765. doi: 10.4081/idr.2018.7765
17. Pyrgos V, Shoham S, Walsh T. Pulmonary zygomycosis. *Semin Respir Crit Care Med.* (2008) 29:111–20. doi: 10.1055/s-2008-1063850
18. Lee J, Hyun J, Kang D, Lee H, Park S. Rare complication of bronchoesophageal fistula due to pulmonary mucormycosis after induction chemotherapy for acute myeloid leukemia: a case report. *J Med Case Rep.* (2016) 10:195. doi: 10.1186/s13256-016-0991-7
19. Hamilos G, Samonis G, Kontoyiannis D. Pulmonary mucormycosis. *Semin Respir Crit Care Med.* (2011) 32:693–702. doi: 10.1055/s-0031-1295717
20. Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids Res.* (2001) 29:e45. doi: 10.1093/nar/29.9.e45
21. Forbes J, Knox N, Peterson C, Reimer A. Highlighting clinical metagenomics for enhanced diagnostic decision-making: a step towards wider implementation. *Comput Struct Biotechnol J.* (2018) 16:108–20. doi: 10.1016/j.csbj.2018.02.006
22. Chiu C, Miller S. Clinical metagenomics. *Nat Rev Genet.* (2019) 20:341–55. doi: 10.1038/s41576-019-0113-7

23. Naccache S, Federman S, Veeraraghavan N, Zaharia M, Lee D, Samayoa E, et al. A cloud-compatible bioinformatics pipeline for ultrarapid pathogen identification from next-generation sequencing of clinical samples. *Genome Res.* (2014) 24:1180–92. doi: 10.1101/gr.171934.113
24. An J, Liao X, Xiao T, Qian S, Yuan J, Ye H, et al. Clinical characteristics of recovered COVID-19 patients with re-detectable positive RNA test. *Ann Transl Med.* (2020) 8:1084. doi: 10.21037/atm-20-5602
25. Tsyrkunou A, Ellison R, Akalin A, Wiederhold N, Sutton D, Lindner J, et al. Multifocal Rhizopus microsporus lung infection following brush clearing. *Med Mycol Case Rep.* (2014) 6:14–7. doi: 10.1016/j.mmcr.2014.08.001
26. Cornely O, Alastruey-Izquierdo A, Arenz D, Chen S, Dannaoui E, Hochhegger B, et al. Global guideline for the diagnosis and management of mucormycosis: an initiative of the European Confederation of Medical Mycology in cooperation with the Mycoses Study Group Education and Research Consortium. *Lancet Infect Dis.* (2019) 19:e405–21. doi: 10.1016/S1473-3099(19)30312-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Sun, Li, Chen, Ma, Han, Liu, Wen, Ren and Ma. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 22 October 2021
doi: 10.3389/fmed.2021.656066

Case Report: A *Candida* Meningitis in an Immunocompetent Patient Detected Through the Next-Generation Sequencing

Xiao-guang Cao[1†], Chuang-wei Yu[2†], Shu-sheng Zhou[1], Yu Huang[1] and Chun-yan Wang[1]*

[1] Department of Emergency Intensive Care Unit (EICU), The First Affiliated Hospital of University of Science and Technology of China (Anhui Provincial Hospital), Hefei, China, [2] Department of Emergency Intensive Care Unit (EICU), TaiHe County People's Hospital, Fuyang, China

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Dhuha Alajmi,
Ministry of Health, Kuwait
Payam Behzadi,
Islamic Azad University, Iran
Lan Yan,
Second Military Medical University, China

***Correspondence:**
Chun-yan Wang
615916339@qq.com

[†] *These authors share first authorship*

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *03 February 2021*
Accepted: *20 September 2021*
Published: *22 October 2021*

Citation:
Cao X-g, Yu C-w, Zhou S-s, Huang Y and Wang C-y (2021) Case Report: A Candida Meningitis in an Immunocompetent Patient Detected Through the Next-Generation Sequencing. Front. Med. 8:656066. doi: 10.3389/fmed.2021.656066

Background: Fungal infections of the central nervous system (CNS) are not commonly seen clinically. Clinical diagnosis of fungal infections often depend on the pathogen culture and the clinical features. This method is time-consuming and insensitive, which can lead to misdiagnosis. The authors introduce an adult patient with fungal infections diagnosed by next-generation sequencing (NGS).

Case: The patient was a 60-year-old male Chinese who had both hypermyotonia of the lower extremities and fever. The auxiliary examinations such as MRI, CT, and cerebrospinal fluid (CSF) analysis showed obvious abnormalities. Because of the difficulties in diagnosis, it was hard to determine the treatment plan. The NGS detected specific sequences of *Candida albicans* in 3 days. The patient was then treated with liposomal amphotericin B and fluconazole. About 3 weeks later, the symptoms of the patient improved significantly and he was discharged from the hospital.

Conclusion: Compared with the routine cultural method, NGS has made a huge advancement in infection diagnosis and targeting antimicrobial therapy for CNS infection.

Keywords: next-generation sequencing, central nervous system infections, *Candida albicans*, fungal infections, antimicrobial therapy

INTRODUCTION

Fungal infections include *Candida* infection, cryptococcal infection, *Pneumocystis carinii* infection. (1). It is well-known that fungal infections are serious opportunistic infectious diseases, although the proportion of patients with fungal infections is not high. Moreover, based on the current epidemiological survey (2), the prevalence of fungal infections is increasing every year. There are at least 15 distinct *Candida* species that cause human disease, but >90% of the invasive disease are caused by the five most common pathogens such as *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (3).

Recently, invasive fungal infections (IFIs) have gained importance in public health due to the increasing number of patients with immunocompromised who are at risk for the infections caused by these opportunistic pathogens (4), especially the fungal infections of the central nervous system (CNS) (5).

Because most of these cases of fungal CNS infection are chronic and develop in patients with an altered immune response, it is difficult for clinicians to diagnose early from the clinical manifestation. The development of fungal CNS infections also mainly depends on the interplay between the health status and fungal virulence factors of the patients.

As we know, in clinical cases, the most common fungal pathogens include *Cryptococcus* spp., *Aspergillus* spp., *Histoplasma capsulatum*, and *Candida albicans*. *Candida* meningitis is the most common fungal pathogen in CNS in clinical cases (6).

Although these diseases are relatively rare, these lethal infections in the CNS give rise to significant morbidity and mortality. Meanwhile, the diagnosis of the fungal infection is often difficult and the treatment is often delayed. There have been catastrophic results because of the misdiagnosis and difficulty in the early diagnosis (5, 6). Patients with cerebrovascular disease are more likely to suffer from the fungal infection of the CNS. The incidence was related to invasive operations, broad-spectrum antibiotics abuse, and the immune system and immunodepression of the patients.

In this case, the pathogens were not found by the cerebrospinal fluid (CSF) and blood culture test. At the same time, we could not get any help from MRI and CT. The treatment of the patients is in a dilemma. Then, the next-generation sequencing (NGS) method was used to detect the pathogen and the result showed that the pathogen was *Candida*.

CASE

A 60-year-old male had a history of fever for half-month (January 1, 2019), diarrhea, 1-week history of somnolence, and extremity of hypertonia. Apart from the past medical history of chronic bronchitis and surgery of hernia, we did not get any meaningful history from his family.

When the symptoms began, he had a fever and the highest body temperature of the patient was 39°C. On the next day, he began to have incontinence and diarrhea. The patient was then sent to the Huainan Xinhua Hospital. The CT of the chest showed that he had pulmonary inflammation; the patient was given the treatment of anti-infection, but the symptoms were not alleviated. About 4 days later, the patient was transferred to the Huainan First People's Hospital and he required the serial lumbar punctures as his intracranial pressure raised persistently. The CSF test showed the abnormal results that CSF protein level and pressure were higher than usual (family members could not provide the accurate results and anti-infective agents). Meanwhile, the body temperature of the patient still fluctuated between 37.5 and 38.1°C. By using the CSF culture and virus antibodies, no pathogen was detected, so the patient continued to receive empiric treatment for viral meningitis and supportive care. Then, the patient developed the symptoms of cough and phlegm, with paroxysmal fever as high as 38.1°C. Because the symptoms of the patients did not improve, he was transferred to the Anhui Provincial Hospital. On arrival, the patient was afebrile and had a blood pressure of 127/80 mm Hg and a heart rate of 72 beats/min. The physical examination showed that he had neck stiffness and hypermyotonia. He had no focal motor or sensor deficits and his nerve examination was normal.

TABLE 1 | Cerebrospinal fluid test.

Open Pressure		80 mmHg
Colors		Transparent
Cell Count	Karyocyte Cell	10 × 10^6/L
	Red Blood Cell	0
	Mononuclear Cell	100%
	Multinucleated Cell	0%
Glucose		2.48 mmol/L
Protein		0.66 g/L
Chloride		115.2 mmol/L
Ig-G		54.6 mg/L
Ig-A		9.50 mg/L
Ig-M		1.31 mg/L

As a result of these data and information, the doctors considered that the patient had viral encephalitis and he was treated with gammopathy, methylprednisolone, and anti-virus therapy. Viral antibodies and β-1,3 glucanase were used to speculate on the type of pathogen again. Combined with the previous examination results of the patients in the Huainan and the current examination results, we did not get the valuable results to identify the pathogens. Therefore, the patient continued to receive empiric treatment for the viral encephalitis and supportive care, but the symptoms were not alleviated. Due to the tetanus antibody that is probable positive, the patient was transferred to the infectious department (January 13, 2019). According to the clinical symptoms, the doctors did not consider the possibility of tetanus. Then, the patient again returned to the neurology department. When the patient arrived, he showed unconsciousness and his oxygen saturation of blood decreased. For further treatment, the patient was sent to the intensive care unit (ICU) (January 15, 2019). Subsequently, the lumbar puncture was performed again, showing an open pressure of 80 mm Hg. The CSF test showed glucose of 2.48 mmol/l, protein of 0.66 g/l, karyocyte count of 10^{10}∼6/L, and chloride of 115.2 mmol/l (**Table 1**). Repetitive serology and CSF studies for the possible organisms continued to be negative. Gram staining and India ink staining did not show any organisms. CSF culture was repeated. While MRI of the brain revealed the abnormal enhancement of meningitis and hemangioma, we did not get any valuable information from MRI.

During this period, the C-reactive protein (CRP) of the patient, mental state, and body temperature gradually decreased after the antibacterial treatment. His sample was sent for pathogen detection by the NGS at the Beijing Genomics Institute (BGI), Wuhan, China. CSF sample from the patient was analyzed by using the BGI PMSeq high-throughput sequencing process for the detection of pathogenic microorganisms. DNA from the CSF sample was extracted by using the TIANamp Micro DNA Kit (Beijing, China). Then, 100 ng of extracted

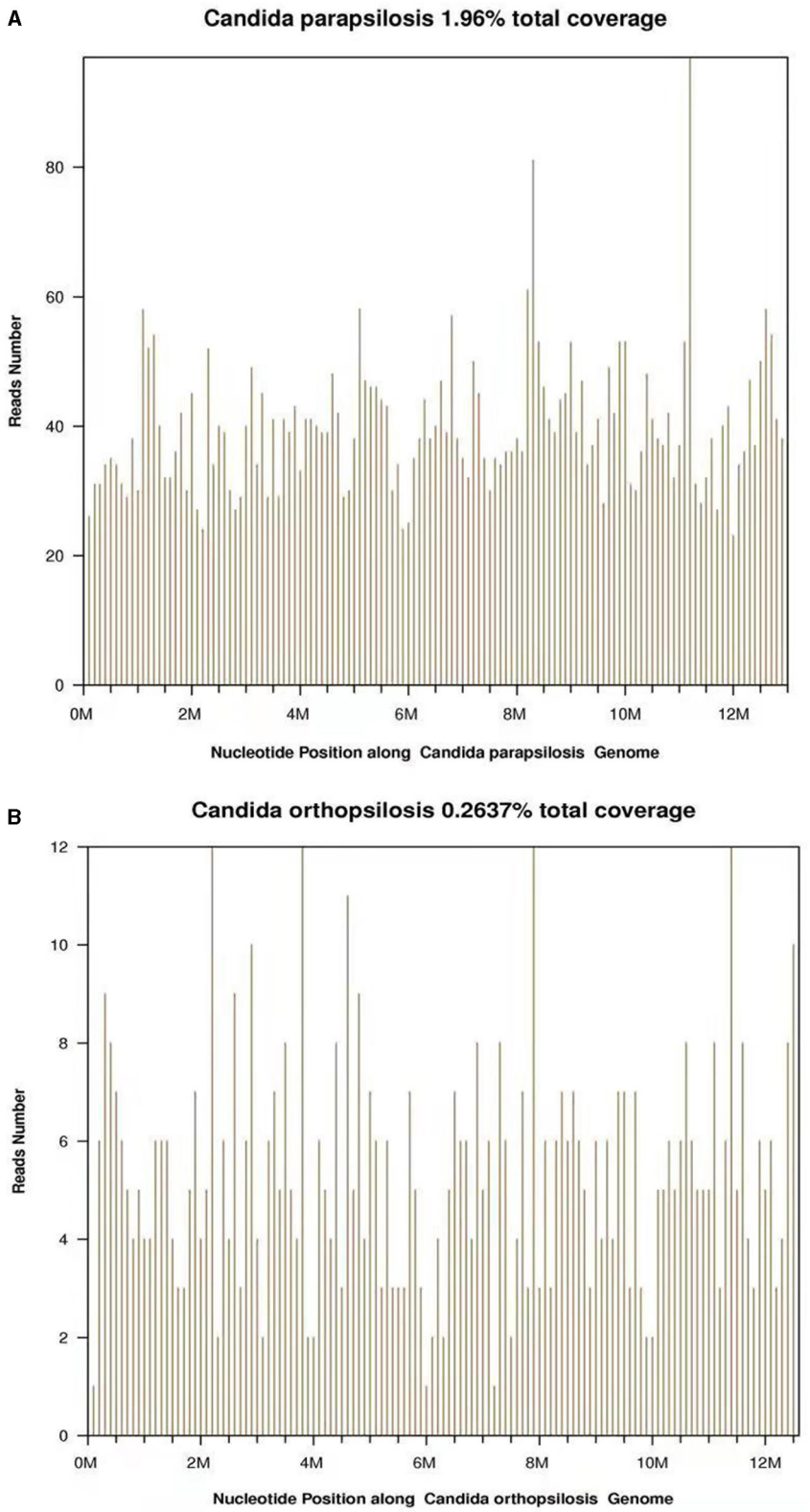


FIGURE 1 | (A) A total of 5,212 reads were mapped to the *Candida parapsilosis* in the reference database. The coverage of the referenced *Candida parapsilosis* genome was 1.96%. **(B)** A total of 675 reads were mapped to the *Candida orthopsilosis* in the reference database. The coverage of the referenced *Candida orthopsilosis* genome was 0.2637%.

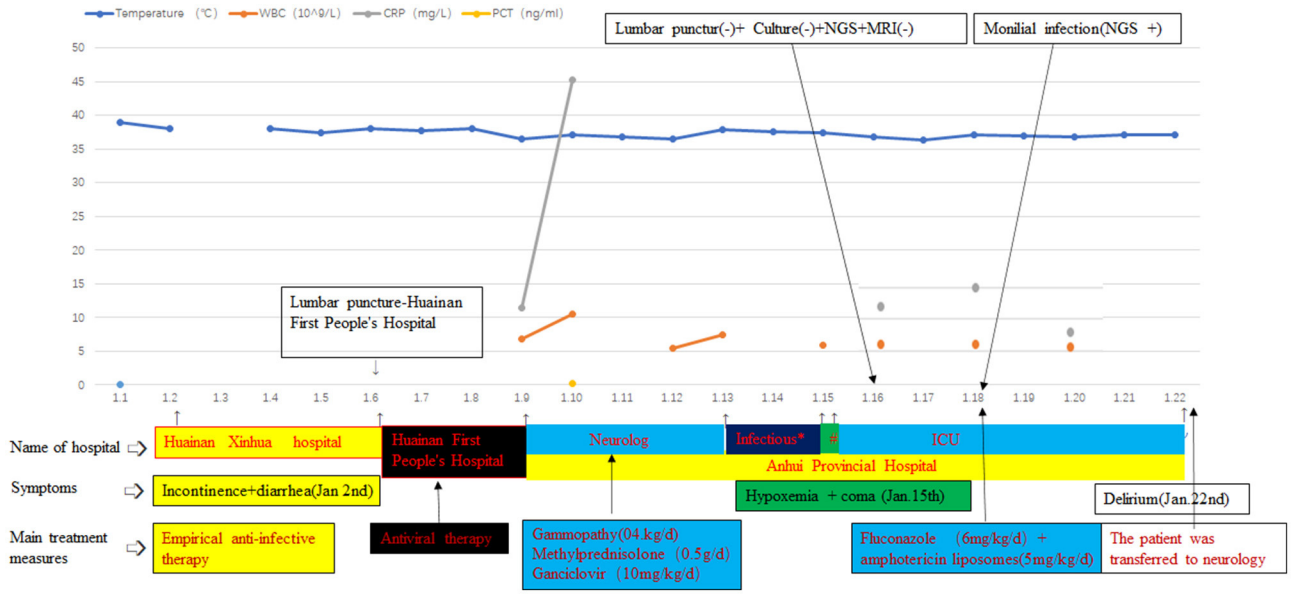


FIGURE 2 | Annotation: "*" indicated the department of infection; "#" indicated that the patient was treated in the neurology department in the afternoon and he was transferred to the intensive care unit (ICU) in the evening; "–" means negative; and "+" means positive. The beginning and end of each color bar indicated the treatment measures during this period.

DNA were subjected to the library building steps including fragmentation, end repair, and sequencing adapter ligation. Then, the DNA library was sequenced on the BGISEQ-500 sequencing platform at the BGI, Wuhan, China. A total of 3,310,880 single-end reads were generated. Then, the reads were processed by using the BGI metagenome analysis platform. After filtering of the human genome sequences, the rest sequences were mapped to a metagenome database including 2,328 bacteria, 199 fungi, 4,189 viruses, and 135 parasites from the National Center for Biotechnology Information (NCBI). Among these reads, 30,451 were mapped to *Candida parapsilosis* (**Figure 1A**) and 2,857 were mapped to *Candida orthopsilosis* (**Figure 1B**), all these are fungal species. The other reads were mapped to the common environmental microbes or laboratory contaminants.

Due to this result, there was a debate among the doctors. Doctors were divided into two groups. One group thought that the foundation of the fungal infection was not sufficient at the current stage and the epidemiological investigation in China showed that the incidence of *Candida* infection in the CNS was low, so they thought that the possibility of contamination of the specimen cannot be ruled out. Because antithyroglobulin antibodies in the patient were positive, combined with thyroid insufficiency and MRI, they considered the possibility of Hashimoto's encephalitis, and the patient should be treated with the hormones. For these reasons, they believed that antifungal therapy was not essential. In the opinion of the other group, although there was no other evidence of the pathogens at present and some clinical symptoms of the patient improved, his muscle tension was still high. Meanwhile, there was no better detection method in our hospital. According to the Infectious Diseases Society of America (IDSA) guidelines (recommendations 28, 32, 33, and 94) (3), the change of the clinical symptoms is the most important basis for the clinicians without basis. Furthermore, the empirical treatment of adults with encephalitis is with drugs within 6 h of suspicion (7), and delay in the diagnosis and inappropriate therapy would result in permanent damage, even also death. It was an unacceptable outcome. Owing to the poor therapeutic effect and symptoms, diagnostic treatment can be initiated and the neurologists also agreed with the treatment. Then, the patient finally received the antifungal treatment with fluconazole combined with the amphotericin liposomes (the dose of fluconazole on the first day was 12, 6 mg/kg/day; amphotericin liposomes: 5 mg/kg/day) (3) and the neurologists and the family of the patient agreed with this treatment. The clinicians monitored the side effects of this treatment regimen such as hypokalemia and renal impairment. Fortunately, after 4 days of treatment, there was a significant improvement in the muscle tone without the side effects. Because of the enclosed environment of the ICU, the patient developed some mental symptoms (restlessness and delirium appeared intermittently). Because the family members are not allowed to accompany in the ICU, the patient was transferred to the neurology unit for further treatment. Doctors hope to treat delirium through the company and psychological comfort of the families of the patient (the hospitalization process is shown in **Figure 2**).

DISCUSSION

In the previous studies, the fungal infections of the CNS were rare clinical cases, but candidemia cases have increased dramatically worldwide (8, 9). Fungal infections can

develop *via* hematogenous dissemination from a distant focus such as central venous catheter and lung (10). The symptoms of *Candida* meningitis are often similar to those in patients with bacterial meningitis such as fever (hyperthermia), headache, neurological symptoms, and meningeal irritation (11).

According to the results of the current research (7), a high index of suspicion is needed in any patient with risk factors such as abdominal surgery, bowel perforation, recent broad-spectrum antibiotic therapy, intravenous drug use, advanced in years, indwelling catheters, low immune function, severe burns, antineoplastic therapy, and long-term glucocorticoid usage (12, 13). The development of fungal infections largely depend on the interplay between the health status of the patients and the virulence of pathogenic fungal species. In many cases, there is no specificity in the clinical symptoms. As we know, fungal meningitis is a common complication of immunosuppression, while *Candida albicans* is the most common pathogen (14, 15). Overall, *albicans* were the most common strains followed by *Candida glabrata* in the clinical cases, accounting for about half of the total cases, but the source of candidemia was unknown in half of all the episodes (16).

In this case, the patient lacked any known risk factors, and the reason for the infection needed further study. According to the current diagnosis, fungal infection can be isolated from blood and CSF, which is the gold standard of the clinical diagnosis. However, the positive rate in the blood cultures and CSF is too low for the clinician. Even if the etiological culture is positive, it will take 3–5 days at the fastest. With respect to the timing of initiation of empirical treatment, some studies are still controversial, with some showing that mortality was lower in the patients who received the antifungal early (7, 17), while other studies showed that there was no significant difference in in-hospital mortality (18, 19).

With most of the research results, the prompt initiation of the antifungal therapy was essential for better outcomes. But in any case, timely and effective treatment with the appropriate anti-infective drugs is the most crucial factor for the best prognosis (20). Providing effective treatment for the patients is the most crying need for the patients and doctors. Meanwhile, if we can get accurate results earlier, the therapeutic efficacy will be better and fewer irrational antifungal drugs will be used. However, in most of the cases, the clinicians did not have adequate laboratory tests at the beginning; they can only judge the condition according to the guidelines, expert consensus, and clinical experience, so as to minimize the possibility of missed diagnosis, misdiagnosis, and overdiagnosis and treatment (3, 7).

The incorrect usage of the antimicrobials will cause the resistance of the pathogen, treatment difficulty, and the tensional doctor-patient relationship, especially in China (21). Fungal cultures of CSF have a bad sensitivity and this method is rarely helpful. Due to the poor sensitivity of the cultures, the diagnosis of fungal infection is very tricky (22). In addition, unlike the other infection, there is not a widely accepted molecular-, antigen-, or nucleic acid-based testing method to expedite the identification of the organism in the medical units, thus delaying the proper treatment (23). Now, diagnoses of these diseases are still tricky because there lacked a fast and reliable means of detection. Delay in the diagnosis and inappropriate therapy can result in permanent damage, even death. It was an unacceptable outcome.

Clinical doctors need another simple and effective method to isolate and detect the pathogen. In the current practice, culture-based methods are still considered as the standard approach of etiological diagnosis of the infection. However, these methods have disadvantages including long turnaround time and practically low sensitivity. Although molecular assays such as PCR-based methods have been established for the pathogen, the detection is limited to the known pathogens listed on the panel.

The NGS is a new laboratory detection technology that directly extracts the nucleic acids from all the pathogens in the samples of the patient for high-throughput sequencing; the NGS is able to detect the thousands of fragments in a laboratory test, therefore this technology can reduce the number of tests and period of detection (24). In the past few years in China, NGS is an emerging innovative technology, which has become highly sensitive in the detection of pathogens (24). NGS has been constantly improving and stepped into clinical practice, providing a powerful tool with advantages including short turnaround time, full-spectrum, and semi-quantitative analysis. In comparison to the routine cultural method, NGS has made a huge advancement in infection diagnosis. NGS had become a new and innovative technology and is gradually applied in clinical practice. In this case, the NGS results showed that pathogens were *Candida parapsilosis* and *Candida orthopsilosis*. They are the different genotypes of *Candida* (25), but only *Candida parapsilosis* is stated in this clinical guideline (3). This also proved the advantages of NGS (19).

The antimicrobial resistance profile of *Candida* in China showed that the isolates were sensitive to the liposomal amphotericin B and fluconazole. This is consistent with the IDSA guidelines (5) and this case indirectly proved the sensitivity and specificity of NGS.

The limitations included few acknowledged criteria of the sequencing result explanation which have been established (26); large-scale study comparing NGS with traditional etiological diagnosis methods which are missing, the sample size of most of the studies is small (27); this study is just a case report, thus, it still needs high-level evidence to support its clinical application; and this method is very sensitive, so strict aseptic operation is required in the detection process, else it is easy to lead to the clinical misdiagnosis (28).

CONCLUSION

Next-generation sequencing technology is a new method of diagnosis. Based on the unique advantages of the NGS technology in the diagnosis of these infectious diseases, this new technology will gradually be applied in clinical practice to benefit a large number of patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: Genome Sequence Archive [BioProject: PRJCA006605/CRP003428; https://bigd.big.ac.cn/gsa/browse/CRA005014].

AUTHOR CONTRIBUTIONS

CY conceived the idea of this article. XC and CW have made the same contributions to the article and they contributed to the preparation of the manuscript and interpreting the data of the patients. All the authors contributed to review, edit, read, and approved the final manuscript.

REFERENCES

1. Donnelly JP, Chen SC, Kauffman CA, Steinbach WJ, Baddley JW, Verweij PE, et al. Revision and update of the consensus definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium. *Clin Infect Dis*. (2019) 6:1367–76. doi: 10.1093/cid/ciz1008
2. Bassetti M, Garnacho-Montero J, Calandra T, Elie A, Arunaloke C, Daniel K, et al. Intensive care medicine research agenda on invasive fungal infection in critically ill patients. *Intensive Care Med.* (2017) 43:1225–38. doi: 10.1007/s00134-017-4731-2
3. Pappas PG, Kauffman CA, Andes DR. Clinical practice guideline for the management of candidiasis: 2016 update by the infectious Diseases Society of America. *J Clin Infect Dis.* (2016) 62:e1–50. doi: 10.1093/cid/civ933
4. Goemaere B, Becker P, Wijngaerden EV, Maertens J, Spriet I, Hendrickx M, et al. Increasing candidemia incidence from 2004 to 2015 with a shift in epidemiology in patients pre-exposed to antifungals. *Mycoses.* (2017) 61:127–33. doi: 10.1111/myc.12714
5. Góralska K, Blaszkowska J, Dzikowiec M. Neuroinfections caused by fungi. *Infection.* (2018) 46:443–59. doi: 10.1007/s15010-018-1152-2
6. Bridges KJ, Li R, Fleseriu M, Cetas JS. Candida meningitis after transsphenoidal surgery: a single-institution case series and literature review. *World Neurosurg.* (2017) 108:41–9. doi: 10.1016/j.wneu.2017.08.115
7. Stoeter DJ, Michael BD, Solomon T, Poole L. Managing acute central nervous system infections in the UK adult intensive care unit in the wake of UK encephalitis guidelines. *J Intensive Care Soc*. (2015) 16:330–8. doi: 10.1177/1751143715587927
8. Camargo T, Marra AR, Silva CV, Cardoso MF, Martino MD, Camargo LF, et al. Secular trends of candidemia in a tertiary care hospital. *Am J Infect Control.* (2010) 38:546–51. doi: 10.1016/j.ajic.2009.12.012
9. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis.* (2004) 39:309–17. doi: 10.1086/421946
10. Mohan S, Jain KK, Arabi M, Shah GV. Imaging of meningitis and ventriculitis. *Neuroimaging Clin N Am.* (2012) 22:557–83. doi: 10.1016/j.nic.2012.04.003
11. de Gans J, van de Beek D. Dexamethasone in adults with bacterial meningitis. *Neuroimaging Clin N Am.* (2012) 22:557–83. doi: 10.1016/j.nic.2012.4.003.
12. Guillermo Q. Epidemiology of candidaemia and invasive candidiasis. A changing face. *Rev Iberoam Micol.* (2014) 31:42–8. doi: 10.1016/j.riam.2013.10.001
13. Barter DM, Johnston HL, Williams SR, Tsay SV, Vallabhaneni S, Bamberg WM. Candida bloodstream infections among persons who inject drugs - Denver metropolitan area, Colorado, 2017-2018. *MMWR Morb Mortal Wkly Rep.* (2019) 68:285–88. doi: 10.15585/mmwr.mm6812a3
14. Miranda EJ, Gonçalves LG, Oscar DS. Cryptococcal meningitis in HIV-negative patient with liver cirrhosis due to hepatitis C. *Braz J Infect Dis.* (2011) 15:399–00. doi: 10.1590/S1413-86702011000400019
15. Tan BH, Chakrabarti A, Li RY, Watcharananan SP, Liu Z, Chindamporn A, et al. Incidence and species distribution of candidaemia in Asia: a laboratorybased surveillance study. *Clin Microbiol Infect.* (2015) 21:946–53. doi: 10.1016/j.cmi.2015.06.010
16. Yeşilkaya A, Azap Ö, Aydin M, Mehtap AO. Epidemiology, species distribution, clinical characteristics and mortality of candidaemia in a tertiary care university hospital in Turkey, 2007-2014. *Mycoses.* (2017) 60:433–9. doi: 10.1111/myc.12618
17. Abbas H, Cifuentes KA, Abbas N, Cindrich R, Singh M. A rare presentation of Cryptococcal meningitis and cerebellitis in an asplenic patient, seronegative for Human Immunodeficiency Virus (HIV). *Am J Case Rep.* (2018) 19:183–86. doi: 10.12659/AJCR.906869
18. Bassetti M, Righi E, Ansaldi F, Rocca DG, Antonelli M, Tumbarello M, et al. A multicenter study of septic shock due to candidemia: outcomes and predictors of mortality. *Intensive Care Med.* (2014) 40:839–45. doi: 10.1007/s00134-014-3310-z
19. Kludze-Forson M, Eschenauer GA, Kubin CJ, Della-Latta P, Simon WL. The impact of delaying the initiation of appropriate antifungal treatment for Candida bloodstream infection. *Med Mycol.* (2010) 48:436–39. doi: 10.3109/13693780903208256
20. Ryuichi H, Yuichi S, Junichi K, Yamamoto S, Kayaba H. Epidemiology, practice patterns, and prognostic factors for candidemia; and characteristics of fourteen patients with breakthrough *Candida* bloodstream infections: a single tertiary hospital experience in Japan. *Infect Drug Resist.* (2018) 31:821–33. doi: 10.2147/IDR.S156633
21. Wei P, Gan D, Qi T, Xu LZ. Continuing violence against medical personnel in China: a flagrant violation of Chinese law. *Biosci Trends.* (2016) 19:240–43. doi: 10.5582/bst.2016.01094
22. Black KE, Baden LR. Fungal infections of the CNS: treatment strategies for the immunocompromised patient. *CNS Drugs.* (2007) 21:293–18. doi: 10.2165/00023210-200721040-00004
23. Li L, Wang L, Zhang C, Chen P, Luo X. A case of *Vibrio vulnificus* related wound infection diagnosed by next-generation sequencing. *ID Cases.* (2019) 24:e00497. doi: 10.1016/j.idcr.2019.e00497
24. Alekseyev YO, Fazeli R, Yang S, Basran R, Miller NS, Remick D, et al. A next-generation sequencing primer—how does it work and what can it do? *Acad Pathol.* (2018) 5:2374289518766521. doi: 10.1177/2374289518766521
25. Arastehfar A, Khodavaisy S, Daneshnia F, Weihua P, Ferry H, Teun B, et al. Molecular identification, genotypic diversity, antifungal susceptibility, and clinical outcomes of infections caused by clinically underrated yeasts, Candida orthopsilosis, and Candida metapsilosis: an Iranian multicenter study (2014–2019). *Front Cell Infect Microbiol*. (2019) 9:264. doi: 10.3389/fcimb.2019.00264
26. Zhong Y, Xu F, Wu J, Marilyn ML. Application of next generation sequencing in laboratory medicine. *Ann Lab Med.* (2021) 41:25–43. doi: 10.3343/alm.2021.41.1.25
27. Wilson MR, Sample HA, Zorn KC, Gabriela M, Randall H, Kevin M, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. *N Engl J Med*. (2019) 380:2327–40. doi: 10.2147/IDR.S56633
28. Thoendel M, Jeraldo P, Greenwood-Quaintance KE, Yao J, Chia N, Hanssen AD, et al. Impact of contaminating DNA in whole-genome amplification kits used for metagenomic shotgun sequencing for infection diagnosis. *J Clin Microbiol.* (2017) 55:1789–801. doi: 10.1128/JCM.02402-16

Author Disclaimer: All the claims expressed in this article are solely those of the authors and they do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Cao, Yu, Zhou, Huang and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Public Health

CASE REPORT
published: 25 October 2021
doi: 10.3389/fpubh.2021.729595

Severe Pneumonia Caused by Infection With *Tropheryma whipplei* Complicated With *Acinetobacter baumannii* Infection: A Case Report Involving a Young Woman

Sheng Wang[1†], *Demeng Xia*[1,2†], *Jianghong Wu*[1†], *Dandan Jia*[1], *Lei Li*[1] *and Shuogui Xu*[1*]

[1] *Department of Emergency, Changhai Hospital, Naval Medical University, Shanghai, China,* [2] *Health Clinic, Liberation Army Unit 91666, Zhoushan, China*

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Guoying Deng,
Shanghai General Hospital, China
Xin Dong,
Shanghai University, China

***Correspondence:**
Shuogui Xu
18516116672@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases–Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *23 June 2021*
Accepted: *29 September 2021*
Published: *25 October 2021*

Citation:
Wang S, Xia D, Wu J, Jia D, Li L and Xu S (2021) Severe Pneumonia Caused by Infection With Tropheryma whipplei Complicated With Acinetobacter baumannii Infection: A Case Report Involving a Young Woman. Front. Public Health 9:729595. doi: 10.3389/fpubh.2021.729595

Whipple's disease is a very rare systemic infectious disease, and very few cases have been reported. However, it can be fatal if not diagnosed and treated appropriately. The major clinical manifestations of this disease are usually digestive and nervous system symptoms. The majority of patients are male and between 40 and 50 years old. Although respiratory symptoms of this disease have rarely been reported, they pose a serious threat to the lives of the patients, especially when they progress to severe pneumonia. During admission to the hospital, *Acinetobacter baumannii* infection makes treatment more difficult. While most patients are middle-aged men, more attention should be given to the diagnosis and treatment of affected young women. To our knowledge, the case presented in the study is the first case of *Tropheryma whipplei* infection that resulted in severe pneumonia and was complicated by *A. baumannii* infection during treatment. We hope that our study can serve as a reference for the diagnosis and treatment of related cases in the future.

Keywords: *Tropheryma whipplei*, severe pneumonia, *Acinetobacter baumannii*, bronchoalveolar lavage, metagenomics next-generation sequencing

BACKGROUND

Tropheryma whipplei infection was first discovered in 1907; it was identified as a rare chronic infectious disease with an annual incidence of 3 in one million population, and a male to female patient ratio of 8 to 1 (1, 2). The most obvious symptom of the prodromal phase of the disease is joint pain, while in the stable phase, it mainly manifests as abdominal pain with diarrhea and weight loss. Later, it develops into myocarditis, pericarditis and inflammation of the nervous system. To date, there have been reports about cases of this disease damaging the eyes, spine and skin (3–5). Because of the involvement of multiple systems, the clinical manifestations are relatively complex, so it is extremely easy to miss the diagnosis and arrive at a misdiagnosis, resulting in inappropriate treatment decisions, resulting in adverse outcomes. Here, we report a rare case of *T. whipplei* infection that led to "severe pneumonia," which was the primary complaint. During hospitalization, the patient became infected with *Acinetobacter baumannii*. After a prompt diagnosis and appropriate treatment, the patient recovered and was discharged. Taking into account the background of the coronavirus disease 2019 (COVID-19) pandemic and

the uniqueness of this disease, we would like to share our team's experience in the diagnosis and treatment of this rare disease with clinicians and other readers.

CASE PRESENTATION

A 23-year-old woman was admitted with chest distress and difficulty breathing. The patient said that 1 week prior, she had symptoms of fever, bloody stools and headache, and oral antibiotics did not relieve the symptoms. On admission, the vital signs observed during the physical examination were as follows: body temperature, 39.6 °C; blood pressure, 82/41 mmHg; pulse, 116 beats/min; and saturated oxygen in arterial blood, 91%. Chest CT results showed that extensive blurring in both lungs, and diffuse inflammation of both lungs was considered (**Figure 1A**). In the context of the COVID-19 pandemic, we first considered the possibility that the patient might have COVID-19; however, only 2 h later, the result of a nucleic acid test of a throat swab was negative, which helped us rule out COVID-19. At the same time, an emergency blood test showed that her white blood cell count was 2.48 × 10^9/L, with a neutrophil percentage of 90.3% and a lymphocytes percentage of only 3.1%. Given the large disparity in the percentages of lymphocytes and neutrophils, we were more inclined to suspect infections with microorganisms other than viruses.

Other examination results were as follows: the levels of procalcitonin (PCT) was 24.55 ng/mL, respectively. According to the above examination results, the patient had a serious infection. Given the patient's high PCT level and clinical symptoms, we considered a bacterial infection, but the low white blood cell and neutrophil levels made it impossible for us to rule out other infections. We have obtained more information about the patient's situation and found that she had lost 5 kg in 3 months and experienced joint pain, but she did not pay attention to these symptoms at the time. Therefore, we had reason to suspect she may be infected with HIV. However, the examination results showed that the HIV antibody level was within normal limit, which forced us to reconsider. Due to the possibility of a fungal or bacterial infection and the need to gain control over that infection immediately, the broad-spectrum antimicrobial drugs imipenem and fluconazole were administered.

On the second day of admission, the blood gas analysis results showed that the patient's arterial oxygen partial pressure had decreased to 50 mmHg, and her partial pressure of carbon dioxide was 75 mmHg. The patient was diagnosed with type I respiratory failure. At the same time, the

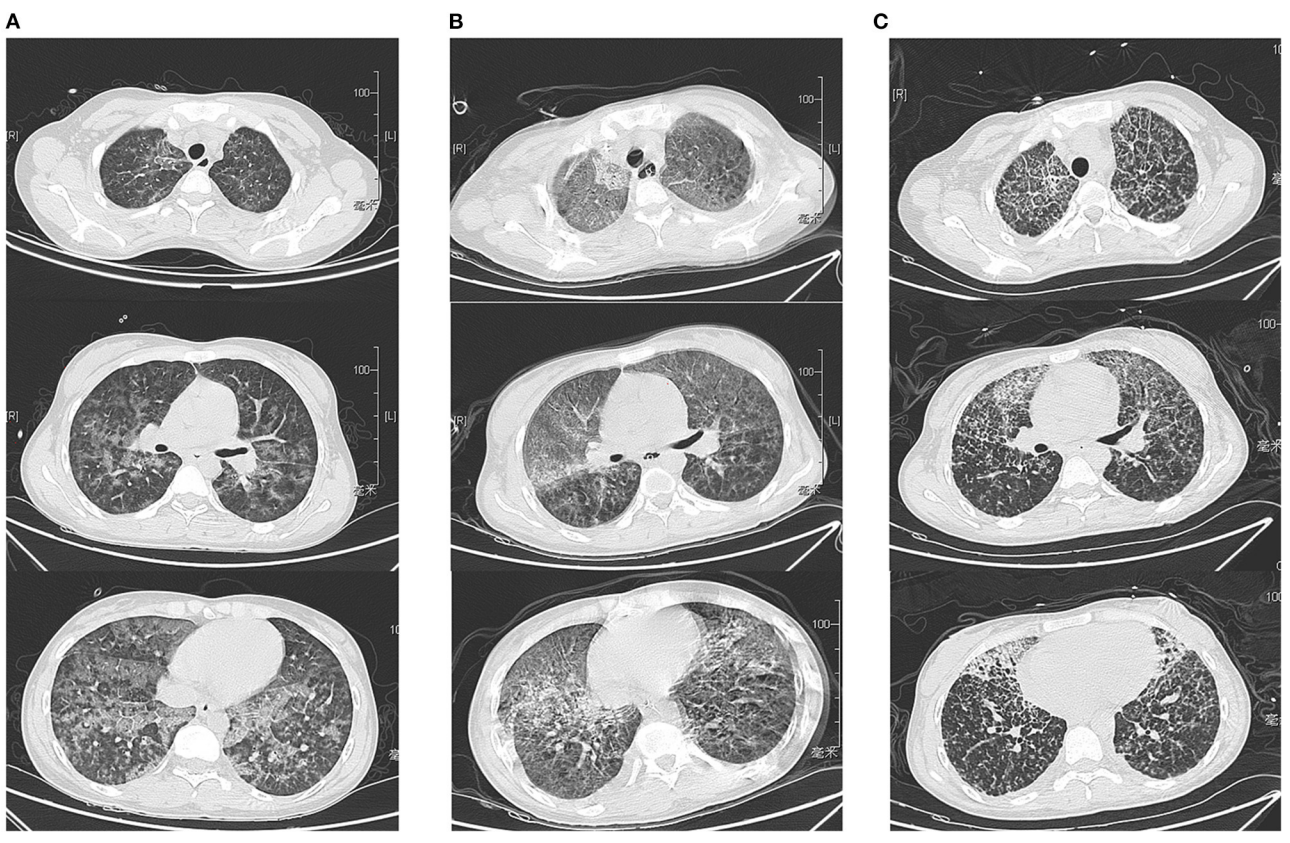
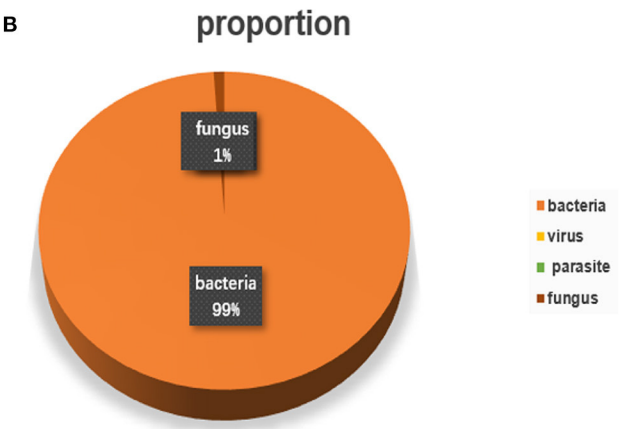


FIGURE 1 | Chest CT results on day 1, day 7, and day 14 after admission. **(A)** Chest CT findings on the first day of admission revealed diffuse pneumonitis throughout the lungs. **(B)** The patient's chest CT on day 7 showed a worsening of the infection (due to the severity of the disease, the patient could not hold her breath well, so the images were not clear). **(C)** On the 14th day of admission, the patient's chest CT results showed slight improvement after treatment.



C

1. Bacteria

	genus		species		
type	Latin name	sequence number	Latin name	sequence number	relative abundance
G+	*Tropheryma*	*5668*	*Tropheryma whipplei*	*5668*	*99.4664%*

Note: G- is Gram-negative bacteria, G+ is Gram-positive bacteria.

FIGURE 2 | Results of the next-generation sequencing of bronchoalveolar lavage fluid. **(A)** Bronchoalveolar lavage fluid. **(B)** The results showed that the relative abundance of *Tropheryma whipplei* was 99.4664%. The number of sequences was 5668. **(C)** The proportions of bacteria, viruses, parasites and fungi.

patient's oxygen saturation level decreased from 90 to 70%. Therefore, endotracheal intubation with mechanical ventilation was performed, and the synchronized intermittent mandatory ventilation (SIMV) was selected as the ventilator mode. The tidal volume was 350 ml (her current weight was 50 kg), the positive end-expiratory pressure (PEEP) was 3 cmH_2O, the oxygen concentration was 35%, and the frequency was 12 times/min. The patient's oxygen saturation was maintained at 100%. At that point, the patient had met the diagnostic standard for severe pneumonia. In addition, the patient's liver function and renal function were impaired to varying degrees: her creatinine level was 293 μmol/L, her alanine aminotransferase level was 66 U/L, her aspartate aminotransferase level was 121 U/L, her platelet count was significantly reduced to 37×10^9/L, and her urine volume was also reduced to < 0.5 ml/kg/h. However, all the above indexes had been normal before disease onset. The patient was given conventional treatments such as inhaled oxygen, fluid replacement, antitussive treatment, diuresis and liver protection, and antibiotics continued to be administered. Her blood pressure remained at approximately 110/70 mmHg on dopamine.

Considering that the results of blood and sputum cultures take time to be returned and substantial wet rales could be heard in both lungs on auscultation, we performed fibreoptic bronchoscopy to enable us to identify the pathogen and provide the appropriate treatment as soon as possible. A large amount of white mucous was found in the airway during the procedure, and bronchoalveolar lavage fluid (BALF) (**Figure 2A**) was obtained for next-generation sequencing (NGS). The gene sequencing results for the pathogenic microorganisms were as follows: the relative abundance of *T. whipplei* was *99.4664%,* and the sequence number was *5,668* (fungi, parasites, chlamydia, mycoplasma, mycobacterium and other types of bacteria were not detected) (**Figures 2B,C**). After identifying the pathogen as a gram-positive bacterium, fluconazole was discontinued, and imipenem was maintained. In the following week, we re-examined the patient's laboratory parameters (**Figures 3**, **4**), and the patient's overall performance had slightly improved.

However, on the seventh day of admission, her PCT value decreased significantly. However, there was a slight rebound in her white blood cell count, and the patient's body temperature also remained at approximately 38 degrees Celsius and did not return to normal. Even more striking was the fact that her chest CT findings worsened (**Figure 1B**). At the same time, the drug sensitivity results of the bacterial culture were obtained. Unfortunately, the results of the bacterial culture of patients showed that the main pathogenic bacterium had changed from *T. whipplei* to carbapenem-resistant *A. baumannii*, which was resistant to all tested antibiotics.

After careful consideration, treatment with the previous antibiotics was stopped, and we made the decision to treat the

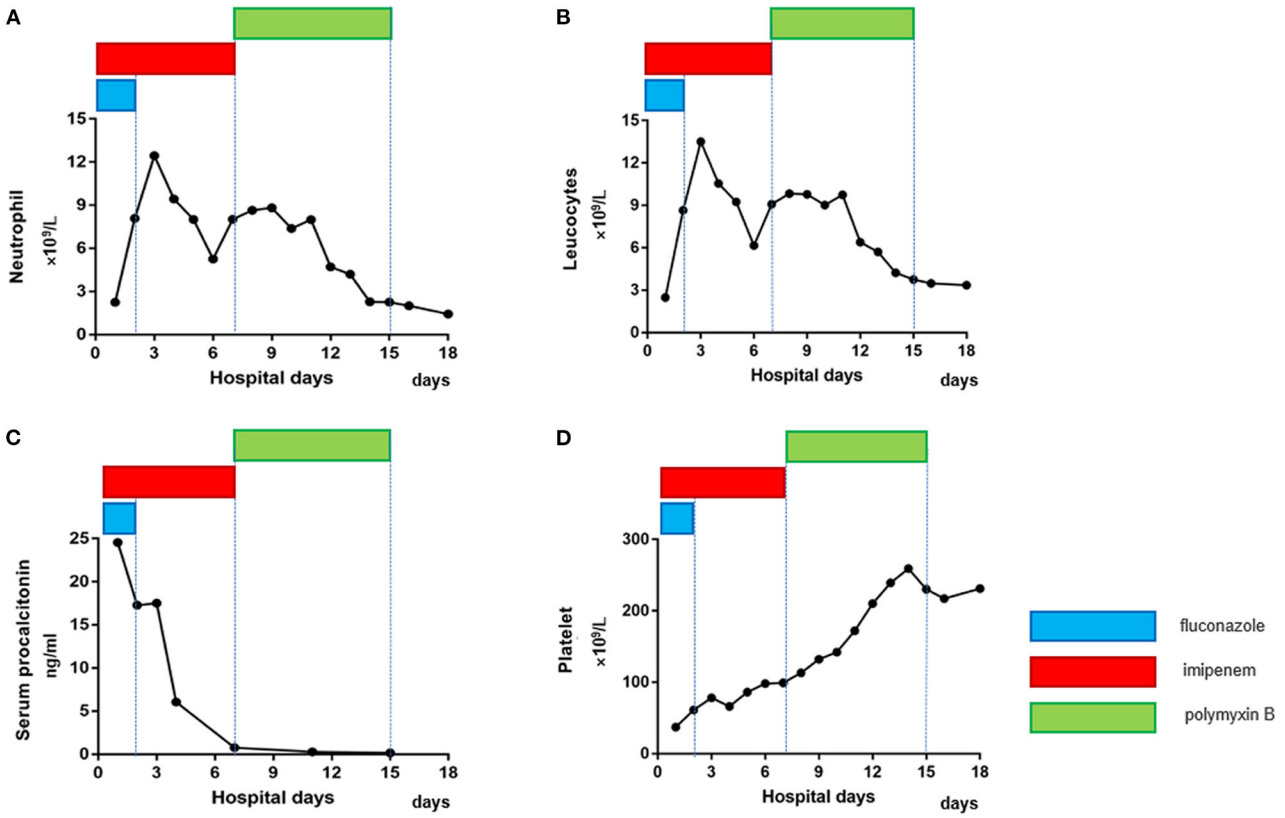


FIGURE 3 | Dynamic changes in infection indexes and platelet counts in the patient after admission. **(A, B)** The neutrophil and leucocyte counts decreased significantly 7 days after admission. **(C)** The serum procalcitonin level continued to decline. **(D)** The platelet count continued to increase. Eventually, the patient's indicators returned to normal (different colors represent different medications, and the length of the lines represents the duration of treatment with the medication).

patient empirically by adding polymyxin B to our treatment plan, given that there were no other drugs available because the *A. baumannii* was resistant to all tested antibiotics. Fortunately, the patient's condition gradually returned to normal. Her liver function, kidney function (**Figure 4**), platelet count and infection indexes improved significantly (**Figure 3**), although her chest CT scans showed that the infection in the lungs had improved only slightly after treatment (**Figure 1C**). Twelve days after admission, the patient's respiratory function had improved, and she was weaned from the ventilator and extubated. All antibiotics were discontinued on the 15th day after admission, and the patient was able to get out of bed and perform simple activities 16 days after admission. She was discharged 18 days after admission.

DISCUSSION

Severe pneumonia is a common disease encountered in clinical practice, but its diagnosis and treatment are often challenging, especially when pneumonia is cause by infections with rare or unknown pathogens. The patient we admitted was treated for symptoms such as hypotension, high fever, dyspnoea and low white blood cell count and was initially diagnosed with severe pneumonia. After the test results were obtained, viral infections, including infection with HIV, were ruled out. Considering that bacterial cultures take a long time, we obtained bronchoalveolar lavage fluid and performed next-generation sequencing. At present, nearly all infectious agents contain DNA or RNA genomes, making sequencing an attractive approach to pathogen detection. Next-generation sequencing has emerged as means of detecting and taxonomically characterizing microorganisms in clinical samples from patients (6). At present, for patients with severe infections and unknown causative pathogen, early next-generation sequencing is necessary to guide the selection of a reasonable treatment plan as soon as possible. The rapid, sensitive results of next-generation sequencing make it far superior to microbial culture. In addition, studies have shown that next-generation sequencing has apparent advantages for the detection of viruses and co-infections, *at present, it has been fully used in the diagnosis of rare bacteria* (7). Finally, in this case, infection with *T. whipplei* was confirmed in only 1 day.

Tropheryma whipplei is a gram-positive actinomycete that is a symbiotic bacterium and is widespread in the external environment. It is unlikely to cause disease in normal people, although it can cause infections in patients with partial immune deficiency. *Tropheryma whipplei* causes a chronic infectious disease known as Whipple's disease, which was first described

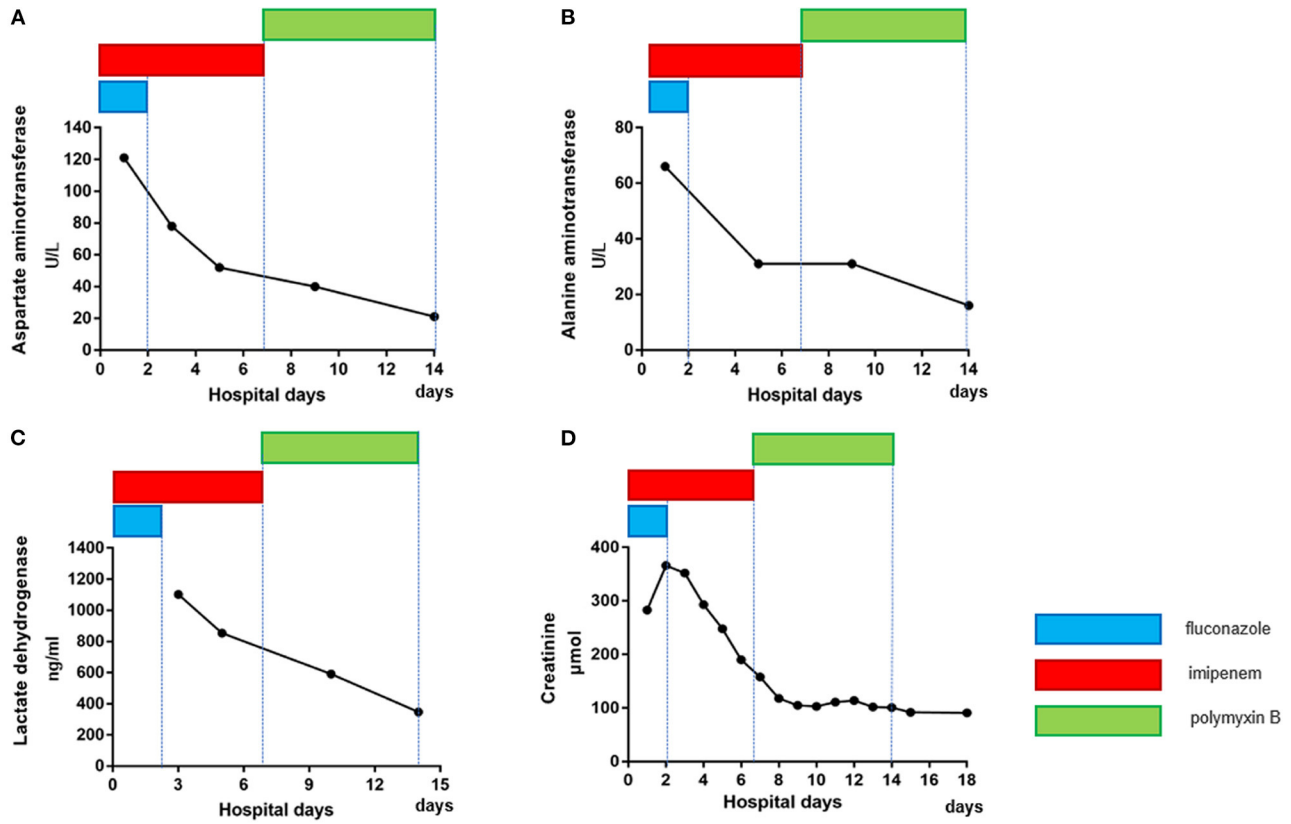


FIGURE 4 | Dynamic changes in liver and kidney function in the patient after admission. After appropriate treatment, the aspartate aminotransferase **(A)**, alanine aminotransferase **(B)**, lactate dehydrogenase **(C)** and creatinine **(D)** levels of the patient gradually returned to normal (different colors represent different medications, and the length of the lines represents the duration of treatment with the medication).

in 1907 by G.H. Whipple and has an annual incidence of 3 in one million population (1). Classic Whipple's disease mainly involves the digestive system and is characterized by a tetrad of symptoms: arthralgia, weight loss, diarrhea, and abdominal pain. Gastrointestinal findings are the most common clinical manifestations that are identified in approximately 90–95% of patients (8). Due to the large proportion of cases involving the digestive system, there are now relatively mature diagnostic and therapeutic measures. A definite diagnosis of classic Whipple's disease is generally made by observing the massive infiltration of foamy macrophages containing dense PAS-positive granules in the lamina propria on histopathological examination of the biopsy material obtained from the small intestine (9). However, cases involving the respiratory system are rare. Existing studies have shown that respiratory infection is reported in only 13–14% of cases (10, 11) and usually involve pleural effusion and/or pulmonary hypertension (8). However, our patient presented with dyspnoea, chest distress and cough as the main symptoms, which is very rare in the literature. Another concern was the age and sex of the patient. According to an Italian study (1), the disease is more likely to occur in middle-aged white males, and the incidence in females is relatively low, with female patients accounting for only 30%. However, a study in the United States (12) found that the sex ratio is changing and that currently there is no significant difference in incidence between men and women. Nevertheless, this requires us to pay more attention to the atypical manifestations of the disease and the diversity of the patient population in clinical practice to improve our diagnostic success and treatment of patients.

To date, the treatment of Whipple's disease with pulmonary symptoms as the main complaints is not yet mature and has not been standardized. In our case, based on the next-generation sequencing results, we changed the treatment regimen in a timely manner, and the patient's condition showed significant improvement. On the 8th day, however, the patient's condition changed again, and the bacterial culture showed *A. baumannii*. This is a gram-negative 0 bacteria common in nosocomial infections, especially in intensive care units (ICUs) and immunocompromised patients with central venous catheters; it is often multi-drug resistant (MDR) and usually infects the respiratory tract, especially in those on mechanical ventilation (13). Antibiotics that are usually effective against *A. baumannii* infections include polymyxins E and B, sulbactam, piperacillin/tazobactam, tigecycline, aminoglycosides and carbapenems. The patient in our case had nearly all of

the risk factors for infection with *A. baumannii*. Unfortunately, in this case, the *A. baumannii* was not sensitive to *any of the tested antibiotics*. We made the decision to treat the patient empirically by adding polymyxin B to our treatment plan. To our surprise, the patient's white blood cell and neutrophil counts returned to normal after a few days, and her lung CT showed improvement. Finally, all antibiotics were discontinued on the 15th day of admission, and the patient was discharged on the 18th day. This also suggests that pathogens that show resistance to antibiotics can sometimes still be treated with empiric therapy. Of course, we cannot deny that early drug sensitivity testing is essential for infection control in cases of serious infections. In addition, we believe that the patient's recovery was also due to her young age, which was conducive to the recovery of her physiological function.

CONCLUSIONS

To our knowledge, this is the first case of severe pneumonia caused by infection with *T. whipplei* complicated with *A. baumannii* infection. The atypical natures of both the affected system and the patient deserve our attention. In terms of diagnosis, next-generation sequencing is relatively faster, supporting the early diagnosis of serious infections. As one of six main pathogens involved in nosocomial infections, *A. baumannii* also poses a clinical challenge; therefore, the timely performance of drug sensitivity tests and the selection of appropriate antibiotics are important. We hope that our report can serve as a reference for the diagnosis and treatment of this disease, and more clinical data are expected to be published to provide more support.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SW and DX collected and analyzed the data and wrote the manuscript. JW, DJ, LL, and SX designed the study and revised the manuscript. All authors have read and approved the final manuscript.

REFERENCES

1. Biagi F, Balduzzi D, Delvino P, Schiepatti A, Klersy C, Corazza GR. Prevalence of Whipple's disease in north-western Italy. *Eur J Clin Microbial Infect Dis.* (2015) 34:1347–8. doi: 10.1007/s10096-015-2357-2
2. Ojeda E, Cosme A, Lapaza J, Torrado J, Arruabarrena I, Alzate L. Whipple's disease in Spain: a clinical review of 91 patients diagnosed between 1947 and 2001. *Rev Esp Enferm Dig.* (2010) 102:108–23. doi: 10.4321/S1130-01082010000200006
3. Martins MDL, Abreu P, Caldeira A, Pinto JD, Sousa R. Whipple Disease and ankylosing spondylitis-a case report. *J clin rheumatol.* (2020). doi: 10.1097/RHU.0000000000001466. [Epub ahead of print].
4. Borretta L, Walsh NM, Bakowsky V, Arnason T, Croul S, Pasternak S. A case of Whipple Disease with cutaneous manifestations. *Am J Dermatopathol.* (2021) 43:e104–6. doi: 10.1097/DAD.0000000000001925
5. Testi I, Tognon MS, Gupta V. Ocular Whipple disease: report of three cases. *Ocul Immunol Inflamm.* (2019) 27:1117–20. doi: 10.1080/09273948.2018.1518461
6. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol.* (2019) 14:319–38. doi: 10.1146/annurev-pathmechdis-012418-012751
7. Fang X, Mei Q, Fan X, Zhu C, Yang T, Zhang L, et al. Diagnostic value of metagenomic next-generation sequencing for the detection of pathogens in bronchoalveolar lavage fluid in ventilator-associated pneumonia patients. *Front Microbiol.* (2020) 11:599756. doi: 10.3389/fmicb.2020.599756
8. T. Marth. Systematic review: Whipple's disease (Tropheryma whipplei infection) and its unmasking by tumour necrosis factor inhibitors. *Aliment Pharmacol Ther.* (2015) 41:709–24. doi: 10.1111/apt.13140
9. Lagier JC, Fenollar F, Lepidi H, Giorgi R, Million M, Raoult D. Treatment of classic Whipple's disease: from in vitro results to clinical outcome. *J Antimicrob Chemother.* (2014) 69:219–27. doi: 10.1093/jac/dkt310
10. Marth T. Whipple's disease. *Acta Clin Belg.* (2016) 71:373–8. doi: 10.1080/17843286.2016.1256586
11. Obst W, von Arnim U, Malfertheiner P. Whipple's disease. *Viszeralmedizin.* (2014) 30:167–72. doi: 10.1159/000363781
12. Elchert JA, Mansoor E, Abou-Saleh M, Cooper GS. Epidemiology of Whipple's disease in the USA between 2012 and 2017: a population-based national study. *Dig Dis Sci.* (2019) 64:1305–11. doi: 10.1007/s10620-018-5393-9
13. Vázquez-López R, Solano-Gálvez SG, Juárez Vignon-Whaley JJ, Abello Vaamonde JA, Padró Alonzo LA, Rivera Reséndiz A, et al. Acinetobacter baumannii Resistance: a real challenge for clinicians. *Antibiotics.* (2020) 9:205. doi: 10.3390/antibiotics9040205

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Wang, Xia, Wu, Jia, Li and Xu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 28 October 2021
doi: 10.3389/fmed.2021.736064

Case Report: Streptococcus Suis Meningitis Diagnosed in a HIV-Infected Patient With Cryptococcal Meningitis Using Next-Generation Sequencing

Yirui Xie, Bing Ruan, Guanjing Lang and Biao Zhu**

State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The Department of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Jun Chen,
Fudan University, China
Yong Gao,
University of Science and Technology of China, China

****Correspondence:***
Yirui Xie
1312019@zju.edu.cn
Biao Zhu
zhubiao1207@zju.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *04 July 2021*
Accepted: *06 October 2021*
Published: *28 October 2021*

Citation:
Xie Y, Ruan B, Lang G and Zhu B (2021) Case Report: Streptococcus Suis Meningitis Diagnosed in a HIV-Infected Patient With Cryptococcal Meningitis Using Next-Generation Sequencing. Front. Med. 8:736064. doi: 10.3389/fmed.2021.736064

Background: Streptococcus suis has been recognized as a zoonotic pathogen that may cause infections in humans. Although rarely described, it is not surprising that both cryptococcal and streptococcus suis meningitis infections can co-exist in a HIV-infected patient with a low CD4 count. However, a fast and accurate diagnose of meningitis of multipathogenic infections is challenging. In this report, we describe such a case of a HIV-infected patient with meningitis of multipathogenic infections.

Case Presentation: The patient was a 34-year-old Chinese male who was diagnosed with cryptococcal meningitis and HIV at the same time about 1 year ago. During the same time period, he had received (with good compliance) fluconazole and tenofovir-lamivudine- dolutegravir based antiretroviral therapy (ART). However, symptom of progressively worsening occipital headache appeared after he was exposed to a truck which was used for transporting pigs. Initial workup indicated an increase of the cerebrospinal fluid (CSF) opening pressure (OP) and an increase in the number of lymphocytes and proteins in CSF. A magnetic resonance imaging (MRI) scan revealed that partial cerebellar surface enhancement. The cryptococcus capsular antigen test of CSF was positive. The results of the India Ink microscopy for cryptococcus, nucleic acid of CMV and EBV and mycobacterium tuberculosis (MTB) tests of CSF were negative. The results of the bacteria and fungi smear and culture of CSF were also negative. Eventually, streptococcus suis was detected using next-generation sequencing (NGS) in CSF. The diagnosis of Streptococcus suis meningitis was made based on the patient's contact history with carrier pigs and the clinical findings addressed above. The treatment of 2 weeks of intravenous ceftriaxone and 1 week of oral moxifloxacin resulted in improvement of the condition of CSF. The anti-fungal treatment using fluconazole continued until the CFS OP went down to a normal level and the cryptococcus capsular antigen test of CSF was negative 6 months later.

Conclusion: This case highlights that NGS might be beneficial to HIV-infected patients

who have meningitis with negative CSF culture results. Multiple etiologies for such condition in the immunocompromised patients must be taken into consideration and early stage NGS is recommended.

Keywords: next-generation sequencing, meningitis, streptococcus suis, cryptococcus, cerebrospinal fluid, HIV

INTRODUCTION

Compared to patients with monomicrobial meningitis, patients infected with multiple types of meningitis are more difficult to be diagnosed (1). However, a fast and accurate diagnosis of the pathogens causing the meningitis is challenging because of the limitations of current conventional tests (2). Sometimes, it is still difficult to draw the correct conclusion based on the test results. Streptococcus suis is a gram-positive, capsulated, hemolytic, facultative anaerobic coccus. It has recently been recognized as a zoonotic pathogen that may cause infections in humans in occupational contact with pigs and/or pork, with meningitis as its most common clinical presentation (2–5). Although rarely described, it is not of surprise that both cryptococcal and streptococcus suis meningitis infections can co-exist in a patient infected with human immunodeficiency virus (HIV) and with a low CD4 count. Next-generation sequencing (NGS), a method with highly sensitive detecting function for analyzing the microbiome, can provide additional valuable information for the detection of pathogens. Here, we present a report of a HIV-infected patient who had cryptococcal meningitis infection for about 1 year and was then diagnosed with streptococcus suis meningitis using NGS, even though his blood and CSF cultures test results were both negative.

CASE REPORT

A 34-year-old Chinese male visited his local tertiary hospital due to progressively worsening occipital headache for the past 4 days, without showing any other symptoms of fever, projectile vomiting and so on. His medical history showed that he had had cryptococcal meningitis and HIV infection for about 1 year, and he had been receiving (with good compliance) fluconazole and tenofovir-lamivudine-dolutegravir based antiretroviral therapy (ART) during the same time period. The levels of the inflammatory markers were normal (Leukocytes $5.61^{*}10^{9}$ cells/mL, C-reactive protein 7 mg/dL). His neurological signs showed no abnormal indications. Head Computed Tomography (CT) scan revealed no abnormalities. The lumber puncture was performed and the test result of CSF is shown in **Table 1**. It shows rising levels of OP, lymphocytes and protein in CSF. The cryptococcus capsular antigen test result of CSF was positive but the results for the gram staining and culture study were negative. The patient was diagnosed with cryptococcal meningitis and was given fluconazole (400 mg, QD) continuously and TMP-SMX (0.48, QD) for 1 week by the doctor at the local hospital. However, his headache problem was not resolved. Then he came to our institution, the Infectious Disease Department of the First Affiliated Hospital, School of Medicine, Zhejiang University, and was hospitalized. Upon arrival, the patient's body temperature was 36.7 C, pulse rate was 95 beats/min, blood pressure was 138/97 mmHg, respiratory rate was 23 breaths/min, and finger pulse SpO2 was 98%. He was suffering from headache but his neurological signs were normal. The laboratory results are as follows: white blood cell count 7.0×10^{9}/L, with a neutrophil ratio of 42%, hemoglobin 150 g/L, and platelet count 294×10^{9}/L; C-reactive protein (CRP)1.8 mg/L; CD4 count 171 cells/μL; normal blood biochemical; negative 1,3-beta-D-glucan test (G test) and galactomannan test (GM test) results (0.1 μg/L). The IgG antibodies of Epstein-Barr virus (EBV) and Human *Cytomegalovirus* (CMV) were positive, while the IgM antibodies and the test of the nucleic acid of EBV and CMV were negative. The test result for mycobacterium tuberculosis (MTB) was negative based on the blood test with T-SPOT, and the result for cryptococcus capsular antigen test of the blood was positive. The chest CT scan and echocardiography results were normal. The lumber puncture was performed on day 2 and the opening pressure was 285 (cm H2O). The test result of CSF is shown in **Table 1**. The cryptococcus capsular antigen test of CSF was positive. An India Ink microscopy on the CSF sample for cryptococcus was also performed and the result was negative. The results of the blood and CSF smears of bacteria and fungi were negative. The results of the acid-fast staining of CSF smear and GeneXpert were both negative for MTB. The test of the nucleic acid of CMV and EBV in CSF was negative. Based on the negative gram staining and culture study results of CSF provided by the local hospital and the above results, the CSF sample was sent to the laboratory for conventional tests and NGS test (using the Illumina Platform, IngeniGen. Ltd, Zhejiang, China) for pathogen-induced meningitis verification. Contrast-enhanced and diffusion-weighted MRI (3.0T) was performed on day 3, and the partial cerebellar surface enhancement was revealed (**Figure 1**), which matched the manifestation of cryptococcal meningitis, and no abnormal signals were detected in the cerebral parenchyma. Two days later, the NGS results showed that streptococcus suis (6 reads) and streptococcus mitis (1 reads) were found in CSF. The results of the blood and CSF culture of bacteria and fungi were still negative on day 5. So, we inquired the patient's medical history again, and he told us he had had close contact with a truck transporting pigs and had choked on water in a water park 2 weeks before his admission. Based on the test reports and his contact history, the diagnosis of streptococcus suis infection was made and intravenous Ceftriaxone (2.0, QD) was given. The patient's headache problem was resolved gradually. A second lumber puncture performed on day 12 revealed improved conditions of CSF (see **Table 1**), with negative gram staining and culture study results. Ceftriaxone was given to the patient for another 2 weeks and then oral moxifloxacin

TABLE 1 | Cerebrospinal fluid profile evolution throughout treatment.

LP date / CSF characteristic	2020.9.5 (Local hospital)	Day 2 2020.9.17	Day 12 2020.9.28	Day 17 020.10.3	Day 42 2020.10.28	Day 70 2020.11.25	Day 98 2020.12.23	Day 187 2021.03.22
Opening pressure (cm H2O)	ND	285	250	210	220	220	210	140
Color	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless
Turbidity	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Cell count (/μL)	410	500	240	120	66	28	22	50
Neutrophils (%)	5	15	4	20	20			15
Lymphocytes (%)	90	80	92	80	80			80
Monocytes and others (%)		5	2					5
Protein (g/L)	ND	1.312	1.3172	1.471	0.753	0.675	0.629	0.401
Glucose (mmol/L)	3.1	2.7	2.7	2.5	2.7	3.7	3.3	4.2
Chlorine (mmol/L)	121	118	119	123	117	127	124	126
CrAg test	+	+	+	−	−	−	−	−
Lactate dehydrogenase (U/L)	ND	37	26	21	21	27	18	18
Adenylate deaminase (U/L)	ND	4.3	2.9	1.8	1.9	0.9	0.1	0.9
India Ink microscopy	−	−	−	−	−	−	−	−
CSF culture	−	−	−	−	−	−	−	−

CSF, Cerebrospinal fluid; LP, Lumbar puncture; CrAg, Cryptococcal capsular polysaccharide antigen; ND, No data; "+": Positive; "-": Negative.

(0.4, QD) for 2 days. The third lumber puncture was performed on day 17, showing continuous conditional improvement of CSF. The patient was discharged in stable condition on day 21 after 1 week of receiving moxifloxacin. Three weeks later, we performed the fourth lumber puncture to confirm that the meningitis infection was cleared, but the patient's OP level was still abnormal, so fluconazole was given continuously until the OP level and cryptococcus capsular antigen test turned normal 6 months later.

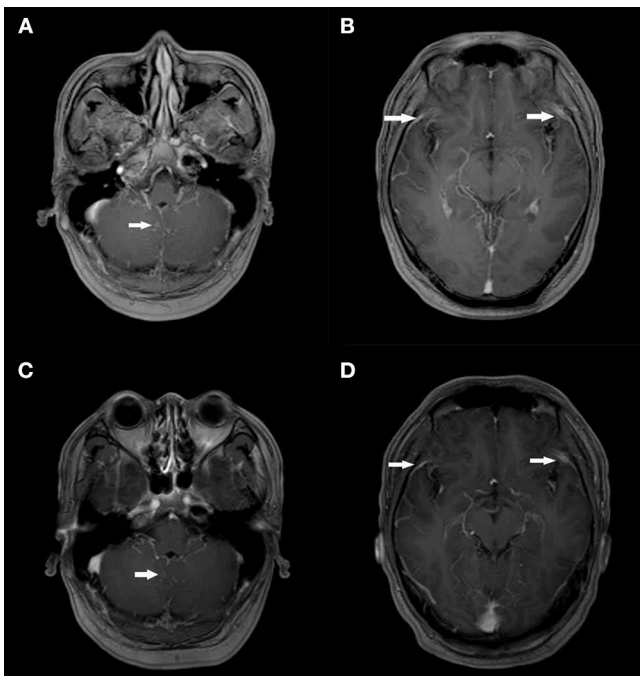


FIGURE 1 | Contrast-enhanced and Diffusion-weighted Cranial MRI Scans on Day 3 of Admission and after 6 Months. On the third day of admission, post-contrast T1 MRI showed partial cerebellar surface enhancement **(A,B)** and get better at 6 months later after discharge **(C,D)**.

DISCUSSION

The rate of AIDS-related opportunistic infections has decreased dramatically due to the success in achieving viral suppression and immune reconstitution combined with the effective and wide use of ART (6). The coexistence of fungal and bacterial meningitis in patients is rarely reported, so here we would like to address the case of the recent diagnosis of streptococcus suis meningitis using NGS in a patient who had prior diagnosed with HIV infection and cryptococcal meningitis about 1 year ago.

In the developed countries, the prevalence of cryptococcal meningitis has dropped amongst HIV patients as it can be diagnosed in the early stage, but the percentage is still exceedingly high in the more resource-limited environments (7). In this case, fluconazole was used and ART initiated 2 weeks later. The therapy went on for about 1 year and the patient's condition was stable but his cryptococcus capsular antigen test result of CSF was positive during the regular review. What's more, symptom of progressively worsening occipital headache appeared after the patient had contact with a truck which carried pigs. The routine blood and CSF test results were negative, and the diagnosis of streptococcus suis meningitis was finally confirmed using NGS on CSF.

Streptococcus suis has recently been recognized as a zoonotic pathogen that may cause infections in people with risk of

occupational exposure to pigs and/or pork (3, 4). Meningitis is the most commonly resulted clinical presentation, followed by septicemia, endocarditis, arthritis, enteritis, spondylodiscitis, endophthalmitis, uveitis and peritonitis (3, 5). Streptococcus suis infection has no specific clinical symptoms in the early stage. Usually, Streptococcus suis meningitis starts with the typical presentation of meningeal symptoms such as fever, headache, nausea, and vomiting, as well as neurological symptoms, such as dizziness and balance disorder. The central nervous system imaging is the standard procedure in most cases. The diagnosis of purulent meningitis can be confirmed based on the CSF examination and the bacteria culture of CSF and blood (5). Typically, Streptococcus suis meningitis presented a neutrophilic CSF inflammatory response. However, this patient had cryptococcal meningitis infection and presented a lymphocytic CSF inflammatory response, hence clear conclusion could not be drawn in this regard. The patient's symptoms of headache and lymphocytic CSF inflammatory response might be caused by the co-occurrence of streptococcus suis meningitis and cryptococcal meningitis on top of his existing HIV infection and relatively low CD4 count. However, further investigation needs to be conducted toward the exact mechanism at work. The conventional culture has been used in the clinical process. However, these conventional tests have its limitations in terms of sensitivity, speed, and spectrum for pathogen detection (8). The routine blood and CSF culture results were negative in the two times of lumbar punctures conducted on the patient. So, a prompt and accurate diagnosis of meningitis of multipathogenic infections in HIV-infected patients is still difficult to achieve. NGS, as a highly sensitive, culture-independent and unbiased method, can identify all potentially known, new or unexpected pathogens (9, 10).

NGS has been successfully used as a diagnostic tool for various infectious diseases in the immunocompromised hosts (11–13). Previous study showed that multiple pathogens were identified by NGS in CSF from more than 15.9% (14/88) of HIV-infected patients who were suspected to have CNS infection with negative result by conventional tests. This indicated that multipathogenic CNS infections are not uncommon in people living with HIV and NGS is capable to identify multiple pathogens in a single test in an unbiased manner (1). Although there is one previous report about the application of NGS in the identification of streptococcus suis septicemia in a patient whose blood bacterial cultures results were negative (14), its application in diagnosing streptococcus suis meningitis in HIV-infected patients with cryptococcal meningitis history has never been addressed until now. Most patients with streptococcus suis meningitis respond well to treatment of broad-spectrum intravenous antibiotics such as penicillin, ampicillin and cefotaxime (5). Although the numbers of the NGS sequence read for streptococcus suis was low, the diagnosis of streptococcus suis meningitis was confirmed in this patient's case based on his contact history with carrier pigs and the clinical findings. The 2 weeks treatment of intravenous ceftriaxone and 1 week of oral Moxifloxacin resulted in the continuous improvement of the condition of CSF.

This case demonstrates that NGS might be beneficial to help diagnose HIV-infected patients who have meningitis with negative CSF cultures results and are more likely to be infected by multiple pathogens. Multiple etiologies for such condition in immunocompromised patients must be taken into consideration and early stage NGS is recommended. However, the application of NGS in clinical diagnosis is still challenging due to the consideration of the cost-effectiveness and the standardization of the entire procedure, from sample collection to result interpretation (15). The result interpretation of NGS requires caution, and other clinical, laboratory and radiological findings must be taken into consideration as well.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YX collected the data and wrote the manuscript. GL and BZ analyzed and interpreted the patient data. BR and BZ reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Natural Science Foundation of China (Young Scientist Fund, 81500491) and the National Science Foundation of China (Major Research Plan, 2018ZX10715-014). These funding agencies have no role in the design, data collection, analysis or interpretation of the research or in the writing of the manuscript.

REFERENCES

1. Chen J, Zhang R, Liu L, Qi T, Wang Z, Song W, et al. Clinical usefulness of metagenomic next-generation sequencing for the diagnosis of central nervous system infection in people living with HIV. *Int J Infect Dis.* (2021) 107:139–44. doi: 10.1016/j.ijid.2021.04.057
2. Wilson MR, Sample HA, Zorn KC, Arevalo S, Yu G, Neuhaus J, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. *N Engl J Med.* (2019) 380:2327–40. doi: 10.1056/NEJMoa1803396
3. Feng Y, Zhang H, Wu Z, Wang S, Cao M, Hu D, et al. Streptococcus suis infection: an emerging/reemerging challenge of bacterial

infectious diseases? *Virulence.* (2014) 5:477–97. doi: 10.4161/viru.28595
4. Dutkiewicz J, Sroka J, Zajac V, Wasiński B, Cisak E, Sawczyn A, et al. Streptococcus suis: a re-emerging pathogen associated with occupational exposure to pigs or pork products. Part I – epidemiology. *Ann Agric Environ Med.* (2017) 24:683–95. doi: 10.26444/aaem/79813
5. Hlebowicz M, Jakubowski P, Smiatacz T. Streptococcus suis meningitis: epidemiology, clinical presentation and treatment. *Vector Borne Zoonotic Dis.* (2019) 19:557–62. doi: 10.1089/vbz.2018.2399
6. Masur H. Recurring and emerging questions related to management of HIV-related opportunistic infections. *Top Antivir Med.* (2018) 26:79–84.
7. Spec A, Powderly WG. Cryptococcal meningitis in AIDS. *Handbook Clin Neurol.* (2018) 152:139–50. doi: 10.1016/B978-0-444-63849-6.00011-6
8. Mangioni D, Peri AM, Rossolini GM, Viaggi B, Perno CF, Gori A, et al. Toward rapid sepsis diagnosis and patient stratification: what's new from microbiology and omics science. *J Infect Dis.* (2020) 221:1039–47. doi: 10.1093/infdis/jiz585
9. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol Mech Dis.* (2019) 14:319–38. doi: 10.1146/annurev-pathmechdis-012418-012751
10. Camargo JF, Ahmed AA, Lindner MS, Morris MI, Anjan S, Anderson AD, et al.. Next-generation sequencing of microbial cell-free DNA for rapid noninvasive diagnosis of infectious diseases in immunocompromised hosts. *F1000Res.* (2020) 8:1194. doi: 10.12688/f1000research.19766.2
11. Zhang Y, Ai JW, Cui P, Zhang WH, Wu HL, Ye MZ. A cluster of cases of pneumocystis pneumonia identified by shotgun metagenomics approach. *J Infect.* (2019) 78:158–69. doi: 10.1016/j.jinf.2018.08.013
12. Wang J, Han Y, Feng J. Metagenomic next-generation sequencing for mixed pulmonary infection diagnosis. *BMC Pulm Med.* (2019) 19:252. doi: 10.1186/s12890-019-1022-4
13. Xie Y, Ruan B, Jin L, Zhu B. Case report: next-generation sequencing in diagnosis of pneumonia due to pneumocystis jirovecii and cytomegalovirus in a patient with HIV infection. *Front Med.* (2021) 8:13653294. doi: 10.3389/fmed.2021.653294
14. Dai Y, Chen L, Chang W, Lu H, Cui P, Ma X. Culture-negative streptococcus suis infection diagnosed by metagenomic next-generation sequencing. *Front Public Heal.* (2019) 7:379. doi: 10.3389/fpubh.2019.00379
15. Miller S, Chiu C, Rodino KG, Miller MB. Point-counterpoint: should we be performing metagenomic next-generation sequencing for infectious disease diagnosis in the clinical laboratory? *J Clin Microbiol.* (2020) 58:e01739–19. doi: 10.1128/JCM.01739-19

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Xie, Ruan, Lang and Zhu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Public Health

ORIGINAL RESEARCH
published: 11 November 2021
doi: 10.3389/fpubh.2021.755228

The Early Diagnosis of Scrub Typhus by Metagenomic Next-Generation Sequencing

Xianghong Liu[1†], *Ye Zhang*[2†], *Jun Zhang*[3], *Zheng Lou*[2], *Han Xia*[2*] *and Zhijuan Lu*[1*]

[1] *Department of Neurology, Ganzhou People's Hospital, Ganzhou, China,* [2] *Department of Scientific Affairs, Hugobiotech Co., Ltd., Beijing, China,* [3] *Emergency Department, Ganzhou People's Hospital, Ganzhou, China*

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Rahul Narang,
All India Institute of Medical Sciences Bibinagar, India
Abhijit Chaudhury,
Sri Venkateswara Institute of Medical Sciences, India
Fangyou Yu,
School of Medicine, Tongji University, China

***Correspondence:**
Han Xia
xiahan@hugobiotech.com
Zhijuan Lu
janezi86@163.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *08 August 2021*
Accepted: *06 October 2021*
Published: *11 November 2021*

Citation:
Liu X, Zhang Y, Zhang J, Lou Z, Xia H and Lu Z (2021) The Early Diagnosis of Scrub Typhus by Metagenomic Next-Generation Sequencing. Front. Public Health 9:755228. doi: 10.3389/fpubh.2021.755228

Introduction: Scrub typhus is a mite-borne infection widespread in Southeast Asia, with clinical symptoms such as fever, chills, skin rash, eschar at the bite site, and other signs of acute febrile illness. The *Rickettsia* pathogen (*Orientia tsutsugamushi*) is always difficult to be diagnosed at an early stage by traditional clinical diagnostic methods, especially for patients without typical eschar. This greatly increases the mortality of patients with scrub typhus. A new approach should be introduced to improve its clinical diagnosis.

Methods: During May 2018 to March 2021, 13 samples from 10 patients with suspected scrub typhus were collected. Metagenomic next-generation sequencing (mNGS) and other diagnostic methods (including serology using Weil–Felix reaction and indirect immunofluorescence test (IIFT) for scrub typhus and respiratory tract profile IgM as well as culture for routine bacteria) were used to identify the pathogens in this study.

Results: The results of mNGS were all positive, with mapped reads of *O. tsutsugamushi* ranging from 1 to 460. Eight patients (80%) were diagnosed as scrub typhus. The other two were diagnosed as suspected scrub typhus due to the limited number of reads of the pathogen (one and two, respectively). According to clinical evidences, nine of the 10 patients were finally diagnosed as scrub typhus, except for patient 9 (suspected scrub typhus by mNGS with one specific reads of the pathogen) diagnosed as acute exacerbation of chronic obstructive pulmonary disease. For the five scrub typhus patients without typical eschar, mNGS gave all positive results (4–460 specific reads). For other methods, only Weil–Felix reaction of one patient detected the pathogen. In addition, the respiratory tract profile (IgM) detected various pathogens, but all were confirmed to be false positive.

Conclusions: mNGS performed better than conventional clinical methods to early diagnose scrub typhus. This approach can be routinely carried out for early and precise diagnosis in clinical infections, especially for those hard to be identified by traditional diagnostic methods.

Keywords: scrub typhus, *Orientia tsutsugamushi*, early diagnosis, metagenomic next-generation sequencing, eschars

INTRODUCTION

Scrub typhus, also known as tsutsugamushi disease, is an acute febrile illness caused by *Orientia tsutsugamushi* (also referred to as *Rickettsia tsutsugamushi* or *Rickettsia orientalis*) (1). The pathogen is a kind of natural obligate intracellular parasite, which can be transmitted to humans by the bite of larval stage of certain kinds of trombiculid mites known as chiggers (2, 3). The disease was first described in Japan and estimated to have one million global cases per year, which seriously influenced the public health of Southeast Asia (3–5). Scrub typhus occurs seasonally, and most infections happen from July to August and from October to November in China (6). During 2006–2017, approximately 4,500 scrub typhus cases were diagnosed in Jiangxi Province, and more than 120,000 patients with scrub typhus were found in 30 provinces in China (7).

The clinical manifestations of this disease include fever, chills, skin rash, and eschar at the bite site. Patients with scrub typhus may also experience headache, myalgia, cough, generalized lymphadenopathy, nausea, vomiting, and abdominal pain (8). In severe cases, it may cause many complications of the lungs, brain, kidney, meninges, and heart, such as acute respiratory distress syndrome, acute kidney failure, encephalitis, gastrointestinal bleeding, meningitis, myocarditis, and pneumonia (9–11). The median mortality of scrub typhus is 6% in untreated patients and 1.45% in treated patients (12, 13). Its mortality can be much higher due to the complications in severe cases. For example, it can reach 14% in brain infections and 24% in patients with multiple organ dysfunction (13, 14). However, the early diagnosis of this infection is difficult, especially in patients without characteristic eschar. In fact, the presence of eschar ranges from 7 to 80% in different populations (15). Other rickettsial diseases may also cause eschar on body of patients, for example, Rocky Mountain spotted fever (16). The scrub typhus is usually misdiagnosed, due to its similar early symptoms to other acute febrile diseases. It needs a series of laborious techniques such as serology, biopsy, culture, and PCR to diagnose, however, all of which are insufficient in the diagnosis (17). For example, Weil–Felix reaction is fast and relatively easy to carry, but the results are always unfaithful (18). The cultural method is dangerous and is not available in most laboratories, which even takes an average of 4 weeks for identification (19). 16S rRNA and groEL gene real-time PCR performs high specificity, but the sensitivity has been reported to be from 45 to 82% (20).

Metagenomic next-generation sequencing (mNGS) is an unbiased approach and, theoretically, it detects all possible pathogens in one clinical sample. It is especially suitable for the diagnosis of rare, novel, and atypical etiologies of complicated infectious diseases (21). Compared to the routine diagnostic methods, mNGS always costs less time (<24 h) and can determine millions of base pairs untargeted sequences in a single experiment (22). As a rapid microbiological diagnostic method for infectious diseases, mNGS had been recently used in clinical practice due to its high efficiency, sensitivity, and cost-effectiveness (21). mNGS had also been introduced in the diagnosis of scrub typhus (23, 24).

In this study, blood and cerebrospinal fluid (CSF) samples of 10 patients with suspected scrub typhus were collected. mNGS and traditional test method were used for the early identification of the pathogen. We aim to evaluate the effectiveness of mNGS for the diagnosis of scrub typhus.

MATERIALS AND METHODS

Patient Recruitment

A total of 10 patients with suspected scrub typhus were admitted to Ganzhou People's Hospital during May 2018 to March 2021. Most of the patients were farmers. Their symptoms included repeated fever (from 3 days to half a month), generalized lymphadenopathy, skin rash and eschar, abdominal pain, myalgia, headache, nausea, vomiting, and blurred vision. Most patients (9 out of 10) suffered repeated fever, and four patients had characteristic eschar. Blood tests were conducted immediately after hospitalization. Complete blood count including leukocytes, neutrophils, and lymphocytes counts were assessed. Liver and kidney function tests and coagulation tests of all patients were measured. The patients also received chest and abdomen computed tomography (CT) scanning and abdominal Color Doppler. Five patients received lumbar punctures, and Pandy's tests of all CSF samples were performed.

Clinical Diagnostics

The body fluid samples of the suspected scrub typhus patients were collected, including blood, CSF, and pleural fluid. Widal reaction and Weil–Felix reaction were used to identify the presence of typhoid and rickettsia. Smear and blood cultures were also used for the detection of probable pathogens. The indirect immunofluorescence test (IIFT) of respiratory tract profile (IgM) (EUROIMMUN, China), including respiratory syncytial virus, adenovirus, influenza A virus, influenza B virus, parainfluenza virus, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, anti-Coxsackievirus A type, anti-Coxsackievirus B type, Echo virus, and *Legionella pneumophila* were looked for. The suspected human cytomegalovirus, herpes simplex virus, and Epstein–Barr virus, which can cause similar clinical symptoms, were also analyzed using PCR in some patients.

mNGS and qPCR Detection

A total of seven blood, five CSF samples, and one pleural fluid sample were collected for mNGS and qPCR analysis. Blood samples were collected and stored at 4°C. CSF and pleural fluid samples were drawn from patients and stored at −80°C. The samples were transmitted on dry ice for PACEseq mNGS detection (Hugobiotech, Beijing, China). QIAamp DNA Micro Kit (QIAGEN) was used to extract DNA according to its manual. DNA libraries were built using QIAseq Ultralow Input Library Kit (Illumina) based on the instructions of the manufacturer. We then analyzed the quality of all libraries using Qubit (Thermo Fisher) and Agilent 2,100 Bioanalyzer (Agilent Technologies). Libraries with high quality were finally sequenced on Nextseq 550 platform (Illumina). Reads <35 bp or with low quality (Q < 30) were removed from the raw data. All human host DNA were filtered out by alignment to human reference database. The

clean reads were finally aligned to Microbial Genome Databases (ftp://ftp.ncbi.nlm.nih.gov/genomes/) using software BWA. The probable pathogens and their mapped reads numbers were reported. qPCR was also performed using *O. tsutsugamushi* real-time PCR Kit (Liferiver) based on its manual (25) on Applied Biosystems 7,500 Real-Time PCR System (ABI). The detection results between qPCR and mNGS were compared using the Chi-square test. The value of mNGS in early diagnosing scrub typhus was evaluated.

RESULTS

Patient Characteristics

Demographic features and baseline data of the patients are shown in **Supplementary Table 1**. Of the 10 patients, 6 were women and 4 were men. The mean age was 64.8 years (44–82 years). Seven out of 10 patients were farmers. For the other three patients, one was only known to live in the countryside, the other two had no relevant information. Half of the patients experienced underlying diseases, including diabetes, atrial fibrillation, thyroid nodules, chronic bronchitis, and coronary heart disease. Fever was the main symptom, which ranged from 3 to 15 days in 9 patients. The concomitant symptoms included generalized lymphadenopathy, abdominal pain, myalgia, headache, nausea, vomiting, and blurred vision. Iconography studies showed hepatosplenomegaly in six patients and pulmonary infections in eight patients. Dysfunctions of multiple organs were found in these patients, including lungs (three respiratory failures), heart (four heart failures), liver (eight liver damages), and kidneys (one kidney damage). Pandy's tests of CSF found that two out of seven patients presented positive, indicating infections in meninges. The main symptoms of the patients were similar to those in acute febrile diseases. Only four patients (40%) had ulcer or eschar on body, which helped their diagnosis as suspected scrub typhus. The clinical characteristics of patients are shown in **Table 1**.

Conventional Detection Results

Widal reaction and Weil–Felix reaction were employed for seven patients. Only one was detected to be scrub typhus with a four-fold rise in titers by Weil–Felix reaction. The sensitivity of Weil–Felix reaction was 11.1%. To detect other probable pathogens, blood culture and smear of nine patients were performed. The results were all negative. The respiratory tract profile (IgM) was used in five patients to detect probable respiratory tract infections. Only one patient (patient 3) was negative, the other four provided positive results. The detected pathogens included the respiratory syncytial virus and parainfluenza virus in patient 1, *Legionella* in patient 4, *Mycoplasma pneumoniae* in patient 6, and influenza A virus and parainfluenza virus in patient 9. However, the following nucleic acid tests of throat swab indicated that these results were all false positive. In addition, PCR analysis of human cytomegalovirus, herpes simplex virus, and Epstein–Barr virus in three patients showed all negative. The detailed results of conventional detection are shown in **Table 2**.

mNGS and qPCR Results

Thirteen samples of the 10 patients were used for mNGS, including blood, CSF, and pleural fluid. The unique reads of *O. tsutsugamushi* were detected in all samples (**Supplementary material**). Eight patients were diagnosed as scrub typhus by mNGS. The number of unique sequences of *O. tsutsugamushi* ranged from 4 to 460. Two patients were diagnosed as suspected scrub typhus due to the small number of *O. tsutsugamushi* reads (one and two reads, respectively) with medium confidence. The sensitivity of mNGS was 100%. qPCR was subsequently used to confirm the results of mNGS. Only one sample (the pleural fluid of patient 8) was detected to be positive (**Figure 1**). The sensitivity of qPCR was 11.1%. Interestingly, this sample was also detected with the most pathogen reads (460 reads) by mNGS. The mNGS and qPCR results are shown in **Table 3** and **Supplementary Figures**.

mNGS results of different sample types were also compared in this study. No significant difference of specific reads of the pathogen was found in mNGS results between blood samples (7) and CSF samples (5) ($p > 0.05$). Of patient 2 and patient 7, the blood and CSF samples were collected at the same time for mNGS. The number of pathogen reads detected in patient 2 from blood and CSF samples were 4 and 10, respectively. And for patient 7, there were 50 and 24 specific reads of the pathogen detected in blood and CSF samples, respectively. It seemed that there was no significant difference between the samples of blood and CSF for mNGS of this pathogen. The

TABLE 1 | The clinical characteristics of the patients of this study.

Patient	Main symptoms	Skin rash and eschar	Hepatosplenomegaly	Swollen lymph nodes
Patient 1	Repeated fever (∼10 days)	No	Yes	NA
Patient 2	Fever, abdominal pain, and palpitations (3 days)	No	No	Neck
Patient 3	Repeated fever (5 days), and myalgia (∼10 days)	Yes	Yes	Neck
Patient 4	Repeated fever (7 days)	Yes	No	Right groin
Patient 5	Fever (10 days)	Yes	Fatty liver	NA
Patient 6	Fever (15 days)	Yes	Splenomegaly	Double armpit
Patient 7	Fever (7 days), dizziness, nausea, and vomiting	No	Splenomegaly	Double armpit
Patient 8	Fever (∼10 days), and abdominal pain (one month)	No	Splenomegaly	NA
Patient 9	limbs fatigue (7 days), and blurred vision	No	Liver cyst	Lungs, retroperitoneum, and mesenteric root
Patient 10	Fever, and headache (9 days)	No	Yes	NA

TABLE 2 | The results of diagnosis by different methods.

Patient	Widal reaction	Weil Felix reaction	Blood cultures and smear	Respiratory pathogen spectrum
Patient 1	Negative	Negative	Negative	Respiratory syncytial virus, and parainfluenza virus
Patient 2	Negative	Negative	Negative	NA
Patient 3	Negative	Negative	Negative	Negative
Patient 4	Negative	Negative	Negative	*Legionella*
Patient 5	NA	NA	Negative	NA
Patient 6	Negative	Negative	Negative	*Mycoplasma pneumoniae*
Patient 7	NA	NA	Negative	NA
Patient 8	Negative	Negative	Negative	NA
Patient 9	NA	NA	NA	Influenza A virus
Patient 10	Negative	Positive	Negative	NA

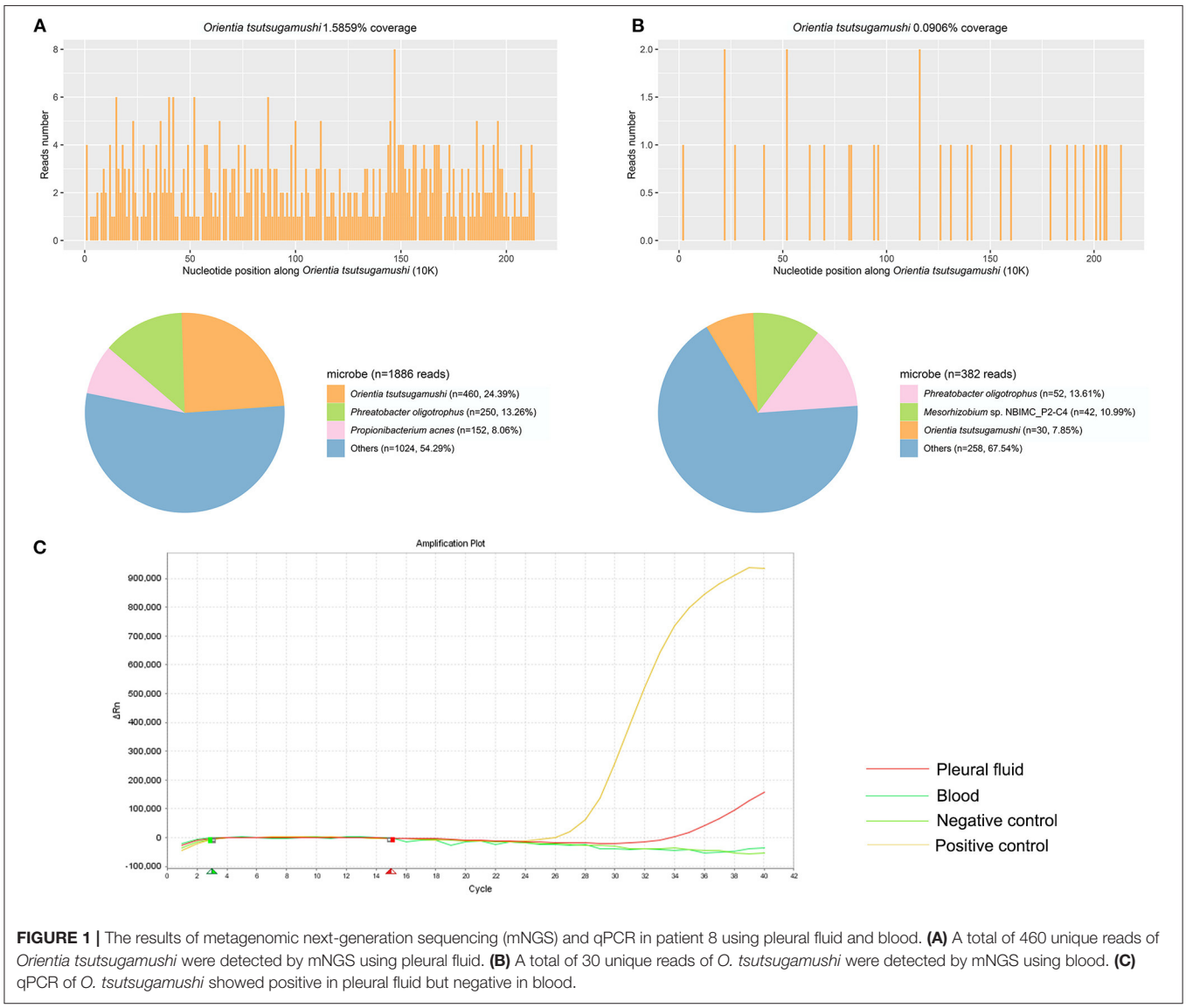


FIGURE 1 | The results of metagenomic next-generation sequencing (mNGS) and qPCR in patient 8 using pleural fluid and blood. **(A)** A total of 460 unique reads of *Orientia tsutsugamushi* were detected by mNGS using pleural fluid. **(B)** A total of 30 unique reads of *O. tsutsugamushi* were detected by mNGS using blood. **(C)** qPCR of *O. tsutsugamushi* showed positive in pleural fluid but negative in blood.

TABLE 3 | The types of samples and the results of mNGS and qPCR.

Patient	Type of sample	Number of reads by mNGS	qPCR results
Patient 1	CSF	10	Negative
Patient 2	CSF	10	Negative
	Blood	4	Negative
Patient 3	Blood	4	Negative
Patient 4	Blood	32	Negative
Patient 5	Blood	6	Negative
Patient 6	Blood	2	Negative
Patient 7	CSF	24	Negative
	Blood	50	Negative
Patient 8	Pleural fluid	460	Positive
	Blood	30	Negative
Patient 9	CSF	1	Negative
Patient 10	CSF	66	Negative

CSF, cerebrospinal fluid; mNGS, metagenomic next-generation sequencing.

efficiencies of mNGS using blood and pleural fluid samples were also compared in patient 8. A total of 460 pathogen reads were detected using pleural fluid sample, while only 30 were found using blood sample.

Of the 10 patients, 9 were finally diagnosed as scrub typhus. While patient 9 (only one unique read of *O. tsutsugamushi*) was identified to be acute exacerbation of chronic obstructive pulmonary disease with coronary heart disease, chronic heart failure (NYHA level 3), silicosis, and old tuberculosis. Patient 6 was also diagnosed as suspected scrub typhus by mNGS with media confidence due to its low detection of pathogen reads, but there was eschar on his body. He was also confirmed to be scrub typhus by final clinical evidences. In addition, except one patient who did not suffer scrub typhus, five scrub typhus patients without typical eschar were all confirmed by mNGS, which was performed with high sensitivity and specificity in the early diagnosis of scrub typhus.

Upon admission, the patients with typical eschar were treated with doxycycline directly. While for the patients without eschar, bacterial infections were first considered. Cephalosporins were used during the treatments, but the effects were limited. When mNGS results came out, their treatments were adjusted immediately to doxycycline. The symptoms of the patients improved gradually, and their temperatures returned to normal in 1–3 days. All the patients fully recovered and most of them were discharged in 7–10 days.

DISCUSSION

In this study, we enrolled 10 patients with suspected scrub typhus, nine of whom were finally diagnosed as this disease. Concomitant pneumonia, dysfunctions of multiple organs, and encephalitis were found in these patients, of which pneumonia was the most common one. Scrub typhus is widespread in China and has become a burden on public health and economy. The deteriorated infection can also cause multiple-organ failure and even death. The mortality of scrub typhus in severe cases or due to improper treatment or misdiagnosis can reach 30–70% (12). Therefore, a better method of timely distinction is always needed.

Weil–Felix is the simplest and fastest serological test, which is often used to detect all kinds of rickettsial diseases. However, the result is notoriously unreliable due to its low positive rate in the early diagnosis (26). In this study, Weil–Felix test was used for seven patients and only one was found positive. The sensitivity of Weil–Felix reaction was very low. Culture was always applied to identify pathogens of different infections, but it had limitations for some microorganisms. The culture of *O. tsutsugamushi* was dangerous and not conducted in this study. The respiratory tract profile (IgM) always gave false-positive results in this study. Considering the untypical symptoms of scrub typhus, this disease was hard to be early diagnosed in older patients and those with no obvious eschar on body, especially when the patients had underlying diseases. The scrub typhus could be always misdiagnosed to other acute febrile diseases, such as malaria, leptospirosis, typhoid, and dengue fever (27, 28). Notably, in our study, 6 out of 10 patients were without eschar and could not be diagnosed by routine clinical methods. In addition, a diagnosis of simple pulmonary infection might have happened due to the presence of *Legionella* and *Mycoplasma pneumoniae* by the respiratory tract profile (IgM) combined with iconography evidences in two patients. Fortunately, mNGS provided faithful results and helped the diagnosis of these patients. The patients received timely specific treatment and were finally discharged with a good prognosis.

qPCR was previously considered as a good tool for the detection of scrub typhus and was usually used in the diagnosis of this disease (20). However, in this study, qPCR performed with a quite low detection rate, with only the pleural fluid sample of patient 8 showing positive with the cycle threshold (CT) at 34.2 cycles. Nevertheless, her blood sample displayed a negative qPCR result. Interestingly, we found that the pleural fluid sample with a positive qPCR result also had the largest number of pathogen reads by mNGS among all the 13 samples. All other samples that were positive with mNGS test were negative for the qPCR test, indicating a better level of detection of mNGS than qPCR methods. The positive detection rate of Weil–Felix reaction in our study was also quite low. One probable reason for the significantly lower sensitivity of qPCR and Weil–Felix reaction than mNGS ($p < 0.01$) was that the early detection was performed within 2 weeks (3–15 days) of the onset of symptoms. The patients only suffered mild symptoms of scrub typhus, and the content of the pathogen in the samples might be not enough to get positive results. For example, Weil–Felix reaction only detected a titer of 1:20 in patient 4.

Compared with the conventional methods, mNGS showed a higher sensitivity. In this study, all the samples of the patients were positive, with the number of *O. tsutsugamushi* reads ranging from 1 to 460. The results of eight patients showed high confidence level, which indicated scrub typhus. The patient with two unique reads of the pathogen was also confirmed as scrub typhus due to the eschar on body and final clinical evidences. Only the patient with one specific read was identified to be other disease. mNGS was successfully applied to the diagnosis

of clinical cases, including immunocompromised, meningitis, pneumonia, and sepsis (29–31). It demonstrated a better ability in the diagnosis of infections by rare, novel, and complex pathogens (21).

Different types of samples including CSF, blood, bronchoalveolar lavage fluid, tissue, pleural fluid, throat swab, and other body fluid were used for mNGS, among which, CSF and blood samples were more common. In this study, we compared the performance of blood, CSF, and pleural fluid samples for mNGS in the same patient. In patient 2, the detected number of reads of *O. tsutsugamushi* using CSF sample was higher than blood sample. While in patient 7, the result was opposite. We did not see any difference of mNGS by CSF and blood samples. However, in patient 8, pleural fluid sample performed better than blood sample. More samples of different types from the same patients are needed for further analysis.

There are some limitations. The sample size of this study is small. The disease is not common. Only 4,500 scrub typhus cases were diagnosed in Jiangxi Province during 2006–2017. Its symptoms are atypical, and most infected patients are farmers, who prefer to receive treatments in local hospitals. This may cause a large amount of misdiagnoses and miss diagnoses. In addition, due to the relatively high price of mNGS, most patients did not receive mNGS detection. Moreover, although conventional diagnostic methods, including serology and qPCR were detected, the results were limited. Fortunately, when considering the presence of the typical symptom (eschar) and positive results from traditional methods of this disease, six (four with eschar, one only with positive Weil–Felix result, and one only with positive qPCR result) of the nine patients could be diagnosed with scrub typhus, which further confirmed the mNGS detection.

CONCLUSIONS

The precise and timely treatment is important for the survival of patients with scrub typhus. But the early diagnosis of this infection is difficult, especially for patients without eschar. Misdiagnosis and miss diagnosis of the disease often occur due to its similar symptoms with other acute febrile illnesses. In this study, mNGS was introduced for the early diagnosis, which performed faster and more sensitive than the other clinical diagnosis methods. Thus, mNGS can be a promising technology for early and precise diagnosis in clinical infections, for scrub typhus, and for those hard to be identified by traditional diagnosis methods.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Review Committee of Ganzhou People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XL, YZ, and ZLu designed and drafted the manuscript. XL and JZ involved in the clinical care and management of the patients. ZLo and HX analyzed the mNGS data. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

ACKNOWLEDGMENTS

We thank Fuhua Peng of Department of Neurology, The Third Affiliated Hospital of Sun Yat-Sen University for the help of our paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.755228/full#supplementary-material

Supplementary Figures 1–11 | The mNGS results of patients 1–10 are shown in **Supplementary Figures 1–10**. The qPCR results were shown in **Supplementary Figure 11**.

Supplementary Table 1 | Demographic features and baseline data of the patients in this study are shown in **Supplementary Table 1**. The detailed information of the detected reads of *Orientia tsutsugamushi* are shown in **Supplementary Tables**.

REFERENCES

1. Salje J. Orientia tsutsugamushi: a neglected but fascinating obligate intracellular bacterial pathogen. *PLoS Pathog.* (2017) 13:e1006657. doi: 10.1371/journal.ppat.1006657
2. Watt G, Parola P. Scrub typhus and tropical rickettsioses. *Curr Opin Infect Dis.* (2003) 16:429–36. doi: 10.1097/00001432-200310000-00009
3. Kelly DJ, Fuerst PA, Ching WM, Richards AL. Scrub typhus: the geographic distribution of phenotypic and genotypic variants of Orientia tsutsugamushi. *Clin Infect Dis.* (2009) 48:S203–30. doi: 10.1086/596576
4. Xu G, Walker DH, Jupiter D, Melby PC, Arcari CM. A review of the global epidemiology of scrub typhus. *PLoS Negl Trop Dis.* (2017) 11:e0006062. doi: 10.1371/journal.pntd.0006062
5. Palm TA. Some Account Of A Disease Called "Shima-Mushi," or "Island-Insect Disease," by the natives of Japan; peculiar, it is believed, to that country, and hitherto not described. *Edinb Med J.* (1878) 24:128–32.
6. Ren J, Sun J, Wang Z, Ling F, Shi X, Zhang R, et al. Re-emergence of scrub typhus in Zhejiang Province, southern China: a 45-year population-based surveillance study. *Travel Med Infect Dis.* (2019). doi: 10.1016/j.tmaid.2019.05.013

7. Yue Y, Ren D, Liu X, Wang Y, Liu Q, Li G. Spatio-temporal patterns of scrub typhus in mainland China, 2006-2017. *PLoS Negl Trop Dis.* (2019) 13:e0007916. doi: 10.1371/journal.pntd.0007916
8. Woodruff R. The oxford textbook of palliative medicine. *J Pain Palliat Care Pharmacother.* (2016) 30:335–6. doi: 10.1080/15360288.2016.1246509
9. Griffith M, Peter JV, Karthik G, Ramakrishna K, Prakash JA, Kalki RC, et al. Profile of organ dysfunction and predictors of mortality in severe scrub typhus infection requiring intensive care admission. *Indian J Crit Care Med.* (2014) 18:497–502. doi: 10.4103/0972-5229.138145
10. Rajapakse S, Weeratunga P, Sivayoganathan S, Fernando SD. Clinical manifestations of scrub typhus. *Trans R Soc Trop Med Hyg.* (2017) 111:43–54. doi: 10.1093/trstmh/trx017
11. Mahajan SK, Mahajan SK. Neuropsychiatric manifestations of scrub typhus. *J Neurosci Rural Pract.* (2017) 8:421–6. doi: 10.4103/jnrp.jnrp_44_17
12. Taylor AJ, Paris DH, Newton PN. A systematic review of mortality from untreated scrub typhus (Orientia tsutsugamushi). *PLoS Negl Trop Dis.* (2015) 9:e0003971. doi: 10.1371/journal.pntd.0003971
13. Bonell A, Lubell Y, Newton PN, Crump JA, Paris DH. Estimating the burden of scrub typhus: a systematic review. *PLoS Negl Trop Dis.* (2017) 11:e0005838. doi: 10.1371/journal.pntd.0005838
14. Peter JV, Sudarsan TI, Prakash JA, Varghese GM. Severe scrub typhus infection: clinical features, diagnostic challenges and management. *World J Crit Care Med.* (2015) 4:244–50. doi: 10.5492/wjccm.v4.i3.244
15. El Sayed I, Liu Q, Wee I, Hine P. Antibiotics for treating scrub typhus. *Cochrane Database Syst Rev.* (2018) 9:CD002150. doi: 10.1002/14651858.CD002150.pub2
16. Kelman P, Thompson CW, Hynes W, Bergman C, Lenahan C, Brenner JS, et al. Rickettsia parkeri infections diagnosed by eschar biopsy, Virginia, USA. *Infection.* (2018) 46:559–63. doi: 10.1007/s15010-018-1120-x
17. Abdad MY, Abou Abdallah R, Fournier PE, Stenos J, Vasoo S. A concise review of the epidemiology and diagnostics of rickettsioses: rickettsia and orientia spp. *J Clin Microbiol.* (2018) 56:17. doi: 10.1128/JCM.01728-17
18. Lim C, Blacksell SD, Laongnualpanich A, Kantipong P, Day NP, Paris DH, et al. Optimal cutoff titers for indirect immunofluorescence assay for diagnosis of scrub typhus. *J Clin Microbiol.* (2015) 53:3663–6. doi: 10.1128/JCM.01680-15
19. Koh GC, Maude RJ, Paris DH, Newton PN, Blacksell SD. Diagnosis of scrub typhus. *Am J Trop Med Hyg.* (2010) 82:368–70. doi: 10.4269/ajtmh.2010.09-0233
20. Paris DH, Blacksell SD, Newton PN, Day NP. Simple, rapid and sensitive detection of Orientia tsutsugamushi by loop-isothermal DNA amplification. *Trans R Soc Trop Med Hyg.* (2008) 102:1239–46. doi: 10.1016/j.trstmh.2008.04.040
21. Goldberg B, Sichtig H, Geyer C, Ledeboer N, Weinstock GM. Making the leap from research laboratory to clinic: challenges and opportunities for next-generation sequencing in infectious disease diagnostics. *MBio.* (2015) 6:e01888–15. doi: 10.1128/mBio.01888-15
22. Forbes JD, Knox NC, Ronholm J, Pagotto F, Reimer A. Metagenomics: the next culture-independent game changer. *Front Microbiol.* (2017) 8:1069. doi: 10.3389/fmicb.2017.01069
23. Wu J, Wu Y, Huang M. Metagenomic next-generation sequencing helped diagnose scrub typhus without eschar: A Case report. *Int J Infect Dis.* (2020) 90:1–4. doi: 10.1016/j.ijid.2019.10.020
24. Chen J, Zheng XD Dai QH, Hong WL Li YP, Chen R, et al. Diagnosis of severe scrub typhus infection by next-generation sequencing:a case report. *BMC Infect Dis.* (2020) 20:270. doi: 10.1186/s12879-020-04991-y
25. Sonthayanon P, Chierakul W, Wuthiekanun V, Phimda K, Pukrittayakamee S, Day NP, et al. Association of high Orientia tsutsugamushi DNA loads with disease of greater severity in adults with scrub typhus. *J Clin Microbiol.* (2009) 47:430–4. doi: 10.1128/JCM.01927-08
26. Pradutkanchana J, Silpapojakul K, Paxton H, Pradutkanchana S, Kelly DJ, Strickman D. Comparative evaluation of four serodiagnostic tests for scrub typhus in Thailand. *Trans R Soc Trop Med Hyg.* (1997) 91:425–8. doi: 10.1016/S0035-9203(97)90266-2
27. Morch K, Manoharan A, Chandy S, Chacko N, Alvarez-Uria G, Patil S, et al. Acute undifferentiated fever in India: a multicentre study of aetiology and diagnostic accuracy. *BMC Infect Dis.* (2017) 17:665. doi: 10.1186/s12879-017-2764-3
28. Wangrangsimakul T, Althaus T, Mukaka M, Kantipong P, Wuthiekanun V, Chierakul W, et al. Causes of acute undifferentiated fever and the utility of biomarkers in Chiangrai, northern Thailand. *PLoS Negl Trop Dis.* (2018) 12:e0006477. doi: 10.1371/journal.pntd.0006477
29. Blauwkamp TA, Thair S, Rosen MJ, Blair L, Lindner MS, Vilfan ID, et al. Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease. *Nat Microbiol.* (2019) 4:663–74. doi: 10.1038/s41564-018-0349-6
30. Langelier C, Kalantar KL, Moazed F, Wilson MR, Crawford ED, Deiss T, et al. Integrating host response and unbiased microbe detection for lower respiratory tract infection diagnosis in critically ill adults. *Proc Natl Acad Sci U S A.* (2018) 115:E12353–E62. doi: 10.1073/pnas.1809700115
31. Wilson MR, O'Donovan BD, Gelfand JM, Sample HA, Chow FC, Betjemann JP, et al. Chronic meningitis investigated via metagenomic next-generation sequencing. *JAMA Neurol.* (2018) 75:947–55. doi: 10.1001/jamaneurol.2018.0463

Conflict of Interest: YZ, ZL, and HX are employed by Hugobiotech Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Liu, Zhang, Zhang, Lou, Xia and Lu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 24 November 2021
doi: 10.3389/fmed.2021.773145

Case Report: Fascioliasis Hepatica Precisely Diagnosed by Metagenomic Next-Generation Sequencing and Treated With Albendazole

Yaling Zhang[1], *Huan Xu*[2], *Yi Liu*[1], *Juan Kang*[1], *Hairu Chen*[2], *Zhiyi Wang*[1*†] and *Dachuan Cai*[1*†]

[1] Department of Infectious Diseases, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China, [2] Vision Medicals Center for Infection Diseases, Guangzhou, China

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Majid Fasihi Harandi,
Kerman University of Medical Sciences, Iran
Lin Chen,
Jinhua Central Hospital, China

***Correspondence:**
Zhiyi Wang
13808341817@163.com
Dachuan Cai
cqmucdc@cqmu.edu.cn

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 09 September 2021
Accepted: 26 October 2021
Published: 24 November 2021

Citation:
Zhang Y, Xu H, Liu Y, Kang J, Chen H, Wang Z and Cai D (2021) Case Report: Fascioliasis Hepatica Precisely Diagnosed by Metagenomic Next-Generation Sequencing and Treated With Albendazole. Front. Med. 8:773145. doi: 10.3389/fmed.2021.773145

The clinical manifestations of fascioliasis hepatica in humans are unspecific. Traditional diagnosis relies on evidence of live parasites or eggs in the bile or feces. However, due to similar imaging manifestations, they are often misdiagnosed as malignant tumors. Here, we report a case of a 43-year-old woman with fever and space-occupying liver disease. Liver biopsy, parasite-specific antibody screening, and stool testing did not find any pathogens. Therefore, metagenomic next-generation sequencing (mNGS) and routine microbiological examinations were performed. Finally, *Fasciola hepatica* was only identified by mNGS. The body temperature of the patient and the eosinophil count remained normal, and the space-occupying liver lesions were significantly absorbed after more than 7 months of treatment with albendazole. The details of this case highlight the timely use of mNGS to identify parasites and judge therapeutic effects after treatment, providing important help for clinical decision-making.

Keywords: case report, fascioliasis hepatica, metagenomic next-generation sequencing (mNGS), precision treatment, albendazole

INTRODUCTION

Fascioliasis hepatica is an infection caused by a trematode of the liver, *Fasciola hepatica*, that particularly affects sheep, goats, and cattle (1). The distribution of humans infected with *F. hepatica* depends on the intermediate hosts. Due to the habitat requirements of the intermediate hosts, the distribution may be patchy (2, 3). France, the United Kingdom, the former Soviet Union, Cuba, and other countries have reported numerous cases. However, the number of patients in China is less (3), mainly scattered in the Liaoning, Jining, Heilongjiang, Jiangxi, Yunnan, Guangdong, and Guangxi provinces. More importantly, there are no cases reported in Chongqing until now. The incubation period and clinical manifestations of human fascioliasis are different and are divided into two stages. In the acute phase, the main manifestations are fever, anemia, hepatomegaly, and a significantly elevated blood eosinophils count (3–7). While in the chronic phase, it manifests as jaundice, ascites, and

even spreads to the extrahepatic organs through the bloodstream (8–11). The clinical outcomes of critically ill patients are always poor. So, the confirmation of the diagnosis is necessary. In non-endemic areas, diagnosis of fascioliasis hepatica can be difficult because the disease is not often encountered and the symptoms may be confused with other hepatic or biliary disorders, such as liver abscess, liver tuberculosis, and so on. Additionally, the radiological findings in *F. hepatic* infection may present with multiple nodular lesions, subcapsular low density, and solitary nodular lesions, etc. resulting in dilemma or confusion. Presently, metagenomic next-generation sequencing (mNGS) based on high-throughput sequencing has been widely used to screen for broad-spectrum pathogens, including *Mycobacterium Tuberculosis*, *Toxoplasma gondii*, and other atypical pathogens. Here, we report the first case of fascioliasis hepatica identified by mNGS in Chongqing, China.

CASE DESCRIPTION

A 43-year-old female, whose height was 160 cm and weight 46 kg [body mass index (BMI) 18.0], was admitted due to 2 months of repeated fever and space-occupying lesions in the liver on May 15, 2020 (day-1). The highest body temperature appeared at night but returned to normal in the morning, with dry cough, shortness of breath, and dull pain in the liver area. The physical examination of the whole body showed no obvious positive signs, there were no abdominal tenderness and percussion pain, and no enlarged superficial lymph nodes were palpated. So, the patient still could live and work properly. She lived in Chongqing city, whose economic situation is medium as a furniture sales staff. She visited Guangzhou province 6 months ago on business for 2 days. She denied any contact with livestock and eating wild vegetables, raw fish, shrimp, and so on, but drank tap water every day. The local hospital prescribed empirical antimicrobial therapy with azithromycin and Chinese herbal medicines; however, the treatment failed to relieve the symptoms of the patient. Thus, the patient was transferred to our hospital for further evaluation. An outline of the episodes is described in **Figure 1**. Chest CT scan showed no infection in the lungs, while mass-like shadows in the liver were observed on the abdominal CT scan. Routine blood parameters revealed elevated white blood cell and eosinophil count (**Figure 2**).

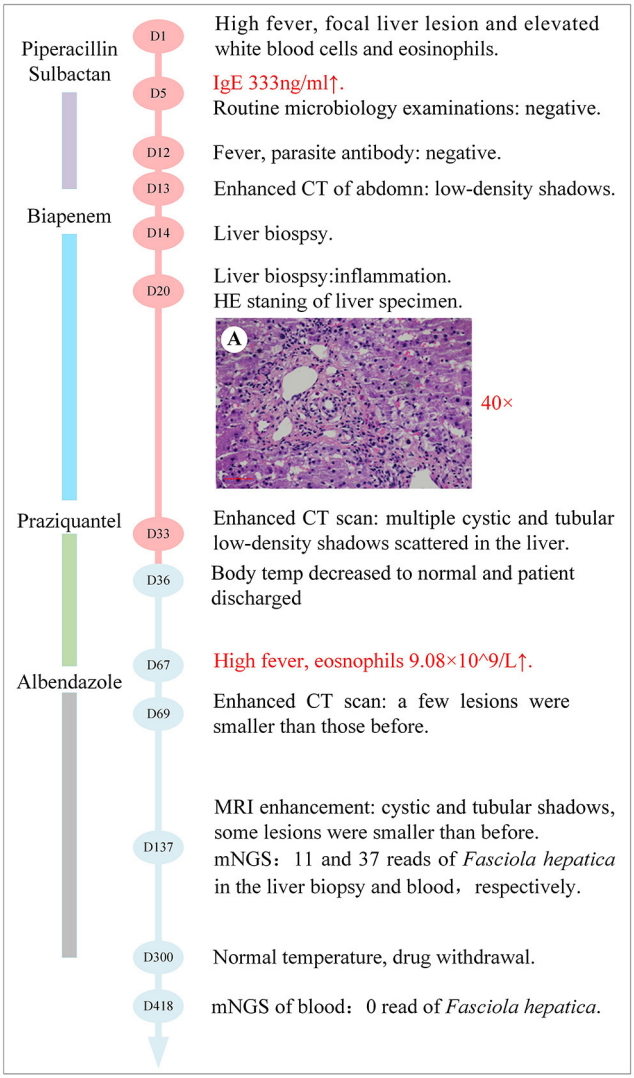


FIGURE 1 | The clinical course of the patient (schematic). Hematoxylin and eosin staining of the liver specimen shows some punctate necrosis on the lobule, apoptotic bodies in the hepatic cord, fatty degeneration of hepatocytes, mild inflammation in the portal area, and eosinophil infiltration in the inflammatory necrosis area.

Enhanced MRI of the upper abdomen showed the increased liver volume, uneven liver parenchyma signal, liver interstitial edema, and accumulated fluid in the abdominal cavity on hospital day 2 (**Figure 3A**). Laboratory tests showed elevated IgE (333 ng/mL), white blood cell, and eosinophil count on hospital day 5. Microbial tests, including blood cultures, stool examination, and stool precipitation, did not detect any pathogens. Eggs were not detected by direct fecal smear and fecal washing precipitation. Due to excessive pharyngeal reflex during gastroduodenal tube placement, two attempts failed to obtain the bile of the patient. The diagnosis of tuberculosis was excluded for the negative results of the chest CT scan, tuberculin skin test, tuberculosis IgG and IgM antibody screening, and T-Spot examination. Meanwhile, ovarian tumors, liver tumors, gastrointestinal tumors, and hematological tumors were also excluded temporarily as the negative results of abdominal enhanced CT and tumor marker detection such as AFP, CA-50, CEA, TAP, CA-125, CA-199, CA-242, and CA-724 and so on. The serum parasite IgG antibody, including schistosome, paragonimiasis, cysticercosis, trichinella spiralis, liver fluke, sparganosis, and hydatid IgG antibody detected by ELISA were negative on hospital day 12. Piperacillin-sulbactam was used as an antibacterial treatment for 10 days from hospital days 4–13.

However, the body temperature of the patient fluctuated several times, and the eosinophil count was not significantly reduced (**Figure 2**). So, an enhanced CT of the lower abdomen was performed on hospital day 13, which showed multiple

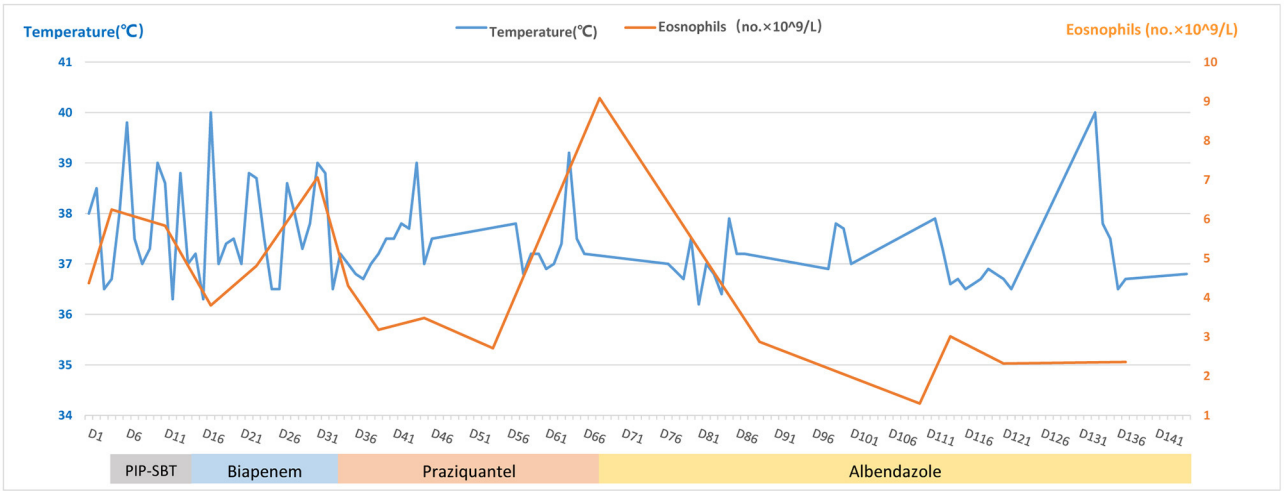


FIGURE 2 | Timeline with relevant data from the episode of care; curves of body temperature and eosinophils count. The blue line shows the body temperature values. The orange line shows the eosinophil counts. Horizontal thick lines show the medications administered. PIP-SBT, Piperacillin-sulbactam.

shadows of abnormal density in the liver and a small amount of effusion, suggesting the possibility of infectious and neoplastic lesions (**Figure 3B**). Because of the high fever, biapenem was adjusted to be used to cover drug-resistant bacteria for more than 2 weeks from hospital day-14 to day-32.

To further confirm the diagnosis, an ultrasound-guided percutaneous liver biopsy was conducted on hospital day-14. The hepatic histopathology reported mild inflammation in the portal area and eosinophil infiltration in the inflammatory necrotic area, with no evidence of malignancy, liver abscess, and parasitic infection on hospital day 20 (**Figure 1**).

As treatment with antibiotics was ineffective (long-term fever, significantly elevated eosinophils count, intrahepatic lesions), the enhanced MRI of the upper abdominal was re-examined on hospital day 33, which indicated no absorption of intrahepatic lesions (**Figure 3C**). Meanwhile, combined with Chongqing epidemiological studies of *Clonorchis sinensis*, parasite infection was suspected and praziquantel was used as treatment. The body temperature decreased, and the patient was discharged on day 36.

During the period of hospital days 33 and 67, the patient still had repeated fever with a maximum temperature of 39°C (**Figure 2**). An enhanced MRI of the upper abdominal showed that the liver shadow had less absorption (**Figure 3D**). So, albendazole was adjusted as the treatment on the follow-up day. Albendazole was orally taken 0.8 g once a day for half a month. As the body temperature of the patient decreased, the dose of albendazole was decreased to 0.6 g for half a month after drug withdraw. On the follow-up day, the dose of albendazole was not adjusted and the patient was prescribed to take albendazole according to taking half a month and stopping for half a month. Firstly, the peak temperature of the body decreased significantly. Secondly, the eosinophil count decreased and ultimately returned to normal. However, there was still a high fever up to 40°C (**Figure 2**), occasionally. Meanwhile, the enhanced MRI of the upper abdominal reexamined on follow-up day 137 showed that there was no significant absorption of the lesions compared with the previous (**Figure 3E**). So far, after a total of 106 days of empirical anti-parasitic treatment, the body temperature and eosinophil count decreased significantly, but the imaging manifestations did not improve significantly.

To identify the pathogen, liver tissue collected on hospital day 14 and peripheral blood on follow-up day 137 were sent for mNGS with the written consent of the patient. The total reads of the liver tissue and blood were 6.93 and 30,379 Mb, respectively. Meanwhile, the reads mapped to microbes in the liver tissue and blood were 934,425, and 29,009. The number of species-specific reads aligning to the Fasciola hepatica genome in the liver biopsy and blood was 3 and 51, respectively (**Figure 4**). Finally, the disease was diagnosed with fascioliasis hepatica with triclabendazole as the first-line drug (4). However, for the sake of the unavailability of triclabendazole under the epidemic of COVID-19 in China and other countries, and the partial effectiveness of albendazole on the fascioliasis hepatica which significantly decreased the body temperature and eosinophil count of the patient, albendazole was used with a prolonged treatment time following the previous way.

Eventually, after receiving treatment of albendazole for more than 7 months, the body temperature and eosinophil count of the patient normalized with no obvious clinical manifestations. So, albendazole was discontinued on day-300. After more than 3 months after drug withdrawal on follow-up day 148, the enhanced MRI of the upper abdominal showed that the intrahepatic low-density shadow was smaller (**Figure 3F**), and the blood mNGS was negative (**Table 1**), suggesting the albendazole treatment was effective.

DISCUSSION

To our knowledge, this is the first case report of fascioliasis hepatica confirmed by mNGS, which played an important

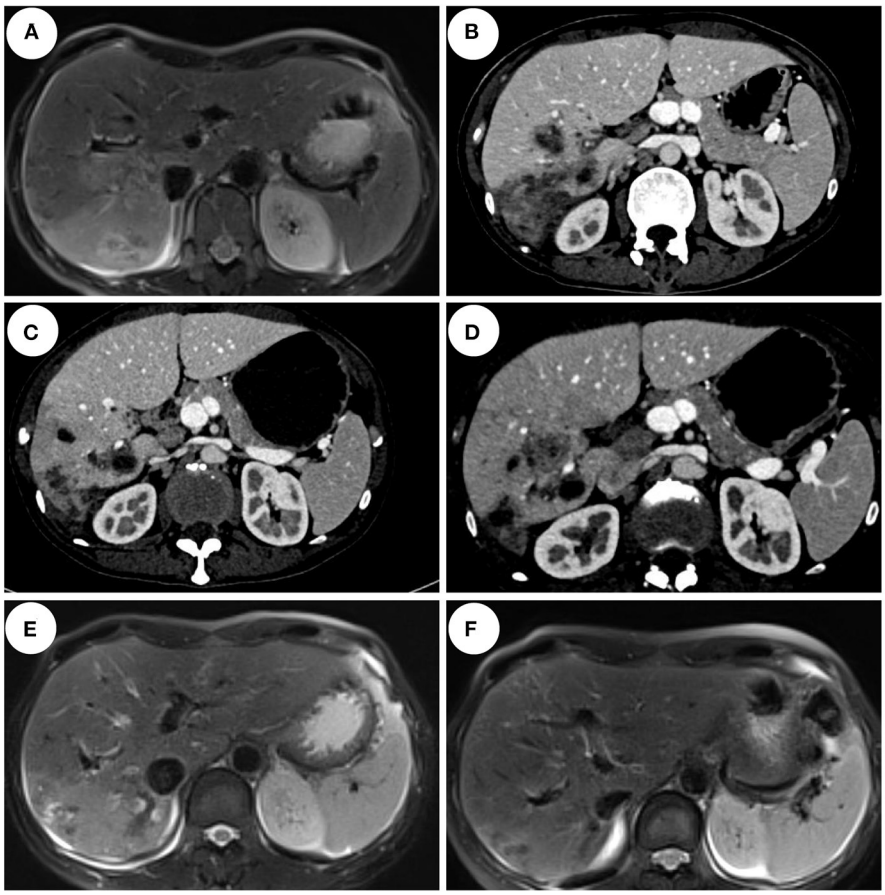


FIGURE 3 | Imaging features of the patient. **(A)** Abdominal MRI enhancement showed that the right lobe of the liver scattered flakes, irregular shape, enhanced uneven enhancement on hospital day 2. **(B)** Abdominal CT enhancement showed liver multiple nodules, mass slightly low-density shadow, edge enhancement on hospital day 13. **(C)** Abdominal CT enhancement showed multiple cystic and tubular low-density shadows scattered in the liver, and some showed edge enhancement on day 33. **(D)** Abdominal CT enhancement showed multiple cystic and tubular low-density shadows scattered in the liver, some showed edge enhancement, and a few lesions were smaller than those before on follow-up day 69. **(E)** Abdominal MRI enhancement showed cystic and tubular shadows in the right lobe of the liver, with irregular shapes and edge enhancement in the enhanced part. Some lesions were smaller than before on follow-up day 137. **(F)** Abdominal MRI enhancement showed the right lobe of the liver scattered flakes, irregular shape, enhanced edge uneven enhancement, lesions significantly reduced on follow-up day 418.

role in this case. Fascioliasis hepatica is often sporadic and has not been reported previously in Chongqing, China. The source of infection is contaminated water containing larva and aquatic plants and is easily ignored by clinicians in medical history collection. The diagnosis of fascioliasis hepatica may be difficult because of the lack of a clear epidemiological history, wide spectrum of differential diagnosis, and low incidence (3). The abnormal laboratory and radiological findings in *F. hepatica* infection may represent hepatitis, liver abscess, malignancy, and infection with parasites such as *Ascariasis* and *Clonorchis sinensis.* More recently, triclabendazole is the drug of choice for its effectiveness against both adult and immature worms (7, 12, 13). As for albendazole, it is partially effective against fascioliasis hepatica. In this case, triclabendazole was not obtained for treatment due to the epidemic of the coronavirus disease 2019 (COVID-19). After treatment with albendazole, the body temperature and eosinophil count of the patient decreased significantly. Albendazole is considered partially effective. In this particular case, both the MRI and CT of the upper abdomen showed abnormal uneven enhancement density shadows that are more likely to be considered as infectious lesions, but tumor lesions cannot be excluded. This may be related to the atypical imaging of fascioliasis hepatica. Such lesions pose a diagnostic dilemma or confusion between malignancy and infection (3). Also, there was no finding of parasites or eggs in the hepatic tissue, which was destroyed when making sliced specimens. Currently, diagnosis is confirmed only by demonstrating live parasites or eggs in the bile or feces, through Fecal Sedimentation, Modified Kato-Katz thick smear method, and Mercury-aldehyde iodine concentration method, but with a relatively low positive rate (4, 5). An examination of the precipitation or centrifugation of the duodenal fluid has a relatively high positive rate (14). Also, demonstrating live parasites or eggs by laparotomy, laparoscopy biopsy, and other histopathologic examination is less used in the clinic for the larger trauma.

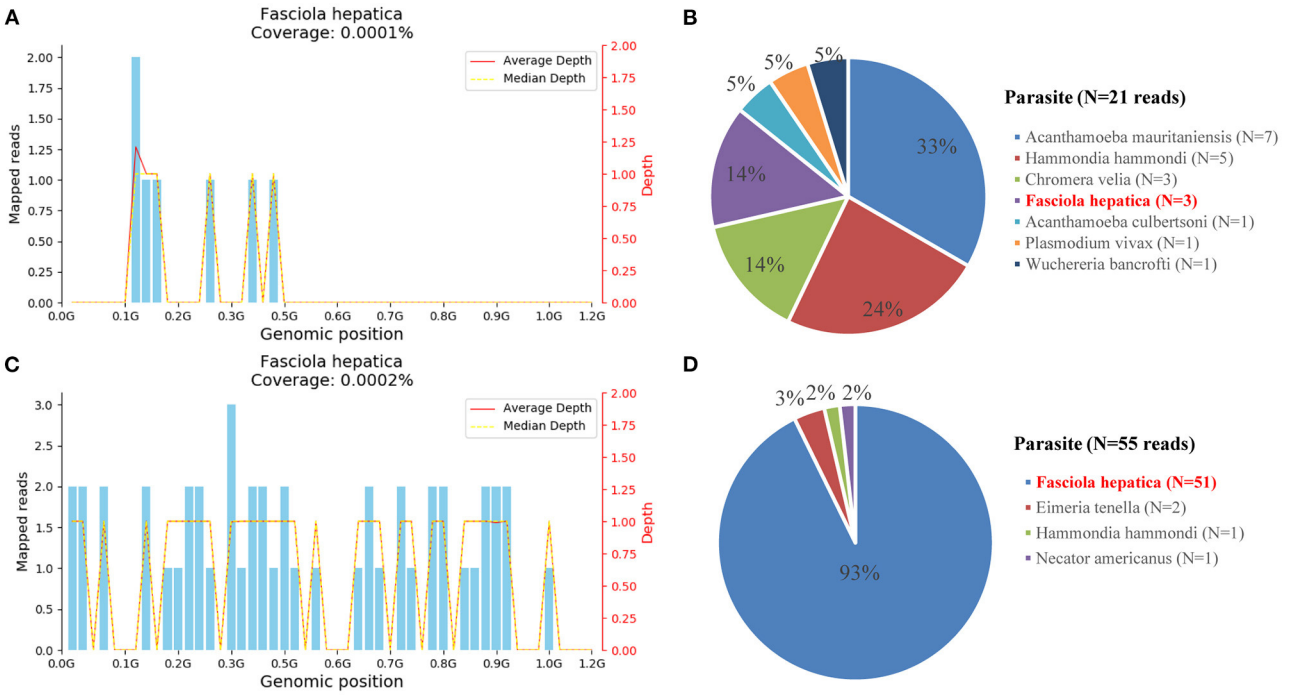


FIGURE 4 | Diagnosis of Fasciola hepatica infection using metagenomic next-generation sequencing (mNGS). **(A)** The reads mapped to the Fasciola hepatica genome with coverage of 0.0001% in the liver tissue. **(B)** The distribution of species-specific reads aligning to the parasite in the liver tissue. **(C)** The reads mapped to the Fasciola hepatica genome with coverage of 0.0002% in the blood. **(D)** The distribution of species-specific reads aligning to the parasite in the blood.

TABLE 1 | Results of metagenomic next-generation sequencing (mNGS) in the liver tissue and blood.

Time of mNGS detection	Sample type	Species-specific reads of *Fasciola hepatica*	Other microbes (Species-specific reads)
Day 138	Liver tissue (collected on day 14)	3	*Methyloversatilis Discipulorum* (389, 801)
	Peripheral blood	51	*Klebsiella variicola* (72) *Torque teno virus* (5)
Day 418	peripheral blood	0	-

However, there is no egg in the stool of the patient in the acute stage. The acute stage of fascioliasis hepatica begins with the slow migration of *F. hepatica* through the liver parenchyma; the mature flukes digest and consume hepatocytes, dig tunnels and caves, and reside in the liver for months, causing hepatitis, chills, fever, right upper abdominal pain, abdominal distension, and loss of appetite (4, 5, 7, 9, 15). While in the chronic stage, the worm moves to the bile duct, and the inner wall is digested, causing bile duct dilatation, cholangitis, cholelithiasis, and liver pain, jaundice, and other discomforts (9–11). But the clinical manifestations are atypical and difficult to diagnose. Therefore, it requires more diagnostic tools to assist in the diagnosis. In the acute stage, serum immunological examination, such as ELISA, indirect fluorescent antibody test (IFA), indirect hemagglutination test (IHA), and counter-current immune-electrophoresis (CIE), can be used as identification tools (16–19). But the serological tests may have similar results with other schistosome infections. Therefore, it is crucial to find early etiological evidence.

Metagenomic next-generation sequencing, with fast detection speed, broad coverage, and high accuracy, is a new type of less biased detection method (20–22), which can detect thousands to millions of nucleotides fragments at once (20, 21, 23, 24). The suspected pathogen can be detected by the mNGS, through the flow of sequencing, data cleaning, error detection, and database comparative analysis. At present, mNGS has been widely used in clinics, including, respiratory tract infection (25), central nervous system infection (26), joint infection (27, 28), hepatic tuberculosis (29), etc. Metagenomic next-generation sequencing has many advantages in identifying hard-to-culture, atypical, parasite, and rare pathogens, such as *Mycobacterium Tuberculosis* and *Toxoplasma gondii*. On the other side, it also has shortcomings such as its high cost, complex process, specimen contamination, etc. *Fasciola hepatica* was detected both in the blood and liver tissues of this case, with corresponding treatment, the clinical symptoms and imaging manifestations were recovered. The peripheral blood was sent for mNGS again after drug withdrawal, and no reads of *Fasciola hepatica* were detected. It proved the effectiveness and specificity of mNGS for the detection of parasites. We believe that the application of mNGS

in pathogen identification will guide the clinical diagnosis and treatment, and then greatly improve the accuracy and benefit patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Written informed consent was obtained from the patient for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ZW and HX contributed to the conception and design of the study. YZ and HX collected the data and wrote the manuscript. HX, YL, JK, and HC analyzed and interpreted the patient data. ZW and DC reviewed the manuscript. All authors read through and approved the final manuscript.

FUNDING

This work was supported by the Natural Science Foundation of Chongqing CSTC (No. cstc2020jcyj-msxmX0015).

ACKNOWLEDGMENTS

We thank Sandeep Kumar Karn for critical language editing.

REFERENCES

1. Mas-Coma S, Valero MA, Bargues MD. Fascioliasis. *Adv Exp Med Biol.* (2019) 1154:71–103. doi: 10.1007/978-3-030-18616-6_4
2. Parkinson M, O'Neill SM, Dalton JP. Endemic human fasciolosis in the Bolivian Altiplano. *Epidemiol Infect.* (2007) 135:669–74. doi: 10.1017/S095026880600728X
3. Mas-Coma MS, Esteban JG, Bargues MD. Epidemiology of human fascioliasis: a review and proposed new classification. *Bull World Health Organ.* (1999) 77:340–6. doi: 10.1046/j.1365-3156.1999.00418.x
4. Lim JH, Mairiang E, Ahn GH. Biliary parasitic diseases including clonorchiasis, opisthorchiasis and fascioliasis. *Abdom Imaging.* (2008) 33:157–65. doi: 10.1007/s00261-007-9326-x
5. Koç Z, Ulusan S, Tokmak N. Hepatobiliary fascioliasis: imaging characteristics with a new finding. *Diagn Interv Radiol.* (2009) 15:247–51. doi: 10.4261/1305-3825.DIR.1851-08.2
6. Van Beers B, Pringot J, Geubel A, Trigaux JP, Bigaignon G, Dooms G. Hepatobiliary fascioliasis: noninvasive imaging findings. *Radiology.* (1990) 174:809–10. doi: 10.1148/radiology.174.3.2406786
7. Teichmann D, Grobusch MP, Göbels K, Müller HP, Koehler W, Suttorp N. Acute fascioliasis with multiple liver abscesses. *Scand J Infect Dis.* (2000) 32:558–60. doi: 10.1080/003655400458884
8. Acosta-Ferreira W, Vercelli-Retta J, Falconi LM. Fasciola hepatica human infection. Histopathological study of sixteen cases. *Virchows Arch A Pathol Anat Histol.* (1979) 383:319–27. doi: 10.1007/BF00430249
9. Bektaş M, Dökmeci A, Cinar K, Halici I, Oztas E, Karayalcin S, et al. Endoscopic management of biliary parasitic diseases. *Dig Dis Sci.* (2010) 55:1472–8. doi: 10.1007/s10620-009-0850-0
10. Aksoy DY, Kerimoglu U, Oto A, Erguven S, Arslan S, Unal S, et al. Infection with Fasciola hepatica. *Clin Microbiol Infect.* (2005) 11:859–61. doi: 10.1111/j.1469-0691.2005.01254.x
11. Lefryekh R, Bensaad A, Bensardi F, Elhattabi K, Bouali M, Daif B, et al. Hepatic fascioliasis presenting with bile duct obstruction: a case report. *Pan Afr Med J.* (2017) 28:44. doi: 10.11604/pamj.2017.28.44.11532
12. Webb CM, Cabada MM. Recent developments in the epidemiology, diagnosis, and treatment of Fasciola infection. *Curr Opin Infect Dis.* (2018) 31:409–14. doi: 10.1097/QCO.0000000000000482
13. Karabuli TA, Shaikhani MA, Karadaghi SH, Kasnazan KH. Education and imaging. Hepatobiliary and pancreatic: fascioliasis. *J Gastroenterol Hepatol.* (2009) 24:1309. doi: 10.1111/j.1440-1746.2009.05955.x
14. Ministry of Health of the People 's Republic of China. Handbook on Prevention and Treatment of Intestinal Parasitosis. Fuzhou: Fujian Education Press. (1996) p. 56–60.
15. Kim KA, Lim HK, Kim SH, Lee WJ, Lim JH. Necrotic granuloma of the liver by human fascioliasis: imaging findings. *Abdom Imaging.* (1999) 24:462–4. doi: 10.1007/s002619900540
16. Abdul-Fattah MM, Yousef SM, Nasr ME, Fouad MA, Abdul-Wahab SE. Indirect fluorescent antibody test in diagnosis of acute fasciolitic syndrome. *J Egypt Soc Parasitol.* (1992) 22:261–4.
17. Ali, N.M. Development and evaluation of a dipstick assay in diagnosis of human fasciolosis. *Parasitol Res.* (2012) 110:1649–54. doi: 10.1007/s00436-011-2678-8
18. Azab M. el-S, el Zayat EA. Evaluation of purified antigens in haemagglutination test (IHA) for determination of cross reactivities in diagnosis of fascioliasis and schistosomiasis. *J Egypt Soc Parasitol.* (1996) 26:677–85.
19. Mas-Coma S, Bargues MD, Valero MA. Diagnosis of human fascioliasis by stool and blood techniques: update for the present global scenario. *Parasitology.* (2014) 141:1918–46. doi: 10.1017/S0031182014000869
20. Street TL, Sanderson ND, Atkins BL, Brent AJ, Cole K, Foster D, et al. Molecular diagnosis of orthopedic-device-related infection directly from sonication fluid by metagenomic sequencing. *J Clin Microbiol.* (2017) 55:2334–47. doi: 10.1128/JCM.00462-17
21. Ruppé E, Lazarevic V, Girard M, Mouton W, Ferry T, Laurent F, et al. Clinical metagenomics of bone and joint infections: a proof of concept study. *Sci Rep.* (2017) 7:7718. doi: 10.1038/s41598-017-07546-5
22. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis.* (2018) 67:S231–40. doi: 10.1093/cid/ciy693
23. Wang H, Lu Z, Bao Y, Yang Y, de Groot R, Dai W, et al. Clinical diagnostic application of metagenomic next-generation sequencing in children with severe nonresponding pneumonia. *PLoS ONE.* (2020) 15:e0232610. doi: 10.1371/journal.pone.0232610
24. Simner PJ, Miller S, Carroll KC. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. *Clin Infect Dis.* (2018) 66:778–88. doi: 10.1093/cid/cix881
25. Walker TM, Kohl TA, Omar SV, Hedge J, Elias CDO, Bradley P, et al. Whole-genome sequencing for prediction of Mycobacterium tuberculosis drug susceptibility and resistance: a retrospective cohort study. *Lancet Infect Dis.* (2015) 15:1193–202. doi: 10.1016/S1473-3099(15)00062-6
26. Sakiyama Y, Kanda N, Higuchi Y, et al. New type of encephalomyelitis responsive to trimethoprim/sulfamethoxazole treatment in Japan. *Neurol Neuroimmunol Neuroinflamm.* (2015) 2:e143. doi: 10.1212/NXI.0000000000000143
27. Yao M, Zhou J, Zhu Y, Zhang Y, Lv X, Sun R, et al. Detection of listeria monocytogenes in CSF from three patients with

meningoencephalitis by next-generation sequencing. *J Clin Neurol.* (2016) 12:446–51. doi: 10.3988/jcn.2016.12.4.446
28. Cai Y, Fang X, Chen Y, Huang Z, Zhang C, Li W, et al. Metagenomic next generation sequencing improves diagnosis of prosthetic joint infection by detecting the presence of bacteria in periprosthetic tissues. *Int J Infect Dis.* (2020) 96:573–8. doi: 10.1016/j.ijid.2020.05.125
29. Ai JW Li Y, Cheng Q, Cui P, Wu HL, Xu B, Zhang WH. Diagnosis of local hepatic tuberculosis through next-generation sequencing: Smarter, faster and better. *Clin Res Hepatol Gastroenterol.* (2018) 42:178–81. doi: 10.1016/j.clinre.2018.04.007

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Zhang, Xu, Liu, Kang, Chen, Wang and Cai. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

BRIEF RESEARCH REPORT
published: 15 December 2021
doi: 10.3389/fcimb.2021.764142

Metagenomic Analysis Identifying a Rare *Leishmania* Infection in an Adult With AIDS

Pingping Song[1†], Shuai Chen[2†], Xiaoyu Tan[1], Yanjun Gao[1], Juanjuan Fu[1], Zhiqing You[1], Chengtan Wang[1], Qigang Zhao[1] and Feng Pang[1]*

[1] Department of Clinical Laboratory, Liaocheng People's Hospital, Liaocheng, China, [2] Department of Clinical Laboratory, Liaocheng Third People's Hospital, Liaocheng, China

Leishmania belongs to a genus of the protozoan parasites that causes leishmaniasis, and includes cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). In this case, *Leishmania* amastigotes were found on cytomorphology examination of the bone marrow specimen, followed by 1,076 *Leishmania donovani* reads using metagenomic next generation sequencing (mNGS). Since being definitely diagnosed with VL/HIV coinfection, the patient was treated with liposomal amphotericin B as the parasite-resistant therapy and was discharged after clinical cure. But nearly a year later, on the mNGS follow-up, *L. donovani* was detected in the patient's blood plasma specimen with 941 reads, suggesting that a relapse of leishmaniasis had occurred. These results indicate that leishmaniasis still exists in China and may represent a public health concern. This case could be helpful in the differential diagnosis of leishmaniasis, and for determining disease progression, prevention, and control of vectors and reservoir hosts.

Keywords: leishmaniasis, *Leishmania*, AIDS, HIV, mNGS, rapid diagnosis

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Tuo Yang,
University of California, San Diego, United States
Xue Zhang,
Wistar Institute, United States

***Correspondence:**
Feng Pang
pangfeng_lc.hosp@outlook.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *25 August 2021*
Accepted: *23 November 2021*
Published: *15 December 2021*

Citation:
Song P, Chen S, Tan X, Gao Y, Fu J, You Z, Wang C, Zhao Q and Pang F (2021) Metagenomic Analysis Identifying a Rare Leishmania Infection in an Adult With AIDS. Front. Cell. Infect. Microbiol. 11:764142. doi: 10.3389/fcimb.2021.764142

INTRODUCTION

Leishmaniasis, a zoonotic disease caused by *Leishmania* that is spread by the sand fly species. It is a poverty-related disease with two main clinical forms: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) (Palumbo, 2009). An estimated 0.9–1.3 million new cases of leishmaniasis per year are reported in nearly 100 countries and regions (Bora, 1999; Gramiccia and Gradoni, 2005; Ready, 2014). As a global public health concern, *Leishmania* mainly affects some of the poorest individuals in the world and is related to malnutrition, poor housing conditions, population displacement, lack of financial resources, and a weakened immune system (Gramiccia, 2011). VL has existed in China for at least 120 years (Han et al., 2018; Han et al., 2021). From 2002 to 2011, a total of 3,169 cases of visceral leishmaniasis were reported in China, with approximately 140 to 509 cases diagnosed per year (Lun et al., 2015). This suggests that leishmaniasis is not extinct in China, and has the potential to cause a public health problem.

As a major worldwide public health concern, the human immunodeficiency virus (HIV) infection is present in all countries and regions. There are about approximately 36.9 million individuals with HIV, and 2.0 million new infections are reported every year (Molina et al., 2003). It is clear that there is an overlap between leishmaniasis transmission and acquired immune deficiency

syndrome (AIDS), resulting in an increasing number of cases of *Leishmania*–HIV coinfection (Puig and Pradinaud, 2003; Correa Soares et al., 2020).

Progress in the diagnosis of leishmaniasis depends on the development of effective methods and the discovery of suitable biomarkers. There are several approaches that have been used in the diagnosis of leishmaniasis, such as microscopic examination, culture, serologic diagnosis, and molecular approaches (Pace, 2014; von Stebut, 2017). However, these methods have similar limitations, including the non-routine availability of reagents. More importantly, physicians rarely consider the infection of such rare pathogens. Recently developed mNGS can overcome the limitations of traditional diagnostic tests. This new technology identifies all pathogens directly from the sample through a single run in a hypothesis-free and culture-independent manner. Studies have shown that mNGS provides more sensitive findings than the traditional culture method in clinical conditions such as immunodeficiency syndrome, blood stream, respiratory, and general infections (Wang et al., 2021). More importantly, due to unbiased sampling, mNGS is theoretically able to identify not only known pathogens but also unexpected pathogens and even discover novel organisms (Han et al., 2019; Zhang et al., 2020).

It should be noted that mNGS also has some limitations such as human genome contamination and possible environmental microbial contamination. The vast majority of reads obtained in mNGS are derived from the human host. This would impede the overall analytical sensitivity of mNGS for pathogen detection. Host depletion methods or targeted sequencing may help to partially mitigate this disadvantage. Because mNGS alone cannot define whether the detected microbe is the causative pathogen or environmental microorganism, a multidisciplinary approach by clinicians, microbiologists, and the lab technicians is required to interpret the result. The application of sequencing technology is shifting from research to clinical laboratories due to rapid technological developments and substantially reduced costs. Before this new approach could be considered as a first-line diagnostic test in *Leishmania* infection, the costs and availability should be further improved.

The patient in this case showed a co-infection with *Leishmania* and HIV, which was diagnosed by bone marrow microscopic examination (**Figure 1**) and mNGS. To our knowledge, this is a rare report of leishmaniasis diagnosed by mNGS in China.

mNGS PROTOCOL

DNA was extracted from 600 µl of bone marrow or plasma using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH, Beijing, China). We extracted the DNA from the negative and positive quality control sample along with the test specimens. Then, DNA libraries were constructed through DNA-fragmentation, end-repair, adapter-ligation, and PCR amplification. The constructed library was qualified by Agilent 2100 (Agilent Technologies, Santa Clara, CA) and using the Qubit 2.0 Fluorometer (Invitrogen, USA). The qualified double-strand DNA library was transformed into a single-stranded circular DNA library through DNA-denaturation and circularization. DNA nanoballs (DNBs) were generated from single-stranded circular DNA using rolling circle amplification (RCA). The DNBs were qualified using Qubit 2.0 Fluorometer. Qualified DNBs were loaded into the flow cell and sequenced (50 bp, single-end) on the BGISEQ-50 platform.

High-quality sequencing data were generated by removing low-quality and short reads (length <35 bp) using in-house software, followed by computational subtraction of human host sequences mapped to the human reference genome (hg19) using Burrows–Wheeler Alignment. After the removal of low complexity reads, the remaining data were classified by simultaneously aligning to Pathogens metagenomics Database

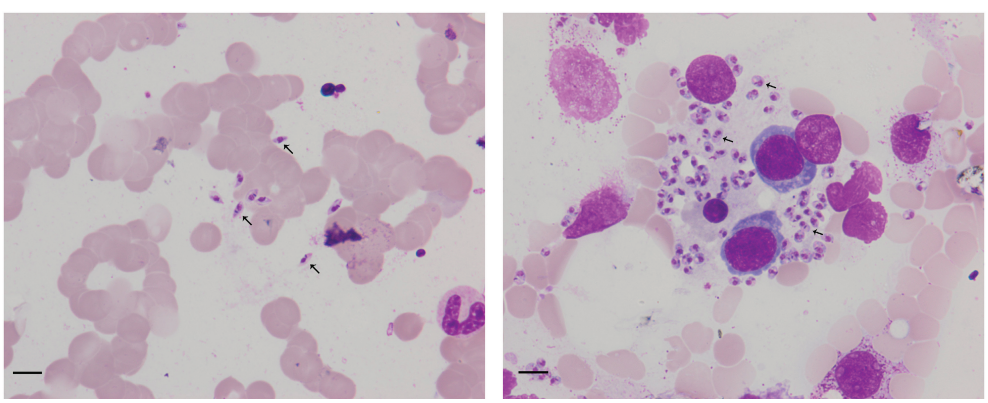


FIGURE 1 | Arrowheads show *Leishmania* amastigotes in bone marrow cytology, which are oval and 2.9–5.7 × 1.8–4.0 µm in size. Scale bar = 10 µm. The cytoplasm is stained lilac or purple-blue with Wright's stain and contains a large round nucleus. The nucleus (red or lavender) is located at the front of the worm and accounts for a third to a half of the worm's length.

(PMDB), consisting of viruses, bacteria, fungi, and parasites. The four Microbial Genome Databases were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/). RefSeq contains 4,945 whole-genome sequences of viral taxa, 6,350 bacterial genomes or scaffolds, 1,064 fungi, and 234 parasites associated with human diseases. The number of unique alignment reads was calculated and standardized to get the number of reads stringently mapped to pathogen species (SDSMRN) and the number of reads stringently mapped to pathogen genus (SDSMRNG). The amount of sequencing data produced by bone marrow and blood plasma mNGS after each step is shown in **Figures 2**, **3**.

The first sequencing detection identified 95,335 (among a total 23,910,670) sequence reads (0.3987%) matching to the parasite metagenomics database (**Figure 2B**), and the *Leishmania* genome reads (8,4831, 88.98%) were predominant among the parasites species, which corresponded to 1,076 *L. donovani* reads covering a high percentage (4.58%).Similarly, in the second detection, we obtained 145,456 (of a total 27,400,143) sequence reads belonging to the *Leishmania* genus genome, and there were 941 *L. donovani* uniquely aligned sequences corresponding to the *Leishmania* genome with a high coverage percentage (4.23%). As expected, no *Leishmania* read was detected from control sample.

RESULTS

An adult female was diagnosed with "HIV infection, liver cirrhosis" on admission with symptoms of fatigue and splenomegaly. A specific pathogen infection was suspected, and then the diagnosis of AID with visceral leishmaniasis was confirmed by bone marrow microscopy and mNGS. A relapse of leishmaniasis occurred in the follow-up reviews with mNGS nearly one year later.

CASE DESCRIPTIONS

The patient, a 54-year-old woman, was admitted to the infection ward of the Liaocheng People's Hospital, China, for "recurrent fatigue, cough, pitting edema of the lower extremities, chest tightness, and dyspnea especially after activities for two years, sustained cough for two days" in October, 2019. Upon tracing the patient's medical history, the following pieces of information were discovered: this patient was diagnosed with "AIDS" nine

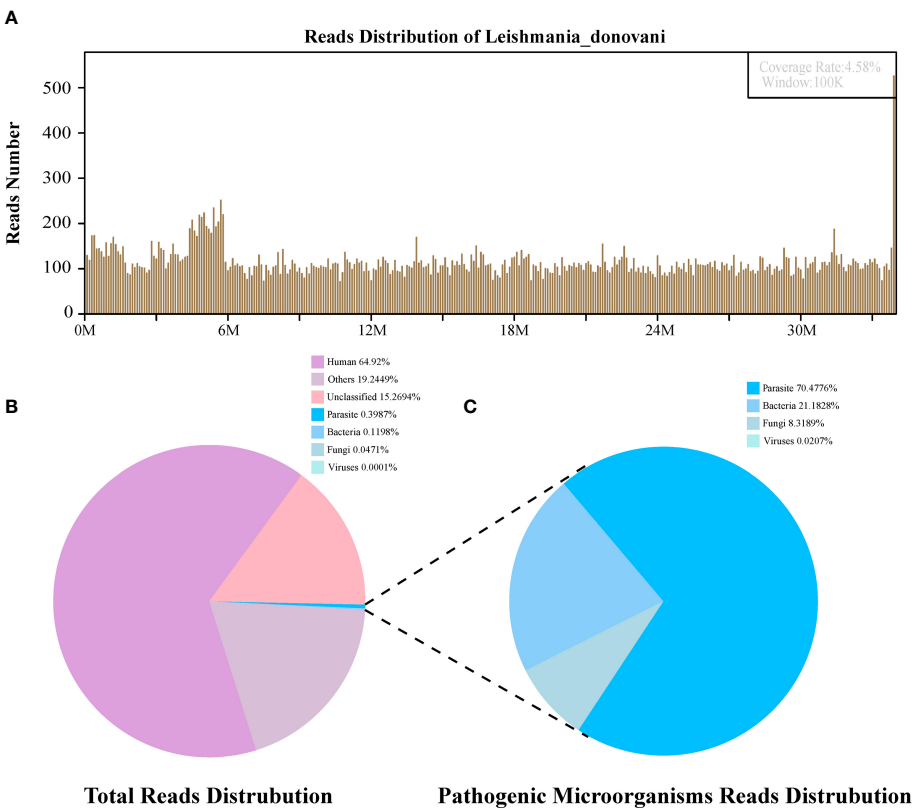


FIGURE 2 | The diagnosis of *Leishmania* infection by Metagenomic next-generation sequencing (mNGS). **(A)** Mapping of *Leishmania donovani* reads on the genome. **(B)** Reads distribution of total DNA in the bone marrow sample. **(C)** Distribution of pathogenic microorganisms reads in the absence of human, others and unclassified reads.

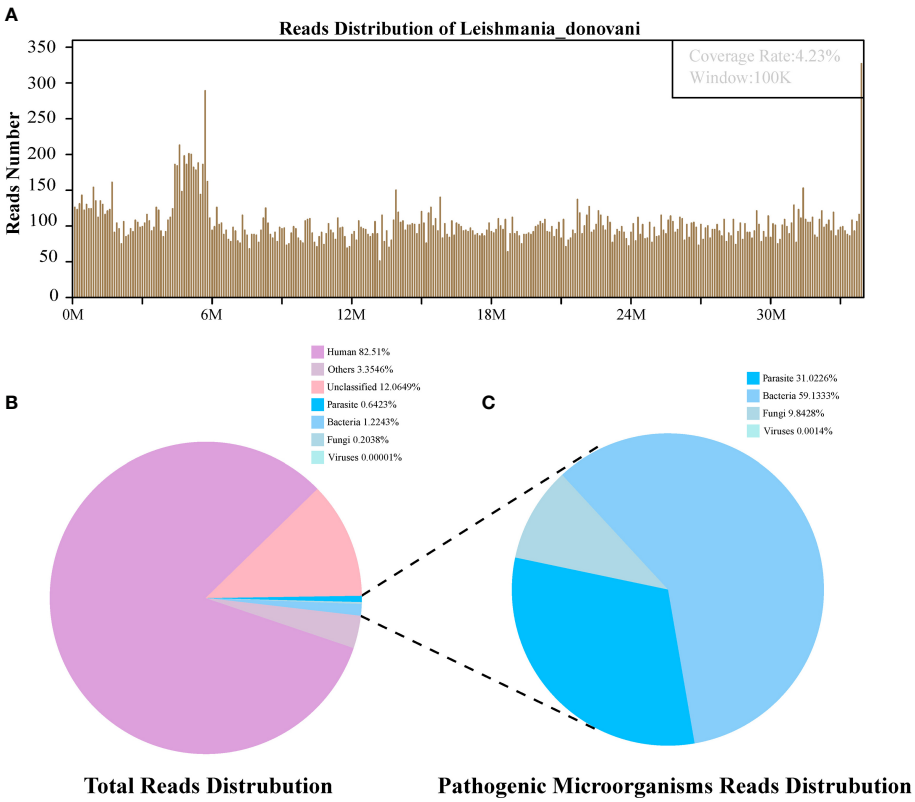


FIGURE 3 | The follow-up diagnosis of *Leishmania* infection by Metagenomic next-generation sequencing (mNGS). **(A)** Mapping of *Leishmania donovani* reads on the genome. **(B)** Reads distribution of total DNA in the blood plasma sample. **(C)** Distribution of pathogenic microorganisms reads in the absence of human, others and unclassified reads.

years ago at the hospital of Xinjiang Province without receiving standard treatment. Four years ago, she was diagnosed with "cirrhosis" due to fatigue and received liver protection treatment at the same hospital. However, she did not take any medications after being discharged from the hospital. She has been using oral anti-HIV drugs (lamivudine, compound sulfonic acid armor oxazole piece, and tenofovir) since 2018.

Born in Liaocheng of Shandong Province, this patient resided in the forest region of Xinjiang Province for more than 10 years, and returned to Liaocheng four years ago. There was no history of smoking, drinking, or toxic exposure. The physical examination revealed the following findings: blood pressure 105/51 mmHg, splenomegaly, and slight pitting edema over both legs. The routine laboratory tests were as follows: blood samples white blood cells (WBC) 1.14×10^9 cells/L, red blood cells (RBC) 1.19×10^9 cells/L, hemoglobin (Hb) 27 g/L, platelets (PLT) 41 $\times 8 \cdot 10^9$ cells/L, procalcitonin 0.34 ng/ml, C-reactive protein 15.15 mg/L; Urine samples: WBC 53 cell/ul and urobilinogen 34 μmol/L (+); Virus screening: hepatitis B surface antigen (HBs-Ag) >250 IU/ml, hepatitis B e antibody (HBe-Ab) 0.66 s/co, hepatitis B core antibody(HBC-Ab) 7.78 s/co, human immunodeficiency virus antibody(HIV-Ab) positive; Biochemical analysis: alanine aminotransferase (ALT)13 IU/L, aspartate aminotransferase (AST) 24 IU/L, albumin (ALB) 18 g/L, globulin (GLB)70 g/L, albumin/globulin (ALB/GLB) 0.26, cholinesterase (CHE) 1115 U/L, total bilirubin (TBIL) 27.2 μmol/L, direct bilirubin (DBIL) 20.1 μmol/L, uric acid (UA) 418 μmol/L, β2–macroglobulin (β2-mb) 5.29 mg/L, cystatin C (CYS-C) 1.14 mg/L, creatine kinase MB subtype(CK-MB) 24 μ/L; T cell subgroup count: CD4 23.01%, CD8 35.23%, CD4/CD8 0.65; Immunoglobulin + complement: C3 0.63 g/L, C4 0.12 g/L, IgG 44.90 g/L, IgA 5.79 g/L, IgM 15.30 g/L; Blood ammonia (AMON): 52.9 μmol/L; abdominal ultrasonography: diffuse liver disease (suggesting early liver cirrhosis), vessel broadening of the main portal vein and splenic vein, unclear wall of the gallbladder, splenomegaly; chest CT: bronchitis, pneumonia in the left lower lobe, multiple nodules in both lungs, mediastinal axillary lymph nodes; Examination of bone marrow cytomorphology: erythroid hyperplasia, higher polychromatic erythroblast, mature red blood Rouleau rank, abundant amastigotes of *L. donovani* around histiocytes (**Figure 1**); mNGS of bone marrow sample: 1,076 reads of *L. donovani* (**Table 1**).

The patient was hospitalized for AIDS and liver cirrhosis, and the relevant examinations revealed that RBC, WBC, and PLT counts decreased in peripheral blood, liver damage and pneumonia. After active treatments (blood transfusion to treat

TABLE 1 | The basic situation of *Leishmania donovani* in twice NGS-based detections.

Sample	Type	Parasite identified	No. of unique reads	No. of *Leishmania* reads	No. of parasite reads	Proportion in parasite reads (%)	Coverage, (%)	Depth
First detection	Bone marrow	*Leishmania donovani*	1,076	84,831	95,335	1.1287%	4.58	1.07
Second detection	Plasma	*Leishmania donovani*	941	145,456	175,991	0.5347%	4.23	1.06

anemia, albumin infusion, anti-HIV therapy, levofloxacin to treat the infection, hepatoprotection to treat jaundice, rising white cells, diuresis, and potassium supplements), these symptoms showed no signs of improvement.

According to the results of the patient's bone marrow cytomorphology examination and sequencing analysis, the patient was diagnosed with "leishmaniasis combined with AIDS". Therefore, in the original treatment regimen, amphotericin B was administered for parasite-resistant therapy. The treatment schedule was the first day 5 mg dose for 6 h by continuous intravenous pumping on October 18, 2019, the dose was gradually increased to 5 mg day by day. On October 23, 2019, the blood parameters showed that the white cells were lower and there were signs liver damage and renal damage. Taking into account the side effects of amphotericin B, the treatment frequency and dose were changed to 20 mg every two days in a single dose (continuous intravenous administration for 6 h) of amphotericin B. We started to use liposomal amphotericin B On 30 October, scheduled for 3 mg/kg/time from the first day to the fourth day (continuous intravenous pumping 4 h), 1.6 mg/kg on day 13 (continuous intravenous pumping 4 h), 1.6 mg/kg on day 20 (continuous intravenous pumping 4 h), totally for 6 times. Then morphological examination of the bone marrow specimen was checked, and *Leishmania* was not detected. The patient's symptoms improved and she was discharged from the hospital.

The patient was readmitted to the hospital for treatment due to fatigue in October 2020. Considering that it might be a relapse of leishmaniasis, mNGS tests for blood plasma specimen were performed, with the consent of her family. The results showed 941 reads of *L. donovani* (**Table 1**). However, considering the high cost of antiparasitic infection, the patient received supportive treatment and was discharged after symptom reduction.

DISCUSSION

In this study, we describe a patient diagnosed with HIV infection, liver cirrhosis on admission to hospital with symptoms of fatigue and splenomegaly. A specific pathogen infection was suspected after collecting demographic information from the patient: a history of residing in the forest region in Xinjiang Province for more than 10 years, clinical symptoms, laboratory test results, imaging examination results, diagnosis and treatment history, and prognostic results. The diagnosis of HIV with *L. donovani* infection was definitely confirmed by mNGS (Tavares et al., 2003). In this study, we diagnosed a rare *Leishmania* infection in an HIV patient by metagenomic analysis. The patient was treated with liposomal amphotericin B and after one year of the initial treatment, mNGS detected 941 *L. donovani* reads from the patient's plasma sample, suggesting a relapse of leishmaniasis. *Leishmania donovani* is a specific pathogen that is not normally present in the environment, serological or PCR reagents for this pathogen are not normally prepared routinely in the laboratory. This patient showed signs of infection on his second admission, so we screened him for possible infectious agents using mNGS, which eventually revealed the recurrence of leishmaniasis. Diagnosis using mNGS for *Leishmania* infection is a novel approach has raised concerns that *Leishmania* is still present in Central China. This finding provides a valuable reference for diagnosis.

Based on previous research, it can be concluded that the clinical manifestations of VL in HIV-infected patients are similar to those of non-coinfected individuals (Puig and Pradinaud, 2003). Typical VL includes intermittent or continuous fever, non-tender hepatosplenomegaly, pancytopenia, leading to anemia, hemorrhages and concurrent infections (de Araujo et al., 2012). However, the atypical form may be misdiagnosed with other opportunistic infections or is diagnosed with substantial delay, so early recognition and treatment are necessary (Nourian et al., 2019). In this case, the patient manifested unusual clinical presentations (fatigue, splenomegaly, anemia, no fever, hemorrhages, or concurrent infections), which frequently occur in individuals with low CD4+ T-cell counts (23.01%) (Bimal et al., 2008; Garg et al., 2009; Santos-Oliveira et al., 2010). Its timely etiologic diagnosis is challenging.

Some elements relevant to the host and environment can affect the prevalence of VL–HIV coinfection (Natalia Souza et al., 2018). *Leishmania* and HIV share an immunopathological mechanism, compromising macrophages and dendritic cells, which is typical of the presence of both pathogens, accelerating the progression of VL and HIV (Vassallo et al., 2014). The impaired immune function of VL/HIV coinfected patients may: (i) favor the reactivation of latent *Leishmania* infection; (ii) induce a more severe presentation of visceral Leishmaniasis; (iii) cause a poorer therapeutic response; and (iv) increase the risk of relapse after treatment (Olivier et al., 2003; Monge-Maillo and Lopez-Velez, 2016).

Therefore, the main challenges in the therapy of VL/HIV coinfection are developing an effective drug that not only resolves the first episode of VL, but also prevents relapses. However, there is rare evidence and data on the optimal therapy for VL/HIV coinfection. Liposomal amphotericin B is a new treatment with the characteristic of relatively high efficiency and low toxicity (Ponte-Sucre et al., 2017; Roatt et al., 2020). The World Health Organization (WHO, 2010) recommended liposomal amphotericin B as a first line therapy

drug for visceral leishmaniasis in African countries (Abongomera et al., 2018). Ultimately, liposomal amphotericin B was used for antiparasitic therapy, and the overall treatment outcome was successful. Side effects emerged involving liver and kidney injury, which were significantly improved by a timely adjustment of the treatment schedule.

As shown previously, leishmaniasis–HIV coinfection presents some difficulties for diagnosis and identification due to atypical clinical manifestations, both in visceral and cutaneous forms. Additional toxicity should be avoided in the treatment of *leishmania*–HIV coinfection, and minimal side effects should be sought. Liposomal amphotericin B is the main drug for the treatment of leishmaniasis. However, dosing is still a significant problem, as there is no consensus on the optimal treatment regimen for different individuals worldwide (Silva Ferreira de Carvalho et al., 2020). An important point to be explored is the search for new active drugs against all species of *Leishmania*. After the clinical cure is obtained, parasites continue to be present in some organs or lesions, and reactivations or relapses of leishmaniasis may occur if immunosuppression is still present.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PS, SC, and CW performed bioinformatics analysis. PS participated in the writing of the manuscript. XT collected cases. YG, JF, and ZY performed mNGS testing. CW analysed and visualised the data. QZ and FP conceived the study and participated in the writing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Medical Science and Technology Development Foundation of Shandong Province, China (Grant No. 2019WS113).

ACKNOWLEDGMENTS

The authors thank the subject who voluntarily participated in this study.

REFERENCES

Abongomera, C., Diro, E., de Lima Pereira, A., Buyze, J., Stille, K., Ahmed, F., et al (2018). The Initial Effectiveness of Liposomal Amphotericin B (AmBisome) and Miltefosine Combination for Treatment of Visceral Leishmaniasis in HIV Co-Infected Patients in Ethiopia: A Retrospective Cohort Study. *PloS Negl. Trop. Dis.* 12 (5), e0006527. doi: 10.1371/journal.pntd.0006527

Bimal, S., Singh, S. K., Sinha, S., Pandey, K., Sinha, P. K., Ranjan, A., et al (2008). Leishmania Donovani: Role of CD2 on CD4+ T-Cell Function in Visceral Leishmaniasis. *Exp. Parasitol.* 118 (2), 238–246. doi: 10.1016/j.exppara.2007.08.009

Bora, D. (1999). Epidemiology of Visceral Leishmaniasis in India. *Natl. Med. J. India* 12 (2), 62–68.

Correa Soares, G. H., Silva, A., Ferreira, L. S. S., Ithamar, J. S., Medeiros, G. A., Pereira, S. R. F., et al (2020). Case Report: Coinfection by Leishmania Amazonensis and HIV in a Brazilian Diffuse Cutaneous Leishmaniasis Patient. *Am. J. Trop. Med. Hyg.* 103 (3), 1076–1080. doi: 10.4269/ajtmh.20-0131

de Araujo, V. E., Morais, M. H., Reis, I. A., Rabello, A., and Carneiro, M. (2012). Early Clinical Manifestations Associated With Death From Visceral Leishmaniasis. *PloS Negl. Trop. Dis.* 6 (2), e1511. doi: 10.1371/journal.pntd.0001511

Garg, R., Barat, C., Ouellet, M., Lodge, R., and Tremblay, M. J. (2009). Leishmania Infantum Amastigotes Enhance HIV-1 Production in Cocultures of Human Dendritic Cells and CD4 T Cells by Inducing Secretion of IL-6 and TNF-Alpha. *PloS Negl. Trop. Dis.* 3 (5), e441. doi: 10.1371/journal.pntd.0000441

Gramiccia, M. (2011). Recent Advances in Leishmaniosis in Pet Animals: Epidemiology, Diagnostics and Anti-Vectorial Prophylaxis. *Vet. Parasitol.* 181 (1), 23–30. doi: 10.1016/j.vetpar.2011.04.019

Gramiccia, M., and Gradoni, L. (2005). The Current Status of Zoonotic Leishmaniases and Approaches to Disease Control. *Int. J. Parasitol.* 35 (11-12), 1169–1180. doi: 10.1016/j.ijpara.2005.07.001

Han, S., Chen, S. B., Yang, Z. H., Feng, Y., and Wu, W. P. (2021). Epidemiology of Leishmania Carriers in Tan Chang County, Gansu Province, China. *Front. Cell. Infect. Microbiol.* 11, 645944. doi: 10.3389/fcimb.2021.645944

Han, D., Li, Z., Li, R., Tan, P., Zhang, R., and Li, J. (2019). mNGS in Clinical Microbiology Laboratories: On the Road to Maturity. *Crit. Rev. Microbiol.* 45 (5-6), 668–685. doi: 10.1080/1040841X.2019.1681933

Han, S., Wu, W. P., Chen, K., Osman, I., Kiyim, K., Zhao, J., et al (2018). Epidemiological Survey of Sheep as Potential Hosts for Leishmania in China. *BMC Vet. Res.* 14 (1), 378. doi: 10.1186/s12917-018-1701-z

Lun, Z. R., Wu, M. S., Chen, Y. F., Wang, J. Y., Zhou, X. N., Liao, L. F., et al (2015). Visceral Leishmaniasis in China: An Endemic Disease Under Control. *Clin. Microbiol. Rev.* 28 (4), 987–1004. doi: 10.1128/CMR.00080-14

Molina, R., Gradoni, L., and Alvar, J. (2003). HIV and the Transmission of Leishmania. *Ann. Trop. Med. Parasitol.* 97 (Suppl 1), 29–45. doi: 10.1179/000349803225002516

Monge-Maillo, B., and Lopez-Velez, R. (2016). Treatment Options for Visceral Leishmaniasis and HIV Coinfection. *AIDS Rev.* 18 (1), 32–43.

Natalia Souza, D. G., Vera Demarchi, A., Regina Maia, S., Thelma, O., and Lucia Maria Almeida, B. (2018). Unusual Clinical Manifestations of Leishmania (L.) Infantum Chagasi in an HIV-Coinfected Patient and the Relevance of ITS1-PCR-RFLP: A Case Report. *Iran. J. Parasitol.* 13 (4), 655–660.

Nourian, M., Heidari, A., Tajali, S., Ghasemi, E., and Mohebali, M. (2019). Paediatric Visceral Leishmaniasis: A Retrospective Study on Clinical Manifestations, Demographic Features and Laboratory Findings of Hospitalised Cases in Iran Between 2006 and 2016. *Trop. Doct.* 49 (1), 59–61. doi: 10.1177/0049475518811513

Olivier, M., Badaro, R., Medrano, F. J., and Moreno, J. (2003). The Pathogenesis of Leishmania/HIV Co-Infection: Cellular and Immunological Mechanisms. *Ann. Trop. Med. Parasitol.* 97 (Suppl 1), 79–98. doi: 10.1179/000349803225002561

Pace, D. (2014). Leishmaniasis. *J. Infect.* 69 (Suppl 1), S10–S18. doi: 10.1016/j.jinf.2014.07.016

Palumbo, E. (2009). Current Treatment for Cutaneous Leishmaniasis: A Review. *Am. J. Ther.* 16 (2), 178–182. doi: 10.1097/MJT.0b013e3181822e90

Ponte-Sucre, A., Gamarro, F., Dujardin, J. C., Barrett, M. P., Lopez-Velez, R., Garcia-Hernandez, R., et al (2017). Drug Resistance and Treatment Failure in

Leishmaniasis: A 21st Century Challenge. *PloS Negl. Trop. Dis.* 11 (12), e0006052. doi: 10.1371/journal.pntd.0006052

Puig, L., and Pradinaud, R. (2003). Leishmania and HIV Co-Infection: Dermatological Manifestations. *Ann. Trop. Med. Parasitol.* 97 Suppl 1, 107–114. doi: 10.1179/000349803225002589

Ready, P. D. (2014). Epidemiology of Visceral Leishmaniasis. *Clin. Epidemiol.* 6, 147–154. doi: 10.2147/CLEP.S44267

Roatt, B. M., de Oliveira Cardoso, J. M., De Brito, R. C. F., Coura-Vital, W., de Oliveira Aguiar-Soares, R. D., and Reis, A. B. (2020). Recent Advances and New Strategies on Leishmaniasis Treatment. *Appl. Microbiol. Biotechnol.* 104 (21), 8965–8977. doi: 10.1007/s00253-020-10856-w

Santos-Oliveira, J. R., Giacoia-Gripp, C. B., Alexandrino de Oliveira, P., Amato, V. S., Lindoso, J. A., Goto, H., et al (2010). High Levels of T Lymphocyte Activation in Leishmania-HIV-1 Co-Infected Individuals Despite Low HIV Viral Load. *BMC Infect. Dis.* 10, 358. doi: 10.1186/1471-2334-10-358

Silva Ferreira de Carvalho, I. P., Peixoto, H. M., Sierra Romero, G. A., and Fernandes de Oliveira, M. R. (2020). Treatment With Liposomal Amphotericin B for All Confirmed Cases of Human Visceral Leishmaniasis in Brazil: A Budget Impact Analysis. *Value Health Reg. Issues* 23, 77–84. doi: 10.1016/j.vhri.2020.05.006

Tavares, C. A., Fernandes, A. P., and Melo, M. N. (2003). Molecular Diagnosis of Leishmaniasis. *Expert Rev. Mol. Diagn.* 3 (5), 657–667. doi: 10.1586/14737159.3.5.657

Vassallo, M., Moranne, O., Ambrosetti, D., Jeandel, P. Y., Pomares, C., Cassuto, E., et al (2014). Visceral Leishmaniasis Due to Leishmania Infantum With Renal Involvement in HIV-Infected Patients. *BMC Infect. Dis.* 14, 561. doi: 10.1186/s12879-014-0561-9

von Stebut, E. (2017). [Leishmaniasis : Diagnosis and Therapy]. *Der Hautarzt; Z. fur Dermatologie Venerologie und verwandte Gebiete* 68 (7), 548–552. doi: 10.1007/s00105-017-4001-9

Wang, C., Li, A., Shi, Q., and Yu, Z. (2021). Metagenomic Next-Generation Sequencing Clinches Diagnosis of Leishmaniasis. *Lancet* 397 (10280), 1213. doi: 10.1016/S0140-6736(21)00352-4

WHO (2010). Control of the Leishmaniases. *World Health Organ Tech Rep Ser* xii-xiii (949), 1–186.

Zhang, H. C., Zhang, Q. R., Ai, J. W., Cui, P., Wu, H. L., Zhang, W. H., et al (2020). The Role of Bone Marrow Metagenomics Next-Generation Sequencing to Differential Diagnosis Among Visceral Leishmaniasis, Histoplasmosis, and Talaromycosis Marneffei. *Int. J. Lab. Hematol.* 42 (2), e52–ee4. doi: 10.1111/ijlh.13103

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Song, Chen, Tan, Gao, Fu, You, Wang, Zhao and Pang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

ORIGINAL RESEARCH
published: 17 December 2021
doi: 10.3389/fmed.2021.779981

Case Report: Metagenomics Next-Generation Sequencing for Diagnosing Cerebral Infarction and Infection Caused by Hematogenous Disseminated Mucormycosis in a Patient With Acute Lymphoblastic Leukemia

*Bingbing Wen, Lisheng Cai, Yun Cai and Xin Du**

Department of Hematology, The Second People's Hospital of Shenzhen, The First Affiliated Hospital of Shenzhen University, Shenzhen, China

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Aiming Pang,
Chinese Academy of Medical Sciences and Peking Union Medical College, China
Mohamed Abdelwahab Hassan,
Charité University Medicine Berlin, Germany

***Correspondence:**
Xin Du
duxingz@medmail.com.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *20 September 2021*
Accepted: *30 November 2021*
Published: *17 December 2021*

Citation:
Wen B, Cai L, Cai Y and Du X (2021) Case Report: Metagenomics Next-Generation Sequencing for Diagnosing Cerebral Infarction and Infection Caused by Hematogenous Disseminated Mucormycosis in a Patient With Acute Lymphoblastic Leukemia. Front. Med. 8:779981. doi: 10.3389/fmed.2021.779981

Disseminated mucormycosis, a serious complication, is associated with high mortality in patients with acute leukemia after chemotherapy. Blood cultures are always negative because of recurrent empirical antifungal treatments. The identification of pathogens is important for diagnosis and therapy. In this case report, we diagnosed culture-negative disseminated mucormycosis with *Rhizomucor miehei* infection leading to cerebral infarction in a patient with leukemia using metagenomics next-generation sequencing (mNGS) form peripheral blood, cerebral spinal fluid, and bronchoalveolar lavage fluid. mNGS technology can be applied to precisely diagnose culture-negative disseminated mucormycosis.

Keywords: mucormycosis, metagenomics next-generation sequencing, *Rhizomucor miehei*, cerebral infarction, cerebral infection, hematogenous disseminated, leukemia

INTRODUCTION

Disseminated mucormycosis, characterized by necrosis and infarction of tissues due to invasion of vasculature by hyphae, is a rare disease that commonly occurs in severely immunosuppressed patients (1, 2). Disseminated mucormycosis is associated with a high mortality rate of approximately 96% in patients with acute leukemia after chemotherapy (3–5). Positive culture and histological evidence for mucormycosis from blood or tissue biopsies are the key diagnostic criteria (6–8). However, positive blood culture results perform poorly at the onset of clinical symptoms and change of videography suspected to be mucormycosis (9–13). The positive culture rate is only 38% for cerebral mucormycosis (14) and 50% for all types of mucormycosis (3). Besides, biopsy specimen is not always feasible in most vulnerable populations. Therefore, molecular diagnostic technology as an adjuvant method are used to improve the early identification of the causative pathogens and guide treatment for patients.

Metagenomic next-generation sequencing (mNGS), a new molecular diagnostic technology, detects microbial nucleic acids with genomics method contained in samples. Its most significant advantage is the lack of requirement for culture and premise hypothesis (14, 15). mNGS has been

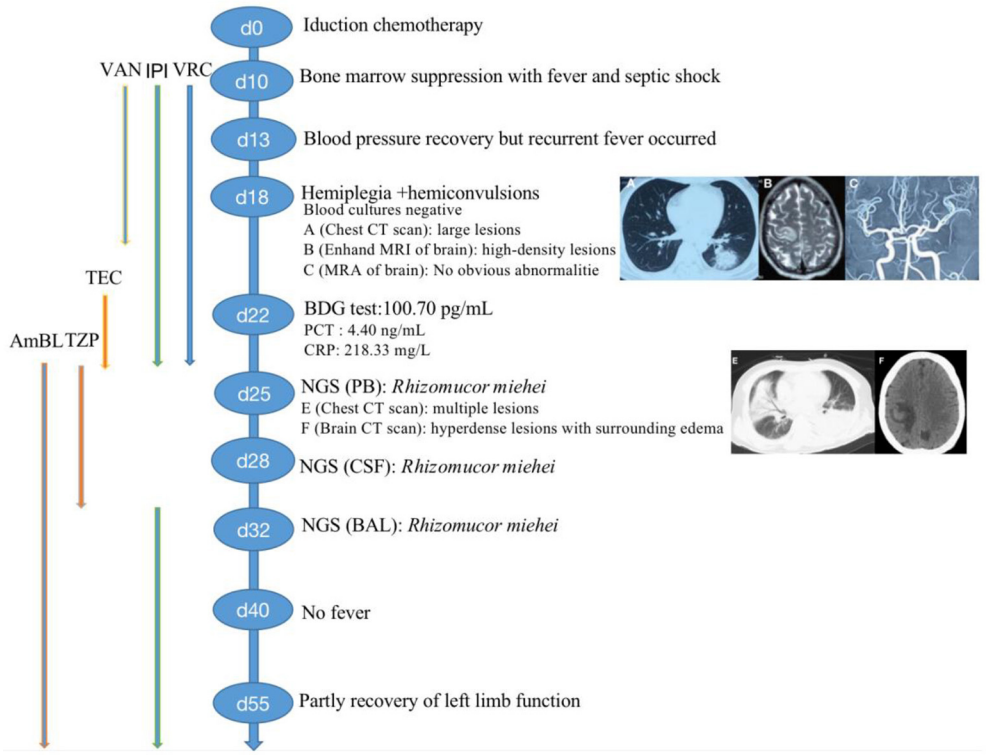


FIGURE 1 | Clinical course of the patient (schematic). BDG, (1,3)-b-D-glucan; PCT, procalcitonin; CRP, c-reactive protein; CT, computed tomography; MRI, magnetic resonance imaging; MRA, magnetic resonance angiography; VRC, voriconazole; IPI, Imipenem; VAN, Vancomycin; TEC, teicoplanin; AmBL, liposome-associated amphotericin B; mNGS, metagenomics next-generation sequencing; PB, peripheral blood; CSF, cerebral spinal fluid; BAL, bronchoalveolar lavage fluid.

applied in infectious diseases including mucormycosis to identify causative pathogens (16, 17). We present a case of culture-negative mucormycosis with *Rhizomucor miehei* infection leading to cerebral infarction diagnosed by mNGS of peripheral blood (PB), cerebral spinal fluid (CSF) and bronchoalveolar lavage fluid (BAL).

CASE DESCRIPTION

A 44-year-old man with acute lymphoblastic leukemia (ALL) underwent induction chemotherapy (IC) (day 0). An outline of the episodes is showed in **Figure 1**. Bone marrow suppression with fever and septic shock occurred on day 10. Patient suffered serious infection, and empirical treatments were used with imipenem (IPI, 1 g, every 8 h, intravenous injection), vancomycin (VAN, 1 g, every 12 h, intravenous injection) and voriconazole (VRC, 200 mg, every 12 h, intravenous injection) as broad-spectrum antibacterial and antifungal prophylaxis and empirical treatments were used with broad-spectrum antibiotics including antifungal prophylaxis. The patient's blood pressure recovered but recurrent fever occurred after 3 days. At that time, a series of cultures of peripheral blood (PB) were negative from day 10 to day 28. No pathogen could be detected, and the patient experienced neutropenia from day 10 to day 25 (**Figure 1**). However, hemiplegia and hemiconvulsions suddenly occurred on patient's right limb, and computed tomography (CT) scans of patient's brain showed a hyperdense lesion with surrounding edema, which was highly suspected as cerebral infarction in the right parietal lobe and small hypodense areas in the left and right parietal lobes. No obvious abnormality was showed by magnetic resonance angiography (MRA) of patient's brain, but CT scans of the lung showed multiple hyperdense lesions on day 18 (**Figure 1**). Febrile neutropenia in patients after chemotherapy with cerebral symptoms may be highly indicative of infections in the brain (18, 19).

Treatment was continued VRC and IPI, and changed to teicoplanin (TEC, 400 mg, daily, intravenous injection) as antibacterial and antifungal drugs Treatment was changed to voriconazole (VRC) and imipenem as antifungal and antibacterial methods after a positive result for the test of (1,3)-β-D-glucan (100.70 pg/mL, Guangzhou Zhaokang Biotechnology Co., Ltd) on day 22. However, the fever was unresponsive and the lesions in the brain and lung were more serious a week later. The procalcitonin (PCT) level was elevated to 4.40 ng/mL, and c-reactive protein (CRP) reached 218.33 mg/L. Aminoleucine

Abbreviations: ALL, Acute lymphoblastic leukemia; AmBL, Liposome-associated amphotericin B; BAL, Bronchoalveolar lavage fluid; CSF, Cerebral spinal fluid; CT, Computed tomography; mNGS, Metagenomics next-generation sequencing; PB, Peripheral blood; VRC, Voriconazole.

TABLE 1 | Pathogenic microorganisms detected in peripheral blood, cerebral spinal fluid, and bronchoalveolar lavage fluid specimen of patient by next-generation sequencing.

Genus			Species	
Name	**Sequence number**	**Relative abundance (%)**	**Name**	**Sequence number**
1.Peripheral blood				
Rhizomucor	41500	99.94	*Rhizomucor miehei*	40895
2. Cerebral spinal fluid				
Rhizomucor	1	0.35	*Rhizomucor miehei*	1
3. Bronchoalveolar lavage fluid				
Rhizomucor	7	28.00	*Rhizomucor miehei*	7
Aspergillus	13	52.00	*Aspergillus flavus*	4
Aspergillus	13	52.00	*Aspergillus terreus*	4
Elzabethkingia	125838	94.66	*Elzabethkingia anophelis*	85818
Stenotrophomonas	2511	1.89	*Stenotrophomonas maltophilia*	2404

TABLE 2 | Cerebrospinal fluid test on day 28.

Parameter (unit)	Result	Normal range
Colors	Transparent	Transparent
Pandy test	Negative	Negative
Red blood cell (10^6/L)	0	N/A
Wite blood cell (10^6/L)	0	(0-8) x 10^6/L
Glucose (mmol/L)	3.10	2.5–4.5
Chloride (mmol/L)	126.3	120–132
CSF protein (g/L)	374.5	150–450
Tumor cell	Negative	Negative

transferase (ALT) and aspartate transaminase (AST) reached 145 U/L and 242 U/L, respectively. Other drugs were applied to protect patient's liver function. *Rhizomucor miehei* infection was shown by mNGS (Genskey Medical Technology Co., Ltd, Beijing, China. NextSeq 500) of PB with high relative abundance about 99.94% on day 25 (**Table 1**), and liposome-associated amphotericin B (AmBL, 100 mg, daily, intravenous injection) was immediately used as antifungal therapy from day 25 to day 55. The patient's body temperature returned to normal (36–37°C) after 3 days of treatment and his complete blood count (CBC) recovered from neutropenia on day 28. Infection with *Rhizomucor miehei* was also proven by mNGS both in CSF with relative abundance about 0.35% on day 28 and BAL with relative abundance about 28% on day 35 (**Table 1**), and the routine and biochemical examinations of CSF were negative (**Table 2**). After 2 and 3 weeks of antifungal treatment, CT scans showed that lesions were obviously absorbed both in the brain (**Figure 2**) and the lung (**Figure 3**), and PCT and CRP were also recovered following antifungal therapy (**Figure 4**). Finally, the patient's general condition improved, and his right limb function partly recovered on day 55. He requested to go back to the local hospital to continue antifungal therapy due to his family reasons.

After 2 months of follow-up by telephone, we learned that patient changed to receive antifungal treatment with amphotericin B (AmB, 150 mg, daily, intravenous injection) and posaconazole (Pos, 300 mg, daily, orally) for other 2 months, because patient's liver had recovered to normal function. Besides, significant reduction in the size of the lesions on imaging tests of this patient were reported. The next chemotherapy for ALL was also prepared to carried out. The antifungal drug regimen was well-tolerated and achieved a remarkable effect.

DISCUSSION

Mucormycosis occurred during the period of neutropenia after chemotherapy for ALL in this patient. Cerebral infarction and fungal encephalitis are caused by hematologic dissemination with poor outcomes. Only a few case reports on disseminated mucormycosis have been reported, and cerebral manifestations of mucormycosis have been recorded even less frequently (20–22).

The high mortality rate in patients with disseminated mucormycosis necessitates rapid diagnosis (3–5). However, very low positive rates of blood cultures from patients with ALL after chemotherapy were observed due to empirical antifungal therapy (23). Pagano, L. et al. showed that only 4 leukemia patients (∼10%) were diagnosed ante-mortem among 116 patients with recorded infections of pulmonary filamentous fungal (24). Another study showed that approximately 35% of 37 patients with histologically recorded mucormycosis and hematological malignancies ware diagnosed ante-mortem (20). CT scans of the lung and the brain provide significant information's for the diagnosis of invasive fungi (25, 26). However, it is difficult to distinguish the kind of fungal infections by these imaging methods.

mNGS, a high-throughput screening method, is an emerging and relatively effective diagnostic method to detect pathogens that are culture-independent, especially for non-cultivable or uncommon microbiological organisms (27, 28). In addition, mNGS is used to detect pathogens by sequencing of extracted DNA from specimens, which is a non-culture-based and fast method (29). In this case study, mNGS of PB, CSF, and BAL was well employed to diagnose disseminated mucormycosis infection

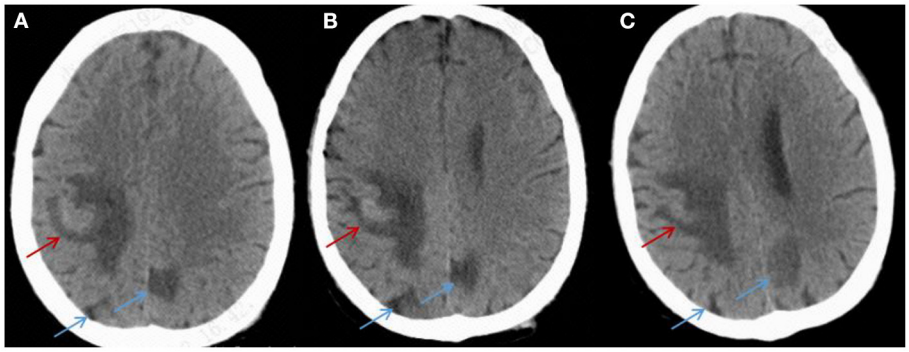


FIGURE 2 | CT imaging for the patient's brain. **(A)** On day 25, a CT scan of the brain showed hyperdense lesions with surrounding edema in the right parietal lobe (red arrow) and hypodense areas (blue arrow) in the left and right parietal lobes. **(B,C)** After 2 and 3 weeks of treatment, a CT scan showed that the lesions were absorbed in the left and right brain (red arrow and blue arrow).

FIGURE 3 | CT imaging for the patient's lung. **(A)** On day 25, a CT scan of the lung showed multiple inflammatory lesions (red arrow) and small amount of bilateral pleural effusion in both lungs. **(B)** After 2 weeks of treatment, a CT scan showed that the lesions were absorbed in the right side of patient's lung. **(C)** After 3 weeks of treatment, a CT scan showed that lesions were significantly absorbed in the right side of patient's lung (red arrow) and smaller amount of bilateral pleural effusion in the side of patient's lung than that after 2 weeks of treatment.

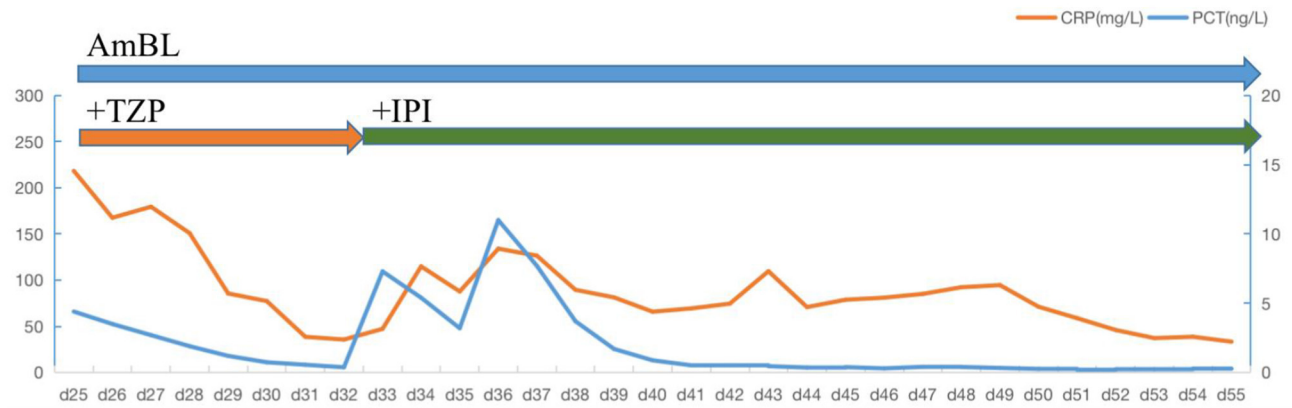


FIGURE 4 | Daily course of the patient's treatment. Horizontal thick blue lines show the medications administered; VRC, voriconazole; AmBL, Liposome-associated amphotericin B; TZP, piperacillin-tazobactanm; IPI, Imipenem; PCT, procalcitonin; CRP, C-reactive protein.

leading to cerebral infarction. In addition, high sequence of elizabethkingia and stenotrophomonas was detected by mNGS of BAL later. It could be a small amount of bacteria hidden in the infusion port, which caused the patient's second infection. Infusion port of patient was removed, and IPI was applied immediately again. PCT and CRP was controlled later.

In general, early initiation of antifungal therapy improves the outcome of mucormycosis infection. A previous study showed that 70 patients with hematologic malignancy and infection of mucormycosis who experienced delayed amphotericin B therapy (starting therapy more than 6 days after diagnosis) were reported to have two-fold increase in mortality rate at 12 weeks after diagnosis compared to early treatment (82.9% versus 48.6%) (30). In the present case study, mNGS, a fast and non-culture-dependent method, was used to diagnose mucormycosis infection, and the initial therapy of AmBL was

performed immediately to treat this disease. The patient's general condition improved, and he eventually partly recovered his left limb function.

mNGS technology, with the advantages of speed and precision, is a selective method for culture-negative diagnosis of the fungal infection, and it can be applied to identify microbes in other symptoms in the future. Besides, some factors such as warm ischemia can interpreted NGS data, which may be the obstacles of NGS. In addition, febrile neutropenia in patients after chemotherapy with cerebral symptoms may be highly indicative of infections in the brain.

CONCLUSION

mNGS testing of PB, CSF, and BAL is a reliable and fast method for precisely diagnosing culture-negative disseminated mucormycosis. Early identification of infectious pathogens can guide antifungal therapy in such patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Second People's Hospital of Shenzhen. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XD contributed to conception and design of the study. LC and YC analyzed and collected data. BW drafted the manuscript. All authors contributed to interpretation of the results, revised the manuscript, and read and approved the final manuscript.

FUNDING

This study was supported by Shenzhen Key Medical Discipline Construction Fund (SZXK008).

ACKNOWLEDGMENTS

This work was grateful for Shenzhen Second People's Hospital.

REFERENCES

1. Kauffman CA Malani AN. Zygomycosis: an emerging fungal infection with new options for management. *Curr Infect Dis Rep.* (2007) 9:435–40. doi: 10.1007/s11908-007-0066-4
2. Lee FY, Mossad SB, Adal KA. Pulmonary mucormycosis: the last 30 years. *Arch Internal Med.* (1999) 159:1301–9. doi: 10.1001/archinte.159.12.1301
3. Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, Schaufele RL, et al. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. *Clin Infect Dis.* (2005) 41:634–53. doi: 10.1086/432579
4. Kontoyiannis DP, Lionakis MS, Lewis RE, Chamilos G, Healy M, Perego C, et al. Zygomycosis in a tertiary-care cancer center in the era of Aspergillus-active antifungal therapy: a case-control observational study of 27 recent cases. *J Infect Dis.* (2005) 191:1350–60. doi: 10.1086/428780
5. Petrikkos G, Skiada A, Lortholary O, Roilides E, Walsh TJ, Kontoyiannis DP. Epidemiology and clinical manifestations of mucormycosis. *Clin Infect Dis.* (2012) 54:S23–34. doi: 10.1093/cid/cir866
6. Machouart M, Larché J, Burton K, Collomb J, Maurer P, Cintrat A, et al. Genetic identification of the main opportunistic Mucorales by PCR-restriction fragment length polymorphism. *J Clin Microbiol.* (2006) 44:805–10. doi: 10.1128/JCM.44.3.805-810.2006
7. Hammond SP, Bialek R, Milner DA, Petschnigg EM, Baden LR, Marty FM. Molecular methods to improve diagnosis and identification of mucormycosis. *J Clin Microbiol.* (2011) 49:2151–3. doi: 10.1128/JCM.00256-11
8. Walsh TJ, Gamaletsou MN, McGinnis MR, Hayden RT, Kontoyiannis DP. Early clinical and laboratory diagnosis of invasive pulmonary, extrapulmonary, and disseminated mucormycosis (zygomycosis). *Clin Infect Dis.* (2012) 54:S55–60. doi: 10.1093/cid/cir868
9. Meyerowitz EA, Sanchez S, Mansour MK, Triant VA, Goldberg, MB. Isolated cerebral mucormycosis in immunocompetent adults who inject drugs: case reports and systematic review of the literature. *Open Forum Infect Dis.* (2020) 7:ofaa552. doi: 10.1093/ofid/ofaa552
10. Droll A, Kunz F, Passweg JR, Michot M. Cerebral mucormycosis in a patient with myelodysplastic syndrome taking corticosteroids. *Br J Haematol.* (2004) 126:441. doi: 10.1111/j.1365-2141.2004.05080.x
11. Lackner M, Caramalho R, Lass-Flörl C. Laboratory diagnosis of mucormycosis: current status and future perspectives. *Fut Microbiol.* (2014) 9:683–95. doi: 10.2217/fmb.14.23
12. Dhakar MB, Rayes M, Kupsky W, Tselis A, Norris G. A cryptic case: isolated cerebral mucormycosis. *Am J Med.* (2015) 128:1296–9. doi: 10.1016/j.amjmed.2015.08.033
13. Hazama A, Galgano M, Fullmer J, Hall W, Chin, L. Affinity of mucormycosis for basal ganglia in intravenous drug users: case illustration and review of literature. *World Neurosurg.* (2017) 98:872e871–3. doi: 10.1016/j.wneu.2016.11.130
14. Kerezoudis P, Watts CR, Bydon M, Dababneh AS, Deyo CN, Frye JM, et al. Diagnosis and treatment of isolated cerebral mucormycosis: patient-level data meta-analysis and mayo clinic experience. *World Neurosurg.* (2019) 123:425–434.e425. doi: 10.1016/j.wneu.2018.10.218
15. Chiu CY, Miller S. A clinical metagenomics nature reviews. *Genetics.* (2019) 20:341–55. doi: 10.1038/s41576-019-0113-7
16. Martin-Blais R, Pathak S, Fitzwater S, Dawson DW, Sisk AE, Farmer DG, et al. Intestinal mucormycosis initially identified by next-generation sequencing of cell-free DNA. *Trans Infect Dis.* (2021) 23:e13656. doi: 10.1111/tid.13656
17. Liu Y, Zhang J, Han B, Du L, Shi Z, Wang C, et al. Case report: diagnostic value of metagenomics next generation sequencing in intracranial infection caused by Mucor. *Front Med.* (2021) 8:682758. doi: 10.3389/fmed.2021.682758
18. Higo T, Kobayashi T, Yamazaki S, Ando S, Gonoi W, Ishida M, et al. Cerebral embolism through hematogenous dissemination of pulmonary mucormycosis complicating relapsed leukemia. *Int J Clin Exp Pathol.* (2015) 8:13639–42.
19. Eucker J, Sezer O, Lehmann R, Weber JR, Graf B, Denkert C, et al. Disseminated mucormycosis caused by Absidia corymbifera leading to cerebral vasculitis. *Infection.* (2000) 28:246–50. doi: 10.1007/s150100070047
20. Pagano L, Ricci P, Tonso A, Nosari A, Cudillo L, Montillo M, et al. Mucormycosis in patients with haematological malignancies: a retrospective clinical study of 37 cases. *Br J Haematol.* (1997) 99:331–6. doi: 10.1046/j.1365-2141.1997.3983214.x
21. Hagensee ME, Bauwens JE, Kjos B, Bowden RA. Brain abscess following marrow transplantation: experience at the Fred Hutchinson Cancer Research Center, 1984-1992. *Clin Infect Dis.* (1994) 19:402–8. doi: 10.1093/clinids/19.3.402

22. El-Ani AS, Dhar V. Disseminated mucormycosis in a case of metastatic carcinoma. *Am J Clin Pathol.* (1982) 77:110–4. doi: 10.1093/ajcp/77.1.110
23. Telenti A, Roberts GD. Fungal blood cultures. *Eur J Clin Microbiol Infect Dis.* (1989) 8:825–31. doi: 10.1007/BF02185855
24. Pagano L, Ricci P, Nosari A, Tonso A, Buelli M, Montillo M, et al. Fatal haemoptysis in pulmonary filamentous mycosis: an underevaluated cause of death in patients with acute leukaemia in haematological complete remission. A retrospective study and review of the literature Gimema Infection Program (Gruppo Italiano Malattie Ematologiche dell'Adulto). *Br J Haematol.* (1995) 89:500–5. doi: 10.1111/j.1365-2141.1995.tb08355.x
25. Heussel CP, Kauczor HU, Heussel GE, Fischer B, Begrich M, Mildenberger P, et al. Pneumonia in febrile neutropenic patients and in bone marrow and blood stem-cell transplant recipients: use of high-resolution computed tomography. *J Clin Oncol.* (1999) 17:796–805. doi: 10.1200/JCO.1999.17.3.796
26. McAdams HP, Rosado de Christenson M, Strollo DC, Patz Jr EF. Pulmonary mucormycosis: radiologic findings in 32 cases. *AJR.* (1997) 168:1541–8. doi: 10.2214/ajr.168.6.9168721
27. Lefterova MI, Suarez CJ, Banaei N, Pinsky B. A next-generation sequencing for infectious disease diagnosis and management: a report of the association for molecular pathology. *J Molec Diagnost.* (2015) 17:623–34. doi: 10.1016/j.jmoldx.2015.07.004
28. Besser J, Carleton HA, Gerner-Smidt P, Lindsey RL, Trees E. Next-generation sequencing technologies and their application to the study and control of bacterial infections. *Clin Microbiol Infect.* (2018) 24:335–41. doi: 10.1016/j.cmi.2017.10.013
29. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infec Dis.* (2018) 67:S231–s240. doi: 10.1093/cid/ciy693
30. Chamilos G, Lewis RE, Kontoyiannis DP. Delaying amphotericin B-based frontline therapy significantly increases mortality among patients with hematologic malignancy who have zygomycosis. *Clin Infec Dis.* (2008) 47:503–9. doi: 10.1086/590004

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Wen, Cai, Cai and Du. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 23 December 2021
doi: 10.3389/fmed.2021.752523

Diffuse Large B-Cell Lymphoma of the Mandible Diagnosed by Metagenomic Sequencing: A Case Report

Kaili Liu[1†], Yang Gao[2†], Jiawei Han[3], Xu Han[4], Yingqi Shi[4], Chao Liu[4] and Jie Li[1*]*

[1] Department of General Medical Ward, First Affiliated Hospital of Soochow University, Suzhou, China, [2] School of Biological Science and Medical Engineering, Beihang University, Beijing, China, [3] Department of Neurosurgery, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [4] Department of Medical, Hangzhou Matridx Biotechnology Co., Ltd, Hangzhou, China

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UConn Health, United States

Reviewed by:
Nitya Singh,
University of Florida, United States
Rajesh Kashyap,
Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), India

***Correspondence:**
Chao Liu
liuchao@matridx.com
Jie Li
lijie@suda.edu.cn

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 03 August 2021
Accepted: 06 December 2021
Published: 23 December 2021

Citation:
Liu K, Gao Y, Han J, Han X, Shi Y, Liu C and Li J (2021) Diffuse Large B-Cell Lymphoma of the Mandible Diagnosed by Metagenomic Sequencing: A Case Report. Front. Med. 8:752523. doi: 10.3389/fmed.2021.752523

Introduction: Non-Hodgkin lymphoma (NHL) has a much higher incidence rate than Hodgkin lymphoma. Approximately 40% NHL occurs in extranodal tissues or organs, and its clinical manifestations are often nonspecific. Primary bone NHL involving the mandible is an uncommon NHL that is characterized by fever, gum swelling and toothache. Therefore, it is often misdiagnosed as oral diseases.

Case Presentation: A 52-year-old female had recurrent fever for more than 1 month, with numbness in her left jaw and toothache. PET/CT showed an uptake area in the left mandible, suggesting microbial infections. However, antibacterial, and antiviral treatment were ineffective. Furthermore, metagenomic sequencing of plasma reported no pathogens, but instead showed significant copy number variations of multiple chromosomes, which highly suggested the existence of tumor. Finally, diffuse large B-cell lymphoma (DLBCL) was diagnosed by mandibular biopsy, and the patient was transferred to Hematology department for chemotherapy.

Conclusion: mNGS not only assists rapid etiological diagnosis, but also helps rule out infection and diagnose malignant neoplasm.

Keywords: non-Hodgkin lymphoma, mandibular NHL, diffuse large B-cell lymphoma, copy number variations, metagenomic next-generation sequencing, fever of unknown origin

INTRODUCTION

Lymphomas are a group of malignant neoplasms originated from lymphoid hematopoietic system and the second most common malignancy of the head and neck region (1–3). Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) are the two main categories of lymphomas, the latter of which occurs more frequently (4). NHL patients usually present with painless lymphadenopathy, and might or might not have other symptoms including fevers, fatigue and drenching night sweats (5). NHL is primarily a disease of the lymph nodes, but it can also involve organs outside the lymphoid system such as the stomach, skin, lung, central nervous system, and oral cavity (6). Primary bone NHL involving the mandible, an uncommon oral cavity NHL, accounts for 0.6% of extranodal lymphomas (7). The clinical manifestations of Primary bone NHL involving the mandible are toothache, swelling, tooth loosening and pathological fracture. In early stage, it is

often misdiagnosed as dental pulp disease, causing a delay in the diagnosis and treatment (7). Most Primary bone NHL involving the mandible are diffuse large B-cell lymphoma (DLBCL), which develops rapidly and has poor prognosis (8).

Metagenomic Next-generation Sequencing (mNGS) can unbiasedly sequence nucleic acids of both human and microbial origin from clinical samples. It is a hypothesis-free and culture-free technique that has been widely used in diagnosing infectious diseases (9). A retrospective study of patients with fever of unknown origin showed that the positive rate of mNGS in plasma samples was significantly higher than traditional culture (61.0 vs. 19.5%) (10). Meanwhile, the sequences of human DNA derived from mNGS can be used to detect chromosomal copy number variations, which might be useful for the diagnosis of cryptogenic malignancies (11).

Here we report a case of mandibular DLBCL in a 52-year-old female with recurrent fever and toothache, in which microbial infections were excluded based on mNGS results. Moreover, cancer was suspected due to abnormal copy number of the patient's chromosomes via analysis of host DNA sequences, which was later confirmed by mandible biopsy.

CASE REPORT

Case Presentation

A 52-year-old female presented with recurrent fever for more than 1 month, with a highest temperature of 38.8°C and chills. The patient experienced numbness in her left mandible and slight pain in her left teeth. The blood routine examination was normal, C-reactive protein (CRP) was 7.33 mg/L, erythrocyte sedimentation rate (ESR) was 42 mm/h, rheumatoid factor was 5.7 IU/ml, anti-o was 89 IU/ml, anti-nuclear antibody, alpha work, Epstein Barr virus, cytomegalovirus DNA, tumor biomarkers were negative. The head (including the mandible region), chest, and abdomen CT showed no active lesions, and echocardiography showed no abnormality. Prior to hospitalization, the patient had a full-body positron emission tomography (PET) scan performed, which revealed an elevated glucose metabolism in the left alveolar branch of the mandible (standardized uptake value (SUV) max = 5.87) (**Figure 1**). In addition, multiple small nodules were seen in both lungs and exudative changes in the lower lobes of both lungs and splenomegaly were observed. These findings were also confirmed by chest, abdomen, and pelvis enhanced CT. As a result, microbial infection was primarily suspected. Following 4 weeks of antibacterial and antiviral treatment, the patient still had recurrent fever, which was accompanied by perspiration. The blood routine showed the red blood cell count was $3.10*10^{12}$/L, hemoglobin was 86 g/L, neutrophil was $1.77*10^{9}$/L, lymphocyte was $0.71*10^{9}$/L, CRP was 16.6 mg/L.

Urine routine examination showed that urine occult blood was weakly positive. Stool routine, blood transfusion routine, blood culture, urine culture, humoral immunity, Widal test, Ana antibody spectrum, antineutrophil cytoplasmic antibody, G test, and EBV-DNA were negative. GM test (0.59) and T-spot.TB test were positive. The biochemical examination showed normal liver and kidney function, albumin 26.7 g/L, ESR 46 mm/h, coagulation function showed D-Dimer was 0.73 mg/L, and prothrombin time was 15.30 s. Tumor biomarker test showed ferritin was 363.36 ng/ml, and serum immunofixation electrophoresis was negative. Ultrasound examination showed no obvious abnormalities of superficial lymph nodes, esophagus, and heart. However, abdominal ultrasound showed substantial echo at the lower pole of spleen, and accessory spleen was considered.

Initial Treatment

The patient was first administered with ticarcillin and moxifloxacin. Nine days after treatment, the fever was still present that peaked at 38.6°C. The blood routine and biochemical examination showed no apparent changes. The serum protein electrophoresis and free light chain quantitative were negative. The antibiotics were then escalated to linezolid combined with sulperazon and tinidazole. Three days later, the patient's symptoms did not improve, and thus antibiotics were withdrawn.

MNGS Testing

Due to little effect of antibiotic treatment, the patient's peripheral blood was collected for mNGS. No pathogen was detected, suggesting a likelihood of non-infectious disease. Meanwhile, the chromosomal copy number analysis showed significant alterations (both duplications and deletions) in chromosomes 1, 5, 6, 11, and 14, which indicated the presence of malignant tumor DNA (**Figure 2**). The same CNV analysis was repeated using buccal swab that showed no abnormalities, suggesting the CNVs were not due to inherited diseases.

Follow-Up and Outcomes

The patient underwent bone marrow biopsy. The morphological examination showed that the percentage of erythroid cells was slightly higher, and iron staining showed that iron utilization was impaired. Hemophagocytes were sporadically seen in the bone marrow. A biopsy of the left mandible was taken, and the immunohistochemical results showed cells positive for CD20, CD79a, Bcl-2, CD5 and c-myc, and Ki-67 value-added index was 90% (**Figure 3**). In addition, CD10, kappa, lambda, CD138, CD38, CD3, Desmin, MyoD1, Myogenin, CD99, CK, Syn, EMA, CyclinD1, Bcl-6, MUM1, CD30 and Sox11 were negative. The pathological findings suggested that the tumor was malignant B-cell lymphoma (diffuse large B-cell type). The patient was diagnosed with DLBCL and transferred to the Hematology department for immunochemotherapy [rituximab 600 mg d1, cyclophosphamide 1.1 g d2, doxorubicin 40 mg d2, vindesine 4 mg d2, and prednisone 15 mg d1-5 (R-CHOP)]. Five cycles of R-CHOP were administered and another full-body PET-CT was performed, which showed a decline of glucose metabolism in the left mandible region (**Supplementary Figure 1**). Adjuvant radiation therapy was initiated following the conclusion of chemotherapy and a total of 15 sessions were performed, after which the patient was discharged. During the most recent follow up visit, the patient had no fever, no cough, no abdominal pain, normal diet, and sleep.

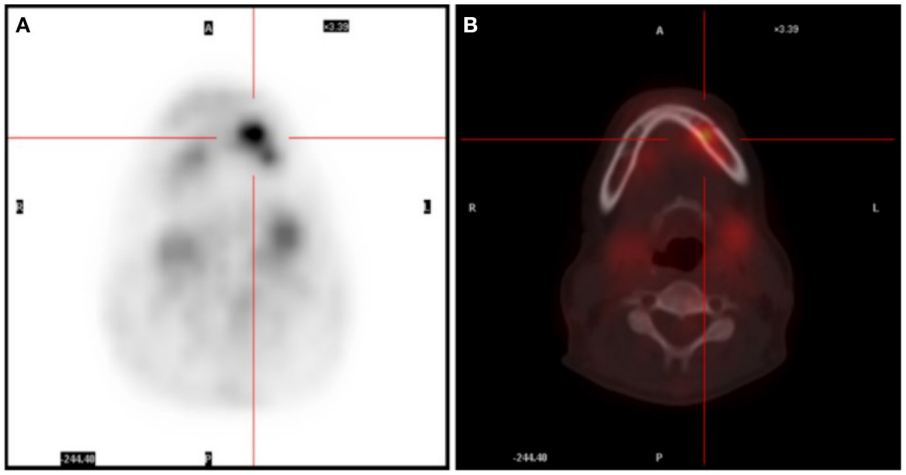


FIGURE 1 | (A) PET scan showing an area with glucose uptake in the facial region. **(B)** PET/CT scan revealing the uptake area located in the left mandible.

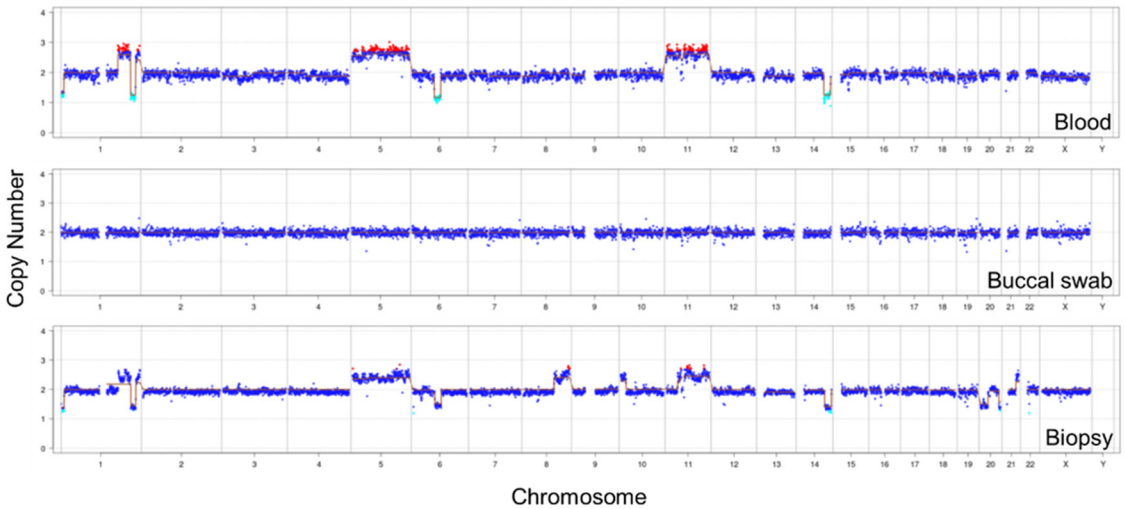


FIGURE 2 | CNV analysis of the patient's autosomes and sex chromosomes. Both plasma and mandibular biopsy samples showed large CNVs on chromosomes 1, 5, 6, 11, and 14. The same analysis using buccal swab showed normal copy numbers.

DISCUSSION

Approximately 40% of NHL occurred in extranodal tissues or organs (12, 13), and the most common sites of occurrence was gastrointestinal tract, followed by head and neck (14). A retrospective study revealed that of 381 patients with NHL (100 were extranodal), 16 (16%) had maxillary or mandibular involvement and most of which were DLBCL (15). DLBCL is the most common type of NHL, accounting for 40% of all cases. It is a type of cancer involving distinct clinical, morphological, immunohistochemical features and prognosis (7). Primary bone NHL involving the mandible is often characterized by swelling, pain, abnormal sensation, loose teeth, and pathological fracture. Patients are usually treated in the Department of Stomatology and often misdiagnosed with oral diseases. In this case, the patient received dental root canal treatment and anti-infection treatment with negligible improvement of symptoms.

Fever of unknown origin can be attributed to many factors, including infection (40%), neoplastic disease (20%), collagen-vascular disease (15%) and so on (16). A retrospective study found that tuberculosis was the most common cause of infectious fever and lymphoma was the most common cause of neoplastic fever (17). In this case, the hemogram and blood biochemistry of the patient were basically normal, CRP and ESR were slightly elevated, indicating inflammatory reaction. Routine etiological tests were negative except for T-spot.TB test. However, chest CT showed no signs of tuberculosis infection. And GM test was positive for the first time but negative when repeated.

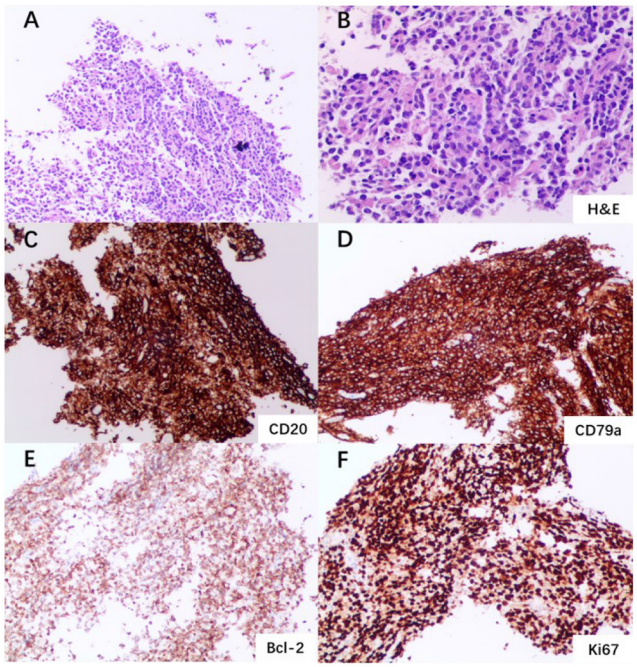


FIGURE 3 | Left mandible biopsy and immunohistochemistry. **(A)** Multiple fragments of a squamous mucosa and soft tissue with a dense lymphoid infiltrate (×100 magnification). **(B)** Aggregate of atypical lymphoid cells (H&E staining; ×200 magnification). **(C)** Immunohistochemical staining showing sheets of large mononuclear lymphoid cells positive for CD20 (×100 magnification). **(D)** Sheets of large mononuclear lymphoid cells positive for CD79a (×100 magnification). **(E)** Cells positive for Bcl-2 staining (×100 magnification). **(F)** Cells positive for Ki67 staining (×100 magnification).

Combined with the lack of pathogens in mNGS results, microbial infection was mostly ruled out. In addition, the full-body PET scan showed increased glucose metabolism in the left mandible, and ferritin and tumor specific growth factor were elevated in serum. However, tumor was not suspected prior to mNGS because abscess, inflammation and dental caries could also lead to increased glucose metabolism, and the specificity of tumor biomarkers was low.

mNGS has been predominantly used for diagnosing infectious diseases, even though most sequences in clinical samples originate from the host. By analyzing human reads, one could obtain potentially valuable information and use it to aid diagnosis.

Copy number variation (CNV) is the change of copy number in one or more genes. Genomic instability is one of the hallmarks of cancer (18). Multiple studies have shown that DNA copy number variation (CNV) is an important component of genetic variation involved in the development and progression of many types of cancer. Chromosomal CNVs detected by sequencing-based tests have been recently used for early diagnosis of colorectal cancer (19), breast cancer (20), and hematologic malignancies (21). Therefore, mNGS has the potential to diagnose both microbial infections and tumor since it unbiasedly sequences DNA of both host and microbial origin. In Cui et al. (22) detected CNV in clinical samples and cell lines of DLBCL patients, and the results showed that CNV was related to the prognosis of patients and might be an important feature of tumor development. Shotgun metagenomic sequencing is suitable for CNV detection across all chromosomes, which has good specificity and reproducibility (23).

The gold standard for tumor diagnosis is biopsy and pathological staining, which requires an invasive procedure that typically takes 3–7 days. In addition, biopsy is not always performed especially when tumor is not primarily considered. Compared to that, mNGS can be performed on a variety of clinical samples and report results in hours. Although the detection of chromosomal CNVs by mNGS cannot directly lead to a specific cancer diagnosis, it could prompt more focused diagnostic testing for a tumor. By incorporating pathogen detection and copy number analysis, mNGS can provide multiple levels of diagnostic clues. It helps optimize the sensitivity of tumor diagnosis and has significance in differential diagnosis for patients suspected of infections, especially when empirical antibiotic therapy fails.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra, accession number PRJNA752259.

ETHICS STATEMENT

Written informed consent was obtained from the relevant individual(s), and/or minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KL is the primary physician that provided diagnosis and treatment of the patient. YG collected and analyzed clinical and sequencing data. JH, XH, and YS performed metagenomic sequencing experiments and CNV analysis. CL and JL wrote the manuscript. All authors have read and approved the final version of the manuscript.

FUNDING

This project was supported by the Science and Technology Plan Projects of Suzhou (grant no. SYS2020099).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.752523/full#supplementary-material

REFERENCES

1. Coskunses FM, Cilasun Ü, Celik Topcu P, Tokuc B. Primary diffuse large B-cell lymphoma of the mandible: a case report. *Gerodontology.* (2020) 37:307–11. doi: 10.1111/ger.12470
2. Agrawal MG, Agrawal SM, Kambalimath DH. Non-Hodgkins lymphoma of maxilla: a rare entity. *Natl J Maxillofac Surg.* (2011) 2:210–3. doi: 10.4103/0975-5950.94485
3. Cazzola M. Introduction to a review series: the 2016 revision of the WHO classification of tumors of hematopoietic and lymphoid tissues. *Blood.* (2016) 127:2361–4. doi: 10.1182/blood-2016-03-657379
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin.* (2020) 70:7–30. doi: 10.3322/caac.21590
5. Armitage JO, Gascoyne RD, Lunning MA, Cavalli F. Non-Hodgkin lymphoma. *Lancet.* (2017) 390:298–310. doi: 10.1016/S0140-6736(16)32407-2
6. Takahashi H, Tsuda N, Tezuka F, Okabe H. Primary extranodal non-Hodgkin's lymphoma of the oral region. *J Oral Pathol Med.* (1989) 18:84–91. doi: 10.1111/j.1600-0714.1989.tb00742.x
7. Siqueira JM, Fernandes PM, de Oliveira ACF, Vassallo J, Alves FA, Jaguar GC. Primary diffuse large B-cell lymphoma of the mandible. *Autops Case Rep.* (2019) 9:e2019109. doi: 10.4322/acr.2019.109
8. Rodrigues-Fernandes CI, de Souza LL, Santos-Costa SFD, Pontes HAR, de Almeida OP, Vargas PA, et al. Clinicopathological analysis of oral diffuse large B-cell lymphoma, NOS: a systematic review. *J Oral Pathol Med.* (2019) 48:185–91. doi: 10.1111/jop.12802
9. Chiu CY, Miller SA. Clinical metagenomics. *Nat Rev Genet.* (2019) 20:341–55. doi: 10.1038/s41576-019-0113-7
10. Hogan CA, Yang S, Garner OB, Green DA, Gomez CA, Dien Bard J, et al. Clinical impact of metagenomic next-generation sequencing of plasma cell-free DNA for the diagnosis of infectious diseases: a multicenter retrospective cohort study. *Clin Infect Dis.* (2021) 72:239–45. doi: 10.1093/cid/ciaa035
11. Gu W, Talevich E, Hsu E, Qi Z, Urisman A, Federman S, et al. Detection of cryptogenic malignancies from metagenomic whole genome sequencing of body fluids. *Genome Med.* (2021) 13:98. doi: 10.1186/s13073-021-00912-z
12. Mendonça EF, Sousa TO, Estrela C. Non-Hodgkin lymphoma in the periapical region of a mandibular canine. *J Endod.* (2013) 39:839–42. doi: 10.1016/j.joen.2013.01.007
13. Oueslati Y, Chebil RB, Abidi I, Sriha B, Khochtali H, Oualha L, et al. Mandibular non-Hodgkin's lymphoma: two observations of a challenging disease. *Pan Afr Med J.* (2020) 37:102. doi: 10.11604/pamj.2020.37.102.23770
14. Fuessinger MA, Voss P, Metzger MC, Zegpi C, Semper-Hogg W. Numb chin as signal for malignancy-primary intraosseous diffuse large b-cell lymphoma of the mandible. *Ann Maxillofac Surg.* (2018) 8:143–6. doi: 10.4103/ams.ams_163_17
15. Etemad-Moghadam S, Tirgary F, Keshavarz S, Alaeddini M. Head and neck non-Hodgkin's lymphoma: a 20-year demographic study of 381 cases. *Int J Oral Maxillofac Surg.* (2010) 39:869–72. doi: 10.1016/j.ijom.2010.03.029
16. Jacoby GA, Swartz MN. Fever of undetermined origin. *N Engl J Med.* (1973) 289:1407–10. doi: 10.1056/NEJM197312272892607
17. Zhou G, Zhou Y, Zhong C, Ye H, Liu Z, Liu Y, et al. Retrospective analysis of 1,641 cases of classic fever of unknown origin. *Ann Transl Med.* (2020) 8:690. doi: 10.21037/atm-20-3875
18. Shlien A, Malkin D. Copy number variations and cancer. *Genome Med.* (2009) 1:62. doi: 10.1186/gm62
19. Xu JF, Kang Q, Ma XY, Pan YM, Yang L, Jin P, et al. A novel method to detect early colorectal cancer based on chromosome copy number variation in plasma. *Cell Physiol Biochem.* (2018) 45:1444–54. doi: 10.1159/000487571
20. Kumaran M, Cass CE, Graham K, Mackey JR, Hubaux R, Lam W, et al. Germline copy number variations are associated with breast cancer risk and prognosis. *Sci Rep.* (2017) 7:14621. doi: 10.1038/s41598-017-14799-7
21. Lenaerts L, Vandenberghe P, Brison N, Che H, Neofytou M, Verheecke M, et al. Genomewide copy number alteration screening of circulating plasma DNA: potential for the detection of incipient tumors. *Ann Oncol.* (2019) 30:85–95. doi: 10.1093/annonc/mdy476
22. Cui W, Cai Y, Wang W, Liu Z, Wei P, Bi R, et al. Frequent copy number variations of PI3K/AKT pathway and aberrant protein expressions of PI3K subunits are associated with inferior survival in diffuse large B cell lymphoma. *J Transl Med.* (2014) 12:10. doi: 10.1186/1479-5876-12-10
23. Liang D, Peng Y, Lv W, Deng L, Zhang Y, Li H, et al. Copy number variation sequencing for comprehensive diagnosis of chromosome disease syndromes. *J Mol Diagn.* (2014) 16:519–26. doi: 10.1016/j.jmoldx.2014.05.002

Conflict of Interest: XH, YS, and CL are employees of Hangzhou Matridx Biotechnology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Liu, Gao, Han, Han, Shi, Liu and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Public Health

CASE REPORT
published: 04 January 2022
doi: 10.3389/fpubh.2021.771154

Case Report: Next-Generation Sequencing in Diagnosis of Atypical Aspiration Pneumonia

Quan Zhang[1], *Wei Xu*[2], *Fei Peng*[1], *Si Lei*[1], *Yanjun Zhong*[3], *Jun Yi*[4]* and *Shangjie Wu*[1]*

[1] Pulmonary and Critical Care Medicine, The Second Xiangya Hospital, Central South University, Changsha, China, [2] Department of Neurology, The First People's Hospital of Chenzhou, Chenzhou, China, [3] Critical Care Medicine, The Second Xiangya Hospital, Central South University, Changsha, China, [4] Department of Gastroenterology, Xiangya Hospital of Central South University, Changsha, China

Since the pandemic of Corona Virus Disease 2019 (COVID-19), especially in the centers most affected, the symptoms such as fever, cough, myalgia or fatigue, and radioactive signs typically related to COVID-19 like ground-glass opacity (GGO) often distract the attention of physicians from other diseases. Aspiration pneumonia and COVID-19 share similarities in some aspects. There may be risk of misdiagnosis in the case of considering radiological patterns of pneumonia. Early diagnosis and treatment often greatly improve prognosis. We herein reported a case of 40-year-old patient who underwent chest CT scan with the discovery of ground-glass opacity, intralobular reticular opacity and interlobular septal thickening, consolidation, and air bronchogram sign, which were mainly located in the middle and upper lobes of the right lung. It was considered to be infection related pneumonia based on the negative reverse transcription-PCR (RT-PCR) result of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The next-generation sequencing (NGS) of bronchoalveolar lavage fluid (BALF) was performed and detected nucleic acid sequences of *Klebsiella sp.* Consequently, the patient accepted sensitive intravenous antibiotics therapy for 13 days and had a remarkable clinical and radiological improvement. His case was followed up through imaging procedures. Because of possible radiologic and clinical similarities between aspiration and COVID-19 pneumonia, COVID-19 can be of some value in proposing a differential diagnosis of aspiration pneumonia. Clinicians could suggest a correct diagnosis by careful examination of the CT images together with attention to the clinical history and judicious utilization of NGS, especially.

Keywords: COVID-19, aspiration pneumonia, next generation sequencing—NGS, case report, differential diagnosis

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Armelia Sari Widyarman,
Trisakti University, Indonesia
Ye Gao,
Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, China

***Correspondence:**
Shangjie Wu
wushangjie@csu.edu.cn
Jun Yi
junyee1989@163.com

Specialty section:
This article was submitted to Infectious Diseases–Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: *06 September 2021*
Accepted: *06 December 2021*
Published: *04 January 2022*

Citation:
Zhang Q, Xu W, Peng F, Lei S, Zhong Y, Yi J and Wu S (2022) Case Report: Next-Generation Sequencing in Diagnosis of Atypical Aspiration Pneumonia. Front. Public Health 9:771154. doi: 10.3389/fpubh.2021.771154

INTRODUCTION

Since the outbreak of Corona Virus Disease 2019 (COVID-19), millions of families worldwide have been invaded, and the development of economy, education, health, and other industries in many countries has been affected in varying degrees. In absence of specific therapeutic vaccines or drugs, early detection and isolation becomes essential against the novel coronavirus. However, in that current emergency, with limitations of sample collection or transportation and kit performance,

the total positive rate of reverse transcription-PCR (RT-PCR) for throat swab samples was reported to be about 30 to 60% at initial presentation (1). Therefore, radiologists revealed that chest CT can provide benefit for diagnosis of COVID-19. Despite its high sensitivity, CT findings are unspecific and may overlap with other diseases (2). However, the symptoms such as fever, cough, myalgia or fatigue, and radioactive signs typically related to COVID-19, like ground-glass opacity (GGO), often distract the attention of physicians from other diseases.

Aspiration pneumonia is a common disease caused by aspiration or inhalation of irritant substances. It is estimated to account for 5–15% of people diagnosed with community-acquired pneumonia (CAP) and is expected to occupy a larger proportion in the aging society over the next coming decades (3, 4). In general, its CT findings are simply characterized by gravity-dependent lung opacity. However, there are some similarities between aspiration pneumonia and COVID-19 pneumonia. Bilateral subpleural patches of GGO, multifocal patchy consolidation, especially in a peripheral distribution with bilateral, and multifocal lower lung involvement have been described as typical for the diagnosis of COVID-19 pneumonia in suspected cases (5, 6). Such presentations are also fairly common in aspiration pneumonia. These changes, which had been present in up to 74% of the patients, make the discrimination more difficult (7). Therefore, how to realize an effective differentiation of the two diseases and make an initial decision for appropriate allocation of the patients to the COVID or non-COVID wards or intensive care units (ICUs) was a challenge we encountered, especially in the current situation of the persistent outbreak of COVID-19 pandemic. In this context, an early, timely, and accurate diagnosis is utmost important for the treatment of potentially catastrophic and life-threatening infection along with for the prognosis of patients with infection. Here, we report one case of aspiration pneumonia in the atypical site, which was confirmed to be infected by *Klebsiella pneumoniae* (*Klebsiella sp.*). Clinical characteristics and findings of high-resolution thoracic CT of the patient are also described.

CASE PRESENTATION

A 40-year-old Chinese man with smoking (30 cigarettes per day) and drinking (300 ml 52% Chinese rice wine per day) habit for over 10 years, and with 2-month exposure history in COVID-19 epidemic area of Wuhan hospital presented to our Emergency Department on June 28, 2020. The chief complaints of this patient were fever and cough for two days, along with ten bouts of abdominal pain and diarrhea. In addition, the patient experienced shortness of breath since the morning of the day of consultation and still had a fever. For the monitoring of vital signs at admission, the patient presented with a blood pressure of 78/46 mmHg, pulse rate of 122 beats/min, respiratory rate of 35 breaths/min, body temperature of 39°C, and oxygen saturation of 79% on room air. Further physical examination revealed dry skin but cold limbs, several spider nevi in the neck, and moist crackles in the right lung on auscultation. For his epidemic history, the emergency physician immediately prescribed throat swab test for COVID-19. The results of routine laboratory tests showed white blood cell count of 3.73×10^9/L (94% neutrophils), platelet count of 32×10^9/L, procalcitonin of 66.5 ng/ml, creatinine of 182.7 μmol/L, alanine aminotransferase (ALT) of 175 U/L, aspartate aminotransferase (AST) of 416 U/L, lactic acid of 5.6 mmol/L, and Na+ of 132 mmol/L with no rest abnormalities in electrolytes.

The patient was provided with chest CT, with the observation of large pieces of high-density shadows particularly dominant in the middle and upper lobes of right lung (**Figure 1A**). The patient appeared with a pale face, was restless, and showing signs of severe respiratory distress and was thus provided with endotracheal intubation for ventilation. Subsequently, when the RT-PCR result for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) returned negative the second morning, the patient was admitted to ICU for further monitoring and therapy. At first, the vomiting history of the patient was not collected due to sedation. Intravenous norepinephrine and hydrocortisone (100 mg q12 h) were given due to the lack of fluid infusion space to reverse shock. Under the view of bronchofiberscopy, the patient was found to have moderate stenosis of right middle and upper lung bronchus and suctioned with copious yellow bloody secretions from the airways. We chose meropenem (1 g q12 h) and moxifloxacin (0.4 g qd) as the antibiotics for empirical antibacterial treatment, for it was difficult to judge whether the pathogen was gram-positive or gram-negative bacteria. At the same time, the blood sample and bronchoalveolar lavage fluid (BALF) were collected to perform metagenomic next-generation sequencing (NGS) and bacterial culture on the first day. The bioinformatics pipeline included (1) standard sample quality filtering, (2) elimination of replicate reads, (3) removal of low-complexity reads matching human genome sequence, and (4) classification of reads by simultaneously aligning to Pathogens metagenomics Database, which was downloaded from latest RefSeq:NCBI reference consisting of 4,945 whole genome sequence of viral taxa, 6,350 bacterial genomes or scaffolds, 1,064 fungi related to human infection, and 234 parasites associated with human diseases.

After 48 h on admission, both the NGS of BALF and whole blood sample revealed *Klebsiella sp.*, which was consistent with the bacterial culture result of BALF 3 days later. The other micro-organisms detected in the NGS were not regarded as responsible for the invasive pulmonary infection (**Table 1**). Taking into account the clinical manifestations, rapid organ damage, the NGS test, and CT results, the patient was diagnosed with aspiration pneumonia infected by *Klebsiella sp.*. Accordingly, we suspended moxifloxacin. After 8 days of monotherapy with meropenem (1 g q8 h), the patient received an adjusted treatment by piperacillin/tazobactam (4.5 g q8 h) after the body temperature of the patient returned to normal, improvement of laboratory findings, and withdrawal of the ventilator. His mental state had markedly improved and the patient was successfully transferred from the ICU to the

Abbreviations: COVID-19, Corona virus disease 2019; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; GGO, Ground-glass opacity; CT, Computed tomography; BALF, Bronchoalveolar lavage fluid; RT-PCR, reverse transcriptase-polymerase chain reaction.

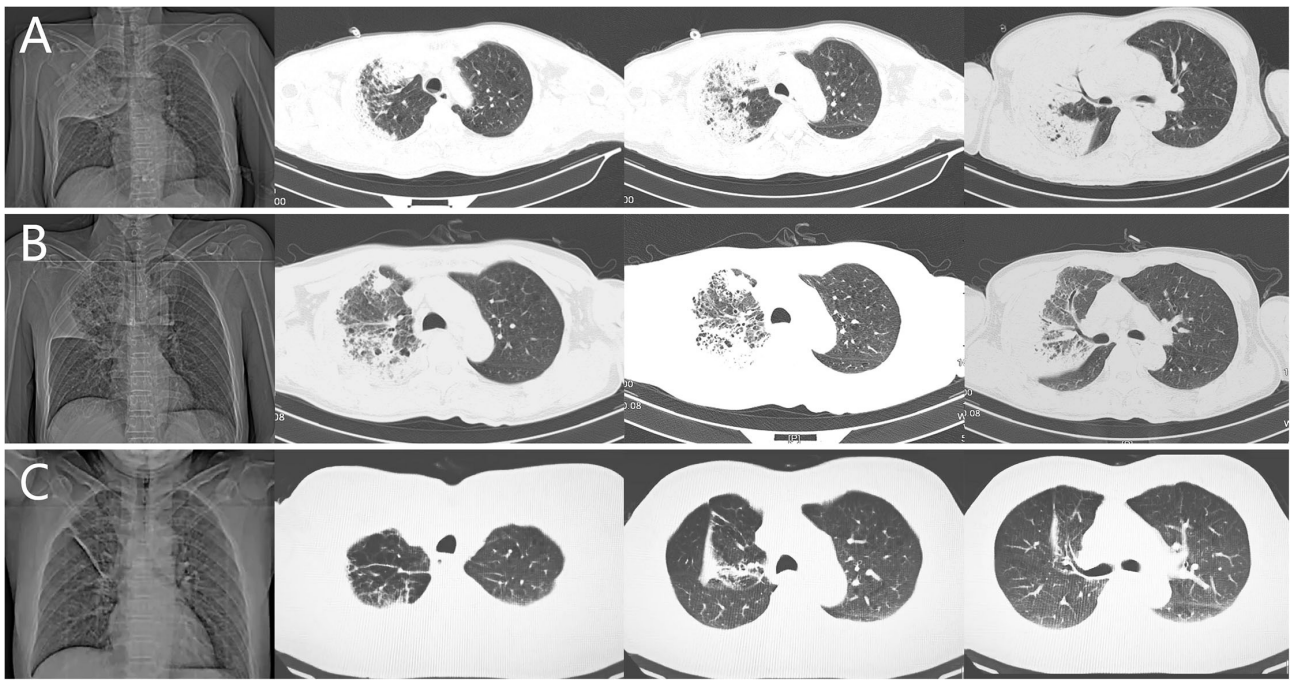


FIGURE 1 | Serial roentgenograms and chest CT images of the patient we reported. **(A)** Transverse thin-section CT of right upper lobe showed ground-glass opacity (GGO), intralobular reticular opacity and interlobular septal thickening. Consolidation and the air bronchogram signs were also presented in the right middle lobe of the patient (on admission). **(B)** Chest CT of the right upper lobe revealed consolidation with cavitation and GGO, with the air bronchogram signs partially absorbed (on day 12 after admission). **(C)** Follow-up CT images on July 4, 2021. The consolidation with cavitation of the right upper lobe were nearly absorbed, but fiber strands were left in the right upper and middle lobe.

TABLE 1 | The results of next-generation sequencing (NGS) in the case.

SPECIES	BALF		Blood	
Name	**Sequence number***	**Relative abundance**	**Sequence number***	**Relative abundance**
Klebsiella pneumoniae	23623	7.71%	897	4.77%
Klebsiella quasipneumoniae	693	0.23%	26	0.14%
Klebsiella aerogenes	671	0.22%	18	0.10%
Klebsiella variicola	670	0.22%	24	0.13%
Klebsiella oxytoca	164	0.05%		
Escherichia coli	603	0.20%	30	0.16%
Salmonella enterica	164	0.11%		
Citrobacter freundii	84	0.03%		
Cedecea neteri	71	0.02%		

*The sequence number of the strict comparison of the microorganism detected at the level of species.

respiratory ward on July 8, 2020. Finally, he was smoothly discharged 3 days later when CT scans were in remission (**Figure 1B**) even though he was left with slight paroxysmal dry cough (**Figure 2**). Long-term follow-up is currently ongoing to monitor changes in the lung lesion of the patient (**Figure 1C**) and the patient still has symptoms of intermittent cough 1 year later.

DISCUSSION AND CONCLUSIONS

In the setting of COVID-19 pandemic, considering that the patient was living in the epidemic area for 2 months, emergency physicians carried out RT-PCR of SARS-CoV-2 at the very beginning without any hesitation. While waiting for PCR results, the chest CT of the patient showed some radiological signs related to COVID-19, such as GGO, consolidation, and intralobular reticular opacity. Of course, they deployed a single isolation room for the patient. However, during this period, the patient deteriorated and had to receive ventilation. Notably, the treatment and monitoring were relatively limited in the Emergency Department. When the patient was admitted to ICU and received more direct examines along with the NGS test, it was only then that we further proposed a possible diagnosis of aspiration pneumonia despite how no definite history of aspiration was provided and no specific inhalant was found under fiberoptic bronchoscope. Coincidentally, after repeated questioning later on, the patient finally admitted to having several bouts of drunken vomiting 3 days before the onset of symptoms.

Aspiration pneumonia mainly occurs in patients with neurological diseases, disturbance of consciousness, esophageal diseases, trauma, etc., which may cause the irritant substances to enter the respiratory tract and induce secondary inflammation, resulting in an impaired natural defense mechanism. Aforementioned conditions contribute to a large number of admissions to emergency departments. There are currently

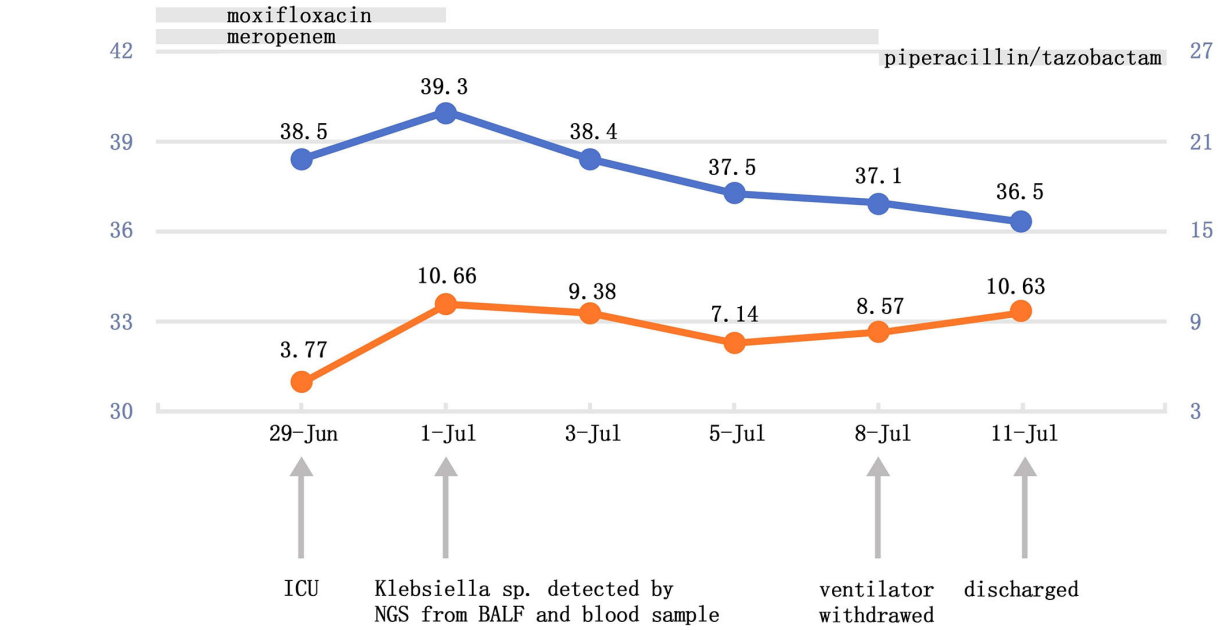


FIGURE 2 | Timeline with relevant data from the case in our hospital; curves of body temperature and leukocyte counts. The arrows below indicate major events. Blue line shows body temperature values. Orange line shows leukocyte counts. ICU, critical care unit; NGS, next-generation sequencing, BALF, bronchoalveolar lavage fluid.

multiple difficulties for clinical management of aspiration pneumonia, such as the lack of accurate diagnostic standards. The challenge in specifically diagnosing aspiration pneumonia is that for many patients in the community who are at risk for macroaspiration, there is difficulty to get the history of events that occurred in the days leading up to the onset of fever, cough, and infiltrating shadows displayed by chest radiograph. To some extent, chest CT is used to evaluate pulmonary lesions, which is of great importance in the diagnosis of aspiration disorders. Studies have shown that CT manifestations of aspiration pneumonia mainly include atelectasis, centrilobular nodules, bronchiolectasis, consolidation, "tree in bud," and GGO (8). In addition, these cases can be classified into the following patterns of pneumonia based on CT: lobar pneumonia, bronchopneumonia, bronchiolitis, or others (e.g., pneumonia with interstitial changes, abscess formation) (7). However, these CT findings have been categorized as "atypical signs" of COVID-19 by Radiological Society of North America (RSNA) (9). In view of the diversity and complexity, the imaging findings of aspiration pneumonia are numerous with the absence of specificity. However, due to the special anatomical position of the right bronchus, it is suggested that the location sites of pulmonary findings are more prevalently distributed in the right lung (10) and in lower lung lobes (7). Posterior segments of the upper lobes and superior segments of the lower lobes are frequently involved when patients aspirate in the recumbent position, whereas the bibasilar segments, particularly, the right middle lobe and lingular segment, are affected in patients in their upright position (11–13). Quite a few cases occur in the right middle and upper lobes at the same time, which, however, is the case of our report.

At present, the pathogenic bacteria of aspiration pneumonia have changed from anaerobic to gram-negative bacteria, and the most common bacteria are, in fact, enteral gram-negative species, including *Escherichia coli*, *Klebsiella sp.*, and *Pseudomonas sp.* (14). According to a retrospective study from Japan, CT findings of GGO was most frequent in patients with concurrent pneumonia than those with Klebsiella pneumonia alone, followed by consolidation and intralobular reticular opacity (15). In previous studies, alcoholism was the most commonly associated condition, followed by a smoking habit, cardiac disease, and malignant disease (15, 16). Our case was nearly the same as reported in a recent study as well (17). Interestingly, our case is consistent with two of these conditions described above. Nevertheless, *Klebsiella sp.* are major pathogens in healthcare-associated infections, and their importance appears to be increasing. It has been reported that patients with Klebsiella sp. chest infection frequently suffer a rapid fatal outcome (18). In terms of CT manifestations of *Klebsiella sp.* pneumonia, the previous report mostly showed necrosis or cavity (19), due to this bacteria being associated with pyogenic lung necrosis and pathological processes that result in frequent cavitation. But recent studies revealed that CT findings in patients with acute *Klebsiella sp.* pneumonia consisted mainly of GGO (100%), consolidation (91.4%), and intralobular reticular opacity (85.9%), which were found in the periphery (96%) of both sides of the lungs (72.2%) and were often associated with pleural effusion (53%) (16). In terms of treatment, antibiotics

are mainly administrated according to PK/PD principle, but it is necessary to be alert to carbapenem-resistant *Klebsiella sp*. Furthermore, no randomized controlled trials have so far reported the positive effect of glucocorticoid, and it is not recommended in the routine treatment of aspiration pneumonia (20). Moreover, glucocorticoids have been shown to promote the *in vitro* growth of Streptococcus pneumoniae and certain gram-negative rods (21). However, in the treatment process of this patient, glucocorticoid was applied to prevent shock rather than pneumonia.

Next-generation sequencing (NGS) may serve as a perfect one-step tool to study a broad range of pathogen characteristics and is applicable on identifying multiple known or new pathogens (viruses, bacteria, fungi, or parasites) from cultures or directly from clinical samples (22). In addition, NGS is also helpful in detection of novel resistance genes in bacteria, both in current and in historical strain collections. Novel variants of antimicrobial resistance genes, disease transmission, and virulence can be identified or predicted using NGS and further experiments (23). In this case, the patient was initially suspected with COVID-19 on admission and given isolation. Meanwhile, based on the physical condition of the patient being classified as an emergency, to some extent, the treatment of patients has been delayed. After all, the Gram-negative bacteria, Klebsiella sp., that caused shock were quickly identified through NGS technology detection, and the patient was saved by antibiotics targeted at the pathogenic bacteria.

In summary, it is difficult to differentiate COVID-19 pneumonia from other types of pneumonia. There may be a risk of misdiagnosis based on radiological patterns of pneumonia only. Therefore, it is not generally recommended to adopt routine CT screening for diagnosis or exclusion of COVID-19, especially under the condition of not-well-controlled COVID-19 pandemic. Correctly triaging these patients is necessary to reduce the risk of infection transmission and alleviate the pressure of emergency department within hospital. On the other hand, our case highlights the importance of clinician awareness of early pathogen diagnosis by NGS of infectious diseases, antibiotic treatment shall be adopted in a timely manner to decrease mortality and improve prognosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Second Xiangya Hospital, Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QZ and FP were involved in the clinical care of the patient. QZ and WX designed the case report, created figures, and drafted the manuscript. YZ and SL reviewed the manuscript. JY and SW contributed to the revision of the manuscript and led the scientific discussion. All authors read and approved the final manuscript.

FUNDING

This study was funded by Emergency Project of Prevention and Control for COVID-19 of Central South University (Grant No. 160260005) and COVID-19 Emergency Project of Changsha Science and Technology Bureau (Grant No. kq2001052).

REFERENCES

1. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA*. (2020) 323:1843–4. doi: 10.1001/jama.2020.3786
2. Bai HX, Hsieh B, Xiong Z, Halsey K, Choi JW, Tran TML, et al. Performance of radiologists in differentiating COVID-19 from Non-COVID-19 viral pneumonia at chest CT. *Radiology*. (2020) 296:E46–54. doi: 10.1148/radiol.2020200823
3. Sanivarapu RR, Gibson J. *Aspiration Pneumonia*. FL: StatPearls Treasure Island (2021).
4. DiBardino DM, Wunderink RG. Aspiration pneumonia: a review of modern trends. *J Crit Care*. (2015) 30:40–8. doi: 10.1016/j.jcrc.2014.07.011
5. Kanne JP. Chest CT Findings in (2019). Novel Coronavirus (2019-nCoV) infections from Wuhan, China: key points for the radiologist. *Radiology*. (2020). 295:16–7. doi: 10.1148/radiol.2020200241
6. Chung M, Bernheim A, Mei X, Zhang N, Huang M, Zeng X, et al. CT Imaging Features of (2019). Novel Coronavirus (2019-nCoV). *Radiology*. (2020). 295:202–7. doi: 10.1148/radiol.2020200230
7. Komiya K, Ishii H, Umeki K, Kawamura T, Okada F, Okabe E, et al. Computed tomography findings of aspiration pneumonia in 53 patients. *Geriatr Gerontol Int*. (2013) 13:580–5. doi: 10.1111/j.1447-0594.2012.00940.x
8. Scheeren B, Marchiori E, Pereira J, Meirelles G, Alves G, Hochhegger B. Pulmonary computed tomography findings in patients with chronic aspiration detected by videofluoroscopic swallowing study. *Br J Radiol*. (2016) 89:20160004. doi: 10.1259/bjr.20160004
9. Simpson S, Kay FU, Abbara S, Bhalla S, Chung JH, Chung M, et al. Radiological Society of North America Expert Consensus Statement on Reporting Chest CT Findings Related to COVID-19. endorsed by the society of thoracic radiology, the American college of radiology, and rsna - secondary publication. *J Thorac Imaging*. (2020) 35:219–27. doi: 10.1097/RTI.0000000000000524
10. Lin L, Lv L, Wang Y, Zha X, Tang F, Liu X. The clinical features of foreign body aspiration into the lower airway in geriatric patients. *Clin Interv Aging*. (2014) 9:1613–8. doi: 10.2147/CIA.S70924
11. Kim M, Lee KY, Lee KW, Bae KT. MDCT evaluation of foreign bodies and liquid aspiration pneumonia in adults. *AJR Am J Roentgenol*. (2008) 190:907–15. doi: 10.2214/AJR.07.2766
12. Marik PE. Aspiration pneumonitis and aspiration pneumonia. *N Engl J Med*. (2001) 344:665–71. doi: 10.1056/NEJM200103013440908
13. Bartlett JG, Gorbach SL. The triple threat of aspiration pneumonia. *Chest*. (1975) 68:560–6. doi: 10.1378/chest.68.4.560
14. Shinohara Y, Origasa H. Post-stroke pneumonia prevention by angiotensin-converting enzyme inhibitors: results of a meta-analysis of five studies in Asians. *Adv Ther*. (2012) 29:900–12. doi: 10.1007/s12325-012-0049-1

15. Okada F, Ando Y, Honda K, Nakayama T, Ono A, Tanoue S, et al. Acute Klebsiella pneumoniae pneumonia alone and with concurrent infection: comparison of clinical and thin-section CT findings. *Br J Radiol.* (2010) 83:854–60. doi: 10.1259/bjr/28999734
16. Okada F, Ando Y, Honda K, Nakayama T, Kiyonaga M, Ono A, et al. Clinical and pulmonary thin-section CT findings in acute Klebsiella pneumoniae pneumonia. *Eur Radiol.* (2009) 19:809–15. doi: 10.1007/s00330-008-1233-1
17. Ishiguro T, Uozumi R, Yoshioka H, Nishida T, Takayanagi N. Comparison between Patients with Chest Infection due to Klebsiella spp. and Streptococcus pneumoniae. *Intern Med.* (2020) 59:611–8. doi: 10.2169/internalmedicine.3531-19
18. Jong GM, Hsiue TR, Chen CR, Chang HY, Chen CW. Rapidly fatal outcome of bacteremic Klebsiella pneumoniae pneumonia in alcoholics. *Chest.* (1995) 107:214–7. doi: 10.1378/chest.107.1.214
19. Moon WK, Im JG, Yeon KM, Han MC. Complications of Klebsiella pneumonia: CT evaluation. *J Comput Assist Tomogr.* (1995) 19:176–81. doi: 10.1097/00004728-199503000-00002
20. Mandell LA, Niederman MS. Aspiration Pneumonia. *N Engl J Med.* (2019) 380:651–63. doi: 10.1056/NEJMra1714562
21. Dickson RP, Erb-Downward JR, Huffnagle GB. Homeostasis and its disruption in the lung microbiome. *Am J Physiol Lung Cell Mol Physiol.* (2015) 309:L1047–55. doi: 10.1152/ajplung.00279.2015
22. Lefterova MI, Suarez CJ, Banaei N, Pinsky BA. Next-Generation Sequencing for Infectious Disease Diagnosis and Management: A Report of the Association for Molecular Pathology. *J Mol Diagn.* (2015) 17:623–34. doi: 10.1016/j.jmoldx.2015.07.004
23. Gwinn M, MacCannell D, Armstrong GL. Next-Generation Sequencing of Infectious Pathogens. *JAMA.* (2019) 321:893–4. doi: 10.1001/jama.2018.21669

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Zhang, Xu, Peng, Lei, Zhong, Yi and Wu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 06 January 2022
doi: 10.3389/fcimb.2021.727594

Clinical Symptoms and Outcomes of Severe Pneumonia Caused by *Chlamydia psittaci* in Southwest China

Fuxun Yang[1†], Jiajia Li[1†], Bo Qi[2†], Longfei Zou[3], Zongming Shi[4], Yu Lei[1], Jun Li[5], Xiaoxiu Luo[1], Fan Zeng[1], Sen Lu[1], Xiaobo Huang[1], Rongan Liu[1*] and Yunping Lan[1*]*

[1] Department of ICU, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, China, [2] Department of Intensive Care Unit, 903 Hospital, Mianyang, China, [3] Department of ICU, Affiliated Hospital of Southwest Medical University, Luzhou, China, [4] Department of ICU, Neijiang People's Hospital Southwest Medical University, Sichuan, China, [5] Department of ICU, Shifang City People's Hospital Affiliated to North Sichuan Medical College, Shifang, China

Here, we aimed to retrospectively analyze the clinical characteristics of 27 patients with severe pneumonia caused by *Chlamydia psittaci* between January 2019 and April 2021 in southwest China. To this end, we collected data on the exposure history, clinical symptoms, laboratory examination, imaging characteristics, evolution, etiology, treatment, and outcomes to suggest a better diagnosis and prevention system. Our results showed that a metagenomic next-generation sequencing test could provide early diagnosis. All patients were sensitive to quinolones and tetracyclines, and the recovery rate was relatively high. Overall, all patients were in critical condition with moderate to severe acute respiratory distress syndrome and shock. In conclusion, early diagnosis of pneumonia caused by *C. psittaci* depends on effective molecular testing, and most patients recover after treatment.

Keywords: *Chlamydia psittaci*, pneumonia, clinical symptoms, mNGS, ICU

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Hirdesh Kumar,
National Institute of Allergy and Infectious Diseases (NIH), United States
Li Wang,
First Affiliated Hospital of Chengdu Medical College, China

****Correspondence:***
Yunping Lan
lanyunping929@163.com
Rongan Liu
frog007-119@qq.com
Xiaobo Huang
drhuangxb@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *19 June 2021*
Accepted: *13 December 2021*
Published: *06 January 2022*

Citation:
Yang F, Li J, Qi B, Zou L, Shi Z, Lei Y, Li J, Luo X, Zeng F, Lu S, Huang X, Liu R and Lan Y (2022) Clinical Symptoms and Outcomes of Severe Pneumonia Caused by Chlamydia psittaci in Southwest China. Front. Cell. Infect. Microbiol. 11:727594. doi: 10.3389/fcimb.2021.727594

1 INTRODUCTION

Chlamydia psittaci is a gram-negative bacterium with a size between bacteria and viruses. It widely exists in wild birds or poultry such as parrots, wild pigeons, chickens, and ducks. Ten genotypes are known that correspond to different hosts and virulence, causing zoonotic infectious diseases (Knittler and Sachse, 2015). Humans are usually infected by inhaling the organisms in dry feces or bird feather dust when caged birds spread their wings. Cage cleaning may pose a risk of infection. Bird bites, mouth to beak contact, and even brief contact, such as visiting bird parks, are also related to the spread of this infection (Hinton et al., 1993). The typical clinical symptoms include fever, chills, headache, dry cough, gastrointestinal problems, severe pneumonia, endocarditis, jaundice, and nervous system complications (Johnston et al., 2000; Hogerwerf et al., 2017; Rybarczyk et al., 2019). There are only a few reports that of clustered cases in families and hospitals, indicating the possibility of human-to-human transmission (Wallensten et al., 2014). *C. psittaci* pneumonia has been recognized worldwide, including in the United States, United Kingdom, Europe, Middle East, and Australia, and was previously thought to cause 1% of the cases of community-acquired

pneumonia (Petrovay and Balla, 2008). However, the exact incidence and prevalence of psittacosis are difficult to determine or may even be underestimated owing to the lack of routine tests and the differences in sensitivity and specificity of common diagnostic tests (Rybarczyk et al., 2020). Pneumonia caused by *C. psittaci* usually has mild symptoms, but some patients can progress to severe disease and even die of respiratory failure and multiple organ failure (Branley et al., 2014). For critically ill patients, successful treatment depends on accurate diagnosis and appropriate support. With the rapid development of molecular methods, especially metagenomic next generation sequencing (mNGS), the early diagnosis of diseases is possible, and the number of related cases reported has increased (Shi et al., 2021; Teng et al., 2021; Yuan et al., 2021). In this study, we summarize the epidemiology, clinical characteristics, laboratory data, and clinical outcomes of 27 cases of severe pneumonia caused by C. *psittaci* diagnosed by mNGS, to provide a basis for effective diagnosis and treatment.

2 METHODS

2.1 Setting

This study was approved by the Ethics Committee of Sichuan Provincial People's Hospital (No. 2020-111), and all data were anonymous before analysis. The study was carried out in accordance with the Declaration of Helsinki. The data of the study were obtained from the comprehensive ICU of four tertiary A hospitals in southwest China, with more than 280 ICU beds in total.

2.2 Study Design

In this retrospective study, we included 27 cases of severe *C. psittaci* pneumonia diagnosed by mNGS in the ICU of four tertiary A hospitals in southwest China from May 2018 to March 2020. The diagnostic criteria of *C. psittaci* pneumonia refer to previous literature reports (Su et al., 2021). We recorded data on age, sex, exposure history, comorbidities, onset course, symptoms, signs, imaging, laboratory examination, method of diagnosis, treatment, and clinical outcomes.

2.3 mNGS Detection Method

A 1.5–3 mL bronchoalveolar lavage fluid (BALF) sample from each patient was collected according to standard procedures. A 1.5 mL microcentrifuge tube with 0.6 mL sample, enzyme, and 1 g 0.5 mm glass bead were attached to a horizontal platform on a vortex mixer and agitated vigorously at 2800–3200 rpm for 30 min. A 0.3 mL sample was separated into a new 1.5 mL microcentrifuge tube and DNA was extracted using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH) according to the manufacturer's recommendation. Then, DNA libraries were constructed through DNA-fragmentation, end-repair, adapter-ligation, and PCR amplification. Agilent 2100 was used for quality control of the DNA libraries. Quality qualified libraries were sequenced by BGISEQ-50/MGISEQ-2000 platform (Jeon et al., 2014). High-quality sequencing data were generated by removing low-quality reads, followed by computational subtraction of human host sequences mapped to the human reference genome (hg19) using Burrows–Wheeler Alignment (Li and Durbin, 2009) The remaining data, by removal of low-complexity reads, were classified by simultaneously aligning to four Microbial Genome Databases, consisting of bacteria, fungi, viruses, and parasites.

The classification reference databases were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/). RefSeq contained 4,945 whole genome sequences of viral taxa, 6,350 bacterial genomes or scaffolds, 1,064 fungi related to human infection, and 234 parasites associated with human diseases.

2.4 Statistical Method

Statistical analyses were performed using R software version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 20.0 (IBM Corporation, Armonk, NY). Categorical data were summarized using frequencies and percentages. Continuous data were summarized using median and interquartile range (IQR). Unpaired Wilcoxon's rank-sum test was used to test differences for continuous variables and Fisher's exact test was used for categorical variables. Survival rate was calculated and plotted by Kaplan-Meier method, and univariate analysis was performed by log-rank test. All reported p-values were two-sided and statistical significance was set at $p < 0.05$.

3 RESULTS

3.1 Epidemiology

The average age of the patients was 60 years (35–87 years). One woman was in her third trimester. Except for four patients who kept pigeons and six who had contact with poultry in open markets, the remaining patients had no clear history of exposure to birds (**Table 1**).

3.2 History and Clinical Manifestations

Among the 27 patients, 10 had hypertension, 11 had cirrhosis, one had diabetes, one had pneumoconiosis, and one had gout. The symptoms of most patients were high fever (≥39°C), cough with or without expectoration, dizziness, headache, and disturbance of consciousness (**Table 1**). The median time from admission to diagnosis was 11 days (2–20 days), and the median time from admission to respiratory failure was 11 days (1–16 days). Eleven patients (40.7%) developed sepsis and acute respiratory failure upon admission to the intensive care unit (ICU).

3.3 Auxiliary Examination

3.3.1 Laboratory Tests

All patients were tested for *Chlamydia trachomatis* IgM on admission, and eight were positive (**Table 2**). Most patients had normal white blood cells but elevated hs-CRP(high-sensitivity C-reactive protein), lymphocytopenia, hyponatremia, and abnormal liver enzymes. The APTT, D-Dimer, MYO and HS-TNTI of the death group and the survival group were statistically significant.

TABLE 1 | Basic characteristics of patients (n = 27) included in the study.

	All (N=27)	survivor (N=23)	Non-survivor (N=4)	P
gender, male, n (%)	19 (70.4%)	17 (73.9%)	2 (50.0%)	0.334
Age, Median [Min, Max]	60.0 [35.0, 87.0]	60.0 [35.0, 87.0]	67.0 [54.0, 83.0]	0.895
Exposure to bird, n (%)	10 (37.0%)	9 (39.1%)	1 (25.0%)	0.594
Time from onset to diagnosis, Median [Min, Max]	11.0 [2.00, 20.0]	10.0 [2.00, 20.0]	13.0 [9.00, 17.0]	0.501
Time from onset to respiratory failure, Median [Min, Max]	7.00 [1.00, 16.0]	7.0[1.0,16.0]	6.5 [6.0,14.0]	0.896
Admission symptoms				
Cough, n (%)	19 (70.4%)	16 (69.6%)	3 (75.0%)	0.826
Backache, n (%)	1 (3.7%)	1 (4.3%)	0 (0%)	0.852
fever, n (%)	23 (85.2%)	20 (87.0%)	3 (75.0%)	0.542
dyspnea, n (%)	22 (81.5%)	19 (82.6%)	3 (75.0%)	0.719
headache, n (%)	10 (37.0%)	8 (34.8%)	2 (50.0%)	0.565
diarrhea, n (%)	3 (11.1%)	3 (13.0%)	0 (0%)	0.605
Characteristics at ICU admission				
APACHE II, Median [Min, Max]	16.0 [8.00, 35.0]	15.0 [8.00, 35.0]	20.0 [17.0, 26.0]	0.411
≤10	6 (22.2%)	6 (26.1%)	0	0.251
10-15	4 (14.8%)	4 (17.4%)	0	
>15	17 (63.0%)	13 (56.5)	4 (100%)	
SOFA, Median [Min, Max]	5.00 [2.00, 9.00]	5.00 [2.00, 9.00]	8.00 [6.00, 8.00]	0.199
PaO2/FIO2 ratio, mm Hg, Median [Min, Max]	162 [46.0, 248]	166 [46.0, 248]	99.0 [69.0, 135]	0.393
>200	5 (18.5%)	5 (21.7%)	0	0.277
100-200	16 (59.3%)	14 (60.9%)	2 (50%)	
<100	6 (22.2%)	4 (17.4%)	2 (50%)	

CI, confidence interval; OR, odds ratio.

3.3.2 Imaging Examination

Chest imaging showed that early lesions were mainly involved in a single lobe of the right lung, with inflammatory exudation and local consolidation. As the disease progressed, inflammatory exudates developed in both lobes with pleural effusion (**Figures 1A–C**). One patient was discharged with residual pulmonary interstitial changes, and the rest had absorbed inflammatory pleural exudate.

3.3.3 mNGS Results

All patients underwent fibrobronchoscopy, and they were tested based on BALF, mNGS, and routine microbiology within 3 days of admission to the ICU. mNGS using BALF samples showed the presence of *Helicobacillus* (7–15234 copies) (**Supplementary Material**) in all patients, *Candida albicans* in six patients, *Corynebacterium striatum* in three patients, and *Streptococcus pneumoniae* in two patients. mNGS testing of blood samples from five patients revealed the presence of *Chlamydia psittaci*.

3.4 Treatment Process

All patients were treated with quinolones, macrolides, or tetracycline antibiotics. Among them, 15 patients were treated with class III drugs before diagnosis (14 quinolones, six levofloxacin, eight moxifloxacin; Macrolide 1 (azithromycin 1)). After diagnosis, 27 patients had their medication adjusted accordingly as follows: 17 cases (63%) were quinolones (levofloxacin: six cases, moxifloxacin: eight cases); there were six cases of tetracycline treatment, accounting for 22.2% (one case of minocycline, five cases of doxycycline), and five cases of macrolide treatment, accounting for 18.5% (five cases of azithromycin). One patient received a combination of azithromycin and doxycycline. Among the 27 patients, 21 patients had fever. The duration of fever withdrawal was approximately 1–4 d, with an average of 2.3 d, accompanied by a significant decrease in hs-CRP, and the effect was significant. Five patients were treated with methylprednisolone (80–320 mg, bid) for inflammatory exudation of the chest early in the disease and did not show spread of infection. Six patients (22.2%) developed nosocomial infections, and one patient (3.7%) had carbapenem-resistant *Klebsiella pneumoniae* and carbapenem-resistant *Acinetobacter baumannii* in the lungs and blood, increasing the risk of death.

Among the 27 patients, five patients had mild acute respiratory distress syndrome (ARDS), 16 patients had moderate ARDS, and six patients had severe ARDS (**Table 3**). All patients received mechanical ventilation, including 14 patients with invasive mechanical ventilation and 23 patients with non-invasive mechanical ventilation. In 10 patients, it was difficult to maintain oxygenation during the treatment of non-invasive ventilator-assisted ventilation, and thus, treatment was replaced with invasive mechanical ventilation. All patients receiving invasive mechanical ventilation were treated with a small tidal volume protective pulmonary ventilation, sedation and analgesia, and prone ventilation as recommended in ARDS treatment guidelines. Three patients with severe ARDS developed extremely severe ARDS during the course of 8–12 d and were given intravenous extracorporeal membrane oxygenation for 6, 10 and 16 d, respectively. In the course of treatment, 4 patients were treated with CRRT because of renal function injury, of which 3 cases died and 1 patient survived and discharged without residual renal function injury.

3.5 Clinical Outcomes

Overall, the majority of patients had a good prognosis (**Table 4**). Of the 27 patients, 23 patients survived, and one patient had

TABLE 2 | Laboratory test.

Laboratory findings	All (N=27)	survivor (N=23)	Non-survivor (N=4)	P
procalcitonin, Median [Min, Max]	1.19 [0.05, 7.23]	1.15 [0.05, 7.23]	2.46 [0.27, 5.72]	0.830
≤0.05	1 (14.8%)	1 (4.3%)	0	0.909
0.05-0.50	7 (25.9%)	6 (26.1%)	1 (25%)	
>0.50	19 (59.3%)	16 (69.6%)	3 (75%)	
IgM positive, n (%)	8 (29.6%)	6 (26.1%)	2 (50.0%)	0.347
Leucocyte, ×10⁹/L, Median [Min, Max]	8.71 [3.65, 24.3]	8.91 [3.90, 24.3]	6.84 [3.65, 11.3]	0.549
<4	2 (7.4%)	1 (4.4%)	1 (25%)	0.346
4-10	17 (63.0%)	15 (65.2%)	2 (50%)	
>10	8 (29.6%)	7 (30.4%)	1 (25%)	
Lymphocyte, × 10⁹/L, Median [Min, Max]	0.56 [0.12, 2.03]	0.520[0.12, 2.03]	0.67 [0.34, 0.86]	0.251
>0.8	4 (14.8%)	3 (13.0%)	1 (25%)	0.315
0.6-0.8	7 (25.9%)	5 (21.7%)	2 (50%)	
<0.6	16 (59.3%)	15 (65.2%)	1 (25%)	
Platelets, × 10⁹/L, Median [Min, Max]	155 [63.0, 350]	159 [63.0, 350]	129 [109, 166]	0.056
<100× 10⁹/L	4 (11.0%)	3 (13.0%)	0	0.444
Hemoglobin, g/dL, Median [Min, Max]	127[72,153]]	122[72,153]	134[122,137]	0.251
Anemia	4 (14.8%)	4 (17.4%)	0	0.366
CRP, Median [Min, Max]	153 [33.7, 274]	153 [33.7, 265]	169 [123, 274]	0.921
>100	23 (85.2%)	19 (82.6%)	4 (100%)	
Albumin (g L-1), Median [Min, Max]	29.7 [24.1, 44.7]	29.7 [24.1, 44.7]	34.4 [27.2, 40.3]	0.562
<30	14 (48.2%)	12 (47.8%)	2 (50%)	0.936
bilirubin (g L-1), Median [Min, Max]	19.0 [8.60, 69.9]	20.4 [8.60, 69.9]	18.6 [11.9, 19.1]	0.418
>20	12 (55.6%)	12 (47.8%)	0	0.053
APTT (s), Median [Min, Max]	33.4 [26.4, 70.0]	32.2 [26.4, 70.0]	43.8 [27.4, 55.5]	0.108
>40	7 (25.9%)	4 (17.39%)	3 (75%)	0.015
D-dimer (mg/l), Median [Min, Max]	4.31 [0.88, 38.6]	4.31 [0.88, 38.6]	4.33 [2.79, 17.50]	0.019
<1.0	2 (7.4%)	2 (8.7%)	0	0.511
1.0-2.0	4 (14.8%)	4 (17.4%)	0	
>2.0	21 (77.8%)	17 (73.9%)	4 (100%)	
CK-MB (ng/ml)	1.20 [0.30, 23.4]	1.00 [0.30, 20.7]	9.00 [1.40, 23.4]	
>2.0	6 (22.2%)	4 (17.4%)	2 (50%)	0.148
MYO (ng/ml)	121 [17.0, 35400]	81.4 [17.0, 2250]	448 [21.7, 35400]	
>300	7 (25.9%)	4 (17.4%)	3 (75%)	0.015
HS-TNTI (ng/L)	18.9 [0.260, 130]	18.9 [0.260, 130]	78.9 [22.6, 129]	
>50	6 (22.2%)	3 (13.0%)	3 (75%)	0.006
mNGS-results	152 [7.00, 5350]	4530 [337, 15200]	337 [7.00, 15200]	
>1000	9 (33.3%)	6 (26.1%)	3 (75%)	0.055

ECMO, extracorporeal membrane oxygenation; MV, mechanical ventilation; CRRT, continuous renal replacement therapy; PCT, procalcitonin; CRP, C-reactive protein.

residual pulmonary interstitial changes without significant decreased activity tolerance. None of the surviving patients had serious complications during follow-up. Four patients died from secondary nosocomial infections. The vital signs of three patients were not maintained after intravenous extracorporeal membrane oxygenation support therapy, including one pregnant woman. All three patients recovered well and were discharged easily. No complications occurred in the follow-up of 3 months. In the case of the pregnant woman, after admission, even though we carried out routine fetal monitoring of the patient, death of the fetus occurred due to various factors, such as severe hypoxia, antimicrobial agents, sedation and analgesia, medical treatment, and the use of anticoagulant drugs, and 2 d after tracheal intubation, labor was induced with the informed consent of the patient's family.

In the pregnant woman's case, even though we conducted regular fetal monitoring after the patient was admitted to hospital, owing to various factors such as severe hypoxia, anti-infective drugs, sedation and analgesia, and the use of anticoagulant drugs after ECMO, fetal death was found *in utero*, 2 days after endotracheal intubation, and labor was induced with the informed consent of the patient's family. Univariate logistic regression of death factors in the study cohort showed that APTT ≥40 s, Myoglobin(MYO) ≥ 300ng/mL and Hypersensitive troponin I(HS-TNTI) ≥ 50ng/L were risk factors for death in severe patients (**Table 3**).

4 DISCUSSION

As previously reported, nearly 63.7% of patients with *C. psittaci* infection are male (Yung and Grayson, 1988), which was in agreement with the rate in our study (69% male).The majority of infections reported in previous studies were in adults between the ages of 30 and 60 years (Shi et al., 2021), as was the case in our cohort. Usually, 90% of the patients have a history of exposure to poultry (Saeki et al., 1996), but in our study, only 31% of the patients reported a contact. Our analysis showed that fever was the most common symptom in patients—results that were consistent with those reported in a previous study (Yung and Grayson, 1988). Besides, nine patients showed neurological

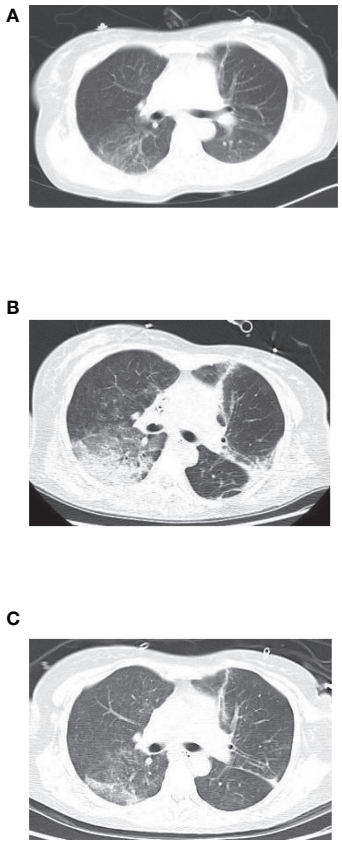


FIGURE 1 | **(A)** Before treatment. **(B)** Before metagenomic next-generation sequencing (mNGS). **(C)** After the treatment.

TABLE 3 | Treatment and outcomes.

	All (N=27)	survivor (N=23)	Non-survivor (N=4)	P
MV, n (%)	11 (40.7%)	7 (30.4%)	4 (100.0%)	0.019
Intubation days, Median [Min, Max]	6.00 [1.00, 17.0]	6.00 [1.00, 17.0]	4.50 [3.00, 10.0]	0.775
ECMO, n (%)	3 (11.1%)	3 (13.0%)	0 (0%)	0.605
CRRT, n (%)	4 (14.8%)	2 (50%)	2 (8.7%)	0.032

MV, Mechanical ventilation; ECMO, extracorporeal membrane oxygenation; CRRT, continuous renal replacement therapy.

symptoms, such as disturbance of consciousness or confusion, whereas 19 patients were diagnosed with hyponatremia. Although the effects of psittacosis on the nervous system were first reported in 1880, the underlying mechanism remains unclear (Macfarlane and Macrae, 1983; Saeki et al., 1996). Four potential pathogenic mechanisms have been proposed: direct invasion of *C. psittaci* to the central nervous system, autoimmune mechanisms, embolism, and electrolyte disorders (Al-Kawi and Madkour, 1986; Vassallo and Shepherd, 1997; Walder et al., 2003). In a previously reported case of encephalopathy (Walder et al., 2003), *C. psittaci* was found in the cerebrospinal fluid of the patient, which may show that the first mechanism is the most possible. Since quinolones or tetracyclines are not able to cross the blood–brain barrier, the prognosis for patients with severe nervous system symptoms is often poor. Indeed, three of the four patients that died in the duration of our study developed neurological symptoms, despite the administration of antibiotics.

All patients showed markedly increased levels of hs-CRP and slightly increased levels of liver enzymes. A previous study reported that *C. psittaci* is more pathogenic and propagates faster than other *Chlamydia* species, causing severe inflammation. Therefore, the increase in white blood cells was not obvious at the early stages of the disease, but that of hs-CRP was rapid, and the results were consistent with those presented in a previous study (Knittler and Sachse, 2015). *C. psittaci* first enters the reticular endothelial cells of the liver and spleen to proliferate after inhalation through the respiratory tract, and consequently, patients with respiratory symptoms also show

TABLE 4 | Univariable logistic regression of factors associated with death risk of critically ill patients with *Chlamydia psittaci*.

	OR	95%CI	P
Age≥65,years	1.06	(0.96,1.16)	0.248
Exposure to bird	0.51	(0.05,5.80)	0.594
Time from onset to diagnosis, days	1.15	(0.87,1.50)	0.321
Time from onset to respiratory failure, days	1.05	(0.79,1.04)	0.722
PCT≥0.5,ng/dl	1.31	(0.12,14.93)	0.826
APACHE II ≥ 25	7.33	(0.36,50.71)	0.196
SOFA	2.05	(0.94,4.46)	0.07
IgM positive	2.83	(0.32,24.81)	0.347
PaO2/FIO2 ratio >100 mmHg	0.21	(0.02,1.97)	0.172
WBC < 4 × 10^9/L	0.14	(0.01,2.87)	0.196
Lym < 0.6 × 10^9/L	5.62	(0.50,63.60)	0.162
Albumin < 30 g L-1	1.09	(0.13,9.12)	0.936
bilirubin ≥20 g L-1	0.94	(0.81,1.09)	0.417
APTT ≥40 s	14.25	(1.16,174.8)	0.038
CK-MB ≥ 2ng/ml	4.75	(0.51,44.5)	0.172
MYO ≥ 300ng/ml	14.25	(1.16,74.8)	0.038
HS-TNTI ≥ 50ng/l	20.0	(1.53,60.7)	0.022
mNGS-results ≥ 1000	8.5	(0.74,98.2)	0.087
Intubation days	0.89	(0.62,1.29)	0.536
CRRT	10.5	(0.92,120.25)	0.059

SOFA, Sequential Organ Failure Assessment.

changes in liver enzymes. A previous study suggested that the diagnosis of *C. psittaci* should be considered when patients aged <60 years with fever, liver enzyme changes, or nervous system symptoms are admitted from early May to early October (Raeven et al., 2016), even if they have no history of contact with birds, to prevent septic shock and ARDS at a later stage.

We found that lymphocytopenia occurred in 93% of critically ill patients. Lymphocytopenia is a significant symptom of patients with severe community-acquired infections. It has been reported that some atypical types of pneumonia can destroy the cytoplasmic components of lymphocytes, leading to lymphocyte apoptosis (Gu et al., 2005). Lymphocytopenia is also common in critically ill patients with psittacosis. Indeed, the four patients that died showed a continuous decrease in the lymphocyte count throughout the course of the disease that was irreversible. Thus, further research is needed to explore the causes of lymphocytopenia caused by *C. psittaci*.

In addition, the increase of MYO and Hs-TNTi caused by chlamydia psittaci was also common in our patients, especially in patients who died. MYO≥300ng/mL and HS-TNTi ≥50ng/L were one of the high-risk death factors in patients with chlamydia psittaci pneumonia. In previous reports, the increase of (Su et al., 2021) CK was a high risk factor for severe chlamydia psittaci pneumonia. Our study further proved that not only CK, MYO and HS-TNTI related to myocardial enzymes were also high risk factors for severe patients, suggesting that myocardial injury caused by Chlamydia psittaci is not rare in clinic. In the clinical treatment, we should be highly alert to such patients to avoid severe cases.

Mechanical ventilation is the primary supportive treatment for critically ill patients. In the present study, all patients were provided oxygen therapy (PaO_2/FiO_2 < 300), whereas six patients with severe ARDS received lung-protective ventilation and prone position ventilation. Three of the latter also received ECMO adjuvant therapy.

A previous study showed that patients with psittacosis had a good prognosis after timely diagnosis and antibiotic treatment. We found that more than 50% of the patients (15/27 cases) were administered quinolones as a preliminary antibiotic treatment that was adjusted after the diagnosis based on the symptoms (Chen et al., 2020). Tetracycline antibiotics are the primary recommendation for *C. psittaci* pneumonia. In our cohort, all patients treated with tetracycline antibiotics were sensitive. However, presently tetracycline resistance is also one of the problems that need to be paid attention in a clinical setting. Tetracycline resistance originated from the environment (Thaker et al., 2010) but is now widely distributed in symbiotic bacteria and pathogenic bacteria, so has been also looking for a solution, clinical if use dehydration tetracycline or analogues inhibiting Tet (X7) (Gasparrini et al., 2020) can prevent the tetracycline antibiotics degradation and save efficacy or develop feasible adjuvant method to against tetracycline resistance by enzymatic inactivated (Markley et al., 2019). Therefore, in patients with severe disease who show tetracycline resistance, we may need to combine macrolides or quinolones. Previous studies showed that quinolones are not effective in patients with psittacosis. In our study, only four patients were treated with quinolones until recovery, whereas the remaining patients were quickly switched to macrolides or tetracyclines after the diagnosis of psittacosis. Most patients showed good efficacy and quick recovery. The four patients that died were administered carbapenems during the preliminary antibiotic treatment, although their procalcitonin was slightly increased at admission. The prolonged use of carbapenems led to multiple drug-resistant bacterial infections, followed by multiple organ failure and death.

Although systemic sex hormone therapy is controversial in ARDS patients, considering severe pneumonia and severe systemic inflammatory reaction, glucocorticoids were used in

all patients, showing good clinical results without any complications. However, additional research and analysis of a higher number of cases is needed to confirm the results.

Clinical diagnosis of *C. psittaci* pneumonia is mainly based on the combination of clinical manifestations and laboratory examination. This theoretical gold standard method of pathogen culture is not routinely tested in most laboratories owing to the infectious nature of *C. psittaci* itself, which requires high levels of biosafety. Currently, commercially available laboratory tests mainly include serological, microbial culture, and nucleic acid amplification tests. The most commonly used serological tests include a complement binding test (CF) and microimmunofluorescent antibody test. The microimmunofluorescent antibody test is the most sensitive and specific serological test for *C. psittaci*, but it is only available in specialized laboratories (Wreghitt, 1993) The CF test is a test traditionally used for diagnosis and requires showing antibody titers at least four times higher than the upper normal limit in repeated serum samples. It has disadvantage of requiring repeated CF tests after 2 weeks and being unable to distinguish between different *Chlamydia* species. Early effective tetracycline therapy might also delay or attenuate the antibody response in critically ill patients. In addition, direct immunofluorescent antibody staining of respiratory secretions (sputum, throat swabs) has been used for rapid diagnosis (Oldach et al., 1993), but more studies are needed to demonstrate its sensitivity and specificity. As a result, molecular approaches are becoming popular. Nucleic acid amplification tests are considered more sensitive and specific than serological antibody tests. Unfortunately, this approach has not been carried out on a clinical scale and has only been conducted in cases of suspected *C. psittaci* outbreaks. With the development of mNGS, a variety of pathogens in different specimens can be quickly and accurately detected, including atypical pathogens, viruses, and fungi, among others, which are difficult to culture. It has the advantage of a wide detection range and no requirement for identifying suspected pathogens in advance and can be used for the diagnosis of lower respiratory tract infections. When treating patients with severe pneumonia, early identification of the pathogen is of utmost importance. With mNGS, etiological results can be obtained within 24 h at the earliest, and tetracycline-based antimicrobial therapy can be adjusted in time to minimize the time required to diagnose psittacosis and the disease process. At the same time, no other pathogens were detected by mNGS, thus reducing the use of unnecessary antibacterial drugs (such as carbapenems and antifungal drugs), effectively reducing the cost and time of hospitalization, and avoiding the emergence of drug-resistant bacteria. Several hospitals in this study were class-A tertiary teaching hospitals, but they only tested for *Chlamydia* IgM. Therefore, in this study, the application of Acer sequencing technology for the diagnosis of *C. psittaci* pneumonia could be used to quickly and specifically identify this species, thus shortening the diagnosis time, identifying other potential pathogens, and allowing for the initiation of targeted antimicrobial therapy in time.

This study had some limitations: 1) data were limited since the number of seriously ill patients was relatively small and 2) the CDC recommends a single IgM antibody titer of 1:16 or higher as a diagnostic test, but unfortunately, our institution does not have this requirement. *C. psittaci* IgM antibody was not detected in any of the patients. 3) Further studies with larger sample sizes are needed to support and confirm our findings.

Overall, for fever of unknown cause combined with exposure history, liver enzymology changes, and nervous system changes, we should be alert to the occurrence of *C. psittaci* pneumonia. Medication should be administered in a timely manner to avoid the later development of ARDS or sepsis or even multiple organ failure. Among all diagnostic methods, mNGS allows for early diagnosis that will help devise effective treatment plans and reduce the recovery time.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital Medical Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FY and FZ wrote the original draft. LZ, ZS, and JuL provided some of the data. XL, RL, YLe, and SL undertook validation, writing, review, and editing. XH, JiL, and YLa undertook review and editing. BQ is responsible for the statistics of the data and the revision of the article. All authors contributed to the article and approved the submitted version.

FUNDING

Funding was provided by the Key research and development project of Science and Technology of Sichuan Province (Grant no. 20ZDYF1870).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.727594/full#supplementary-material

REFERENCES

Al-Kawi, M. Z., and Madkour, M. M. (1986). Brain Stem Encephalitis in Ornithosis. *J. Neurol. Neurosurg. Psychiatry* 49 (5), 603–604. doi: 10.1136/jnnp.49.5.603

Branley, J. M., Weston, K. M., England, J., Dwyer, D. E., and Sorrell, T. C. (2014). Clinical Features of Endemic Community-Acquired Psittacosis. *New Microbes New Infect.* 2 (1), 7–12. doi: 10.1002/2052-2975.29

Chen, X., Cao, K., Wei, Y., Qian, Y., Liang, J., Dong, D., et al. (2020). Metagenomic Next-Generation Sequencing in the Diagnosis of Severe Pneumonias Caused by Chlamydia Psittaci. *Infection* 48 (4), 535–542. doi: 10.1007/s15010-020-01429-0

Gasparrini, A. J., Markley, J. L., Kumar, H., Wang, B., Fang, L., Irum, S., et al. (2020). Tetracycline-Inactivating Enzymes From Environmental, Human Commensal, and Pathogenic Bacteria Cause Broad-Spectrum Tetracycline Resistance. *Commun. Biol.* 3 (1), 241. doi: 10.1038/s42003-020-0966-5

Gu, J., Gong, E., Zhang, B., Zheng, J., Gao, Z., Zhong, Y., et al. (2005). Multiple Organ Infection and the Pathogenesis of SARS. *J. Exp. Med.* 202 (3), 415–424. doi: 10.1084/jem.20050828

Hinton, D. G., Shipley, A., Galvin, J. W., Harkin, J. T., and Brunton, R. A. (1993). Chlamydiosis in Workers at a Duck Farm and Processing Plant. *Aust. Vet. J.* 70 (5), 174–176. doi: 10.1111/j.1751-0813.1993.tb06123.x

Hogerwerf, L., DE Gier, B., Baan, B., and Van Der Hoek, W. (2017). Chlamydia Psittaci (Psittacosis) as a Cause of Community-Acquired Pneumonia: A Systematic Review and Meta-Analysis. *Epidemiol. Infect.* 145 (15), 3096–3105. doi: 10.1017/S0950268817002060

Jeon, Y. J., Zhou, Y., Li, Y., Guo, Q., Chen, J., Quan, S., et al. (2014). The Feasibility Study of non-Invasive Fetal Trisomy 18 and 21 Detection With Semiconductor Sequencing Platform. *PloS One* 9 (10), e110240. doi: 10.1371/journal.pone.0110240

Johnston, W. B., Eidson, M., Smith, K. A., and Stobierski, M. G. (2000). Compendium of Measures to Control Chlamydia Psittaci Infection Among Humans (Psittacosis) and Pet Birds (Avian Chlamydiosis), 2000. *Morb. Mortal. Wkly. Rep. Recomm. Rep.* 49 (RR-8), 3–17. doi: 10.1053/j.jepm.2010.11.007

Knittler, M. R., and Sachse, K. (2015). Chlamydia Psittaci: Update on an Underestimated Zoonotic Agent. *Pathog. Dis.* 73 (1), 1–15. doi: 10.1093/femspd/ftu007

Li, H., and Durbin, R. (2009). Fast and Accurate Short Read Alignment With Burrows-Wheeler Transform. *Bioinformatics* 25 (14), 1754–1760. doi: 10.1093/bioinformatics/btp324

Macfarlane, J. T., and Macrae, A. D. (1983). Psittacosis. *Br. Med. Bull.* 39 (2), 163–167. doi: 10.1093/oxfordjournals.bmb.a071810

Markley, J. L., Fang, L., Gasparrini, A. J., Symister, C. T., Kumar, H., Tolia, N. H., et al. (2019). Semisynthetic Analogues of Anhydrotetracycline as Inhibitors of Tetracycline Destructase Enzymes. *ACS Infect. Dis.* 5 (4), 618–633. doi: 10.1021/acsinfecdis.8b00349

Nieuwenhuizen, A. A., Dijkstra, F., Notermans, D. W., and van der Hoek, W. (2018). Laboratory Methods for Case Finding in Human Psittacosis Outbreaks: A Systematic Review. *BMC Infect. Dis.* 18 (1), 442. doi: 10.1186/s12879-018-3317-0

Oldach, D. W., Gaydos, C. A., Mundy, L. M., and Quinn, T. C. (1993). Rapid Diagnosis of Chlamydia Psittaci Pneumonia. *Clin. Infect. Dis.* 17 (3), 338–343. doi: 10.1093/clinids/17.3.338

Petrovay, F., and Balla, E. (2008). Two Fatal Cases of Psittacosis Caused by Chlamydophila Psittaci. *J. Med. Microbiol.* 57 (Pt 10), 1296–1298. doi: 10.1099/jmm.0.2008/001578-0

Raeven, V. M., Spoorenberg, S. M., Boersma, W. G., van de Garde, E. M., Cannegieter, S. C., Voorn, G. P., et al. (2016). Atypical Aetiology in Patients Hospitalised With Community-Acquired Pneumonia is Associated With Age, Gender and Season; a Data-Analysis on Four Dutch Cohorts. *BMC Infect. Dis.* 16, 299. doi: 10.1186/s12879-016-1641-9

Rybarczyk, J., Versteele, C., Lernout, T., and Vanrompay, D. (2019). Human Psittacosis: A Review With Emphasis on Surveillance in Belgium. *Acta Clin. Belg.* 75 (1), 42–48. doi: 10.1080/17843286.2019.1590889

Rybarczyk, J., Versteele, C., Lernout, T., and Vanrompay, D. (2020). Human Psittacosis: A Review With Emphasis on Surveillance in Belgium. *Acta Clin. Belg.* 75 (1), 42–48. doi: 10.1080/17843286.2019.1590889

Saeki, S., Hirata, I., Fukusako, T., Negoro, K., Nogaki, H., and Morimatsu, M. (1996). A Case of Psittacosis With Psychiatric Symptoms, Abnormal EEG, and Abnormal SPECT. *No To Shinkei* 48 (12), 1141–1145.

Shi, Y., Chen, J., Shi, X., Hu, J., Li, H., Li, X., et al. (2021). A Case of Chlamydia Psittaci Caused Severe Pneumonia and Meningitis Diagnosed by Metagenome Next-Generation Sequencing and Clinical Analysis: A Case Report and Literature Review. *BMC Infect. Dis.* 21 (1), 621. doi: 10.1186/s12879-021-06205-5

Su, S., Su, X., Zhou, L., Lin, P., Chen, J., Chen, C., et al. (2021). Severe Chlamydia Psittaci Pneumonia: Clinical Characteristics and Risk Factors. *Ann. Palliat. Med.* 10 (7), 8051–8060. doi: 10.21037/apm-21-1502

Teng, X. Q., Gong, W. C., Qi, T. T., Li, G. H., Qu, Q., Lu, Q., et al. (2021). Clinical Analysis of Metagenomic Next-Generation Sequencing Confirmed Chlamydia Psittaci Pneumonia: A Case Series and Literature Review. *Infect. Drug Resist.* 14, 1481–1492. doi: 10.2147/IDR.S305790

Thaker, M., Spanogiannopoulos, P., and Wright, G. D. (2010). The Tetracycline Resistome. *Cell Mol. Life Sci.* 67 (3), 419–431. doi: 10.1007/s00018-009-0172-6

Vassallo, M., and Shepherd, R. J. (1997). A Case of Relapsing Psittacosis Associated With a Stroke. *Int. J. Clin. Pract.* 51 (4), 261.

Walder, G., Schönherr, H., Hotzel, H., Speth, C., Oehme, A., Dierich, M. P., et al. (2003). Presence of Chlamydophila Psittaci DNA in the Central Nervous System of a Patient With Status Epilepticus. *Scand. J. Infect. Dis.* 35 (1), 71–73. doi: 10.1080/0036554021000026984

Wallensten, A., Fredlund, H., and Runehagen, A. (2014). Multiple Human-to-Human Transmission From a Severe Case of Psittacosis, Sweden, January-February 2013. *Euro. Surveill.* 23;19 (42), 20937. doi: 10.2807/1560-7917.ES2014.19.42.20937

Wreghitt, T. (1993). Chlamydial Infection of the Respiratory Tract. *Commun. Dis. Rep. CDR Rev.* 3 (9), R119–R124.

Yuan, Y., Zhang, X., and Gui, C. (2021). Detection of Chlamydia Psittaci in Both Blood and Bronchoalveolar Lavage Fluid Using Metagenomic Next-Generation Sequencing: A Case Report. *Medicine (Baltimore)* 100 (27), e26514. doi: 10.1097/MD.0000000000026514

Yung, A. P., and Grayson, M. L. (1988). Psittacosis—A Review of 135 Cases. *Med. J. Aust.* 148 (5), 228–233. doi: 10.5694/j.1326-5377.1988.tb99430.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Yang, Li, Qi, Zou, Shi, Lei, Li, Luo, Zeng, Lu, Huang, Liu and Lan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 07 January 2022
doi: 10.3389/fcimb.2021.768613

Detection of *Nocardia* by 16S Ribosomal RNA Gene PCR and Metagenomic Next-Generation Sequencing (mNGS)

Juanjuan Ding[1]*, *Bing Ma*[2]*, *Xupeng Wei*[3] and *Ying Li*[1]*

[1] Department of Respiratory and Critical Care Medicine, Henan Provincial People's Hospital, Zhengzhou, China, [2] Department of Clinical Laboratory, Henan Provincial People's Hospital, Zhengzhou, China, [3] Department of Respiratory and Critical Care Medicine, Xuchang Central Hospital, Xuchang, China

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Luigi Principe,
Azienda Sanitaria Provinciale di Crotone, Italy
Arryn Craney,
Orlando Health, United States

***Correspondence:**
Juanjuan Ding
dingjuanhai@163.com
Bing Ma
mabing2001@126.com
Ying Li
191880609@qq.com

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 31 August 2021
Accepted: 10 December 2021
Published: 07 January 2022

Citation:
Ding J, Ma B, Wei X and Li Y (2022) Detection of Nocardia by 16S Ribosomal RNA Gene PCR and Metagenomic Next-Generation Sequencing (mNGS). Front. Cell. Infect. Microbiol. 11:768613. doi: 10.3389/fcimb.2021.768613

In this study, the aim was to investigate the discriminatory power of molecular diagnostics based on mNGS and traditional 16S ribosomal RNA PCR among *Nocardia* species. A total of fourteen clinical isolates from patients with positive *Nocardia* cultures and clinical evidence were included between January 2017 and June 2020 in HeNan Provincial People's Hospital. DNA extraction and 16S rRNA PCR were performed on positive cultures, and pathogens were detected by mNGS in these same samples directly. Among the 14 *Nocardia* isolates, four species were identified, and *N. cyriacigeorgica* (8 cases) is the most common species. Twelve of the 14 *Nocardia* spp. isolates were identified by the two methods, while two strains of *N. cyriacigeorgica* were not identified by mNGS. All tested isolates showed susceptibility to trimethoprim-sulfamethoxazole (SXT), amikacin and linezolid. Apart from *Nocardia* species, other pathogens such as *Acinetobacter baumannii, Klebsiella pneumonia, Aspergillus, Enterococcus faecalis, Human herpesvirus*, etc., were detected from the same clinical samples by mNGS. However, these different pathogens were considered as colonization or contamination. We found that it is essential to accurately identify species for determining antibiotic sensitivity and, consequently, choosing antibiotic treatment. 16S rRNA PCR was useful for identification of nocardial infection among species, while this technique needs the clinicians to make the pre-considerations of nocardiosis. However, mNGS may be a putative tool for rapid and accurate detection and identification of *Nocardia*, beneficial for applications of antimicrobial drugs and timely adjustments of medication.

Keywords: nocardiosis, 16S ribosomal RNA gene, polymerase chain reaction, next generation sequencing, species, molecular diagnosis

INTRODUCTION

Nocardia is a genus of aerobic, Gram-positive, branching, filamentous, weakly acid-fast bacteria (Wang et al., 2015). The organisms are saprophytic and are found in water, soil, dust, and vegetable matter. Upon exposure to dust particles, *Nocardia* may become airborne, causing infection by inhalation (Chen et al., 2014). Individuals with poor immune system, for example, patients with lung disorders, diabetes, cancers, HIV/AIDS, chronic alcoholism, connective tissue disorders, organ transplantation, and those undergoing corticosteroid therapy are prone to infections with *Nocardia* spp. (Kandi, 2015). The major clinical symptoms of human nocardiosis include lymphadenitis, lymphangitis, encephalitis, pneumonia, and cutaneous tissue lesions (Condas et al., 2015). Despite its infrequency, pulmonary nocardiosis is clinically severe (Sadamatsu et al., 2017). Recently, nocardiosis has been described as isolated cases and case series in lungs and other sites of immunocompetent patients, representing 33%-56% of total cases. Of note, bronchiectasis, chronic obstructive pulmonary disease (COPD), pulmonary alveolar proteinosis, and asthma are predisposing factors among patients with chronic respiratory diseases (Sadamatsu et al., 2017).

Reports on the incidence of *Nocardia* infection worldwide are limited, which might be caused by difficulties in isolation and incubation of *Nocardia* as well as lack of systematic literatures on *Nocardia* infection in health system (Darazam et al., 2013). There are annually approximate 500-1000 cases of nocardiosis in the United States. 16S rRNA PCR is vital for the accurate identification of *Nocardia* in clinical microbiology laboratories, but this method requires *a priori* knowledge of microorganisms that are suspected to exist in clinical samples under investigation prior to detection (Boers et al., 2019). Unbiased metagenomic next generation sequencing (mNGS) can detect infectious especially the rare or new pathogens (Lu et al., 2020), and compared with traditional methods, it performs better in diagnosis, indicating its application in early diagnosis and management of patients.

However, understanding of nocardiosis is mostly derived from case reports in China, and case series are required for shedding light on nocardiosis. We conducted this study to identify *Nocardia* species using 16SrRNA and mNGS in a tertiary hospital in China. The major aim was to investigate the discriminatory power of molecular diagnostics based on mNGS and traditional 16S ribosomal RNA PCR among *Nocardia* species. A secondary objective was to investigate the species distribution, clinical manifestations, and microbiological characteristics in our hospital.

METHODS

Study Design and Participants

This study was conducted at the HeNan Provincial People's Hospital, a 3900-bed tertiary care teaching hospital in China and all culture-proven cases of nocardiosis from January 2017 to June 2020. Medical records were reviewed for clinical information, including demographics, laboratory and radiological findings, physical examination, symptoms at presentation, antibiotic treatment, and outcome. Isolation of *Nocardia* species from clinical samples including bronchoalveolar lavage, sputum, lung aspiration, and purulent pus as well as pertinent clinical symptoms and imaging findings were required for nocardiosis diagnosis. *Nocardia* species were identified by conventional phenotypic identification methods and Bruker MALDI-TOF MS systems (Bruker Daltonics, Billerica, MA, USA). After the microbial culture dish gave an alarm, parallel specimen were sent to commercial companies for mNGS. 16S rRNA PCR was carried out to identify the species for the culture results. In spite of *Nocardia* being isolated from samples, cases without clinical symptoms and chest radiographical findings were judged as colonization and excluded from analysis. Disseminated nocardiosis was defined as involvement of ≥2 non-contiguous organs or the central nervous system.

16S rRNA PCR and Sequencing

The bacteria isolation and culture protocol were conducted according to a previous study (Larruskain et al., 2011). The species further identification of each *Nocardia* strain was done by sequencing the 16S rRNA gene (1500 bp).Genomic DNA of *Nocardia* was extracted by using Ezup Column Bacteria Genomic DNA Purification Kit (Cat. No. SK8255, Sangon, China). PCR primers were the bacterial 16S rRNA universal primer pairs, 7F and 1540R(5' CAGAGTTTGATCCTGGCT3', 5'AGGAGGTGA TCCAGCCGCA3'), 27F and 1492R(5' AGTTTGATCTTGGC TCAG 3', 5'GGTTACCTTGTTACGACTT 3'). The 16S rRNA gene was amplified by PCR under the thermal cycling conditions of 35 cycles at 94°C for 1 min for denaturation, 60°C for 1 min for primer annealing, and 72°C for 2 min for primer extension. A 3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) with primers used in gene amplification was used for sequencing the PCR products. The resulting DNA sequences were analyzed by the BLAST program.

Metagenomic Next Generation Sequencing and Data Analysis

A 300 μl sample of bronchoalveolar lavage fluid (BALF), sputum, tissue, purulent pus, etc. were collected in DNase/RNase tubes for identifying the potential pathogens. DNA libraries were constructed through transposase mediated methods, and PCR amplification followed (Vision Medicals, China). For measurement of the adapters and the sizes of fragments before sequencing, the quality of the DNA libraries was determined by an Qsep1 bio-fragment analyzer (BiOptic. Inc., La Canada Flintridge, CA). Qualified DNA libraries were pooled together and sequenced on Nextseq 550 Dx sequencing platform (illumina, San Diego, CA). After removal of low-quality, and short (length < 40bp) reads, Burrows-Wheeler Alignment was used for computational subtraction of human host sequences mapped to the human reference genome (hg38 and YH sequences), and the high-quality sequencing data were generated. After removal of low-complexity reads, the remaining data were classified according to four (bacteria, parasites, fungi, viruses) Microbial Genome Databases. The classification reference databases were

downloaded and optimized from public databases including NCBI, EBI or Genbank. At last, the multi-parameters of species in Microbial Genome Databases were calculated and exported. Results were interpreted by professionals with microbiology and clinical background.

Antimicrobial Susceptibility Tests

The *in vitro* antimicrobial susceptibility of all strains was detected using broth microdilution method, as recommended by the CLSI for antimicrobial susceptibility testing of *Nocardia*. Antimicrobials (amikacin, trimethoprim-sulfamethoxazole, linezolid, imipenem, minocycline, cefotaxime, ciprofloxacin, tobramycin) were used for susceptibility testing. Resistance to two or more of the most commonly used drugs (amikacin, ceftriaxone, TMP-SMZ and imipenem) refers to multidrug resistance (Betrán et al., 2016).

RESULTS

Demographic and Clinical Features

In the present study, fourteen patients with positive *Nocardia* cultures and clinical evidence were included. The clinical features of the patients and *Nocardia* strains isolated from samples are shown in **Table 1**. In 12 cases of these patients, only the lung was infected. One case had pulmonary and neurological involvement. The other patient had skin involvement. Male patients accounted for nine cases, and the median age was 56 years (range, 23-75) in all fourteen patients. Chronic lung disease (6 cases), including bronchiectasis (4 cases), COPD (1 case) and interstitial pneumonia (1 case), were most common underlying diseases. Other 7 patients had underlying disease with poor immune system, including solid tumor (3 cases), chronic renal disease (2 cases), diabetes mellitus (1 case) and hematological malignancy (1 case). Only one patient with skin nocardiosis had no underlying disease. Three patients died of the severity of their disease. The most common presenting manifestations were fever (8 cases), cough (7 cases) and sputum production (5 cases), followed by dyspnea (4 cases), hemoptysis (1 cases), chest pain (1 case), cutaneous abscess (1 case). Neurological signs were observed in one patient who has disseminated nocardiosis. All 14 patients underwent chest computed tomography (CT). Bronchiectasis (n=4) and infiltration (n=4) were the most common presentation of chest CT findings, followed by consolidation (n=3), masses (n=3), cavity (n=3), nodules (n=2) and pleural effusion (n=1).

Distribution of *Nocardia* Species

Of the 14 *Nocardia* isolates, 12 isolates were identified by the two methods, while two isolates of *N. cyriacigeorgica* were not identified by mNGS. The isolated *Nocardia* species include *N. cyriacigeorgica* (8 cases), *N. farcinic*a (4 cases), *N. otitidiscaviarum* (1 case) and *N. brasiliensis* (1 case), determined by its 99.93% or 100% sequence similarity to the reference sequence in GenBank (**Table 1**).

Antimicrobial Susceptibility

Tests of antimicrobial susceptibility were performed on these isolates, and the results are provided in **Table 2**. All tested

TABLE 1 | Clinical features of the patients and *Nocardia* strains isolated from samples.

No.	Age	Gender	Underlying disease	Symptoms	Imaging	Specimen	Species by 16S rRNA	Identify	Referrence Assesion	Species by mNGS	Antibiotic treatment	Outcome
1	75	male	Lung cancer	cough	mass	lung aspiration	*N.cyriacigeorgica*	100%	LR215973.1	*N.cyriacigeorgica*	SXT	Recovery
2	53	male	Membranous nephropathy	fever, dyspnea	nodule;infiltration	suputum	*N.cyriacigeorgica*	100%	LR215973.1	*N.cyriacigeorgica*	SXT+IPM	Recovery
3	49	female	Bronchiectasis	fever, cough, sputum production	bronchiectasis, infiltration	BAL	*N. facinica*	100%	MN100049.1	*N. facinica*	SXT	Recovery
4	23	female	Bronchiectasis	cough, sputum production	bronchiectasis, infiltration	BAL	*N. facinica*	100%	MN100049.1	*N. facinica*	SXT	Recovery
5	42	female	Diabetes mellitus	fever, cough, sputum production	consolidation, pleural effusion	BAL	*N.otitidiscaviarum*	100%	KX500116.1	*N.otitidiscaviarum*	SXT	Death
6	68	male	Nephrotic syndrome	fever, hemoptysis, chest pain.neurological signs	cavity	BAL	*N.cyriacigeorgica*	100%	LR215973.1	Unidentified	SXT+IPM+ LZD	Death
7	70	male	COPD	dyspnea	cavity, mass	suputum	*N.brasiliensis*	99.93%	CP022088.2	*N.brasiliensis*	SXT+ LVX	Recovery
8	50	male	Bronchiectasis	fever, cough, suputum production,	bronchiectasis, infiltration	BAL	*N.cyriacigeorgica*	100%	LR215973.1	Unidentified	SXT	Recovery
9	78	male	None	cutaneous abscess	none	purulent pus	*N.cyriacigeorgica*	100%	LR215973.1	*N.cyriacigeorgica*	SXT	Recovery
10	56	female	Interstitial pneumonia	dyspnea,	consolidation	lung aspiration	*N.cyriacigeorgica*	100%	LR215973.1	*N.cyriacigeorgica*	SXT	Recovery
11	68	female	Lung cancer	fever, dyspnea	mass,	suputum	*N.cyriacigeorgica*	100%	LR215973.1	*N.cyriacigeorgica*	SXT	Recovery
12	28	male	Hematological malignancy	fever, cough	consolidation	BAL	*N. facinica*	100%	MN100049.1	*N. facinica*	SXT+IPM+ LZD	Death
13	63	male	Esophageal cancer	fever	nodules,cavity	BAL	*N.cyriacigeorgica*	100%	LR215973.1	*N.cyriacigeorgica*	SXT+ LVX	Recovery
14	57	male	Bronchiectasis	cough, sputum production	bronchiectasis,	BAL	*N. facinica*	100%	MN100049.1	*N. facinica*	SXT	Recovery

BAL, brochoalveolar lavage; COPD, chronic obstructive pulmonary disease; SXT, trimethoprim-sulfamethoxazole; IPM, imipenem; LZD, linezolid; LVX, levofloxacin.

isolates were susceptible to amikacin, trimethoprim-sulfamethoxazole, linezolid. Susceptible isolates were seen for imipenem (10/14), minocycline (8/14), cefotaxime (4/14) and ciprofloxacin (6/14). Multidrug-resistance was found in four isolates including one isolate of *N. cyriacigeorgica* (1/8), one isolate of *N. farcinica* (1/4), one isolate of *N. otitidiscaviarum* (1/1) and one isolate of *N. brasiliensis* (1/1). All these four multidrug-resistant isolates were resistant to imipenem, cefotaxime, ciprofloxacin,

Other Pathogens Detected by mNGS

Other than *Nocardia* species, different pathogens such as *Acinetobacter baumannii*, *Klebsiella pneumonia, Aspergillus, Enterococcus faecalis, Human herpesvirus*, etc., were detected from the same clinical samples by mNGS. Combined with clinical manifestations, conventional culture method and (1, 3) -β-D-glucan and galactomannan (GM) test, these different pathogens were considered as colonization or contamination.

DISCUSSION

Although being considered as an opportunistic infection, nocardiosis also infects immunocompetent hosts. In this study, the most common underlying disease is bronchiectasis, which is consistent with the previous report from China. A study by Yang et al. that analyzed 13 patients infected by nocardiosis showed that twelve cases were diagnosed with pulmonary nocardiosis and bronchiectasis in 6 patients (Yang et al., 2017). Results from other reports are different, showing that COPD is the third most common risk factor, second only to chronic steroid therapy and solid organ transplantation (Kancherla et al., 2019). Nevertheless, bronchiectasis and COPD were deemed as risk factors for pulmonary nocardiosis (Kancherla et al., 2019). Pulmonary structural abnormalities and bacterial colonization on the bronchus facilitate the presence of *Nocardia* (Tomás et al., 2007). Patients with bronchiectasis were increasingly diagnosed with pulmonary nocardiosis. The reasons for the apparent increase are not entirely clear. The increased incidence of nocardiosis with bronchiectasis can be caused by environmental exposures, microbiologic surveillance, and other factors (Woodworth et al., 2017).

Clinical manifestations of nocardiosis lack specificity (Mootsikapun et al., 2005). Our findings indicated that the most common clinical manifestations were fever and cough. In this study, bronchiectasis and infiltration were the most common CT manifestations, followed by consolidation, masses, cavity, nodules and pleural effusion. Different from our study, most patients were observed with cavitation coupled with nodules, masses and consolidations in other reports (Chen et al., 2014). Mehrian et al. found that multiple pulmonary nodules, consolidation, and cavity are the main CT presentations of pulmonary nocardiosis in either immunocompetent patients or those with immune deficit, but these characteristics are not specific (Mehrian et al., 2015). The higher prevalence of bronchiectasis in our study in comparison to other reports is probably the main reason for the difference of computed tomography features.

In consistent with other studies, the *Nocardia* species showed good sensitivity to SXT, linezolid, and amikacin in the present study. Additionally, these isolates showed varying susceptibilities to different antibiotics. Different *Nocardia* species may have different susceptibility profiles, and this information is crucial for providing adequate antimicrobial therapy as well as investigating the epidemiology of *Nocardia* infections (Tamakoshi et al., 2018). When *Nocardia* is isolated from clinical specimens, the species should be identified. The traditional culture and biochemical methods are very time-consuming, resulting in a delayed diagnosis of nocardiosis. However, early application of suitable antibiotherapy, which depends on early detection of the nocardial infection, is vital for decreasing the high death rates of patients with nocardiosis (Couble et al., 2005). 16S rRNA PCR and sequencing offer accurate, inexpensive, rapid, and reliable identification of nocardial infection among species (Rahdar et al., 2021). At present, 16S rRNA PCR and sequencing are used for limited number of pathogens at a time or successful culture of microorganisms from clinical samples was required. In our study, 16S ribosomal RNA gene sequencing was useful for identification and detection of species of *Nocardia*. However, two isolates of *N. cyriacigeorgica* were not identified by mNGS. The possible reason is that mNGS currently has some limitations, such as human background and lack of unified standards for detailed experimental steps. However, since mNGS possesses more sensitivity than 16S rRNA sequencing analysis in diagnosing unspecified pathogens, especially rare pathogens, mNGS was adopted for pathogenic diagnosis of diseases. In our study, mNGS detected other pathogens apart

TABLE 2 | Antimicrobial susceptibility of *Nocardia* species was determined using the standard broth microdilution method.

Antimicrobials	No. of susceptible isolates				
	N. cyriacigeorgica (n=8)	*N. facinica* (n=4)	*N.otitidiscaviarum* (n=1)	*N.brasiliensis* (n=1)	Total (n=14)
Amikacin	8	4	1	1	14
TMP-SMZ	8	4	1	1	14
linezolid	8	4	1	1	14
Imipenem	7	3	0	0	10
Minocycline	3	3	1	1	8
cefotaxime	3	1	0	0	4
Ciprofloxacin	3	3	0	0	6
Tobramycin	6	0	1	1	8

from *Nocardia* species in the same samples, nevertheless, these cases were not considered as mixed infection. The results of mNGS still should be analyzed by clinicians in combination with the clinical features and conventional methods (such as traditional culture, G test, GM test, Aspergillus IgG antibody test, Cryptococcus serum antigen assay, etc.). Almost all pathogens in the samples can be detected by mNGS, while 16S rRNA PCR and sequencing were only applied for detection of a specific pathogen with the matched primers, and under such condition, rare pathogens may be missed, or primers containing mismatches for the tested pathogens may be mistakenly used, which leads to reduction in sensitivity and failure in detection (Gu et al., 2019). Thus, mNGS combined with targeted sequencing may be significantly useful for nonsterile samples, such as those from stool, polymicrobial abscesses, or bronchoalveolar lavage (Gu et al., 2019).

In conclusion, our study analyzed imaging findings, clinical features, drug susceptibility pattern, treatment and outcomes of nocardiosis-infected patients. These results will be promising for early diagnosis and management of patients. We found that 16S rRNA PCR was useful for identification of nocardial infection among species, while this technique needs the pre-considerations of nocardiosis by clinicians. However, mNGS may be advantageous for rapid and accurate detection and identification of *Nocardia*, which promotes applications of appropriate antimicrobial drugs and timely medication adjustments if necessary.

DATA AVAILABILITY STATEMENT

All data have been uploaded to China National GeneBank DataBase (http://db.cngb.org/cnsa), CNP0002551.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Committee of Henan Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JD designed the study, collected and analyzed the data, wrote, and edited the manuscript. BM collected the samples, participated in the next generation sequencing, and edited the manuscript. XW collected the samples and data. YL collected and analyzed the data, wrote, and edited the manuscript.

FUNDING

This work was supported by Project from Science and Technology of Henan Province (132300410061).

ACKNOWLEDGMENTS

We would like to thank Xin Huang for his help to this study. H-X helped us to follow up patients.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.768613/full#supplementary-material

REFERENCES

Betrán, A., Villuendas, M. C., Rezusta, A., Pereira, J., Revillo, M. J., and Rodríguez-Nava, V. (2016). Clinical Significance, Antimicrobial Susceptibility and Molecular Identification of Nocardia Species Isolated From Children With Cystic Fibrosis. *Braz. J. Microbiol.* 47, 531–535. doi: 10.1016/j.bjm.2016.01.029

Boers, S. A., Jansen, R., and Hays, J. P. (2019). Understanding and Overcoming the Pitfalls and Biases of Next-Generation Sequencing (NGS) Methods for Use in the Routine Clinical Microbiological Diagnostic Laboratory. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 1059–1070. doi: 10.1007/s10096-019-03520-3

Chen, J., Zhou, H., Xu, P., Zhang, P., Ma, S., and Zhou, J. (2014). Clinical and Radiographic Characteristics of Pulmonary Nocardiosis: Clues to Earlier Diagnosis. *PloS One* 9, e90724. doi: 10.1371/journal.pone.0090724

Condas, L. A., Ribeiro, M. G., Muro, M. D., de Vargas, A. P., Matsuzawa, T., Yazawa, K., et al. (2015). Molecular Identification and Antimicrobial Resistance Pattern of Seven Clinical Isolates of *Nocardia* Spp. *Rev. Inst Med. Trop. Sao Paulo* 57, 251–256. doi: 10.1590/S0036-46652015000300012

Couble, A., Rodríguez-Nava, V., de Montclos, M. P., Boiron, P., and Laurent, F. (2005). Direct Detection of Nocardia Spp. In Clinical Samples by a Rapid Molecular Method. *J. Clin. Microbiol.* 43, 1921–1924. doi: 10.1128/JCM.43.4.1921-1924.2005

Darazam, I. A., Shamaei, M., Mobarhan, M., Ghasemi, S., Tabarsi, P., Motavasseli, M., et al. (2013). Nocardiosis: Risk Factors, Clinical Characteristics and Outcome. *Iran Red Crescent Med. J.* 15, 436–439. doi: 10.5812/ircmj.2384

Gu, W., Miller, S., and Chiu, C. Y. (2019). Clinical Metagenomic Next-Generation Sequencing for Pathogen Detection. *Annu. Rev. Pathol.* 24, 319–338. doi: 10.1146/annurev-pathmechdis-012418-012751

Kancherla, R., Ramanathan, R. P., Appalaraju, B., and Rajagopala, S. (2019). Pulmonary Nocardiosis Presenting as Exacerbation of Chronic Pulmonary Disease. *Indian J. Crit. Care Med.* 23, 467–474. doi: 10.5005/jp-journals-10071-23270

Kandi, V. (2015). Human Nocardia Infections: A Review of Pulmonary Nocardiosis. *Cureus* 15, e304. doi: 10.7759/cureus.304

Larruskain, J., Idigoras, P., Marimón, J. M., and Pérez-Trallero, E. (2011). Susceptibility of 186 Nocardia Spp. Isolates to 20 Antimicrobial Agents. *Antimicrob. Agents Chemother.* 55, 2995–2998. doi: 10.1128/AAC.01279-10

Lu, R., Zhao, X., Li, J., Niu, P. H., Yang, B., Wu, H. L., et al. (2020). Genomic Characterisation and Epidemiology of 2019 Novel Coronavirus: Implications for Virus Origins and Receptor Binding. *Lancet* 395, 565–574. doi: 10.1016/S0140-6736(20)30251-8

Mehrian, P., Esfandiari, E., Karimi, M. A., and Memari, B. (2015). Computed Tomography Features of Pulmonary Nocardiosis in Immunocompromised and Immunocompetent Patients. *Pol. J. Radiol.* 80, 13–17. doi: 10.12659/PJR.892042

Mootsikapun, P., Intarapoka, B., and Liawnoraset, W. (2005). Nocardiosis in Srinagarind Hospital, Thailand: Review of 70 Cases From 1996-2001. *Int. J. Infect. Dis.* 9, 154–158. doi: 10.1016/j.ijid.2004.06.011

Rahdar, H. A., Bahador, A., Shahraki-Zahedani, S., Karami-Zarandi, M., Soori, T., Jafari, S., et al. (2021). Pulmonary Nocardiosis in Pemphigus Vulgaris Patients From Tehran, Iran. Infect. *Disord. Drug Targets* 21, 78–83. doi: 10.2174/1871526520666191231144607

Sadamatsu, H., Takahashi, K., Tashiro, H., Komiya, K., Nakamura, T., and Sueoka-Aragane, N. (2017). Successful Treatment of Pulmonary Nocardiosis With Fluoroquinolone in Bronchial Asthma and Bronchiectasis. *Respirol Case Rep.* 26, e00229. doi: 10.1002/rcr2.229

Tamakoshi, J., Kimura, R., Takahashi, K., and Saito, H. (2018). Pulmonary Reinfection by Nocardia in an Immunocompetent Patient With Bronchiectasis. *Intern. Med.* 57, 2581–2584. doi: 10.2169/internalmedicine.0531-17

Tomás, R. M., Villanueva, R. M., Calzada, S. R., Durantez, M. S., Tarazona, J. M. V., Alapont, M. M., et al. (2007). Pulmonary Nocardiosis: Risk Factors and Outcomes. *Respirology* 12, 394–400. doi: 10.1111/j.1440-1843.2007.01078.x

Wang, H. K., Sheng, W. H., Hung, C. C., Chen, Y. C., Lee, M. H., Lin, W. S., et al. (2015). Clinical Characteristics, Microbiology, and Outcomes for Patients With Lung and Disseminated Nocardiosis in a Tertiary Hospital. *J. Formos Med. Assoc.* 114, 742–749. doi: 10.1016/j.jfma.2013.07.017

Woodworth, M. H., Saullo, J. L., Lantos, P. M., Cox, G. M., and Stout, J. E. (2017). Increasing Nocardia Incidence Associated With Bronchiectasis at a Tertiary Care Center. *Ann. Am. Thorac. Soc* 14, 347–354. doi: 10.1513/AnnalsATS.201611-907O

Yang, H. Q., Shi, H. Z., and Tong, Z. H. (2017). Retrospective Analysis of 13 Cases of Nocardiosis. *Zhonghua Jie He He Hu Xi Za Zhi* 40, 588–591. doi: 10.3760/cma.j.issn.1001-0939.2017.08.009

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Ding, Ma, Wei and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 12 January 2022
doi: 10.3389/fcimb.2021.783568

The Application of Whole–Exome Sequencing in Patients With FUO

Wanru Guo[1†], Xuewen Feng[1†], Ming Hu[1†], Yanwan Shangguan[1], Jiafeng Xia[1], Wenjuan Hu[1], Xiaomeng Li[1], Zunjing Zhang[2], Yunzhen Shi[3] and Kaijin Xu[1]*

[1] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [2] Department of Respiratory Medicine, Lishui Hospital of Traditional Chinese Medicine (TCM), Lishui, China, [3] Department of Infectious Diseases, Dongyang People's Hospital, Dongyang, China

Background: Fever of unknown origin (FUO) is still a challenge for clinicians. Next-generation sequencing technologies, such as whole exome sequencing (WES), can be used to identify genetic defects in patients and assist in diagnosis. In this study, we investigated the application of WES in individuals with FUO.

Methods: We performed whole-exome sequencing on 15 FUO patients. Clinical information was extracted from the hospital information system.

Results: In 7/15 samples, we found positive results, including potentially causative mutations across eight different genes: *CFTR*, *CD209*, *IRF2BP2, ADGRV 1*, *TYK2*, *MEFV*, *THBD* and *GATA2.*

Conclusions: Our results show that whole-exome sequencing can promote the genetic diagnosis and treatment of patients with FUO.

Keywords: next-generation sequencing, whole exome sequencing, fever of unknown origin, gene mutation, genetic diagnosis

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Changixn Wu,
Shanxi University, China
Balaji Banoth,
St. Jude Children's Research Hospital, United States

****Correspondence:***
Kaijin Xu
zdyxyxkj@zju.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *26 September 2021*
Accepted: *17 December 2021*
Published: *12 January 2022*

Citation:
Guo W, Feng X, Hu M, Shangguan Y, Xia J, Hu W, Li X, Zhang Z, Shi Y and Xu K (2022) The Application of Whole–Exome Sequencing in Patients With FUO. Front. Cell. Infect. Microbiol. 11:783568. doi: 10.3389/fcimb.2021.783568

INTRODUCTION

Fever of unknown origin (FUO) has been a clinical problem since it was defined in 1961. FUO was first proposed by Petersdorf and Beeson in 1961, and was defined as a fever lasting more than 3 weeks with a temperature over 38.3°C on several occasions, that remained undiagnosis after one week of inpatient investigation (Petersdorf and Beeson, 1961). Accurate diagnosis can be challenging because a wide variety of diseases, including some rare diseases, can cause fever. Current methods including laboratory examinations, radiographic modalities and invasive investigations, are of limited use and sometimes cannot fully explain the disease and pathogenesis. Since infectious diseases are still the leading cause of FUO, many studies in recent years have focused on the etiology of the disease, and new technologies such as metagenomic next-generation sequencing (mNGS) have been applied to detect pathogens (Gu et al., 2019; Wright et al., 2021). However, few studies have focused on the role of host factors, which also play an important role in the development of diseases. It is novel for clinicians to explore the causes of fever from the perspective of host factors. Studies have shown that although phenotypic expression is highly variable, some cases of fever are related to gene mutations (Özen et al., 2017). For example, systemic

autoinflammatory syndrome is known to often manifest as fever of unknown origin and is diagnosed by clinical suspicion and genetic testing (Lachmann, 2015). Some autoimmune diseases are also considered to be due to genetic factors (Liu et al., 2015; Erman and Çipe, 2020). Next-generation sequencing technologies (NGS), such as whole exome sequencing (WES), can be used for diagnosis or research to identify genetic mutations associated with the clinical phenotype of patients. As a noninvasive technology, NGS is capable of high-throughput sequencing with high sensitivity at relatively low cost, and it can effectively discover many new genes and pathways related to disease (Levy and Boone, 2019). In this study, we sought to analyze 15 cases of FUO utilizing WES to determine whether WES could be effectively utilized to identify the genetic cause of FUO.

MATERIALS AND METHODS

Study Design and Participants

Fifteen patients hospitalized with FUO were selected in the First Affiliated Hospital of Zhejiang University between September 1, 2019 and September 1, 2021. All patients had fever lasting more than 3 weeks with a temperature over 38.3°C on several occasions, and the diagnosis was unclear after one week of inpatient investigation. Blood samples from these patients were collected for WES, and clinical, radiological, and pathogenic findings, as well as treatment data were extracted from the hospital information system. Ethics approval was obtained from the Institutional Review Board of the First Affiliated Hospital, Zhejiang University School of Medicine. Patients or their guardians signed a written informed consent.

Sequencing

Genomic DNA was extracted from whole blood obtained from the patients and any participating family members. DNA samples were randomly disrupted by the ultrasonic high-performance sample processing system (Covaris). Segments were selected using the Agencourt AMPure XP-Medium kit, and library preparation was performed. Then the DNA of the target gene exon and adjacent splice regions was captured and enriched using a BGI V4 chip. Sequencing was performed on the Mgiseq-2000 sequencing platform. The average effective sequencing depth of the target region was ≥100X, and >95% of target bases, and had coverage >20X. The sequences were compared with the UCSC hg19 human reference genome by BWA. SNP detection and InDel analysis were performed, and then the mutation was selected by filtering through database annotation. The classification of variant pathogenicity is based on the American College of Medical Genetics (ACMG) guidelines.

RESULTS

These patients were between 15-63 years old, and they came to our hospital with different symptoms related to the respiratory system, cardiovascular system, immune system, central nervous system and skin. Seven patients were diagnosed with infectious diseases, including bronchiectasis with infection, Mycobacterium arhus infection, idiopathic pericarditis, Mycobacterium intracellular infection, Scedosporium boydii infection and Mycobacterium avium infection. Three patients were undiagnosed, and other patients were diagnosed as follows: pyoderma gangrenosum, hemophagocytic syndrome, systemic lupus erythematosus (SLE) and hemophagocytic syndrome, periodic fever syndrome, and pulmonary embolism. Patient characteristics are given in **Table 1**.

We analyzed 15 cases of FUO using WES, with 14 submitted as probands only and 1 with two additional family members. The results were divided into three categories: definitive result refers to the pathogenic or likely pathogenic variant in a disease gene associated with the clinical phenotype, possible/probable diagnosis refers to variants in a disease gene possibly related to the clinical phenotype, negative result means that no variants in genes related to the reported phenotype have been found (Retterer et al., 2016). Overall, across the 15 cases, a definitive result was given in 2 cases, a possible/probable result was given in 5 (**Table 2**) and a negative result was given in 8 cases. The heterozygous missense mutation (c.2042A>T p.G lu681Val) in the *CFTR* gene in S-1 and the mutation (c.194_195insT p.Ala66Argfs*119) in the *GATA2* gene in S-15 were classified as likely pathogenic through ACMG guidelines for pathogenicity. The heterozygous missense mutation (c.1049-18A>G) in the *IRF2BP2* gene in S-2, the mutation (c.10601C>T p. Ser3534Leu) in the *ADGRV 1* gene in S-5, the compound heterozygous mutations (c.3083A>G p.Asn1028Ser, c.2590C>T p.Arg864Cys) in the *TYK2* gene in S-7, and the mutation (c.472T>G p.Cys158Gly) in the *THBD* gene in S-12 were all considered to be possibly associated with the reported phenotype. The pathogenicity of these mutations has not been reported and they were classified as a VoUS (variant of unknown significance) according to the ACMG guidelines. The mutation (c.220C>T p.Gln74*) in the *CD209* gene in S-2 and the heterozygous missense mutation (c.442G>C p.Glu148Gln) in the *MEFV* gene in S-11 have been reported previously. They were possibly associated with the reported phenotype and were also classified as a VoUS. WES data also identified two cases with CNVs greater than 1 Mb. A duplicate copy of chromosome 8 was detected in S-4, and approximately 60.8 Mb of suspected chimeric repeat was detected in the 1q21.1-q32.1 segment in S-6. This finding was not previously detected in the ISCA, Decipher and ClinVar databases and requires further study.

DISCUSSION

We have performed clinical and molecular analyses in 15 patients with FUO to investigate the potential use of WES to identify genetic causes of FUO, which has rarely been studied before. The mutational analysis identified that 7 patients (46.7%) had positive results. The mutation detected in S-1 has previously been identified to play a role in bronchiectasis. *CFTR* can regulate

TABLE 1 | Clinical characteristics of patients.

Sample	Age*/sex (M/F)	Diagnosis	Symptoms other than fever	Treatment	Outcome
S-1	63/F	Bronchiectasis with infection	cough and sputum	Anti-infection	Controlled without recurrence
S-2	26/M	Mycobacterium Aarhus infection	cough	Anti-mycobacterial therapy	Improved
S-3	26/M	Undiagnosed	/	Methylprednisolone	Controlled without recurrence
S-4	38/M	Pyoderma gangrenosum	damaged skin	Methylprednisolone	Improved
S-5	19/F	Undiagnosed	cough	NSAIDs; cough medicine	Controlled without recurrence
S-6	50/F	Idiopathic pericarditis	chest distress	Colchicines, Methylprednisolone	Improved
S-7	30/M	Mycobacterium intracellular infection	abdominal pain	Anti-mycobacterial therapy	Cured
S-8	23/F	Hemophagocytic syndrome	rash, joint pain	Methylprednisolone; Ciclosporin	Under treatment
S-9	54/M	Scedosporium boydii infection	headache	Voriconazole	Improved
S-10	21/F	Systemic Lupus Erythematosus; Hemophagocytic syndrome	cough	Etoposide; Ciclosporin	Under treatment
S-11	38/F	Periodic Fever Syndrome	joint pain	NSAIDs	Controlled without recurrence
S-12	47/M	Pulmonary embolism	cough, chest distress	Low molecular heparin, anti-infection	Cured
S-13	48/F	Mycobacterium intracellular infection	lymphadenectasis	Anti-mycobacterial therapy	Cured
S-14	26/F	Undiagnosed	headache	Symptomatic treatment	Under treatment
S-15	15/F	MonoMAC syndrome; Mycobacterium avium infection	cough and sputum	Anti-mycobacterial therapy	Under treatment

M, male; F, female; NSAIDs, nonsteroidal anti-inflammatory drugs.
The symbol "" means age of participant at initial referral.*

TABLE 2 | The positive results of whole exon sequencing.

Sample	The primary results	Chromosomal location	Transcript ID: Mutation (amino acid variation)	ACMG	Relationship between mutations and phenotypes	Literature support
S-1	*CFTR*	chr7:117232263	NM_000492.3:c.2042A>T(p.G lu681Val)	Likely pathogenic	Partially related	Yes
S-2	*CD209* *IRF2BP2*	chr19:7810932 chr1:234743616	NM_021155.3: c.220C>T(p.Gln74*) NM_182972.2: c.1049-18A>G	VoUS	Partially related	Yes
S-5	*ADGRV 1*	chr5:90040914	NM_032119.3: c.10601C>T(p. Ser3534Leu)	VoUS	Partially related	Yes
S-7	*TYK2*	chr19:10463719 chr19:10467271	NM_003331.4: c.3083A>G(p.Asn1028Ser) NM_003331.4: c.2590C>T(p.Arg864Cys)	VoUS	Partially related	Yes
S-11	*MEFV*	chr16:330425-3304 626	NM_000243.2: c.442G>C(p.Glu148Gln)	VoUS	Partially related	Yes
S-12	*THBD*	chr20:23029670	NM_000361.2: c.472T>G(p.Cys158Gly)	VoUS	Partially related	Yes
S-15	*GATA2*	chr3:128205680-128205681	NM_032638.4: c.194_195insT (p.Ala66Argfs*119)	Likely pathogenic	Partially related	Yes

ACMG, American College of Medical Genetics; VoUS, variant of unknown significance.
The symbol "" means nonsense mutations in amino acids.*

airway epithelial surface mucus. Genetic defects of *CFTR* leads to poor mucus cilia clearance, excessive mucus secretion and recurrent infections with virulent pathogens including Pseudomonas aeruginosa, Staphylococcus aureus, and nontuberculous mycobacteria (Perrem and Ratjen, 2019). Studies have shown that heterozygosity for *CFTR* has pathogenic consequences and contributes to the development of bronchiectasis (Tzetis et al., 2001; Casals et al., 2004). This patient had been hospitalized several times with fever, cough and sputum, and had a history of intracellular mycobacterium infection. Therefore, the host factors that contributed to the disease were of great interest to us, and we look forward to finding better treatments. *CFTR* modulators are a new class of drugs that directly solve functional protein defects, and can enhance *CFTR* gene or protein expression levels at the cell surface (Patel et al., 2020). Significant progress has been made in the development, which is expected to improve the prognosis of patients.

S-2 carried two heterozygous missense mutations. The first was in the *CD209* gene (c.220C>T p.Gln74*) and the second was in the *IRF2BP2* gene (c.1049-18A>G). The *CD209* gene encodes the dendritic cell-specific intercellular adhesion molecule-3 binding nonintegrin factor (DC-SIGN) molecule, which is located on the surface of dendritic cells and alveolar macrophages. It is a natural immune recognition receptor associated with tuberculosis. Mycobacterium tuberculosis can bind to DC-SIGN through its cell wall component lipid arabinomannan (mannose-cappedlipoarabinomannan, ManLAM), leading to the infection of dendritic cells and alveolar macrophages (Barreiro et al., 2006).

Studies have shown that *CD209* gene variants may affect the protection against and susceptibility to Mycobacterium tuberculosis infection (da Silva et al., 2014). Keller et al. showed that *IRF2BP2* variant led to failure of B cell differentiation, impaired immunoglobulin secretion and a familial form of common variable immunodeficiency disorder (CVID14) (Keller et al., 2016). The patient was young and had no structural bronchial abnormalities or other risk factors. Therefore, we highly suspect that these mutations compromised immune function and susceptibility to Mycobacterium tuberculosis in this patient.

A novel heterozygous mutation (c.10601C>T p. Ser3534Leu) in *ADGRV 1* was identified in S-5. *ADGRV1* mutation has been seen previously associated with familial febrile seizure 4 (Han et al., 2020), which is a febrile disease. Since the patient in our study also presented with recurrent fever without evidence of infection or common autoimmune disease, we propose that the new mutation in *ADGRV1* may have an impact on the occurrence of febrile disease in this patient. Further research is needed to prove that *ADGRV1* mutation is a significant susceptible gene for febrile disease.

WES revealed compound heterozygous mutations (c.3083A>G p.Asn1028Ser, c.2590C>T p.Arg864Cys) in the *TYK2* gene in S-7. Sanger sequencing showed that both parents were heterozygous for mutations in *TYK2*: c.2590C>T in the mother and c.3083A>G in the father (**Figure 1**). The patient had recurrent fever with nontuberculous mycobacteria invading the bowel, lymph nodes

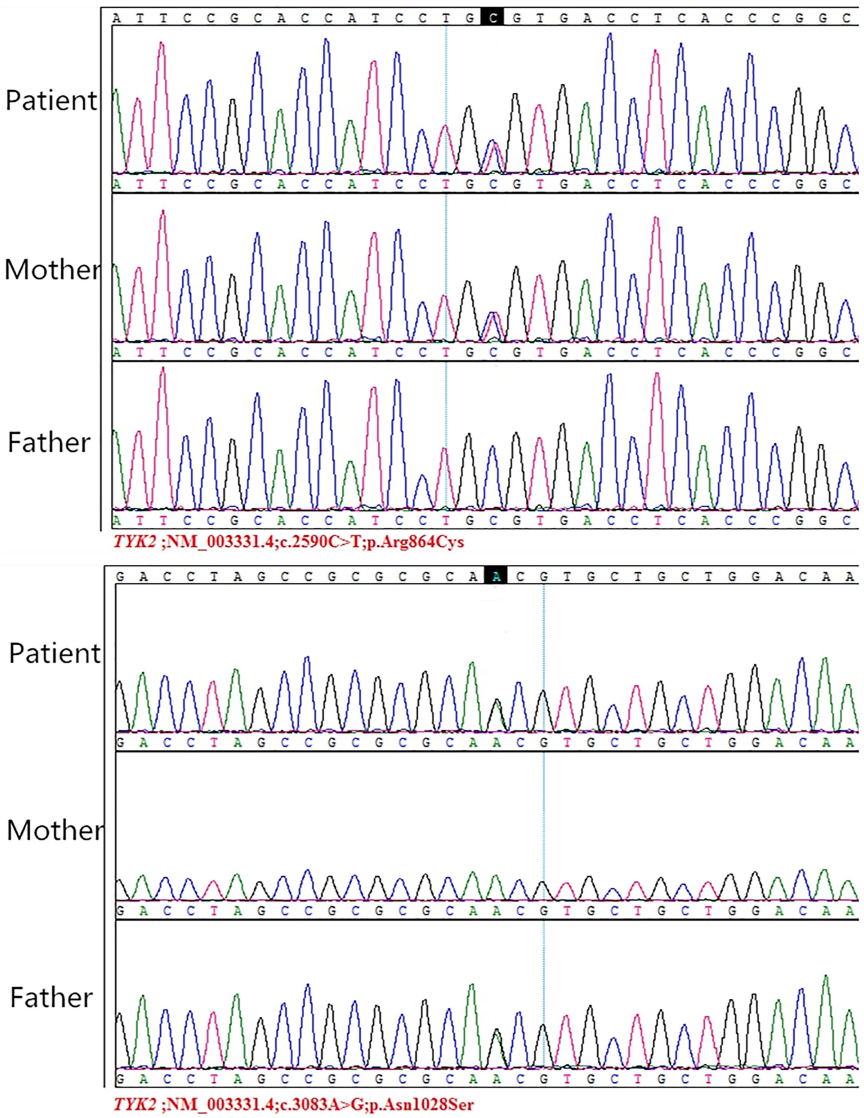


FIGURE 1 | Sanger sequencing of TYK2 mutations in the patient and his parents. Reprinted with permission from Guo, W., Feng, X., Yang, M., Shangguan, Y., Shi, P., Wang, S., et al. (2020). Mycobacterium Intracellulare Infection Associated with TYK2 Deficiency: A Case Report and Review of the Literature. Infect. Drug. Resist. 13, 4347-4353. Creative Commons Attribution - Non Commercial (unported, v3.0) License (https://creativecommons.org/licenses/by-nc/3.0/).

and bronchus. Previous studies have shown that *TYK2* mutations are associated with susceptibility to mycobacterial disease (Kreins et al., 2015; Rosain et al., 2019), which is consistent with this patient. In addition to conventional anti-tuberculosis treatment, injection of recombinant IFN-γ and transduction of the TYK2 gene are new treatment options (Minegishi et al., 2006; Bustamante et al., 2014).

The heterozygous missense (c.442G>C p.Glu148Gln) in the *MEFV* gene in S-11 has been reported to cause disease. Homozygous or compound heterozygous mutations can manifest as normal phenotypes and atypical partial clinical phenotypes of familial Mediterranean fever (FMF), while patients with typical FMF phenotypes are extremely rare (Cekin et al., 2017). These mutations may be conditional disease-causing polymorphic sites. Carrying multiple mutation sites may cause disease, but the condition may be atypical. S-11 presented with recurrent fever with joint pain, and periodic fever syndrome was considered, which is related to the *MEFV* mutation. Colchicine can effectively control FMF, and thalidomide, sulfasalazine, nonsteroidal anti-inflammatory drugs, and biological agents such as IL-1 antagonists and TNF-α inhibitors can also be used (Petrushkin et al., 2016).

S-12 carried a novel mutation (c.472T>G p.Cys158Gly) in the *THBD* gene. Thrombomodulin (THBD) is encoded by the *THBD* gene and expressed in vascular endothelial cells. It is a cofactor for thrombin-mediated activation of the anticoagulant protein C pathway and plays a key role in maintaining the balance of coagulation and anticoagulation (Esmon and Owen, 1981). Studies show that *THBD* mutations can lead to thrombomodulin protein defects, a tendency toward thrombosis, and an increased risk of venous thrombosis (Key and Reiner, 2016; Zhang et al., 2021), which supports the diagnosis of pulmonary embolism in this patient. The patient was admitted to the hospital due to fever, had a history of pulmonary embolism, and without risk factors such as trauma, tumor, long-term bed rest, and varicose veins of the lower extremities. Repeated attacks of pulmonary embolism were harmful to the patient, and the results of WES provide a basis for subsequent prevention. We think that individuals with *THBD* gene defects need to be more vigilant about the risk of thrombosis and take more active preventive and treatment measures.

S-15 carried a novel mutation (c.194_195insT p.Ala66Argfs*119) in the *GATA2* gene. The patient presented with fever of unknown origin, accompanied by cough and sputum. Blood cell count revealed monocytopenia (monocyte count: 10 cells/mm^3). A transbronchial biopsy of the lung showed Mycobacterium avium infection. Pulmonary alveolar proteinosis was also seen in this patient. The combination of these phenomena supported the diagnosis of monocytopenia and mycobacterial infection (MonoMAC) syndrome caused by *GATA2* gene mutations, with nontuberculous mycobacterial infection as the main clinical feature. *GATA2* encodes a hematopoietic transcription factor that controls myeloid differentiation and plays an important role in the proliferation and differentiation of hematopoietic stem cells and multipotential progenitor cells (Ding et al., 2017). *GATA2* mutations not only lead to the reduction of monocytes, NK, B and dendritic cells, but also cause patients to be susceptible to MDS and AML (Camargo et al., 2013). Currently, symptomatic treatment is generally adopted according to the clinical manifestations of patients. Allogeneic hematopoietic stem cell transplantation has been proven to be an effective strategy to restore the patient's hematopoietic and immune functions (Moraes-Fontes et al., 2019).

According to the current literature and related studies, no proven phenotype-related mutations were detected in the remaining eight samples, which may be caused by other mutation sites. As our data accumulate, further investigation of FUO patients in a larger patient cohort is likely to identify genetic mutations not included in the current study. Two of the eight patients were diagnosed as Scedosporium boydii infection and mycobacterium intracellular infection respectively, whose FUO phenotypes was thought to be related to pathogens/pathogen-derived factors.

The diagnosis of FUO remains challenging. Many FUOs caused by inflammatory diseases and rare diseases are particularly difficult to diagnose and require a high degree of clinical suspicion as well as reliable molecular evidence to confirm. Advances in molecular genetic technology have increased the importance of these tests in the diagnosis of FUO. Based on our experience, we recommend WES as an auxiliary test for many indications, including no immunodeficiency found in routine examination, such as HIV, complement, antibodies, and cytokines, but clinically highly suspected immune abnormalities and, suspicion of self-inflammatory disease and deficiencies in inflammation regulation. Importantly, some diagnostic results can affect the treatment options and improve the prognosis. New treatments that target genetic mutations may be more effective than traditional treatments. We believe that WES will play an increasingly important role in the diagnosis and treatment of FUO-related infectious and noninfectious diseases. Despite the rapid development of WES technology, it also has some limitations. First, structural mutations, large fragment insertion mutations, dynamic mutations and complex recombination mutations cannot be detected. Second, the database it uses is not perfect, so the analysis results may not be comprehensive. Additionally, the report takes approximately four weeks, resulting in the lack of timely guidance for disease diagnosis and treatment. Thus, clinical data needs to be further accumulated in future studies. We hope that more global multicenter clinical data and WES results can be uploaded simultaneously, so that more genetic-related diseases can be recognized.

Our research has some limitations. The sample size in our study was modest; thus, larger studies are needed to analyze the role of WES in patients with FUO in the future. We also have not further confirmed the pathogenicity of these mutations discovered by WES and cannot determine whether these mutations are etiologies of these patients. Subsequent separation and functional studies at the PBMC level other than the whole blood level are needed to support our findings.

CONCLUSION

Here, we demonstrate the application of WES in patients with FUO to identify potential disease-causing genetic mutations. Through this analysis, we found positive results in 7 patients,

including 8 gene variants, representing a positive rate of 46.7% in our population (n=7/15). In conclusion, WES successfully detected mutant genes and identified some potential diseases causing fever, which has a complementary role in the diagnosis and treatment of patients with FUO.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

WG, XF, MH, YaS, and KX was involved in the conception and design of the study. WG, MH, YaS, JX, WH, XL, ZZ, and YuS were involved in the collection and assembly of data. WG, XF, and MH were involved in interpreting the data. WG and XF wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Regional Characteristics Of Nontuberculous Mycobacteria In Lishui (grant number 2018zdhz10).

REFERENCES

Barreiro, L. B., Neyrolles, O., Babb, C. L., Tailleux, L., Quach, H., McElreavey, K., et al. (2006). Promoter Variation in the DC-SIGN-Encoding Gene CD209 Is Associated With Tuberculosis. *PLoS. Med.* 3 (2), e20. doi: 10.1371/journal.pmed.0030020

Bustamante, J., Boisson-Dupuis, S., Abel, L., and Casanova, J. L. (2014). Mendelian Susceptibility to Mycobacterial Disease: Genetic, Immunological, and Clinical Features of Inborn Errors of IFN-γ Immunity. *Semin. Immunol.* 26 (6), 454–470. doi: 10.1016/j.smim.2014.09.008

Camargo, J. F., Lobo, S. A., Hsu, A. P., Zerbe, C. S., Wormser, G. P., and Holland, S. M. (2013). MonoMAC Syndrome in a Patient With a GATA2 Mutation: Case Report and Review of the Literature. *Clin. Infect. Dis.* 57 (5), 697–699. doi: 10.1093/cid/cit368

Casals, T., De-Gracia, J., Gallego, M., Dorca, J., Rodríguez-Sanchón, B., Ramos, M. D., et al. (2004). Bronchiectasis in Adult Patients: An Expression of Heterozygosity for CFTR Gene Mutations? *Clin. Genet.* 65 (6), 490–495. doi: 10.1111/j.0009-9163.2004.00265.x

Cekin, N., Akyurek, M. E., Pinarbasi, E., and Ozen, F. (2017). MEFV Mutations and Their Relation to Major Clinical Symptoms of Familial Mediterranean Fever. *Gene* 626, 9–13. doi: 10.1016/j.gene.2017.05.013

da Silva, R. C., Segat, L., da Cruz, H. L., Schindler, H. C., Montenegro, L. M., Crovella, S., et al. (2014). Association of CD209 and CD209L Polymorphisms With Tuberculosis Infection in a Northeastern Brazilian Population. *Mol. Biol. Rep.* 41 (8), 5449–5457. doi: 10.1007/s11033-014-3416-y

Ding, L. W., Ikezoe, T., Tan, K. T., Mori, M., Mayakonda, A., Chien, W., et al. (2017). Mutational Profiling of a MonoMAC Syndrome Family With GATA2 Deficiency. *Leukemia* 31 (1), 244–245. doi: 10.1038/leu.2016.256

Erman, B., and Çipe, F. (2020). Genetic Screening of the Patients With Primary Immunodeficiency by Whole-Exome Sequencing. *Pediatr. Allergy Immunol. Pulmonol.* 33 (1), 19–24. doi: 10.1089/ped.2019.1097

Esmon, C. T., and Owen, W. G. (1981). Identification of an Endothelial Cell Cofactor for Thrombin-Catalyzed Activation of Protein C. *Proc. Natl. Acad. Sci. U. S. A.* 78 (4), 2249–2252. doi: 10.1073/pnas.78.4.2249

Gu, W., Miller, S., and Chiu, C. Y. (2019). Clinical Metagenomic Next-Generation Sequencing for Pathogen Detection. *Annu. Rev. Pathol.* 14, 319–338. doi: 10.1146/annurev-pathmechdis-012418-012751

Han, J. Y., Lee, H. J., Lee, Y. M., and Park, J. (2020). Identification of Missense ADGRV1 Mutation as a Candidate Genetic Cause of Familial Febrile Seizure 4. *Children (Basel).* 7 (9), 144. doi: 10.3390/children7090144

Keller, M. D., Pandey, R., Li, D., Glessner, J., Tian, L., Henrickson, S. E., et al. (2016). Mutation in IRF2BP2 Is Responsible for a Familial Form of Common Variable Immunodeficiency Disorder. *J. Allergy Clin. Immunol.* 138 (2), 544–550.e544. doi: 10.1016/j.jaci.2016.01.018

Key, N. S., and Reiner, A. P. (2016). Genetic Basis of Ethnic Disparities in VTE Risk. *Blood* 127 (15), 1844–1845. doi: 10.1182/blood-2016-03-701698

Kreins, A. Y., Ciancanelli, M. J., Okada, S., Kong, X. F., Ramírez-Alejo, N., Kilic, S. S., et al. (2015). Human TYK2 Deficiency: Mycobacterial and Viral Infections Without Hyper-IgE Syndrome. *J. Exp. Med.* 212 (10), 1641–1662. doi: 10.1084/jem.20140280

Lachmann, H. J. (2015). Autoinflammatory Syndromes as Causes of Fever of Unknown Origin. *Clin. Med. (Lond).* 15 (3), 295–298. doi: 10.7861/clinmedicine.15-3-295

Levy, S. E., and Boone, B. E. (2019). Next-Generation Sequencing Strategies. *Cold. Spring. Harb. Perspect. Med.* 9 (7), a025791. doi: 10.1101/cshperspect.a025791

Liu, J. Z., van Sommeren, S., Huang, H., Ng, S. C., Alberts, R., Takahashi, A., et al. (2015). Association Analyses Identify 38 Susceptibility Loci for Inflammatory Bowel Disease and Highlight Shared Genetic Risk Across Populations. *Nat. Genet.* 47 (9), 979–986. doi: 10.1038/ng.3359

Minegishi, Y., Saito, M., Morio, T., Watanabe, K., Agematsu, K., Tsuchiya, S., et al. (2006). Human Tyrosine Kinase 2 Deficiency Reveals Its Requisite Roles in Multiple Cytokine Signals Involved in Innate and Acquired Immunity. *Immunity* 25 (5), 745–755. doi: 10.1016/j.immuni.2006.09.009

Moraes-Fontes, M. F., Caramalho, Í., Hsu, A. P., Holland, S. M., and Abecasis, M. (2019). MonoMAC Syndrome Caused by a Novel GATA2 Mutation Successfully Treated by Allogeneic Hematopoietic Stem Cell Transplantation. *J. Clin. Immunol.* 39 (1), 4–6. doi: 10.1007/s10875-018-0576-x

Özen, S., Batu, E. D., and Demir, S. (2017). Familial Mediterranean Fever: Recent Developments in Pathogenesis and New Recommendations for Management. *Front. Immunol.* 8, 253. doi: 10.3389/fimmu.2017.00253

Patel, S. D., Bono, T. R., Rowe, S. M., and Solomon, G. M. (2020). CFTR Targeted Therapies: Recent Advances in Cystic Fibrosis and Possibilities in Other Diseases of the Airways. *Eur. Respir. Rev.* 29 (156), 190068. doi: 10.1183/16000617.0068-2019

Perrem, L., and Ratjen, F. (2019). Anti-Inflammatories and Mucociliary Clearance Therapies in the Age of CFTR Modulators. *Pediatr. Pulmonol.* 54 (Suppl 3), S46–s55. doi: 10.1002/ppul.24364

Petersdorf, R. G., and Beeson, P. B. (1961). Fever of Unexplained Origin: Report on 100 Cases. *Med. (Baltimore)* 40, 1–30. doi: 10.1097/00005792-196102000-00001

Petrushkin, H., Stanford, M., Fortune, F., and Jawad, A. S. (2016). Clinical Review: Familial Mediterranean Fever-An Overview of Pathogenesis, Symptoms, Ocular Manifestations, and Treatment. *Ocul. Immunol. Inflamm.* 24 (4), 422–430. doi: 10.3109/09273948.2015.1010012

Retterer, K., Juusola, J., Cho, M. T., Vitazka, P., Millan, F., Gibellini, F., et al. (2016). Clinical Application of Whole-Exome Sequencing Across Clinical Indications. *Genet. Med.* 18 (7), 696–704. doi: 10.1038/gim.2015.148

Rosain, J., Kong, X. F., Martinez-Barricarte, R., Oleaga-Quintas, C., Ramirez-Alejo, N., Markle, J., et al. (2019). Mendelian Susceptibility to Mycobacterial Disease: 2014-2018 Update. *Immunol. Cell. Biol.* 97 (4), 360–367. doi: 10.1111/imcb.12210

Tzetis, M., Efthymiadou, A., Strofalis, S., Psychou, P., Dimakou, A., Pouliou, E., et al. (2001). CFTR Gene Mutations–Including Three Novel Nucleotide Substitutions–and Haplotype Background in Patients With Asthma, Disseminated Bronchiectasis and Chronic Obstructive Pulmonary Disease. *Hum. Genet.* 108 (3), 216–221. doi: 10.1007/s004390100467

Wright, W. F., Simner, P. J., Carroll, K. C., and Auwaerter, P. G. (2021). Progress Report: Next-Generation Sequencing (NGS), Multiplex Polymerase Chain Reaction (PCR), and Broad-Range Molecular Assays as Diagnostic Tools for Fever of Unknown Origin (FUO) Investigations in Adults. *Clin. Infect. Dis*, ciab155. doi: 10.1093/cid/ciab155

Zhang, P., Gong, K., Zhang, L., Xiao, L., Guo, S., Li, Y., et al. (2021). The Associations Between THBD C.1418C>T Polymorphism and Lower Extremity Deep Vein Thrombosis or Endothelial Progenitor Cell. *Int. Angiol.* doi: 10.23736/s0392-9590.21.04596-x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Guo, Feng, Hu, Shangguan, Xia, Hu, Li, Zhang, Shi and Xu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 14 January 2022
doi: 10.3389/fmed.2021.753988

Case Report: Metagenomic Next-Generation Sequencing for Diagnosis of Human Encephalitis and Endophthalmitis Caused by Pseudorabies Virus

Weiqian Yan, Zhiping Hu, Yingchi Zhang, Xiaomei Wu and Hainan Zhang**

Department of Neurology, Second Xiangya Hospital, Central South University, Changsha, China

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UCONN Health, United States

Reviewed by:
Prasanth Manohar,
Zhejiang University-University of Edinburgh Institute, China
Yan-Dong Tang,
Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS), China
Karol Perlejewski,
Medical University of Warsaw, Poland

****Correspondence:***
Xiaomei Wu
wuxiaomei0923@csu.edu.cn
Hainan Zhang
hainanzhang@csu.edu.cn

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *05 August 2021*
Accepted: *13 December 2021*
Published: *14 January 2022*

Citation:
Yan W, Hu Z, Zhang Y, Wu X and Zhang H (2022) Case Report: Metagenomic Next-Generation Sequencing for Diagnosis of Human Encephalitis and Endophthalmitis Caused by Pseudorabies Virus. Front. Med. 8:753988. doi: 10.3389/fmed.2021.753988

Purpose: The objective of our study was to report a case of encephalitis and endophthalmitis caused by pseudorabies virus (PRV), identified using metagenomic next-generation sequencing (mNGS).

Case Presentation: A 54-year-old worker, from a swine slaughterhouse, developed signs of severe encephalitis, including fever, disturbance of consciousness, hypopnea, and status epilepticus, after finger injury at work. The PRV sequences were successfully identified from the blood, cerebrospinal fluid (CSF), and aqueous humor of the patient through mNGS, which was further verified using a Sanger sequencing.

Conclusion: Our case emphasizes the importance of mNGS in early diagnoses of infectious diseases, and gives a clue that PRV can spread across species and infect human. It is necessary to carry out a skin protection and education about disease prevention for people who have close contact with swine.

Keywords: pseudorabies virus, Suid herpesvirus 1, endophthalmitis, next-generation sequencing, encephalitis

INTRODUCTION

Pseudorabies virus (PRV), also called Aujeszky disease virus or Suid herpesvirus 1, belongs to Alphaherpesvirinae subfamily of the family Herpesviridae and is widely distributed around the world. Swine are the main natural hosts and sources of PRV infections (1). The PRV is latent in the peripheral nervous systems (PNS) of infected swine, which shed large quantities of virus in bodily secretions and excretions. The PRV is mainly spread *via* direct contact, and may also be transmitted by water, air, and contaminated fomites (2). There are some reports on human PRV infection based on clinical manifestations and epidemiological history. However, few researches had conclusive evidences for human PRV infection due to the rigorous conditions of viral PCR and viral culture and low accuracy of antigen-antibody test (3–5). In recent years, with the application of metagenomic next-generation sequencing (mNGS) in the diagnosis of infectious diseases, human encephalitis and/or endophthalmitis caused by PRV has been reported, confirming the possibility of cross-species infection of PRV (6–11). The PRV related infection has received increasing attention since the first case of human endophthalmitis caused by PRV was reported in 2018 (6). Given the close relationship between human and swine, we report this case to emphasize the importance of mNGS in early diagnosis and highlight the threat to public health.

CASE PRESENTATION

A 54-year-old male, who worked as a butcher of the slaughterhouse in Hunan Province, China, was admitted to the Neurology Intensive Care Unit (NICU) of Second Xiangya Hospital, Central South University, due to recurrent fever for a duration of 8 days and convulsions with impaired consciousness for 3 days. He obtained some minor cuts on his left hand during the autopsy process of a dead swine 14 days before his initial symptom occurred. The initial symptoms (day 1) presented with cough and fever (the highest temperature of 39°C). No other neurological symptoms were presented at that time. He was then admitted to the local hospital and was diagnosed with upper respiratory tract infection, receiving an antibiotic treatment. However, his condition was not improving. On day 5, after initial symptoms, he developed fuzzy consciousness, headache, fecal, and urinary incontinence. Plain brain computer tomography (CT) was normal. Analysis of cerebrospinal fluid (CSF) showed white blood cells (WBC) of 4×10^6/L (0–5×10^6/L) and protein content of 0.63 g/L (0.15–0.45 g/l) (**Table 1**). The results of CSF further indicated that it was an early phase of viral meningitis. He was given an antiviral treatment (Acyclovir and Ribavirin) and antibiotic therapy (Piperacillin/tazobactam). On day 6, he developed facial and limb convulsions and was treated with anti-epileptic therapy (sodium valproate and midazolam). He was given an emergency orotracheal intubation and ventilator-assisted ventilation, due to shortness of breath. On day 8, after the initial symptoms, he was referred to our hospital due to status epilepticus and disturbance of consciousness. On admission, he presented with a body temperature of 39°C, heart rate of 107 bpm (beats per minute), blood pressure of 155/94 mmHg, and oxygen saturation (SpO_2) of 99% at an inspired oxygen concentration of 30% with mechanical ventilation, and no spontaneous breaths were detected. Glasgow Coma Scale (GCS) was E1VTM1. Pupils were both 3 mm in diameter. The left pupil was sluggishly reactive to light and the reflex of the right one disappeared. Although he had neck stiffness and Kernig signs, long tract signs, such as hyperreflexia, Hoffman's signs, as well as Babinski signs were not detected. Some minor cuts were found on his left hand (**Supplementary Figure 1**). Laboratory findings were WBC 12.01×10^9/L ($3.5–9.5 \times 10^9$/L), hemoglobin (Hb) 127 g/dl (115–150 g/dl), sodium 148.3 mmol/L (135–145 mmol/l), potassium 3.4 mmol/L (3.5–5.5 mmol/l), creatinine 89 μmol/L (62–115 μmol/L), albumin 39.7 g/L (40–55 g/l), alanine transaminase (ALT) 90.6 U/L (9–50 U/l), and aspartate transaminase (AST) 49.3 U/L (15–40 U/l). Serological tests for HIV, TP, hepatitis B/C, and TB (T-SPOT) were negative. The cytomegalovirus antibody IgG (CMV-IgG) of 19.300 IU/mL, herpes simplex virus type I antibody (HSV) IgG of 26.76 COI (positive), rubella virus antibody IgG (RU-IgG) of 20.800 IU/mL, and *Toxoplasma gondii* antibody IgG (TOX-IgG) of <0.130 IU/ml were found. The IgM against all pathogens was negative. Lumbar puncture indicated an opening pressure of 290 mmH_2O (80–180 mmH_2O), CSF showed WBC of 10×10^6/L (0–5×10^6 /L), protein of 0.37 g/L (0.15–0.45 g/L) and glucose of 3.05 mmol/L (synchronous blood glucose of 9.7 mmol/L) (**Table 1**). Besides, CSF latex agglutination test (LAT), microbiological tests including the Gram/acid-fast bacillus (AFB) were negative, as well as the autoimmune encephalitis antibodies including NMDAR, AMPA1, AMPA2, LGI1, CASPR2, GABABR, IgLON5, DPPX, GAD65, GlyR1, DRD2, mGluR5, mGluR1, and Neurexin-3a, which were tested based on a cell-based assay (Kingmed Diagnostics Co., Ltd.). On day 8, Plain CT brain imaging was still normal after initial symptoms (admission day) (**Figure 1A**). With the suspicion of viral meningitis on admission, he was treated with Acyclovir (500 mg, Q8h), 20% Mannitol (125 ml, Q8h), Glycerol fructose (250 ml, Q12h), Piperacillin/tazobactam (4.5 g, Q8h), fluid therapy and nutritional support. The epileptic seizures of the patient were difficult to bring under control, propofol was used intravenously to stop status epilepticus. Meanwhile, oral antiepileptic therapy including Levetiracetam and Oxcarbazepine were applied. On day 11, after initial symptoms, pathogen reads PRV was detected in CSF, blood, and aqueous humor, using PACEseq mNGS, so we accordingly added Phosphonoformate (3 g, Q8h) combined with Acyclovir (500 mg, Q8h) to enhance the antiviral treatment. However, the condition of the patient did not see any significant improvement. On day 15, after initial symptoms, Plain CT brain imaging showed patchy hypo-density in the bilateral frontotemporal lobe (**Figure 1B**). Methylprednisolone Sodium Succinate (120 mg, Qd) was intravenously given for 7 days, followed by oral prednisone (60 mg, Qd) treatment. There was no improvement of the consciousness of the patient. Even when all the sedatives used to control epileptic were stopped, he was still in deep coma. On day 19, CT brain imaging showed multiple patchy hypo-density in right basal ganglia, left thalamus, bilateral frontotemporal lobe, and bilateral insular lobe (**Figure 1C**). On day 24, the lesion site of CT brain is basically the same as before (**Figure 1D**), which indicated that the present treatment might not be effective. During hospitalization, the patient was unable to wean off mechanical ventilation, although the pulmonary infection had been basically healed. Given that the prognosis of the patient was extremely poor, the patient was voluntarily discharged to local hospital for palliative treatment.

mNGS

A PACEseq mNGS detection (Hugobiotech, Beijing, China) was performed. The QIAamp DNA Micro Kit (QIAGEN) was used to extract DNA. The library was then built using QIAseq™ Ultralow Input Library Kit (Illumina, San Diedo, CA, USA). The library quality was assessed using Qubit (Thermo Fisher) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and was finally sequenced on Nextseq 550 platform (Illumina, San Diedo, CA, USA). Short, low-quality, and low-complexity reads were removed from the raw data. The human DNA was also filtered out by alignment to human reference database (hg38) using bowtie 2. The remaining reads were finally aligned to Microbial Genome Databases (ftp://ftp.ncbi.nlm.nih.gov/genomes/) by BWA. A total of 652 unique reads mapped to the Suid alphaherpesvirus 1 genome out of 15,970 microbial reads (**Figure 2A**) with a genome coverage rate of 25.3% (**Figure 2B**) in CSF sample, whereas only one unique

TABLE 1 | Dynamic CSF analysis over the course of hospitalization.

CSF-Variable	Intracranial pressure (mmH_2O)	WBC ($\times 10^6$/L)		Protein (g/L)	Glucose (mmol/L)	Chloride (mmol/L)	CSF-Culture
		Differential count (%)					
		Monocyte	Coenocyte				
5th day	/	4	0	0.63	normal	106.8	/
8th day	290	10	4	0.37	3.05	132.4	(–)
11th day	350	4	6	0.78	3.14	127	(–)
16th day	400	42	6	0.78	3.62	125.5	(–)
19th day	400	55	15	0.69	4.09	131.4	/
23th day	310	45	15	0.80	4.57	144	/

FIGURE 1 | The plain CT brain imaging on the 8th day **(A)**, 15th day **(B)**, 19th day **(C)**, and 24th day **(D)** after initial symptoms.

read of Suid alphaherpesvirus 1 in blood sample was detected, which might be due to the low concentration of viral DNA in blood. Notably, mNGS of aqueous humor sample detected 1,566 unique reads (0.42%, 1,566/368,878) (**Figure 2C**) aligned to the Suid alphaherpesvirus 1 genome with a genome coverage rate of 35% (**Figure 2D**). Furthermore, PCR targeting *glycoprotein B* (*gB*) gene of Suid alphaherpesvirus 1 showed that fragments of about 194 bp in length were successfully amplified from CSF and aqueous humor samples (**Figure 2E**). The identity of the fragment sequence obtained from a Sanger sequencing, against the reference sequence of Suid alphaherpesvirus 1 (GenBank accession no. MK806387.1) was 99% (**Figure 2F**). However, a negative PCR result was found in blood sample due to the low viral load. The above results confirmed the presence of Suid alphaherpesvirus 1 in CSF and aqueous humor samples.

DISCUSSION

Pseudorabies virus (PRV) is an alpha herpesvirus that causes neurological diseases in many wild and domestic animals. The PRV infection is highly prevalent among swine. This virus, which takes swine as its natural host, has been found in humans in 1914, but those cases were debatable because they lacked evidence of antibodies or PRV sequences. Three suspected cases of human PRV infection with positive antibodies were reported in 1987, strongly indicating that PRV could infect human (3). In 2018, Ai et al. reported a case of human endophthalmitis caused by PRV infection, and this is the first case of human PRV endophthalmitis revealed by mNGS and PCR, while PRV was only detected in the aqueous humor in this case (6). Among the reported cases, the main manifestation was severe encephalitis or endophthalmitis, respectively (7–11). While, in our case, PRV, as detected by mNGS in blood, CSF, and aqueous humor samples, exhibited a wider range of infections.

Clinical manifestations and neuroimaging changes of our patient was basically consistent with those of patients with PRV encephalitis as previously reported (7–11). The clinical characteristics of suspected pseudorabies encephalitis (PRE) are as follows: (I) the patients mainly engaged in the production, processing or marketing of live swine and raw pork; (II) acute onset with fever, headache, and other "cold-like symptoms"; (III) severe encephalitis with rapid progress characterized by seizures, disturbance of consciousness, ICU treatment often needed, and even ventilator-assisted breathing; (IV) other systems, such as endophthalmitis; (V) The PRE neuroimaging show necrotizing encephalitis mainly involved gray matter, including limbic system, basal ganglia, and brainstem; (VI) The CSF exhibits characteristics of aseptic meningitis or viral meningitis, including elevated intracranial pressure (ICP), a mild to moderate increase of WBC, lymphocytic inflammation in CSF cytology, elevated CSF protein and so on; (VII) Antiviral drugs, human immunoglobulins and steroid hormones can be tried, but effective antiviral treatments have yet to be determined. In addition to the typical severe cases, there

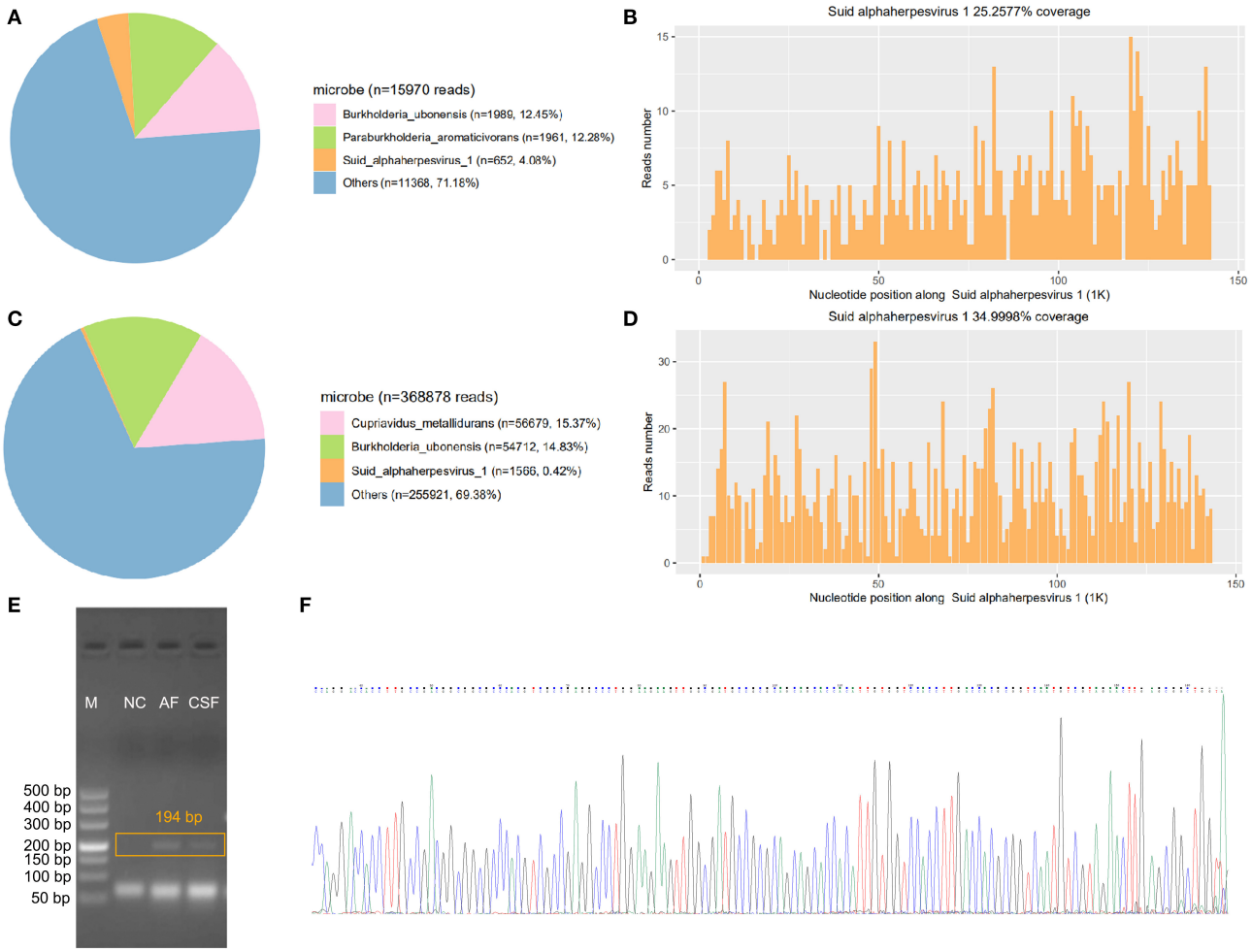


FIGURE 2 | Findings of mNGS and PCR confirmation; **(A,B)** mNGS results of the CSF. **(C,D)** mNGS results of the aqueous humor; **(E)** Gel electrophoresis of PCR products; **(F)** Sanger sequencing result; Genome coverage rate is total length of detected sequences dividing by the length of published genome sequence. The "AH," "CSF," "NC," and "M" represent aqueous humor, cerebrospinal fluid, negative control, and marker, respectively.

are also cases with relatively mild clinical manifestations or incomplete involvement.

In our case, the early diagnosis was delayed, mainly due to the early "cold-like symptoms," which did not arouse the attention of the patient and, thus, early effective treatment was not delivered. In the later stage, the clinical manifestations of the patient progressed to the typical manifestations of severe encephalitis. As the patient rapidly progressed to a coma, the endophthalmitis of the patient was not diagnosed. The PRV in the aqueous humor was found positive in the follow-up body fluid testing. Plain CT is not sensitive enough in the early stage of disease, and as the disease progressed, it showed that the characteristic neuroimaging changes, our patient showed hypodensity lesions in the bilateral frontotemporal lobe, basal ganglia, thalamus, and insular lobe. Repeated CSF tests are suggestive of viral meningitis, mainly with elevated ICP and mild increase of WBC. We used mNGS to place a strict alignment of the PRV in the blood, CSF and aqueous humor of the patient, while the nucleic acid for Herpes simplex virus (HSV) and other DNA viruses were negative. Other clinical virology tests and pathogen tests of the patient were negative, as well as the autoimmune encephalitis antibodies, which helped to exclude other causes of encephalitis.

Antiviral drugs should be used for initial treatment of patients with highly suspected viral encephalitis. Postponing antiviral therapy (more than 48 h) can lead to a poor prognosis, including ocular complications-acute retinal necrosis syndrome (12). Acyclovir, as the main drug for the Herpes simplex encephalitis (HSE), may not have an ideal effect on a severe PRV encephalitis. Accordingly, we added phosphonoformate as the intensive antiviral therapy for this patient. Meanwhile, human immunoglobulins and glucocorticoids can also be tried, and we used methylprednisolone sodium succinate for a short period of time, while the condition of the patient had not been effectively improved. Although we successfully detected PRV by using mNGS and a corresponding antiviral therapy was also

performed, the prognosis of this patient was worse without timely and accurate diagnosis in the early stage of PRV infection, emphasizing the importance of mNGS in early diagnoses.

The mNGS results of the patient demonstrated the presence of PRV in blood, CSF, and aqueous humor, which was a newly identified virus that infects human. In addition, mNGS data also showed high reads number and coverage rate of PRV, which was further confirmed by PCR and Sanger sequencing, validating the credibility of the mNGS technology. Specific viral diagnosis could be achieved by demonstration of viral nucleic acid, or antibody in CSF or isolation of the virus from CSF or brain tissue (12). The sensitivity of traditional CSF diagnostic methods for CNS viral infections is very low, because the short period of viremia and difficulty in obtaining brain tissue through biopsy make it difficult to isolate viruses. However, the state-of-the-art mNGS technology could detect all the pathogens in the clinical sample without a priori and with high sensitivity. It has been more and more accepted as a rapid and precise method for clinical diagnostics of infectious diseases, including sepsis, meningitis, and pneumonia. The mNGS performs a great advantage in clinical diagnosis, especially for rare, new, and mixed infections (13). There are also some limitations in using mNGS. The key limitation of mNGS is that microbial nucleic acids from the patients' samples are dominated by human host background, which limits the overall analytical sensitivity of the approach for pathogen detection, given the relative scarcity of microbial non-human reads that are sequenced. However, mNGS is a revolutionary technology that has disrupted traditional clinical diagnostics on several fronts.

Since 2017, reported cases of PRV encephalitis or endophthalmitis were all from China. In 2020, hSD-1/2019, a human-originated PRV strain, was isolated from an acute human encephalitis case, and showed close phylogenetic relationships and similar etiological characteristics to Chinese PRV variant strains, implying that there may be certain risks of PRV transmission from swine to human (14). It was hypothesized that the PRV detected in our case may be a variant strain rather than PRV classical strain, which can be further confirmed by a whole genome sequencing. Thus, we went back to the slaughterhouse to collect the residual blood from the slaughter knife for further analysis, but PRV virus was not detected. It may be due to the fact that the knife had been exposed to the air for a long time. The swine slaughtered by the patient had been sold on the market, so we can no longer trace the source of the infection. Additionally, novel Chinese PRV variant strain may lead to failure of the standard Bartha-K61 vaccine to protect swine against the PRV (15). Given the current global epidemic of PRV in swine, this "new life-threatening zoonosis" deserves more attention, and the mechanism of human PRV infection requires further investigation.

CONCLUSION

We found that PRV infection can result in prominent central nervous system (CNS) disorders and fatal encephalitis in human. For cases of encephalitis with unknown etiology, if the epidemiology, clinical manifestations, CSF, and brain imaging conform to the PRE, PRV nucleic acid testing and serological testing should be perfected as soon as possible. The mNGS helps to rapidly screen for new viral encephalitis such as PRV from encephalitis of an unknown etiology. The PRE are rare encephalitis which are detectable, treatable, and preventable. People who worked in the animal husbandry and veterinary should increase awareness of self-protection when contacting swine, as broken skin or mucous membrane might make it easy for PRV to transmit from infected animals to human. Establishing accessible diagnostic protocol, determining effective antiviral treatment, exploring the infection pathway and pathogenic mechanism of PRV to human are all the remaining problems to be solved.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available due to ethical and privacy restrictions. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Professional Committee of the Second Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

WY wrote the manuscript. WY, ZH, and YZ helped to collect the clinical data and neuroimages. XW and HZ analyzed the data and made revisions of the manuscript. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was supported by grant of National Natural Science Foundation of China (No. 81801123) to WY.

ACKNOWLEDGMENTS

We would like to thank Han Xia of Department of Scientific Affairs, Hugobiotech Co., Ltd., for the help of technical assistance in mNGS and PCR assay.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.753988/full#supplementary-material

REFERENCES

1. Pomeranz LE, Reynolds AE, Hengartner JC. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. *Microbiol Mol Biol Rev*. (2005) 69:462–500. doi: 10.1128/MMBR.69.3.462-500.2005
2. Tan L, Yao J, Yang Y, Luo W, Yuan X, Yang L, et al. Current status and challenge of pseudorabies virus infection in China. *Virol Sin*. (2021) 36:588–607. doi: 10.22541/au.159179952.28426543
3. Mravak S, Bienzle U, Feldmeier H, Hampl H, Habermehl OK. Pseudorabies in man. *Lancet*. (1987) 1:501–2. doi: 10.1016/S0140-6736(87)92105-2
4. Anusz Z, Szweda W, Popko J, Trybała E. [Is Aujeszky's disease a zoonosis?]. *Przegl Epidemiol*. (1992) 46:181–6.
5. Sawitzky D. Transmission, species specificity, and pathogenicity of Aujeszky's disease virus. *Arch Virol Suppl*. (1997) 13:201–6. doi: 10.1007/978-3-7091-6534-8_19
6. Ai J, Weng S, Cheng Q, Cui P, Li Y, Wu H, et al. Human endophthalmitis caused by pseudorabies virus infection, China, 2017. *Emerg Infect Dis*. (2018) 24:1087–90. doi: 10.3201/eid2406.171612
7. Yang H, Han H, Wang H, Cui Y, Liu H, Ding S. A case of human viral encephalitis caused by pseudorabies virus infection in China. *Front Neurol*. (2019) 10:534. doi: 10.3389/fneur.2019.00534
8. Wang Y, Nian H, Li Z, Wang W, Wang X, Cui Y. Human encephalitis complicated with bilateral acute retinal necrosis associated with pseudorabies virus infection: a case report. *Int J Infect Dis*. (2019) 89:51–4. doi: 10.1016/j.ijid.2019.09.019
9. Yang X, Guan H, Li C, Li Y, Wang S, Zhao X, et al. Characteristics of human encephalitis caused by pseudorabies virus: a case series study. *Int J Infect Dis*. (2019) 87:92–9. doi: 10.1016/j.ijid.2019.08.007
10. Wang D, Tao X, Fei M, Chen J, Guo W, Li P, et al. Human encephalitis caused by pseudorabies virus infection: a case report. *J Neurovirol*. (2020) 26:442–8. doi: 10.1007/s13365-019-00822-2
11. Hu F, Wang J, Peng X. Bilateral necrotizing retinitis following encephalitis caused by the pseudorabies virus confirmed by next-generation sequencing. *Ocul Immunol Inflamm*. (2020) 29:922–5. doi: 10.1080/09273948.2019.1704027
12. Halperin JJ. Diagnosis and management of acute encephalitis. *Handb Clin Neurol*. (2017) 140:337–47. doi: 10.1016/B978-0-444-63600-3.00018-0
13. Guan H, Shen A, Lv X, Yang X, Ren H, Zhao Y, et al. Detection of virus in CSF from the cases with meningoencephalitis by next-generation sequencing. *J Neurovirol*. (2016) 22:240–5. doi: 10.1007/s13365-015-0390-7
14. Liu Q, Wang X, Xie C, Ding S, Yang H, Guo S, et al. A novel human acute encephalitis caused by pseudorabies virus variant strain. *Clin Infect Dis*. (2020) 73:e3690–700. doi: 10.1093/cid/ciaa987
15. Wong G, Lu J, Zhang W, Gao FG. Pseudorabies virus: a neglected zoonotic pathogen in humans? *Emerg Microbes Infect*. (2019) 8:150–4. doi: 10.1080/22221751.2018.1563459

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Yan, Hu, Zhang, Wu and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Public Health

ORIGINAL RESEARCH
published: 21 January 2022
doi: 10.3389/fpubh.2021.738412

Metagenomic Next-Generation Sequencing vs. Traditional Microbiological Tests for Diagnosing Varicella-Zoster Virus Central Nervous System Infection

Yunqi Zhu[1†], Miaomiao Xu[1†], Chengyuan Ding[2†], Zhihang Peng[3], Weixiao Wang[4], Binghu Sun[1], Jian Cheng[1], Chen Chen[1], Wei Chen[4], Hongxia Wei[1] and Zhiliang Hu[1,2*]*

[1] Department of Infectious Disease, The Second Hospital of Nanjing, Nanjing University of Chinese Medicine, Nanjing, China, [2] Center for Global Health, School of Public Health, Nanjing Medical University, Nanjing, China, [3] School of Public Health, Nanjing Medical University, Nanjing, China, [4] Department of Clinical Research Center, The Second Hospital of Nanjing, Nanjing University of Chinese Medicine, Nanjing, China

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Ping Ma,
Tianjin Second People's Hospital, China
Citra Fragrantia Theodorea,
University of Indonesia, Indonesia

***Correspondence:**
Zhiliang Hu
huzhiliang@njucm.edu.cn
Hongxia Wei
wghongxia@sina.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 08 July 2021
Accepted: 03 December 2021
Published: 21 January 2022

Citation:
Zhu Y, Xu M, Ding C, Peng Z, Wang W, Sun B, Cheng J, Chen C, Chen W, Wei H and Hu Z (2022) Metagenomic Next-Generation Sequencing vs. Traditional Microbiological Tests for Diagnosing Varicella-Zoster Virus Central Nervous System Infection. Front. Public Health 9:738412. doi: 10.3389/fpubh.2021.738412

Background: Unbiased metagenomic next-generation sequencing (mNGS) detects pathogens in a target-independent manner. It is not well-understood whether mNGS has comparable sensitivity to target-dependent nucleic acid test for pathogen identification.

Methods: This study included 31 patients with chickenpox and neurological symptoms for screening of possible varicella-zoster virus (VZV) central nervous system (CNS) infection. Microbiological diagnosing of VZV cerebrospinal fluid (CSF) infection was performed on stored CSF samples using mNGS, quantitative and qualitative VZV-specific PCR assays, and VZV IgM antibodies test.

Results: The median age was 30.0 [interquartile range (IQR), 24.3–33.3] years. 51.6% of the patients were men. About 80.6% of the patients had normal CSF white blood cell counts ($\leq 5 \times 10^6$/L). VZV IgM antibodies presented in 16.1% of the CSF samples, and nucleic acids were detectable in 16.1 and 9.7% using two different VZV-specific real-time PCR protocols. Intriguingly, maximal identification of VZV elements was achieved by CSF mNGS ($p = 0.001$ and $p = 007$; compared with qualitative PCR and VZV IgM antibody test, respectively), with sequence reads of VZV being reported in 51.6% (16/31) of the CSF samples. All VZV PCR positive samples were positive when analyzed by mNGS. Of note, human betaherpesvirus 6A with clinical significance was unexpectedly detected in one CSF sample.

Conclusions: Our study suggests that CSF mNGS may have higher sensitivity for VZV detection than CSF VZV PCR and antibody tests, and has the advantage of identifying unexpected pathogens.

Keywords: metagenomic next-generation sequencing, varicella-zoster virus, central nervous system infection, polymerase chain reaction, nucleic acid test

INTRODUCTION

Central nervous system (CNS) infections have led to a substantial morbidity and mortality in clinical practice; however, the etiologic agents remain undetermined in around half of the cases (1, 2). The diagnostic challenge is that more than 100 infectious agents can cause encephalitis, and that the initial neurological manifestations of many CNS infections are non-specific (3–5). It is difficult to measure all the CNS pathogens by traditional microbiological tests, such as morphological, serological, molecular, and culture methods (5). An acceptable, but not optimal, strategy is to target the most possibly causative pathogens based on a clinical judgment. However, in this context, the efficacy of pathogen discovery would largely depend on the clinical experience of the treating physician. Moreover, the etiologies of cases with atypical manifestations or caused by rare pathogens are likely to be unconfirmed.

Unlike the traditional pathogen-detection methods, which require a prior knowledge of the causative agents, the unbiased metagenomic next-generation sequencing (mNGS) identifies pathogens in a target-independent way (6). It has been reported that cerebrospinal fluid (CSF) mNGS helps to detect known as well as unexpected CNS pathogens (7–11). However, it is less clear whether mNGS has comparable sensitivity as compared with traditional pathogen-detection methods for diagnosing a specific CNS infection. Clinicians may still want to know whether pathogen-specific nucleic acid test is needed when an mNGS reveals a negative result for the pathogen of interesting. For the diagnosis of CNS *Mycobacterium tuberculosis* (MTB) infection, studies have shown that mNGS has higher sensitivity as compared with MTB-specific PCR (12, 13). Whether similar finding could be found in viral encephalitis needs to be further studied.

The sensitivity of CSF mNGS for pathogen detection may be greatly influenced by the CSF pleocytosis which leads to a substantial human DNA contamination (14). Since viral encephalitis generally has less inflamed CSF compared with other forms of CNS infections, the advantage of pathogen identification by mNGS may be more prominent in viral encephalitis. In the present study, we aimed to investigate the potency of CSF mNGS for identification of the etiologic agent using a model of varicella-zoster virus (VZV) CNS infection.

METHODS

Patients

This study included 31 patients with suspected VZV CNS infection admitted to the second hospital of Nanjing, China, from August 2018 to November 2020. In Nanjing, the second hospital of Nanjing is the only designated hospital for managing patients with primary VZV infection. Patients with characteristic skin rashes suggestive of varicella and concomitant neurological symptoms were hospitalized for screening of possible VZV CNS involvement. Diagnostic procedures in routine clinical care for those patients included a cerebral MRI and analysis of CSF cell counts, chemistry, and bacterial culture. After obtained written informed consent, additional CSF samples were collected and stored at −80°C until analysis for VZV IgM antibodies, VZV PCR, and unbiased mNGS.

The medical records, such as epidemiological and demographic data, medical history, underlying comorbidities, clinical manifestations, laboratory tests, cerebral imaging findings, treatments, and outcomes, were retrieved from an electronic health record system. This study was approved by the ethics committee of the second hospital of Nanjing (reference number: 2020-LY-kt061).

Metagenomic Next-Generation Sequencing

Cerebrospinal fluid samples were sent for PMSeq-DNA, a commercially available service from BGI-Shenzhen, which detects pathogenic microorganisms through mNGS. In this study, 600 μl CSF sample was mixed with enzyme and glass beads, and was vortexed vigorously at 2,800–3,200 rpm for 30 min. Then, total DNA was extracted from 300 μl each CSF sample using TIANamp Micro DNA Kit (DP316, Tiangen Biotech, Beijing, China) according to the recommendation of manufacturer. DNA libraries were constructed through DNA-fragmentation, end-repair, adapter-ligation, and PCR amplification. After quality control with Agilent 2100 (Agilent Technologies, CA, USA), libraries were sequenced by BGISEQ-50 platform. Low-quality sequence reads and sequence reads mapped to the human reference genome (hg19) were removed. The remaining data were classified by aligning to four Microbial Genome Databases containing bacteria, fungi, viruses, and parasites. The reference databases were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/) which contained 4,945 whole genome sequences of viral taxa, 6,350 bacterial genomes or scaffolds, 1,064 pathogenic fungi genomes, and sequences of 234 parasites associated with human diseases.

Detection of VZV-IgM Antibodies

An ELISA kit (ESR104M, SERION, Friedrich-Bergius-Ring, Würzburg, Germany) was used to measure VZV-IgM antibodies in CSF samples. The ELISA procedure was done following SERION ELISA classic CSF Diagnostics instructions with a modification that the dilution factor of CSF samples was changed from 1:20 to 1:10. As a CSF standard was not included in the kit, the interpretation of ELISA results was based on average optical density (OD) 450 values. The ELISA test was considered negative if the average OD value from duplicate sample wells is <0.1 or was <2-fold of that in the negative control. In our study, CSF samples from nine patients with syphilis-seropositive were used as negative control. Those nine patients were excluded from neurosyphilis based on normal CSF cell counts and chemistry tests, and negative CSF *treponema pallidum* particle agglutination assays. The ELISA assay was considered positive if the average OD value from each of the duplicate sample wells was ≥ 2-fold of that in the negative control.

Real-Time PCR Detection Assay

The VZV nucleic acid was detected by real-time PCR assay. For the qualitative assay, 30 μl of total DNA was extracted from 200 μl CSF sample using TIANamp Micro DNA Kit (DP316,

Tiangen Biotech). Then, the presence of VZV DNA was detected using a commercially available VZV nucleic acid detection kit following the instruction of manufacturer (D2211, Daan Gene, Guangdong, China). Each sample was testes two times. The following PCR program was run in the applied biosystems (ABI) 7500 Thermal Cycler (Applied Biosystems, MA, USA): 50°C for 2 min; 95°C for 15 min; 45 cycles of 94°C for 15 s; and 55°C for 45 s. A cycle threshold (Ct) value of 38 or less was considered positive.

In addition, the CSF samples were sent for absolute quantification of the VZV viral load using an in-house real-time quantification PCR test at a third-party diagnostic company (New Jingpei Diagnostics, Shanghai, China). Then, 100 μl of total DNA was extracted from 200 μl CSF sample using a DNA/RNA extraction Kit (ZTLJB, Tianlong Biotech, Hangzhou, China). Furthermore, 5 μl DNA sample was added to the reaction mix prepared following instruction of QuantiTect Probe PCR Kit (204343, Qiagen, Germany). The primers and probe set for VZV DNA amplification and detection were as follows: forward primer: 5′-TGCAGGGCATGGCTCAGT-3′; reverse primer: 5′-CCCAAGAACCACATGTCCAAC-3′; and probe: 5′-FAM-TCC TCAGTGCGGTGGTTGCCCA-BHQ1-3′ (15). The following PCR program was run in the applied biosystems (ABI) 7500 Thermal Cycler: 50°C for 2 min; 95°C for 15 min; 45 cycles of 94°C for 15 s; and 60°C for 40 s. Standard curve for absolute quantification was generated by amplifying serial dilutions of a plasmid containing VZV DNA segment. The lower limit of detection as evaluated by the service provider was 500 copies/ml.

Statistical Analysis

Continuous variables were expressed as the medians and interquartile ranges (IQRs). Categorical variables were shown as the counts and percentages in each category. Comparison between groups was done using McNemar's test for categorical variables, when appropriate. Statistical analysis was done by using SPSS version 22.0 (IBM, NY, USA). A $p < 0.05$ is considered statistically significant.

RESULTS

The Characteristics of the Patients

The characteristics of the 31 patients admitted for screening of the VZV CNS infection were summarized in **Table 1**. None of the patients previously received chickenpox vaccine or had any evidence of immune deficiency. The diagnosis of primary VZV infection was mainly based on the presence of typical rashes morphologically resembling chickenpox. Serum VZV IgM antibodies were positive in 66.7% (8/12) of the patients. The median age was 30.0 (IQR, 24.3–33.3) years. About 51.6% (16/31) of the patients were men, and 54.8% (17/31) patients had contacted with chickenpox within 4 weeks. The median time from onset of illness to admission was 4.0 (3.0–5.0) days, while from onset of neurological symptoms was 2.0 (1.0–4.0) days. The most common neurological symptom was headache (93.5%, 29/31), followed by nausea (45.2%, 14/31) and vomiting (19.4%, 6/31). Signs of meningeal irritation were not common, with nuchal rigidity seen in 12.9% (4/31) of the patients and Kernig's sign in 3.2% (1/31) of the patient. The majority of the patients (80.6%, 25/31) had CSF white blood cell counts not more than 5 $\times$ 10^6 cells/L.

TABLE 1 | Clinical, laboratory, and imaging characteristics of the 31 patients hospitalized for screening of varicella-zoster virus (VZV) central nervous system (CNS) infection.

	Total (n = 31)
Demographics and clinical characteristics	
Age, years	30 (24.3–33.3)
Sex, male	16 (51.6)
Comorbidity	2 (6.5)
Recently contacted with chickenpox	17 (54.8)
Time from chickenpox onset, days	4.0 (3.0–5.0)
Time from neurological symptoms onset, days	2 (1–4)
Headache	29 (93.5)
Neuasia	14 (45.2)
Vomitting	6 (19.4)
Mental Change	2 (6.5)
Difficulty urinating	2 (6.5)
Nuchal rigidity	4 (12.9)
Kernig's sign	1 (3.2)
Brudzin ski's sign.	0 (0)
Blood laboratory tests	
White blood cell count, $\times 10^9$/L	4.9 (3.9–7.0)
Lymphocyte count, $\times 10^9$/L	1.3 (1.1–2.9)
Lactate dehydrogenase, IU/L	240.0 (202.5–289.5)
C-reactive protein, mg/L	10.8 (2.7–20.9)
Cerebrospinal fluid analysis	
White blood cell count, $\times$ 10^6/L	2.0 (1.0–5.0)
Proteins, mg/L	261.4 (219.7–354.8)
Glucose, mmol/L	3.2 (2.9–3.8)
Lactate dehydrogenase, IU/L	17 (14–21)
Adenosine deaminase	2 (0.1–3.6)
Negative bacterial culture	31 (100)
Brain imaging	
Abnormal magnetic resonance imaging	4 (12.9)

Diagnosis of VZV CNS Infection Through CSF VZV IgM Detection, VZV PCR, and mNGS

At the time of discharge, 41.9% (13/31) of the patients were clinically diagnosed with VZV CNS infection. Of the 13 patients with a clinical diagnosis of VZV CNS infection, 6 had CSF pleocytosis (leukocytes $>5 \times 10^6$ cells/L). The diagnosis of VZV CNS infection in the remaining 7 patients with normal CSF white blood cell counts was made by expert penal after thoroughly evaluating the clinical course and imaging findings of those patients. To explore the microbiological evidence of VZV CNS infection, stored CSF samples were tested for VZV IgM antibodies, VZV PCR, and unbiased mNGS. VZV IgM antibodies were presented in 16.1% (5/31) of the CSF samples.

TABLE 2 | Diagnosis of VZV CNS infection.

	CSF mNGS[a]	
	VZV detected ($n = 16$)	**VZV not detected ($n = 15$)**
Clinical diagnosed VZV CNS infection	7 (43.8)	6 (40.0%)
Positive CSF VZV IgM antibodies	3 (18.8)	2 (13.3)
CSF VZV nucleic acid test (qualitatively)	5 (31.5)	0 (0)
CSF VZV nucleic acid test (quantitatively)	3 (18.8)	0 (0)
Abnormal cerebral MRI	1 (6.3)	3 (20)

CSF, cerebrospinal fluid; mNGS, metagenome next-generation sequencing; VZV, varicella-zoster virus; CNS, central nervous system; MRI, magnetic resonance imaging.
[a]The median [interquartile range (IQR)] sequence reads of CSF mNGS is 34.6 (28.8–48.3) millions.

Varicella-zoster virus nucleic acids were detected in 16.1% (5/31) and 9.7% (3/31) of the CSF samples, respectively, when different real-time PCR reagents were used. VZV nucleic acids were detected in three CSF samples by both qualitative and quantitative VZV PCR. Two CSF samples, with negative quantitative VZV PCR results, tested weak positive for VZV by using qualitative VZV PCR with cycle threshold of 37.88 and 37.93, respectively. Intriguingly, maximal identification of VZV elements was achieved by CSF mNGS ($p = 0.001$ and $p = 007$; compared with qualitative PCR and VZV IgM antibody test, respectively), with sequence reads of VZV being reported in 51.6% (16/31) of the CSF samples (**Table 2**). The VZV relative abundance was provided in **Supplementary Table 1**. All the cases with positive CSF VZV PCR results tested positive for VZV sequence reads with CSF mNGS (**Table 2**).

Pathogens Unexpectedly Identified by mNGS and the Clinical Significance

Of the 16 patients with VZV DNA detected by mNGS, the median VZV read was 6 (IQR, 1–85) (**Figure 1A**); VZV was the only reported pathogen in five CSF samples, and the remaining 11 CSF samples also detected other pathogens, most commonly the CMV (5 CSF samples; range of sequence reads, 1–26) (**Figure 1B**). Pathogens unexpectedly identified by mNGS in the 31 CSF samples were shown in **Table 3**; of which, 5 had HSV-1 sequence reads (range, 1–155). The presence of CMV and HSV-1 was evaluated by two real-time PCR based kits (Sansure Biotech, Hunan, China), and none of the CSF sample showed positive result.

A mNGS of the CSF sample revealed relatively high sequence reads of human betaherpesvirus 6A (HHV-6A) and low sequence reads of VZV (889 and 6, respectively). The patient was admitted to the hospital because of fever and itchy blisters for 6 days and difficulty urinating for 1 day. She had a close contact with her son who was recently diagnosed with chickenpox. CSF analysis demonstrated pleocytosis with leukocytes of 120×10^6 cells/L. A cerebral MRI showed multiple abnormalities in left thalamus, cerebellar vermis, and left cerebral peduncles. At that time, she was clinically diagnosed with VZV CNS infection, and

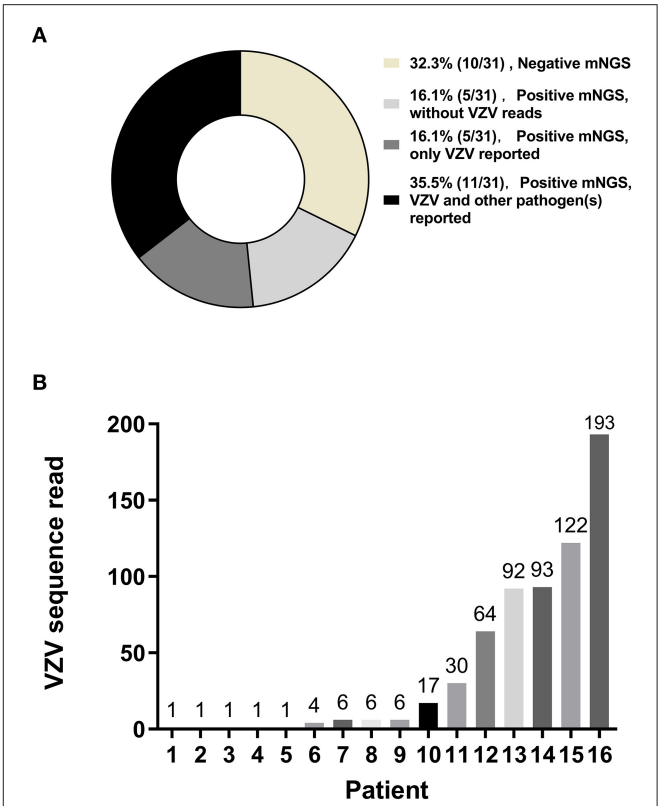


FIGURE 1 | Metagenomic next-generation sequencing (mNGS) of cerebrospinal fluid (CSF) samples. Metagenomic next-generation sequencing was performed on CSF samples from 31 patients with suspected varicella-zoster virus (VZV) central nervous system (CNS) infections. VZV sequence reads were the only sequence reads reported in 16.1% (5/31) of the patients. VZV sequence reads and sequences reads of other pathogens were both reported in 35.5% (11/31) of the patients **(A)**. Of those 16 patients with VZV being detected by CSF mNGS, variable numbers of VZV sequence reads were reported **(B)**.

was treated with acyclovir, intravenous immunoglobulin, and dexamethasone. The clinical condition was gradually improved. After hospitalization for 26 days, the patient was discharged without obvious symptoms. The presence of HHV-6A in the stored CSF sample, which was unexpectedly identified by mNGS, was further confirmed by real-time PCR following the protocol described by Gautheret-Dejean et al. (16).

DISCUSSIONS

Viral encephalitis accounts for 20–50% of microbiologically confirmed cases of encephalitis, with herpes simplex virus being the most common etiologic agent followed by VZV (17). Detection of the specific viral IgM antibodies or nucleic acid in CSF sample is regarded as a confirmation of viral encephalitis (3, 17). As for the VZV encephalitis, those parameters have variably diagnostic sensitivities, possibly due to different patients being enrolled in the studies (18–21). In a study on VZV CNS infections, VZV nucleic acid was detectable in the CSF only in one-fourth of the patients (18). Since CSF VZV viral load is correlated with the severity of VZV CNS infection (22, 23),

TABLE 3 | Pathogens unexpectedly reported by the mNGS.

	No. of positive CSF sample	No. of sequence reads
Cytomegalovirus	5	1, 4, 5, 7, and 26
Herpes simplex virus-1	5	1, 1, 4, 8, and 155
Human polyomavirus-5	2	3 and 3
Candida albicans	2	4 and 10
Mycoplasma species	2	3 and 5
Others: human betaherpesvirus 6A, human papillomavirus-5, *Staphylococcus aureus*, *Rickettsia felis*, *Aspergillus fumigatus*, Torque teno virus 28, human papillomavirus-19, Epstein-Barr virus, and molluscum contagiosum virus	1 for each pathogen	889, 10, 4, 4, 4, 3, 3, 2 and 2, respectively

CSF, cerebrospinal fluid.

patients with mild neurological involvement may have extremely low viral burden below the detection limit of VZV PCR. The presence of VZV IgM antibody in CSF could be analyzed by using the commercial ELISA kits. However, a CSF standard is generally not provided by the manufacturers, which makes setting a cut-off value complicated. This may be one of the reasons that contributes to the low utility of this parameter in diagnosing VZV CNS infection, although a study revealed a low sensitivity of CSF IgM antibody for identification of VZV CNS infection (18).

For the above-mentioned reasons, a negative CSF VZV IgM test or nucleic acid test does not necessarily rule out VZV CNS infection. Therefore, in the present study, we did not arbitrarily define "true positive" or "true negative" cases of VZV CNS infection. In this context, the exact sensitivity of each microbiological test for diagnosing of CNS infection was not calculated. Instead, the magnitude of positive result was compared. Our study showed that, compared with CSF VZV IgM antibody test or CSF VZV PCR test, CSF mNGS identified much higher number of cases with clues of VZV CNS infection. Of note, all the CSF samples with positive VZV PCR results tested positive for VZV sequence reads by using mNGS. Those findings suggest that CSF VZV-specific PCR may not improve the sensitivity of diagnosing VZV CNS infection when mNGS has already been performed on CSF samples.

The mNGS focuses on the whole genome sequence of the pathogen which is broken into small sequence segments that are unbiasedly amplified and measured. Therefore, sequences of a given pathogen are more likely to be detected by mNGS targeting the whole genome of the pathogen, as compared with pathogen-specific PCR which generally targets single or two regions of the genome sequence (24). Moreover, in the case of target gene mutation, pathogen-specific PCR may not be able to amplify the target gene, which leads to a false negative result. The abovementioned reasons could explain why, in our study, mNGS performed better with regard to the detection of VZV sequences compared with PCR methods. Finally, as shown in **Table 1**, the level of CSF white blood cells infiltration is relatively low. In this condition, the sensitivity of mNGS for pathogen detection may be less impacted by human genome contamination.

The main advantage of mNGS is unbiased sampling which enables broad identification of pathogens in a hypothesis-free manner (6). Using this relatively novel strategy, others and we have already diagnosed a number of unexpected or rare cases with infectious etiologies (7, 9, 25–28). In the current study on patients with suspected VZV CNS infection, HHV-6A was unexpectedly identified by CSF mNGS from a patient with obvious neurological symptoms. Since HHV-6A has been shown to be related to neurological diseases (29–33), the presence of HHV-6A in the CSF sample of our patient could be of clinical importance. The finding re-emphasizes that agnostic mNGS is a useful strategy for identification of the etiologic agent when the treating physician is not familiar with the encountered clinical situation.

The mNGS is not a perfect diagnostic strategy in clinical microbiology. The unbiased sampling is not just an advantage but also, to some extent, could be a drawback. First, clinical samples are dominated by human host background. Unwanted human DNA occupies the majority of sequence reads of the mNGS which would limit the sensitivity of this strategy for pathogen identification (6, 34). Second, sequence reads from microorganisms not related to the clinical diseases can also be reported, which complicates the interpretation of the mNGS results. Those microbial contaminants, such as environmental microbes and skin flora, could be generated during sample collection and processing. Even the reagents used for processing may be contaminated by microbial DNA (6, 34). Finally, mNGS does not have the power to distinguish invasive infection from colonization. In addition, it could not differentiate between latent and active infections. In this condition, mNGS results should be interpreted based on the clinical judgement of the treating physicians.

As shown in **Table 3**, trace amounts of microbial nucleic acids, most commonly the CMV and HSV-1, were reported. CMV and HSV-1 are common human pathogens, both of which could cause CNS diseases (3). The mNGS, itself, could not answer whether those microbial DNAs really presented in the CSF samples. Additionally, it is not possible to tell whether those pathogens caused the neurological symptoms of our patients. Those drawbacks of mNGS are not likely to be completely resolved in the near future. At this point, it is advocated that mNGS results are treated as a list of candidate etiological agents which help to narrow the diagnosis. Only when the limitations of the mNGS are fully understood, then the clinicians may properly interpretate the mNGS results.

In conclusion, our study found that CSF mNGS was the most sensitive microbiological test for diagnosing VZV CNS infection, and could unexpectedly identify the causative agent that were not recognized based on traditional diagnostic tests. Perhaps, for the diagnoses of viral encephalitis caused by DNA virus, with continuing improving the methodology of mNGS, pathogen-specific PCR may not be needed when mNGS has already been used for pathogen detection. Nevertheless, pathogen-specific antibody detection may still be performed because it provides different biological information. Finally, although the sensitivity of mNGS can be further increased by technical innovation, the specificity will continue to be a great concern. Clinical judgement

of the treating physicians is very important for interpretating the results.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The raw sequences of mNGS were deposited to the NCBI database under accession numbers PRJNA784638.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Second Hospital of Nanjing. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZH designed this study. YZ, CD, and ZH collected the data. MX, WW, and ZH completed the experiment. MX, ZP, WC, and ZH analyzed the data. BS, JC, and CC were involved in the clinical management of the patients and analyzed the clinical data. This manuscript was initially drafted by YZ, CD, and ZH and then revised by MX, ZP, WW, BS, JC, CC, WC, HW, and ZH. All authors approved the final manuscript.

FUNDING

This study was funded in part by the project of Jiangsu province medical youth talent (QNRC2016059) and the Nanjing medical science and technique development foundation (YKK18153). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.738412/full#supplementary-material

REFERENCES

1. Somand D, Meurer W. Central nervous system infections. *Emerg Med Clin North Am.* (2009) 27:89–100. doi: 10.1016/j.emc.2008.07.004
2. Dubot-Pérès A, Mayxay M, Phetsouvanh R, Lee SJ, Rattanavong S, Vongsouvath M, et al. Management of central nervous system infections, vientiane, laos, 2003-2011. *Emerg Infect Dis.* (2019) 25:898–910. doi: 10.3201/eid2505.180914
3. Tunkel AR, Glaser CA, Bloch KC, Sejvar JJ, Marra CM, Roos KL, et al. The management of encephalitis: clinical practice guidelines by the Infectious Diseases Society of America. *Clin Infect Dis.* (2008) 47:303–27. doi: 10.1086/589747
4. Granerod J, Cunningham R, Zuckerman M, Mutton K, Davies NW, Walsh AL, et al. Causality in acute encephalitis: defining aetiologies. *Epidemiol Infect.* (2010) 138:783–800. doi: 10.1017/S0950268810000725
5. He T, Kaplan S, Kamboj M, Tang YW. Laboratory diagnosis of central nervous system infection. *Curr Infect Dis Rep.* (2016) 18:35. doi: 10.1007/s11908-016-0545-6
6. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Ann Rev Pathol.* (2019) 14:319–38. doi: 10.1146/annurev-pathmechdis-012418-012751
7. Hu Z, Weng X, Xu C, Lin Y, Cheng C, Wei H, et al. Metagenomic next-generation sequencing as a diagnostic tool for toxoplasmic encephalitis. *Ann Clin Microbiol Antimicrob.* (2018) 17:45. doi: 10.1186/s12941-018-0298-1
8. Wilson MR, Sample HA, Zorn KC, Arevalo S, Yu G, Neuhaus J, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. *N Engl J Med.* (2019) 380:2327–40. doi: 10.1056/NEJMoa1803396
9. Fang M, Weng X, Chen L, Chen Y, Chi Y, Chen W, et al. Fulminant central nervous system varicella-zoster virus infection unexpectedly diagnosed by metagenomic next-generation sequencing in an HIV-infected patient: a case report. *BMC Infect Dis.* (2020) 20:159. doi: 10.1186/s12879-020-4872-8
10. Hong NTT, Anh NT, Mai NTH, Nghia HDT, Nhu LNT, Thanh TT, et al. Performance of metagenomic next-generation sequencing for the diagnosis of viral meningoencephalitis in a resource-limited setting. *Open Forum Inf Dis.* (2020) 7:ofaa046. doi: 10.1093/ofid/ofaa046
11. Xing X-W, Zhang J-T, Ma Y-B, He M-W, Yao G-E, Wang W, et al. Metagenomic next-generation sequencing for diagnosis of infectious encephalitis and meningitis: a large, prospective case series of 213 patients. *Front Cell Infect Microbiol.* (2020) 10:88. doi: 10.3389/fcimb.2020.00088
12. Wang S, Chen Y, Wang D, Wu Y, Zhao D, Zhang J, et al. The feasibility of metagenomic next-generation sequencing to identify pathogens causing tuberculous meningitis in cerebrospinal fluid. *Front Microbiol.* (2019) 10:1993. doi: 10.3389/fmicb.2019.01993
13. Yan L, Sun W, Lu Z, Fan L. Metagenomic Next-Generation Sequencing (mNGS) in cerebrospinal fluid for rapid diagnosis of Tuberculosis meningitis in HIV-negative population. *Int J Inf Dis.* (2020) 96:270–5. doi: 10.1016/j.ijid.2020.04.048
14. Ji X-C, Zhou L-F, Li C-Y, Shi Y-J, Wu M-L, Zhang Y, et al. Reduction of human DNA contamination in clinical cerebrospinal fluid specimens improves the sensitivity of metagenomic next-generation sequencing. *J Mol Neurosci.* (2020) 70:659–66. doi: 10.1007/s12031-019-01472-z
15. Persson A, Bergström T, Lindh M, Namvar L, Studahl M. Varicella-zoster virus CNS disease–viral load, clinical manifestations and sequels. *J Clin Virol.* (2009) 46:249–53. doi: 10.1016/j.jcv.2009.07.014
16. Gautheret-Dejean A, Manichanh C, Thien-Ah-Koon F, Fillet AM, Mangeney N, Vidaud M, et al. Development of a real-time polymerase chain reaction assay for the diagnosis of human herpesvirus-6 infection and application to bone marrow transplant patients. *J Virol Methods.* (2002) 100:27–35. doi: 10.1016/S0166-0934(01)00390-1
17. Tyler KL. Acute viral encephalitis. *N Engl J Med.* (2018) 379:557–66. doi: 10.1056/NEJMra1708714
18. Koskiniemi M, Piiparinen H, Rantalaiho T, Eränkö P, Färkkilä M, Räihä K, et al. Acute central nervous system complications in varicella zoster virus infections. *J Clin Virol.* (2002) 25:293–301. doi: 10.1016/S1386-6532(02)00020-3
19. Gregoire SM, Van Pesch V, Goffette S, Peeters A, Sindic CJ. Polymerase chain reaction analysis and oligoclonal antibody in the cerebrospinal fluid from 34 patients with varicella-zoster virus infection of the nervous system. *J Neurol Neurosurg Psychiatry.* (2006) 77:938–42. doi: 10.1136/jnnp.2006.090316
20. Nagel MA, Cohrs RJ, Mahalingam R, Wellish MC, Forghani B, Schiller A, et al. The varicella zoster virus vasculopathies: clinical, CSF, imaging, and virologic features. *Neurology.* (2008) 70:853–60. doi: 10.1212/01.wnl.0000304747.38502.e8
21. De Broucker T, Mailles A, Chabrier S, Morand P, Stahl J-P. Acute varicella zoster encephalitis without evidence of primary vasculopathy in a case-series of 20 patients. *Clin Microbiol Inf.* (2012) 18:808–19. doi: 10.1111/j.1469-0691.2011.03705.x
22. Aberle SW, Aberle JH, Steininger C, Puchhammer-Stöckl E. Quantitative real time PCR detection of Varicella-zoster virus DNA in cerebrospinal fluid in

patients with neurological disease. *Med Microbiol Immunol.* (2005) 194:7–12. doi: 10.1007/s00430-003-0202-1
23. Rottenstreich A, Oz ZK, Oren I. Association between viral load of varicella zoster virus in cerebrospinal fluid and the clinical course of central nervous system infection. *Diagn Microbiol Infect Dis.* (2014) 79:174–7. doi: 10.1016/j.diagmicrobio.2014.02.015
24. Metzker ML. Sequencing technologies—the next generation. *Nat Rev Genet.* (2010) 11:31–46. doi: 10.1038/nrg2626
25. Mongkolrattanothai K, Naccache SN, Bender JM, Samayoa E, Pham E, Yu G, et al. Neurobrucellosis: unexpected answer from metagenomic next-generation sequencing. *J Pediatric Infect Dis Soc.* (2017) 6:393–8. doi: 10.1093/jpids/piw066
26. Brown JR, Bharucha T, Breuer J. Encephalitis diagnosis using metagenomics: application of next generation sequencing for undiagnosed cases. *J Infect.* (2018) 76:225–40. doi: 10.1016/j.jinf.2017.12.014
27. Huang Z, Zhang C, Li W, Fang X, Wang Q, Xing L, et al. Metagenomic next-generation sequencing contribution in identifying prosthetic joint infection due to Parvimonas micra: a case report. *J Bone Joint Inf.* (2019) 4:50–5. doi: 10.7150/jbji.30615
28. Jin W, Miao Q, Wang M, Zhang Y, Ma Y, Huang Y, et al. A rare case of adrenal gland abscess due to anaerobes detected by metagenomic next-generation sequencing. *Ann Transl Med.* (2020) 8:123. doi: 10.21037/atm.2020.01.123
29. Asano Y, Yoshikawa T, Kajita Y, Ogura R, Suga S, Yazaki T, et al. Fatal encephalitis/encephalopathy in primary human herpesvirus-6 infection. *Arch Dis Child.* (1992) 67:1484–5. doi: 10.1136/adc.67.12.1484
30. Ogata M, Satou T, Kawano R, Takakura S, Goto K, Ikewaki J, et al. Correlations of HHV-6 viral load and plasma IL-6 concentration with HHV-6 encephalitis in allogeneic stem cell transplant recipients. *Bone Marrow Transplant.* (2010) 45:129–36. doi: 10.1038/bmt.2009.116
31. Ogata M, Satou T, Kadota J-I, Saito N, Yoshida T, Okumura H, et al. Human Herpesvirus 6 (HHV-6) reactivation and HHV-6 encephalitis after allogeneic hematopoietic cell transplantation: a multicenter, prospective study. *Clin Infect Dis.* (2013) 57:671–81. doi: 10.1093/cid/cit358
32. Leibovitch EC, Jacobson S. Evidence linking HHV-6 with multiple sclerosis: an update. *Curr Opin Virol.* (2014) 9:127–33. doi: 10.1016/j.coviro.2014.09.016
33. Eliassen E, Hemond CC, Santoro JD. HHV-6-associated neurological disease in children: epidemiologic, clinical, diagnostic, and treatment considerations. *Pediatr Neurol.* (2020) 105:10–20. doi: 10.1016/j.pediatrneurol.2019.10.004
34. Simner PJ, Miller S, Carroll KC. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. *Clin Infect Dis.* (2018) 66:778–88. doi: 10.1093/cid/cix881

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Zhu, Xu, Ding, Peng, Wang, Sun, Cheng, Chen, Chen, Wei and Hu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 21 January 2022
doi: 10.3389/fcimb.2021.745156

Evaluations of Clinical Utilization of Metagenomic Next-Generation Sequencing in Adults With Fever of Unknown Origin

Zhang-fan Fu[1†], Hao-cheng Zhang[1†], Yi Zhang[1], Peng Cui[1], Yang Zhou[1,2], Hong-yu Wang[1], Ke Lin[1], Xian Zhou[1], Jing Wu[1], Hong-long Wu[2,3], Wen-hong Zhang[1] and Jing-wen Ai[1]*

[1] Department of Infectious Diseases, Shanghai Key Laboratory of Infectious Diseases and Biosafety Emergency Response, National Medical Center for Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China, [2] BGI PathoGenesis Pharmaceutical Technology Co., Ltd., BGI-Shenzhen, Shenzhen, China, [3] BGI Wuhan Biotechnology, BGI-Shenzhen, Wuhan, China

Introduction: The diagnosis of infection-caused fever of unknown origin (FUO) is still challenging, making it difficult for physicians to provide an early effective therapy. Therefore, a novel pathogen detection platform is needed. Metagenomic next-generation sequencing (mNGS) provides an unbiased, comprehensive technique for the sequence-based identification of pathogenic microbes, but the study of the diagnostic values of mNGS in FUO is still limited.

Methods: In a single-center retrospective cohort study, 175 FUO patients were enrolled, and clinical data were recorded and analyzed to compare mNGS with culture or traditional methods including as smears, serological tests, and nucleic acid amplification testing (NAAT) (traditional PCR, Xpert MTB/RIF, and Xpert MTB/RIF Ultra).

Results: The blood mNGS could increase the overall rate of new organisms detected in infection-caused FUO by roughly 22.9% and 19.79% in comparison to culture (22/96 vs. 0/96; OR, ∞; p = 0.000) and conventional methods (19/96 vs. 3/96; OR, 6.333; p = 0.001), respectively. Bloodstream infection was among the largest group of those identified, and the blood mNGS could have a 38% improvement in the diagnosis rate compared to culture (19/50 vs. 0/50; OR, ∞; p = 0.000) and 32.0% compared to conventional methods (16/50 vs. 3/50; OR, 5.333; p = 0.004). Among the non-blood samples in infection-caused FUO, we observed that the overall diagnostic performance of mNGS in infectious disease was better than that of conventional methods by 20% (9/45 vs. 2/45; OR, 4.5; p = 0.065), and expectedly, the use of non-blood mNGS in non-bloodstream infection increased the diagnostic rate by 26.2% (8/32 vs. 0/32; OR, ∞; p = 0.008). According to 175 patients' clinical decision-making, we found that the use of blood mNGS as the first-line investigation could effectively increase 10.9% of diagnosis

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Yixin Chen,
Xiamen University, China
Qihua Fu,
Shanghai Children's Medical Center, China

***Correspondence:**
Jingwen Ai
jingwenai1990@126.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *21 July 2021*
Accepted: *23 November 2021*
Published: *21 January 2022*

Citation:
Fu Z-F, Zhang H-C, Zhang Y, Cui P, Zhou Y, Wang H-Y, Lin K, Zhou X, Wu J, Wu H-L, Zhang W-H and Ai J-W (2022) Evaluations of Clinical Utilization of Metagenomic Next-Generation Sequencing in Adults With Fever of Unknown Origin. Front. Cell. Infect. Microbiol. 11:745156. doi: 10.3389/fcimb.2021.745156

rate of FUO compared to culture, and the strategy that the mNGS of suspected parts as the second-line test could further benefit infectious patients, improving the diagnosis rate of concurrent infection by 66.7% and 12.5% in non-bloodstream infection, respectively.

Conclusion: The application of mNGS in the FUO had significantly higher diagnostic efficacy than culture or other conventional methods. In infection-caused FUO patients, application of blood mNGS as the first-line investigation and identification of samples from suspected infection sites as the second-line test could enhance the overall FUO diagnosis rate and serve as a promising optimized diagnostic protocol in the future.

Keywords: metagenomic next-generation sequencing, fever of unknown origin, precision diagnose, etiology, application

INTRODUCTION

Since 1961, Petersdorf has defined the fever of unknown origin (FUO) as a fever of 38.3°C or higher for 3 weeks or more on multiple occasions; the etiology cannot be determined despite appropriate investigation even after at least a week in the hospital (Petersdorf and Beeson, 1961). Over 200 disorders have been found in the differential diagnosis of FUO, which was divided into infection, non-infectious inflammatory disease (NIID), cancer, other conditions, and unresolved diagnosis (Mourad et al., 2003; Kucukardali et al., 2008; Horowitz, 2013). Due to the complicated clinical characteristics and absence of laboratory analysis indicators, the disease is difficult to be diagnosed and contributes to a tremendous cost on average of 40,295 US dollars during hospitalization (Szymanski et al., 2020). Because infection is one of the main causes of FUO (Fusco et al., 2019; Naito et al., 2019; Wright and Auwaerter, 2020; Wright et al., 2021), an optimized screening and diagnostic work flow for detecting pathogens of infection-caused FUO is needed.

Since the launch of the second-generation sequencing platform in early 2000, next-generation sequencing (NGS) technology has advanced at a rapid pace in the past 20 years. Nowadays, NGS may be performed for as low as $1,000 per sample (10.5 MB) to an average depth of 400× coverage (Yohe and Thyagarajan, 2017). Metagenomic next-generation sequencing (mNGS) is an inspection method that uses a next-generation sequencing platform to detect all genomes of environmental or patient samples (Chiu and Miller, 2019). Because of its unbiased and high throughput, mNGS has been used to study human microbial flora (Lagier et al., 2018), human pathogens (Gu et al., 2019), environmental microflora (ocean or soil) (Zhao & Bajic, 2015; Fierer, 2017), forensic field (Hampton-Marcell et al., 2017), etc. In addition, recent studies have reported their successful applications of mNGS in a variety of infectious disorders including central nervous system infection (Wilson et al., 2018; Wilson et al., 2019; Zhang et al., 2020), respiratory tract infection (Langelier et al., 2018), focal infection (Zhang et al., 2019), bloodstream infection (Gosiewski et al., 2017), etc. However, research directly evaluating the ability of mNGS in FUO is still limited, although previous study has exhibited its ability in increasing cost efficacy of FUO (Chai et al., 2018). Consequently, in this study, we undertook a retrospective cohort analysis on the overall clinical values of mNGS in the application of FUO patients in the east of China.

MATERIALS AND METHODS

Study Design and Participants

This was a single-center, retrospective cohort study, in which all patients at the Huashan Hospital with the diagnosis of fever of unknown origin from March 7, 2017, to August 1, 2018 were enrolled (**Figure 1**). The final diagnosis of the 175 cases (**Table 1**) were evaluated by the experts group including three experienced physicians. Blood samples were collected after getting the consent of the patients or their surrogates and sent for mNGS and culture synchronously. Other examinations such as blood routine, blood biochemical tests, autoimmune antibody, and conventional pathogenic methods (including blood smear, latex agglutination test, serologic tests, and nucleic acid amplification testing (NAAT) (traditional PCR, Xpert MTB/RIF, and Xpert MTB/RIF Ultra) were performed according to the clinical necessity. If the patients were suspected of having infections other than bloodstream infections, specimens of suspected infected sites will also be sent for mNGS and the above-mentioned methods.

Criteria of Positive Detection Criteria for mNGS Test

Each sample's sequencing results were divided into two tables, one for bacteria/fungi and the other for viruses. The specifically mapped read number (SMRN) of each microbial taxonomy was standardized to SMRN per 20 million (M) of total sequencing reads (SDSMRN, standardized SMRN).

$$\mathrm{SDSMRN} = \mathrm{SMRN} \times 20 \ \text{million}/\text{total reads}.$$

A bacterial/fungal was considered positively detected if (i) it was among the top 10 genera with the highest SDSMRN; (ii) within its genus, it was ranked top; (iii) its SDSMRN >1; and (iv) it was a commonly reported bloodstream infection pathogen.

A virus was judged positively detected if (i) if a virus belonged to the top 3 with the highest SDSMRN and (ii) it had a SDSMRN >5.

Because of the low yield of DNA extraction and relatively limited risk of contamination, pathogens such as *Mycobacterium*

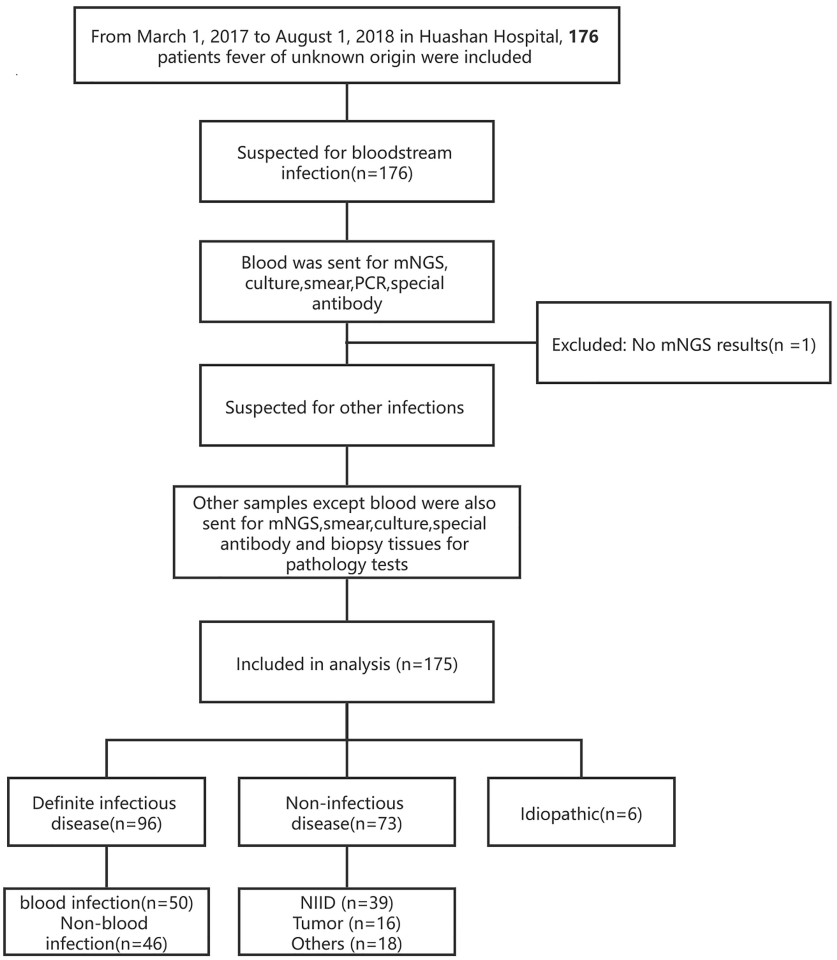


FIGURE 1 | Overview of patients' enrollment workflow. Flowchart of study.

spp., *Nocardia* spp., *Brucella* spp., and others were considered identified if (i) it was one of the top 20 genera with the highest SDSMRN, (ii) it positioned first within its genus, and (iii) it had a SDSMRN >1. For the detection of pathogens within the Enterobacteriaceae family, only the species with highest SDSMRN was considered as a positive detection.

The clinical interpretation of sequencing results was mainly based on the symptoms, physical signs, and uses of drugs. The final diagnosis of the 175 cases was evaluated by the expert's group including three experienced physicians who worked alone.

Criteria of Fever of Unknown Origin

i. Classic fever of unknown origin: a group of diseases that cannot be diagnosed after 1 week of comprehensive physical examination in the outpatient or hospitalization. Fever lasts for more than 3 weeks, and oral temperature is >38.3°C at least three times (or at least three times the temperature fluctuates >1.2°C within 1 day);
ii. Inpatients of fever of unknown origin: The patient had no fever when admitted but had a fever for more than 3 days after admission. The oral temperature was >38.3°C at least three times (or body temperature fluctuates >1.2°C at least three times within 1 day);
iii. Neutropenic patients with fever of unknown origin: The patient has agranulocytosis (neutrophil count $<0.5 \times 10^9$ cells/L); fever that lasts more than 3 days, oral temperature >38.3°C (or body temperature fluctuates >1.2°C, within 1 day); the body fluid specimens were cultured for >48 h, and the results were negative.

Metagenomic Next-Generation Sequencing and Data Analysis

Sample Processing and Nucleic Acid Extraction

Three to five milliliters of the blood sample was drawn in an ethylenediaminetetraacetic acid (EDTA) anticoagulant tube (BD Vacutainer® EDTA tubes) at room temperature within 12 h before plasma separation and centrifuged at 400*g* for 20–30 min at 20°C. The plasma from the patients was transferred to two new sterile 1.8-ml tubes and kept at −80°C until DNA extraction. Other samples of latent infected parts, including sputum,

TABLE 1 | Distribution of 175 FUO cases.

Disease	No.	Disease	No.	Disease	No.	Disease	No.	Disease	No.	Disease	No.
Infection	96*			NIID	39	Tumor	16	Others	18	Idiopathic	6
Bloodstream infections	50	Nasosinusitis	1	Vasculitis	14	Lymphoma	10	Allergic reaction	3		
Lower respiratory infections	17	Severe hepatitis	1	UCTD	6	Thyroid cancer	2	Drug fever	3		
Urinary infections	6	Tuberculous pleurisy	1	Adult-onset Still's disease	6	Leukemia	1	Autoimmune encephalitis	2		
Central nervous system infections	6	Tuberculosis	1	Reactive arthritis	3	Bladder cancer	1	Fatigue syndrome	2		
Biliary duct infections	3	Vertebra infection	1	CTD	3	Bone metastatic cancer	1	Post-infection allergy	2		
Skin and soft tissue infections	3			SLE	2	HLH	1	Autoimmune hepatitis	1		
Endocarditis	3			polymyalgia rheumatica	2			Crohn disease	1		
Abdominal infections	1			Interstitial lung disease	1			Dysfunction of autonomic nerve	1		
Impetigo	1			Rheumatoid arthritis	1			Seizures	1		
Lymphangitis	1			Schnitzler syndrome	1			Systemic inflammatory response syndrome	1		
Lymphnoditis	1							Hashimoto thyroiditis	1		

**Some patients with infectious diseases have more than two infections, so the total number of cases of all infectious diseases exceeds 96.*
UCTD, undefined connective tissue disease; CTD, connective tissue disease; SLE, systemic lupus erythematosus; HLH, hemophagocytic lymph histiocytosis.

bronchoalveolar lavage fluid (BALF), cerebrospinal fluid (CSF), urine, puncture fluid, pleural fluid, NPS, and tissues (lung tissues, bone marrow, lymph nodes, and aortic valve vegetations) were collected according to standard procedures and also stored at −80°C. Before extraction, sputum specimens were liquefied and NPS was submerged in 3 ml preservation solution, while other samples went straight to the next operation.

Each sample was used for DNA extraction. For all liquid samples except plasma, 600 µl was transferred into a fresh 1.5 ml microcentrifuge and mixed with 1 g 0.5 mm BioSpec beads (0.5 mm dia. zirconia/silica, Cat. No. 11079105z) before being agitated vigorously at 2,800–3,200 rpm for 30 min on a horizontal platform on a Vortex-Genie 2 Vortex Mixer 12 (Scientific Industries, USA). Cell-free DNA was extracted directly from plasma without cell-wall-breaking treatment. Total DNA was extracted with TIANamp Micro DNA Kit (DP316, Tiangen Biotech, Beijing, China).

Samples from patients suspected with viral infection at the time of sample collection went through RNA extraction. Total RNA was extracted with QIAamp Viral RNA Mini Kit (52906, Qiagen, China) according to the manufacturer's instructions. With the SuperScript II Reverse Transcription Kit (18064-014, Invitrogen, China), the RNA was reverse transcribed and synthesized into double-stranded complementary DNA (ds cDNA).

DNA libraries were constructed *via* DNA fragmentation, end repair, A-tailing addition, adapter ligation, and PCR amplification. The Agilent 2100 and Qubit 2.0 system was used to performed quality control (200–300 bp, >2 ng/µl), and qualified libraries were sequenced on BGISEQ-2000 platform with single-end 50 bp strategy. At least 20 million reads were produced from each sample. To control the sequencing quality and contamination of each sequencing run, we added positive and negative control (HeLa cell lines with or without *Acinetobacter baumannii*) in each run.

Bioinformation Pipeline

High-quality sequencing data were generated by removing low-quality and short (length <35 bp) reads. Next, the clean reads after quality filtering were mapped to a human reference database including hg19 and Yanhuang genome sequence using Burrows–Wheeler alignment (Version 0.7.10). Remaining reads were aligned to the non-redundant bacterial, virus, fungal, and parasite databases using Burrows–Wheeler alignment (Version 0.7.10). The mapped data were processed for advanced data analysis. The genome databases were downloaded from National Center for Biotechnology Information (NCBI) (ftp://ftp.ncbi.nlm.nih.gov/genomes/). RefSeq contains 4,061 whole genome sequence of viral taxa, 2,473 bacterial genomes or scaffolds, 199 fungi related to human infection, and 135 parasites associated with human diseases. We uploaded the raw data onto China National GeneBank (CNP0000607).

Statistical Analysis

In this article, the pathogens extra detection rate (PEDR) of mNGS and the improved diagnostic rate (IDR) of mNGS were calculated by the following methods:

Pathogens extra detection rate:

$$PEDR_{mNGS/culture} = \frac{true\ \ mNGS\ \ positive\ \ cases - culture\ \ positive\ \ cases}{clinical\ \ definate\ \ infectious\ \ cases};$$

$$PEDR_{mNGS/conventional\ \ methods} = \frac{true\ \ mNGS\ \ positive\ \ cases - culture\ \ positive\ \ cases}{clinical\ \ definate\ \ infectious\ \ cases}$$

where the true mNGS-positive cases were those in consistent with clinical judgement.

Improved diagnostic rate:

$$IDR_{bloodstream\ infection} = IDR_{BSI}$$

$$= \frac{true\ mNGS\ positive\ cases\ BSI - culture\ positive\ cases\ BSI}{suspected\ BSI\ cases};$$

$$IDR_{cocurrent\ infection\ with\ bloodstream\ infection} = IDR_{co-BSI} =$$

$$\frac{true\ mNGS\ positive\ cases\ of\ co-BSI - culture\ positive\ cases\ of\ CO-BSI}{suspected\ co-BSI};$$

$$IDR_{non-bloodstream\ infection} = IDR_{non-BSI} =$$

$$\frac{true\ mNGS\ positive\ cases\ of\ non-BSI - culture\ positive\ cases\ of\ non-BSI}{suspected\ non-BSI}$$

The continuous variants of baseline characteristics were analyzed using the Student's t-test with their odds ratios (ORs) and 95% confidence intervals (95% CIs). We analyzed McNemar test and kappa test to compare differences across subgroups. Statistical analyses and figures were conducted using the SPSS statistical package 12.0 software, Excel 2019, Xmind 8.0 version, and R 4.1.0 version.

RESULTS

General Characteristics of Enrolled Patients

A total of 176 patients with the diagnosis of FUO were enrolled in the study and underwent clinically baseline screening in Huashan Hospital affiliated to Fudan University from March 7, 2017 to August 1, 2018. We excluded one case for low quantity of nucleic acid verification of mNGS and finally enrolled 175 individuals. According to the 175 patients' final diagnosis, 96 of these 175 patients were classified to the group of infectious disease (ID). Seventy-three patients were categorized into the non-infectious disease (NID), and the other six patients were idiopathic group (IG) (**Figure 1**). Additionally, the NID group was further classified to NIID (n = 39), tumor (n = 16), and other diseases (n = 18). All the baseline characteristics of 175 patients are shown in **Table 2**, and there was not any significant difference in baseline data including gender, age, count of white blood cell (WBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and procalcitonin (PST) between ID and NID groups.

Distribution of Identified Pathogens by mNGS

In general, the results of pathogens detected by mNGS showed that the virus was the most prevalent pathogens, in which the most frequently detected was *EBV* and then *CMV* (**Figure 2**). The top identified bacteria were *Mycobacterium tuberculosis* complex and *Escherichia coli*, and we also detected a wide range of fungi, such as *Penicillium marneffei*, *Aspergillus spp*., etc. mNGS was proved to have advantage over culture mainly in the identification of causal agents that cannot be cultured or had time-consuming culture process, such as *EBV*, *Aspergillus spp*., and *MTB* complex. However, mNGS had some more false-positive cases than that in culture in our study (**Supplementary Tables 1A–C**). **Figure 3**

Diagnostic Performance on Pathogens Extra Detection Rate of mNGS, Compared to Culture and Other Conventional Methods in Using Blood Samples in Infection-Caused FUO

In order to evaluate the diagnostic performance of mNGS, we calculated a set of $PEDR_{mNGS/culture}$. The overall $PEDR_{mNGS/culture}$ was 22.9%, $PEDR_{mNGS/culture}$ of bloodstream infection was 38%, but $PEDR_{mNGS/culture}$ of non-bloodstream infection was only 6.5%. We concluded that the mNGS of the FUO patients' blood samples might increase the pathogens extra detection rate of infectious diseases compared to that of culture alone by 22.9% (22/96 vs. 0/96; OR, ∞; p = 0.000) (**Table 3**). Bloodstream infection comprised the largest component of all the diseases, and the blood mNGS test could have a 38% improvement of pathogens extra detection rate compared to culture in the bloodstream infection (19/50 vs. 0/50; OR, ∞; p = 0.000) (**Table 3**), but the pathogens extra detection rate of blood mNGS test in non-bloodstream infection was limited only by 6.5% (**Table 3**).

We further analyzed the consistency between mNGS and culture of blood samples. In both mNGS and culture, 8 of 96 cases tested positive for infectious disease, and 66 of 96 cases were negative. Twenty-two cases were mNGS test positive and

TABLE 2 | The baseline of enrolled patients.

	Overall	Infection	Non-infection	p-values
N	169	96	73	
Age [mean (SD)]	47.87 (16.35)	47.33 (15.43)	48.58 (17.57)	0.626
Gender = Male (%)	103 (60.9)	62 (64.6)	41 (56.2)	0.341
WBC [mean (SD)]	27.98 (140.52)	23.64 (127.94)	34.25 (157.79)	0.641
PLT [mean (SD)]	232.96 (121.58)	241.44 (126.61)	221.03 (114.05)	0.306
Neutrophil [mean (SD)]	5.78 (3.63)	6.14 (3.94)	5.26 (3.08)	0.149
ESR [mean (SD)]	32.13 (23.44)	32.37 (25.00)	31.77 (21.09)	0.877
CRP [mean (SD)]	53.46 (51.94)	56.69 (53.63)	48.59 (49.32)	0.347
Ferritin [mean (SD)]	894.32 (728.53)	910.26 (746.13)	870.84 (707.96)	0.758
PCT [mean (SD)]	1.60 (9.19)	1.67 (8.66)	1.49 (10.00)	0.909
RBC [mean (SD)]	24.35 (198.97)	34.85 (258.34)	9.17 (19.01)	0.426

**The remain six are idiopathic; they do not belong to infection or non-infection.*

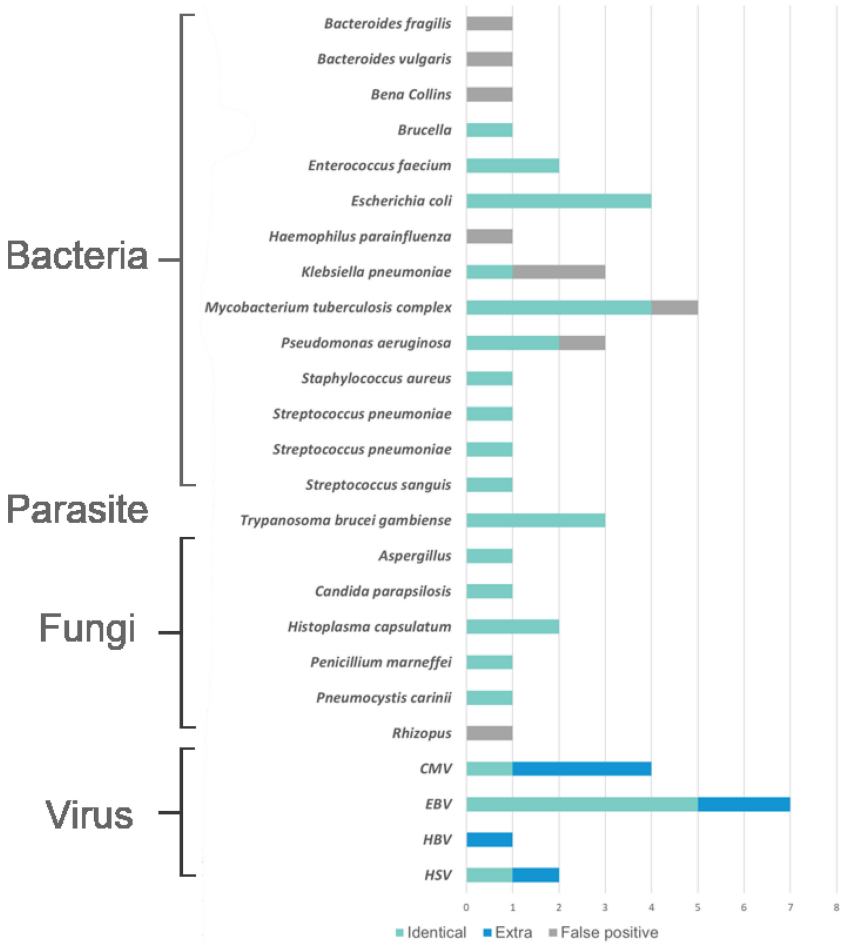


FIGURE 2 | The distribution of pathogens detected by metagenomic next generation sequencing (mNGS). The distributions were divided into four parts, namely, bacteria, parasites, fungi, and viruses, and each part consisted of identical detection (the blue), extra detection (the green), and false positive detection (the gray).

culture negative. None of the samples had only culture-positive results (**Table 3**). In our analysis, the concordance of mNGS and culture was not so consistent (Kappa value = 0.342, p = 0.000; McNemar, p = 0.000), and the possible reason may be that the total diagnostic efficacy of mNGS is higher than that of culture by 22.9% (**Table 3**).

We also evaluated pathogens extra detection rate of mNGS compared to other conventional methods in blood samples (**Table 4**). The total $PEDR_{mNGS/conventional\ methods}$ was 19.79%, the bloodstream infection of $PEDR_{mNGS/conventional\ methods}$ was 32%, and the non-bloodstream infection's $PEDR_{mNGS/conventional\ methods}$ was 6.52%. Overall, the total pathogens extra detection rate of mNGS was higher than that of conventional methods (19/96 vs. 3/96; OR, 6.333; p = 0.001) by 19.79% in all diseases, particularly in bloodstream infection by 32.0% (16/50 vs. 3/50; OR, 5.333; p = 0.004), while the pathogens extra detection rate of blood mNGS in non-bloodstream infection showed limited efficacy by 6.52% (**Table 4**). mNGS was also outstanding in the exclusion of infectious disease than conventional methods, according to the negative results of the tests in the NID (**Tables 3**, **4**).

Diagnostic Performance on Pathogens Extra Detection Rate of mNGS, Compared to Conventional Methods in Using Non-Blood Samples in Infection-Caused FUO

We also obtained a set of $PEDR_{mNGS/conventional\ methods}$ of non-blood samples to evaluate the diagnostic efficacy of mNGS. The total $PEDR_{mNGS/conventional\ methods}$ of non-blood samples in all infections was 20%, and non-bloodstream infection's $PEDR_{mNGS/conventional\ methods}$ was 26.2%, while the bloodstream infection's $PEDR_{mNGS/conventional\ methods}$ was only 7.7%, respectively. The use of mNGS in non-blood samples showed better performance than conventional methods in infectious diseases (9/45 vs. 2/45; OR, 4.5; p = 0.065) and improved the pathogens extra detection rate by 20%. What is more, especially in non-bloodstream infection, the application of non-blood samples mNGS increased the pathogens extra detection rate by

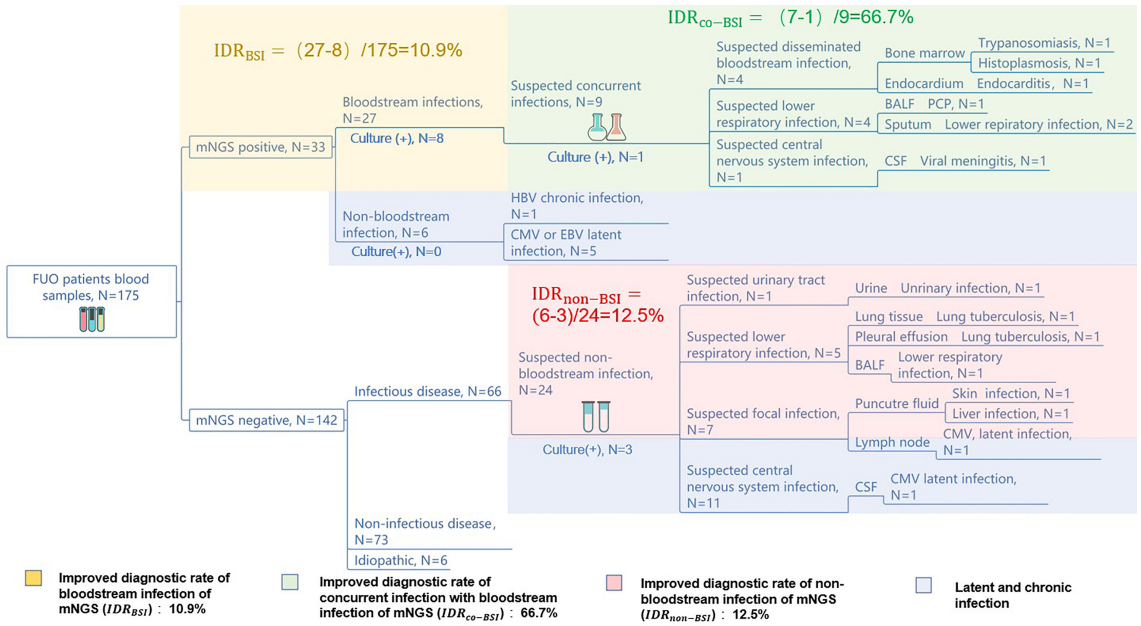


FIGURE 3 | mNGS improves the diagnosis rate of infectious diseases. The diagnostic workflows of the 175 FUO patients' pathogen identification. The yellow, green, red, and purple part show the mNGS' improving diagnostic rate of bloodstream infections, concurrent infection with bloodstream infection, non-bloodstream infection, and latent or chronic infection, respectively. The numbers of improved rate of mNGS were calculated by: $IDR = \frac{true\ mNGS\ positive\ cases - culture\ positive\ cases}{suspected\ infectious\ cases}$,.

26.2% compared to conventional methods (8/32 vs. 0/32; OR, ∞; p = 0.008) (**Table 5**).

The Application of mNGS in Assisting FUO Clinical Decision-Making and Improving Diagnostic Rate

To assist the clinical decision-making, we have summarized a diagnostic work flow (**Supplementary Figure 1**) of the 175 FUO patients comparing mNGS with culture. We have found that the application of blood mNGS as the first-line examination is an effective strategy in the diagnosis of infection-caused FUO, including bloodstream infection, concurrent infection with bloodstream infection, and non-bloodstream infection. We reported an IDR_{BSI} of 10.9%, an $IDR_{CO\text{-}BSI}$ of 66.7%, and an $IDR_{non\text{-}BSI}$ of 12.5%, respectively. Compared to culture, blood mNGS test can improve the diagnostic rate of bloodstream infection by 10.9%. The application of mNGS as the second-line test in the suspected infectious part can also improve the diagnostic rate of the FUO patients by 66.7% among those who have concurrent infection with BSI. Moreover, mNGS can diagnose more non-bloodstream infectious diseases than culture by 12.5%.

Furthermore, mNGS may diagnose the cases of latent or chronic viral infection (HBV, CMV, EBV, etc.), and those pathogens may be not the main cause of FUO but assist the later clinical decision-making.

DISCUSSION

The global burden of febrile illness is still difficult to quantify. Currently, the cause of fever disease is still not identified in approximately 9–51% of patients (Bleeker-Rovers et al., 2007; Shi et al., 2013; Kabapy et al., 2016). Despite advances in diagnostic

TABLE 3 | The diagnostic value of mNGS compared to culture in blood samples in FUO.

Disease	Samples	NGS(+)/culture(+)	NGS(+)/culture(–)	NGS(–)/culture(+)	NGS(–)/culture(–)	$PEDR_{mNGS/culture}$ (%)
Infectious disease	96	8	22	0	66	22.9%
Blood infection	50	8	19	0	23	38.0%
Non-blood infection	46	0	3*	0	43	6.5%
NIID	39	0	1*	0	38	
Tumor	16	0	2*	0	14	
Others	18	0	0	0	18	
Idiopathic	6	0	0	0	6	

These specimens additionally detect viruses such as CMV, EBV, and HBV, and virus chronic infection or in a latent infection is considered.

TABLE 4 | The diagnostic value of mNGS compared to conventional methods in blood samples in FUO.

Disease	Samples	NGS(+)/CM(+)	NGS(+)/CM(–)	NGS(–)/CM(+)	NGS(–)/CM(–)	$PEDR_{mNGS/conventional\ methods}$ (%)
Infectious disease	96	11	19	3	63	19.79%
Blood infection	50	11	16	3	20	32.0%
Non-blood infection	46	0	3*	0	43	6.52%
NIID	39	0	1*	0	38	
Tumor	16	0	2*	0	14	
Others	18	0	0	0	18	
Idiopathic	6	0	0	0	6	

These specimens additionally detect viruses such as CMV, EBV, and HBV, and virus chronic infection or in a latent infection is considered.

methods, the proportion of patients with undiagnosed FUO after the systematic examination has not decreased. The reason may be that with the improvements of medical care and diagnostic tools, diagnosis rates of common diseases are improved. However, cases that meet the classic definition of FUO are becoming increasingly complex (Shi et al., 2013; Robine et al., 2014).

Although the proportion of different causative diseases of FUO has changed over time, infectious factors remain the primary cause (Fusco et al., 2019). The complex infectious diseases pose a challenge for diagnostic and therapeutic determination. Unbiased mNGS represents a powerful tool to fill the gaps in our understanding of the etiology of FUO, thus informing the improvement of diagnostic algorithms. The previous study mentioned above has proved that mNGS can provide a 30%–50% extra detection rate in multiple types of infection such as focal infections, central nervous infections, and respiratory infections.

In this study, we conducted a retrospective cohort study to evaluate the diagnostic value of mNGS techniques in patients who were diagnosed with FUO. The PEDRmNGS/conventional methods of blood mNGS in infectious disease is 19.79%, which is higher than that of conventional methods, particularly in BSI that is 32.0%. Also, the PEDRconventional methods of non-blood mNGS in infection is 20%, which is also higher than that of conventional methods, especially in non-BSI that is 26.2%. Thus, the application of mNGS can help improve the clinical diagnosis by 38% in bloodstream infection and 6.5% in non-bloodstream infection. Our study showed that mNGS may act as a promising tool for the diagnosis of FUO patients. However, the results implied that if only blood samples are tested in patients of FUO, the clinical value of mNGS is relatively low. Only by collecting effective specimens from the part suspected of specific infection can the detection rate of the etiology be improved. The assessment and identification of etiology have improved the medical management of patients with FUO, helping to avoid the administration of unnecessary antibiotic therapy (Wylie et al., 2012).

What is more, mNGS is considered to have the latent to be used as an all-in-one diagnostic test, helping differentiate infections and non-infections, identify causative agents, and discover novel or emerging pathogens (Jerome et al., 2019). However, the expensive cost made mNGS not act as an all-purpose tool to resolve any uncertainty, but rather as a technology that cloud be used in specific contexts, such as seriously ill cases, or as a supplement to some special clinical cases (Chai et al., 2018).

In our study, we found the problem of the low detection rate of RNA virus, intracellular bacteria, and fungi. We listed the probable reasons of the low detection rate of RNA virus as follows. We apply the RNA meta-transcriptomics test process in all respiratory samples and did not apply the process in other samples considering the high expense of the mNGS process and higher RNA virus detection rate in respiratory samples. In

TABLE 5 | The diagnostic value of mNGS compared to conventional methods in non-blood samples in FUO.

Disease	Samples	Sample type	NGS(+)/CM(+)	NGS(+)/CM(–)	NGS(–)/CM(+)	NGS(–)/CM(–)	$PEDR_{conventional\ methods}$ (%)
Infection	45	/	8	9	2	26	20%
bloodstream infection	13	/	0	1	1	11	7.70%
	4	CSF	0	0	0	4	0.00%
	4	Tissue	0	1	1	2	25.00%
	3	Puncture fluid	0	0	0	3	0.00%
	1	Pleural fluid	0	0	0	1	0.00%
	1	BALF	0	0	0	1	0.00%
Non-blood stream infection	32	/	8	8	0	16	26.20%
	12	Tissue	2	3	0	7	25.00%
	9	CSF	1	2	0	6	22.22%
	2	Puncture fluid	2	0	0	0	0.00%
	4	Sputum	1	1	0	2	25.00%
	3	BALF	2	0	0	1	0.00%
	1	Urine	0	1	0	0	100.00%
	1	Pleural fluid	0	1	0	0	100.00%

addition, since we did not remove the human cellular RNA before the mNGS sequencing process, the virus' RNA genome is difficult to be detected compared to the human's cellular RNA yield. Thus, the detection rate of the RNA virus is relatively low in our study. In order to get a higher detection rate of RNA viruses and make a more accurate diagnosis of FUO, we need to perform the RNA meta-transcriptomics procedure to the samples and reassess the diagnostic rate in the further study.

The low detection rate of the fungi was mainly caused by the rigid cell walls; thus, many studies recommended adding bead beating to solve the problem. But at the same time, it also increases the host background of human DNA (Gu et al., 2021). Moreover, some studies are trying to add lyticase to increase the fungi's DNA release.

For the intracellular bacteria, the low detection rate is related to the limited copies of intracellular bacteria in plasma. Some studies have improved the protocol of the whole process by increasing the DNA and RNA's release of samples by resuspension in lysis buffer [10 mM Tris–HCl, 25 mM EDTA, 1% sodium dodecyl sulfate (SDS), 0.8 mg/ml, proteinase K] with a mixture of 1.0 mm zirconia and 0.1 mm of silica/zirconia beads, then going on the instructions of DNA extraction (Batinga et al., 2017). On the other hand, deepening the sequencing coverage of samples can be another method (Zhou et al., 2019).

Our study still had some limitations. First, this is a retrospective study with a small sample size in one single center; for the limited diseases and the patients' number, a larger cohort from some multicenter study may be needed to further confirm our results. Second, there are still six patients who have not finally been diagnosed in our cohort, which implied that the mNGS alone cannot act as the final diagnostic tool for the FUO diseases. To get the final outcome of those patients, we may need follow-up information in a longer period.

In conclusion, this single-center retrospective study found that using mNGS to detect pathogens in FUO holds higher diagnostic sensitivity than culture or other conventional methods. In FUO patients, particularly those suspected of infectious illnesses, utilizing blood mNGS as the first-line investigation can increase the diagnosis rate; using samples from other suspected infection sites as the optional second-line test could further improve diagnostic ability. However, because mNGS technology is expensive and the FUO disease spectrum is relatively broad, including a large number of non-infectious diseases, accurate and effective selection of target samples and specimens could contribute to better efficacy use of mNGS' unbiased and precise characteristics and is the key for significantly improving the patients' diagnostic rate and success rate of treatment. Overall, the application of mNGS in FUO patients serve as a promising optimized diagnostic protocol in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study was approved by the Huashan Hospital ethical committee the methods were carried out in accordance with the approved guidelines of the institution. The ethical number is No. Ky2017-338.

AUTHOR CONTRIBUTIONS

JA conceived and designed the study. ZF and HZ analyzed the data and collected the related clinical information. HWa and KL conducted clinical work associated with the study. YZhou, PC, and HWu provided technical support. YZhang and XZ wrote the draft. JA and WZ made a final revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (82002141), Shanghai Youth Science and Technology Talents Sailing Project (20YF1404300) and Special Projects for Medical Innovation Research (21Y11902100).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.745156/full#supplementary-material

REFERENCES

Batinga, M. C. A., Dos Santos, J. C., Lima, J. T. R., Bigotto, M. F. D., Muner, K., Faita, T., et al. (2017). Comparison of Three Methods for Recovery of Brucella Canis DNA From Canine Blood Samples. *J. Microbiol. Methods* 143, 26–31. doi: 10.1016/j.mimet.2017.08.019

Bleeker-Rovers, C. P., Vos, F. J., de Kleijn, E., Mudde, A. H., Dofferhoff, T. S. M., Richter, C., et al. (2007). A Prospective Multicenter Study on Fever of Unknown Origin: The Yield of a Structured Diagnostic Protocol. *Medicine* 86 (1), 26–38. doi: 10.1097/MD.0b013e31802fe858

Chai, J. H., Lee, C. K., Lee, H. K., Wong, N., Teo, K., Tan, C. S., et al. (2018). Cost-Benefit Analysis of Introducing Next-Generation Sequencing (Metagenomic) Pathogen Testing in the Setting of Pyrexia of Unknown Origin. *PloS One* 13 (4), e0194648. doi: 10.1371/journal.pone.0194648

Chiu, C. Y., and Miller, S. A. (2019). Clinical Metagenomics. *Nat. Rev. Genet.* 20 (6), 341–355. doi: 10.1038/s41576-019-0113-7

Fierer, N. (2017). Embracing the Unknown: Disentangling the Complexities of the Soil Microbiome. *Nat. Rev. Microbiol.* 15 (10), 579–590. doi: 10.1038/nrmicro.2017.87

Fusco, F. M., Pisapia, R., Nardiello, S., Cicala, S. D., Gaeta, G. B., and Brancaccio, G. (2019). Fever of Unknown Origin (FUO): Which Are the Factors Influencing the Final Diagnosis? A 2005-2015 Systematic Review. *BMC Infect. Dis.* 19 (1), 653. doi: 10.1186/s12879-019-4285-8

Gosiewski, T., Ludwig-Galezowska, A. H., Huminska, K., Sroka-Oleksiak, A., Radkowski, P., Salamon, D., et al. (2017). Comprehensive Detection and

Identification of Bacterial DNA in the Blood of Patients With Sepsis and Healthy Volunteers Using Next-Generation Sequencing Method - the Observation of DNAemia. *Eur. J. Clin. Microbiol. Infect. Dis.* 36 (2), 329–336. doi: 10.1007/s10096-016-2805-7

Gu, W., Deng, X., Lee, M., Sucu, Y. D., Arevalo, S., Stryke, D., et al. (2021). Rapid Pathogen Detection by Metagenomic Next-Generation Sequencing of Infected Body Fluids. *Nat. Med.* 27 (1), 115–124. doi: 10.1038/s41591-020-1105-z

Gu, W., Miller, S., and Chiu, C. Y. (2019). Clinical Metagenomic Next-Generation Sequencing for Pathogen Detection. *Annu. Rev. Pathol.* 14, 319–338. doi: 10.1146/annurev-pathmechdis-012418-012751

Hampton-Marcell, J. T., Lopez, J. V., and Gilbert, J. A. (2017). The Human Microbiome: An Emerging Tool in Forensics. *Microb. Biotechnol.* 10 (2), 228–230. doi: 10.1111/1751-7915.12699

Horowitz, H. W. (2013). Fever of Unknown Origin or Fever of Too Many Origins? *N. Engl. J. Med.* 368 (3), 197–199. doi: 10.1056/NEJMp1212725

Jerome, H., Taylor, C., Sreenu, V. B., Klymenko, T., Filipe, A. D. S., Jackson, C., et al. (2019). Metagenomic Next-Generation Sequencing Aids the Diagnosis of Viral Infections in Febrile Returning Travellers. *J. Infect.* 79 (4), 383–388. doi: 10.1016/j.jinf.2019.08.003

Kabapy, A. F., Kotkat, A. M., Shatat, H. Z., and Abd El Wahab, E. W. (2016). Clinico-Epidemiological Profile of Fever of Unknown Origin in an Egyptian Setting: A Hospital-Based Stud-2010). *J. Infect. Dev. Ctries* 10 (1), 30–42. doi: 10.3855/jidc.7198

Kucukardali, Y., Oncul, O., Cavuslu, S., Danaci, M., Calangu, S., Erdem, H., et al. (2008). The Spectrum of Diseases Causing Fever of Unknown Origin in Turkey: A Multicenter Study. *Int. J. Infect. Dis.* 12 (1), 71–79. doi: 10.1016/j.ijid.2007.04.013

Lagier, J. C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., et al. (2018). Culturing the Human Microbiota and Culturomics. *Nat. Rev. Microbiol 16*, 540–550. doi: 10.1038/s41579-018-0041-0

Langelier, C., Zinter, M. S., Kalantar, K., Yanik, G. A., Christenson, S., O'Donovan, B., et al. (2018). Metagenomic Sequencing Detects Respiratory Pathogens in Hematopoietic Cellular Transplant Patients. *Am. J. Respir. Crit. Care Med.* 197 (4), 524–528. doi: 10.1164/rccm.201706-1097LE

Mourad, O., Palda, V., and Detsky, A. S. (2003). A Comprehensive Evidence-Based Approach to Fever of Unknown Origin. *Arch. Intern. Med.* 163 (5), 545–551. doi: 10.1001/archinte.163.5.545

Naito, T., Tanei, M., Ikeda, N., Ishii, T., Suzuki, T., Morita, H., et al. (2019). Key Diagnostic Characteristics of Fever of Unknown Origin in Japanese Patients: A Prospective Multicentre Study. *BMJ Open* 9 (11), e032059. doi: 10.1136/bmjopen-2019-032059

Petersdorf, R. G., and Beeson, P. B. (1961). Fever of Unexplained Origin: Report on 100 Cases. *Medicine (Baltimore)* 40, 1–30. doi: 10.1097/00005792-196102000-00001

Robine, A., Hot, A., Maucort-Boulch, D., Iwaz, J., Broussolle, C., and Sève, P. (2014). Fever of Unknown Origin in the 2000s: Evaluation of 103 Cases Over Eleven Years. *Presse Med.* 43 (9), e233–e240. doi: 10.1016/j.lpm.2014.02.026

Shi, X. C., Liu, X. Q., Zhou, B. T., Zhang, L. F., Ma, X. J., Deng, G. H., et al. (2013). Major Causes of Fever of Unknown Origin at Peking Union Medical College Hospital in the Past 26 Years. *Chin. Med. J. (Engl.)* 126 (5), 808–812.

Szymanski, A. M., Clifford, H., and Ronis, T. (2020). Fever of Unknown Origin: A Retrospective Review of Pediatric Patients From an Urban, Tertiary Care Center in Washington, DC. *World J. Pediatr.* 16 (2), 177–184. doi: 10.1007/s12519-019-00237-3

Wilson, M. R., O'Donovan, B. D., Gelfand, J. M., Sample, H. A., Chow, F. C., Betjemann, J. P., et al. (2018). Chronic Meningitis Investigated via Metagenomic Next-Generation Sequencing. *JAMA Neurol.* 75 (8), 947–955. doi: 10.1001/jamaneurol.2018.0463

Wilson, M. R., Sample, H. A., Zorn, K. C., Arevalo, S., Yu, G., Neuhaus, J., et al. (2019). Clinical Metagenomic Sequencing for Diagnosis of Meningitis and Encephalitis. *N. Engl. J. Med.* 380 (24), 2327–2340. doi: 10.1056/NEJMoa1803396

Wright, W. F., and Auwaerter, P. G. (2020). Fever and Fever of Unknown Origin: Review, Recent Advances, and Lingering Dogma. *Open Forum Infect. Dis.* 7 (5), ofaa132–ofaa132. doi: 10.1093/ofid/ofaa132

Wright, W. F., Simner, P. J., Carroll, K. C., and Auwaerter, P. G. (2021). Progress Report: Next-Generation Sequencing (NGS), Multiplex Polymerase Chain Reaction (PCR), and Broad-Range Molecular Assays as Diagnostic Tools for Fever of Unknown Origin (FUO) Investigations in Adults. *Clin. Infect. Dis.* 2021, ciab155. doi: 10.1093/cid/ciab155

Wylie, K. M., Mihindukulasuriya, K. A., Sodergren, E., Weinstock, G. M., and Storch, G. A. (2012). Sequence Analysis of the Human Virome in Febrile and Afebrile Children. *PloS One* 7 (6), e27735. doi: 10.1371/journal.pone.0027735

Yohe, S., and Thyagarajan, B. (2017). Review of Clinical Next-Generation Sequencing. *Arch. Pathol. Lab. Med.* 141 (11), 1544–1557. doi: 10.5858/arpa.2016-0501-RA

Zhang, H. C., Ai, J. W., Cui, P., Zhu, Y. M., Hong-Long, W., Li, Y. J., et al. (2019). Incremental Value of Metagenomic Next Generation Sequencing for the Diagnosis of Suspected Focal Infection in Adults. *J. Infect.* 79 (5), 419–425. doi: 10.1016/j.jinf.2019.08.012

Zhang, Y., Cui, P., Zhang, H. C., Wu, H. L., Ye, M. Z., Zhu, Y. M., et al. (2020). Clinical Application and Evaluation of Metagenomic Next-Generation Sequencing in Suspected Adult Central Nervous System Infection. *J. Transl. Med.* 18 (1), 199. doi: 10.1186/s12967-020-02360-6

Zhao, F., and Bajic, V. B. (2015). The Value and Significance of Metagenomics of Marine Environments. Preface. *Genomics Proteomics Bioinf.* 13 (5), 271–274. doi: 10.1016/j.gpb.2015.10.002

Zhou, X., Wu, H., Ruan, Q., Jiang, N., Chen, X., Shen, Y., et al. (2019). Clinical Evaluation of Diagnosis Efficacy of Active Mycobacterium Tuberculosis Complex Infection via Metagenomic Next-Generation Sequencing of Direct Clinical Samples. *Front. Cell Infect. Microbiol.* 9, 351. doi: 10.3389/fcimb.2019.00351

Conflict of Interest: Authors YZ and HW were employed by company BGI-Shenzhen.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Fu, Zhang, Zhang, Cui, Zhou, Wang, Lin, Zhou, Wu, Wu, Zhang and Ai. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 25 January 2022
doi: 10.3389/fmed.2021.756592

Next Generation Sequencing for Diagnosis of Leptospirosis Combined With Multiple Organ Failure: A Case Report and Literature Review

Juan Lu[1†], *Juan Hu*[2†], *Shanshan Yu*[1] *and Lanjuan Li*[1*]

[1] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China, [2] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Intensive Care Unit, College of Medicine, The First Affiliated Hospital, Zhejiang University, Hangzhou, China

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Timothy Kudinha,
Charles Sturt University, Australia
Mohamed Helmy,
Agency for Science, Technology and Research (A*STAR), Singapore
Ammar Husami,
Cincinnati Children's Hospital Medical Center, United States
Ana Paula Loureiro,
Estácio de Sá University, Brazil

***Correspondence:**
Lanjuan Li
ljli@zju.edu.cn

[†]These authors share first authorship

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 10 August 2021
Accepted: 23 November 2021
Published: 25 January 2022

Citation:
Lu J, Hu J, Yu S and Li L (2022) Next Generation Sequencing for Diagnosis of Leptospirosis Combined With Multiple Organ Failure: A Case Report and Literature Review. Front. Med. 8:756592. doi: 10.3389/fmed.2021.756592

Introduction: Leptospirosis poses a major threat to human life. The disease spectrum ranges from a nearly undetectable presentation to severe multi-organ dysfunction and death. Leptospirosis is difficult to diagnose by traditional antibody and culture tests. We here present a case of multiple organ failure associated with leptospirosis.

Material and Methods: A 64-year-old woman presented with fatigue and arthralgia, which developed rapidly into multiple organ injuries, and she eventually died of cerebral hemorrhage. Serum antibody test and cultures of blood, sputum, urine, and feces samples were all negative. The patient was diagnosed with leptospirosis by the next-generation sequencing (NGS).

Conclusion: We conclude that leptospirosis is a neglected zoonosis caused by pathogenic *Leptospira* species. New techniques such as NGS are highlighted for early diagnosis. Surveillance for pathogens during diagnosis can provide guidance for clinical treatment and improves prognosis.

Keywords: leptospirosis, next generation sequencing, diagnosis, multiple organ failure, clinical treatment

INTRODUCTION

Leptospirosis is a widely distributed zoonosis and a major public health problem. Its clinical manifestations are complex and diverse; misdiagnosis is common and the fatality rate is high (1). Humans can be infected via direct or indirect contact with animal urine or excreta, making them unintentional and susceptible hosts. Leptospirosis ranges from a mild and self-limiting illness to severe disease and death (2). Early detection, accurate diagnosis, timely treatment, and close observation are essential to improve cure rate and decrease fatality rate (3). The classical bacterial culture process requires a long time (about 13 weeks). Moreover, the antibody detection has poor specificity and tends to yield false positive results (4, 5). New techniques including nucleic acid molecular diagnostic technologies, such as polymerase chain reaction (PCR) or high-throughput sequencing using next-generation sequencing (NGS) have been developed and applied in the detection of leptospirosis, which can provide valuable guidance for clinical treatment (5–8). Here, we present a case of leptospirosis caused by *Leptospira* interrogans associated with symptomatic multi-organ dysfunction.

CASE PRESENTATION

On October 13, 2020, a 64-year-old woman was referred to our hospital with fatigue, low blood pressure, arthralgia, and myalgia, for 1 week. She had a history of untreated hydronephrosis for 4 years and was positive for hepatitis B surface antigen for several decades. She did farm work in the mountain without personal protection 1 week prior to admission to a local hospital.

Her laboratory findings on admission were as follows: white blood cell (WBC) count 15.3 × 10^9/L; platelet (PLT) level 10 × 10^9/L; albumin level 22.7 g/L; alanine transaminase (ALT) level 79 U/L; aspartate aminotransferase (AST) level, 134 U/L; total bilirubin (TBI) level 105.3 μmol/L; direct bilirubin (DBI) level 60.6 μmol/L; creatinine level 87.3 μmol/L; blood urea nitrogen level 16.68 mmol/L; prothrombin time (PT), 18.6 s; activated partial thromboplastin time (APTT) 41.9 s; and D-dimer level 6,320 μg/L. Her blood pressure (120/73 mmHg) and breathing was stable.

Four days later, her blood pressure fell dramatically to 84/58 mmHg, jaundice appeared, and renal function deteriorated despite symptomatic treatment. Subsequently, she was transferred to our hospital and complained of generalized pain without fever and jaundice on the entire body. Physical examination revealed thickened wet rales in both lungs, abdominal distention, and edema in both lower limbs. Neurological examination was negative. Other signs were normal. The Glasgow Coma Scale score was 15/15 and blood pressure was 100/80 mmHg and pulse rate 98 beats/min (on noradrenaline maintenance) in the supine position. Laboratory parameters were markedly elevated: white blood cell count 57.0 x 10^9/L; total bilirubin 254.5 μmol/L; creatinine 263 μmol/L; procalcitonin 10.73 ng/mL; and C-reactive protein 59.3 mg/L. Chest radiography revealed obvious patchy shadows in both lungs. Supportive care included infusion of thrombocytes and plasma, liver protection, and relief of jaundice by high-flow oxygen therapy (60 L/min). Under an initial diagnosis of pneumonia, meropenem (1.0 g three times per day) was prescribed in combination with continuous renal replacement therapy.

On days 2–4 of hospitalization, the patient's condition worsened, and we commenced noninvasive assisted ventilation under high-flow oxygen (60 L/min). The patient's maximum body temperature was 37.2°C. Tracheoscopy revealed extensive hemorrhage in both airways. Brain computed tomography (CT) findings were normal (**Figure 1A1**). Chest CT revealed bilateral patchy shadows and diffuse infiltration and extensive consolidation in the right lung (**Figure 1B1**). Etiological cultures of blood, sputum, urine, and feces samples were performed; serum antibodies against dengue, leptospirosis, and epidemic hemorrhagic fever were tested for, considering the patient's history of farm work in the mountains. Bone marrow puncture was performed due to obviously abnormal blood routine examinations. Samples of sputum, urine, and feces were collected to detect *Leptospira* IgM and IgG antibodies, which were both negative. Multidisciplinary diagnosis and treatment were carried out by the Departments of Hematology, Infectious Disease, Pneumology, and Organ Transplantation, as well as the Intensive Care Unit. PCR was conducted using the diagnostic reagent kits based on the guidelines of the Center for Disease Control (CDC) of Zhejiang Province. Next-generation sequencing (NGS) of blood and sputum samples was also conducted. Supportive organ therapy was initiated. The patient developed multiple organ failure, but the cause was unclear. Entecavir (25 mg per day) and ornithine aspartate (40 mg per day) were administered to lower the high blood ammonia level. Her liver cirrhosis with ascites was evident on abdominal CT, possibly due to the patient's long history of hepatitis B surface antigen-positivity.

NGS revealed *Leptospira interrogans* (four different sequences of leptospires DNA, **Supplementary Material 1**) in blood on day 4 of hospitalization. Two family members who had engaged in farm work also underwent antibody test for leptospirosis, which revealed negative results. Her red blood cell count was decreased, and the free iron level was very high; chronic anemia was apparent. Meropenem was terminated and penicillin was initiated (four injections of 800,000 units per day) combined with dexamethasone (20 mg per day) to prevent the Hector reaction. Repeat chest CT indicated hydrothorax and relief of bilateral exudation (**Figure 1B2**).

On days 5–13 of hospitalization, The test of bone marrow puncture showed no obvious abnormality. However, the patient's liver function deteriorated; she was sedated under non-invasive assisted ventilation and administered continuous renal replacement therapy. Considering the severe jaundice and inflammatory response caused by the pulmonary infection and activation of hepatitis B virus, as well as liver failure, plasmapheresis was performed to remove inflammatory factors and improve liver function on days 8, 9, and 10. Piperacillin-tazobactam (4.5 g three times per day) was commenced because *Pseudomonas aeruginosa* was detected in sputum on day 10. Further sputum cultures revealed *Aspergillus fumigatus*; chest CT revealed improvement of the infiltrates in both lungs except for new patchy shadows in the right upper lung (**Figure 1B3**). Voriconazole (200 mg twice a day) was prescribed as antifungal therapy. The priorities at this time were symptomatic treatments including venous nutritional support, adjustment of the acid-base balance and circulatory parameters, prevention of gastrointestinal bleeding, and protection of organ parenchymae. On day 14, the patient suddenly vomited blood (200 mL) and then lost consciousness; her Glasgow Coma Scale score was suddenly decreased to 3/15. Emergency brain CT revealed hematoceles in both lateral ventricles and hemorrhage in the right paraoccipital lobe that had ruptured the lateral ventricle (**Figure 1A2**). Chest CT was also repeated (**Figure 1B4**). We communicated with the family members about the patient's condition and explained that no further treatment options were available. The patient died 5 h later.

DISCUSSION

Leptospirosis is sporadic in the temperate regions around the world. It is an epidemic-prone disease carrying the causative agents in the damaged mucous membranes, and is generally caused by exposure to contaminated water. The

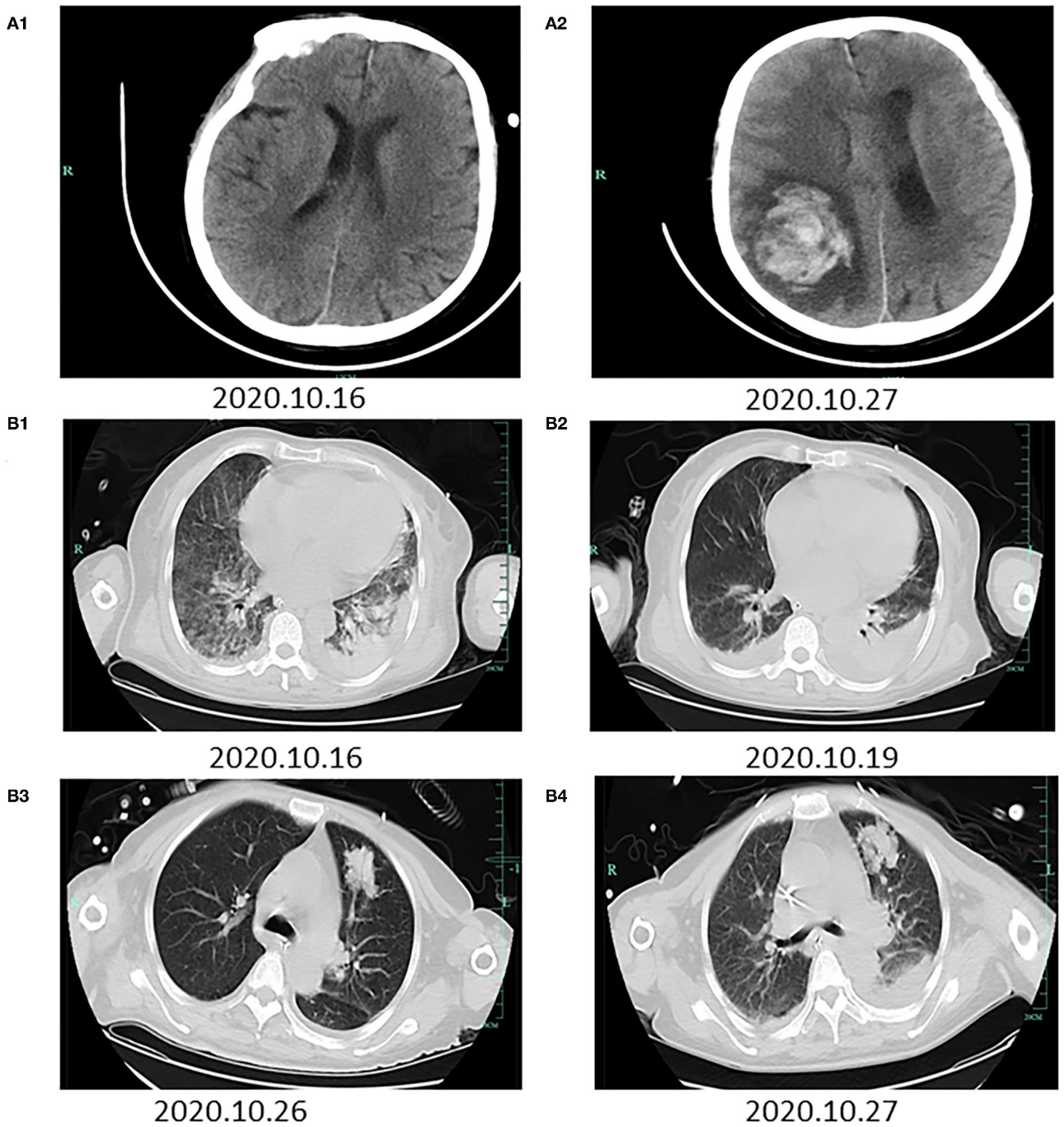


FIGURE 1 | Radiographic images during the hospitalization. The first row of brain CTs **(A1,A2)** was taken on October 16 and 27, 2020, respectively. **(A1)** A normal brain. **(A2)** Hemorrhage in the right paraoccipital lobe that had ruptured the lateral ventricle. The second and third row of chest CTs **(B1–B4)** was taken on October 16, 19, 26, and 27, 2020, respectively. **(B1)** Bilateral patchy shadows and diffuse infiltration and extensive consolidation in the right lung. **(B2)** Hydrothorax and relief of bilateral exudation. **(B3)** New patchy shadows in the right upper lung. **(B4)** Patchy shadows in the right upper lung and bilateral exudation.

clinical manifestations of leptospirosis are complex and easily mimic other life-threatening infections, and diagnosis and differential diagnosis are particularly important. Our patient initially presented with fatigue and arthralgia, followed by rapid multiple organ damage, which rendered early diagnosis difficult. It is crucial to perform early antibody and culture tests. Liver failure was obvious, suggesting the presence of jaundice, coagulation defect, and history of hepatitis B-associated liver cirrhosis. However, other diseases should also be considered because of the abnormal blood tests, poor renal function, and subsequent multiple organ dysfunction. Although leptospirosis antibody results were negative, the presence of pathogens

could not be completely ruled out as leptospires are frequently found in low numbers. Several diagnostic tests are available for leptospirosis including detection of the pathogen and antibodies. These tests are particularly useful in the early stage of this disease and before the use of antimicrobial drugs when bacterial numbers are highest in the blood and urine (9). *Leptospira* culture allows detection of leptospires, but it is technically difficult due to the demand of special growth media (10). Because leptospires are fastidious so as to require enrichment media and long incubation time under *in vitro* conditions independent of infection date, culture is less helpful for clinical diagnosis (11). In the traditional antibody tests, MAT is often used (12). However, identification of the infecting serovar is difficult based on the cross-reactive MAT results on account of laboratory variation and differences in host-specific humoral immune responses (13). Molecular diagnostic tools, such as PCR, have become widely available in commercial diagnostic laboratories for the diagnosis of leptospirosis (14). False-negative results can be encountered if there are low bacterial loads as a result of disease phase, immune response, or administration of antimicrobial drugs (15, 16). Several ELISAs for detecting IgM, IgG, or both antibody types have been developed to detect specific antibodies in leptospirosis. However, its overall sensitivity is lower compared with the MAT, which has not been previously recommended (17, 18).

Patients may benefit from next-generation sequencing (NGS), which is a method that allows for high-throughput, massively parallel sequencing of thousands to billions of DNA fragments independently and simultaneously. Over the past five years, NGS testing has been used not only in basic research but also in clinical diagnosis, especially are applied in clinical microbiology examinations (19). The NGS testing for our case showed the evidence of four different sequences of leptospires DNA (**Supplementary Material 2**). NGS in microbiology examinations can reveal the characteristics of pathogens by morphology and a genomic definition of pathogens (20). If only culture is performed, diagnosis of severe and acute infection may be missed, leading to inadequate or delayed treatment, and increased morbidity and mortality (21). Appropriate antimicrobial treatment in combination with supportive therapy would be beneficial to reducing the mortality from this disease. Due to the difficulty in the cultivation or poor growth rate of some organisms, as well as the use of prophylactic antimicrobial drugs, conventional testing methods used in microbiological diagnosis are limited in pathogen detection, thus compromising the effectivity of antimicrobial drugs (22). From this perspective, NGS is a promising and sensitive method to detect the selected organism types and discover early, new or unexpected organisms (23).

Most deaths from leptospirosis are attributed to renal failure and/or gastrointestinal, pulmonary, or cerebral hemorrhage (24–26) caused by extensive capillary damage including extensive renal necrosis and glomerular atrophy. Some bacterial pathogens produce hemolysins including Sph2, and the expression of Sph2 is highest among epithelial cells (27). *L. interrogans* Sph2 was recently found to trigger inflammatory reaction, cytomembranous injury, and apoptosis by damaging the membranes of blood vessel endothelial cells through increasing intracellular reactive oxygen species (ROS) and decreasing the mitochondrial membrane potential (MMP). Moreover, *Leptospira* increases the permeability and engorgement of blood vascular cells (28–30). In addition, platelet aggregation, a crucial player in the blood coagulation process, is inactivated independent of PI3K/AKT-ERK signaling pathways, thereby causing hemorrhage in tissues (31). Activation of coagulation factor-III extends the coagulation time, and is associated with pulmonary, renal, and cerebral hemorrhage, leading to death due to severe types of leptospirosis (32, 33).

CONCLUSION

Leptospirosis is a neglected zoonosis caused by pathogenic *Leptospira*. It ranges from a mild and self-limiting illness to severe disease and death. The correct diagnosis could be established by actively adopting new examination methods such as NGS, which should be performed early to avoid misdiagnosis. The NGS technology represents a useful opportunity and tool for precise diagnostics to identify pathogens in infectious diseases.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JL and LL structured the text and content. JL and JH wrote and edited the manuscript and generated the figures. SY reviewed the literature. All authors contributed intellectually to manuscript content, read, and agreed to the published version of the manuscript.

FUNDING

This research was funded by the Independent Project Fund of the State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the National Key Research and Development Program of China (Grant No. 2016YFC1101404/3), and Zhejiang Basic Public Welfare Research Program of China (Grant No. LQ20H030012).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.756592/full#supplementary-material

REFERENCES

1. Ghazaei C. Pathogenic leptospira: advances in understanding the molecular pathogenesis and virulence. *Open Vet J.* (2018) 8:13–24. doi: 10.4314/ovj.v8i1.4
2. Guerrier G, D'Ortenzio E. The jarisch-herxheimer reaction in leptospirosis: a systematic review. *PLoS ONE.* (2013) 8:e59266. doi: 10.1371/journal.pone.0059266
3. Noda AA, Grillova L, Mariet JF, Paiffer NB, Ruiz YC, Rodriguez I, et al. A first insight into the genomic diversity of leptospira strains isolated from patients in Cuba. *PLoS ONE.* (2020) 15:e0229673. doi: 10.1371/journal.pone.0229673
4. Alinaitwe L, Kankya C, Allan KJ, Rodriguez-Campos S, Torgerson P, Dreyfus A. Bovine leptospirosis in abattoirs in Uganda: molecular detection and risk of exposure among workers. *Zoonoses Public Health.* (2019) 66:636–46. doi: 10.1111/zph.12616
5. Eldin C, Jaulhac B, Mediannikov O, Arzouni JP, Raoult D. Values of diagnostic tests for the various species of spirochetes. *Med Mal Infect.* (2019) 49:102–11. doi: 10.1016/j.medmal.2019.01.009
6. Mahtab M, Khan F, Azam M, Rizvi M, Sultan A, Shukla I, et al. Molecular characterization and phylogenetic analysis of human pathogenic leptospira species circulating in a tertiary care hospital of Western Uttar Pradesh in India. *Pathog Glob Health.* (2019) 113:275–81. doi: 10.1080/20477724.2019.1685196
7. Barry R, Nissly RH, Feria W, Thirumalapura N, Tewari D, Jayarao BM, et al. A probe-based real-time PCR assay for the detection of *Neospora caninum* in clinical samples from cattle. *Vet Parasitol.* (2019) 269:2–6. doi: 10.1016/j.vetpar.2019.04.002
8. Di Azevedo MIN, Lilenbaum W. An overview on the molecular diagnosis of animal leptospirosis. *Lett Appl Microbiol.* (2021) 72:496–508. doi: 10.1111/lam.13442
9. Fraune CK, Schweighauser A, Francey T. Evaluation of the diagnostic value of serologic microagglutination testing and a polymerase chain reaction assay for Diagnosis of Canine Leptospirosis diagnosis of acute leptospirosis in dogs in a referral center. *J Am Vet Med Assis.* (2013) 242:1373–80. doi: 10.2460/javma.242.10.1373
10. Faucher JF, Hoen B, Estavoyer JM. The management of leptospirosis. *Expert Opin Pharmacother.* (2004) 5:819–27. doi: 10.1517/14656566.5.4.819
11. Rodriguez J, Blais M, Lapointe C, Carioto L, Harel J. Feline leptospirosis: a serologic and urinary PCR survey in healthy cats and in cats with kidney disease. In: *Proceedings of the 30th Annual Congress of the American College of Veterinary Internal Medicine (ACVIM).* New Orleans, LA (2012). p. 790–791.
12. Sykes JE, Hartmann K, Lunn KF, Moore GE, Stoddard RA, Goldstein RE, et al. 2010 ACVIM Small animal consensus statement on leptospirosis: diagnosis, epidemiology, treatment, and prevention. *J Vet Intern Med.* (2011) 25:1–13. doi: 10.1111/j.1939-1676.2010.0654.x
13. Markovich JE, Ross L, McCobb E. The prevalence of leptospiral antibodies in free roaming cats in Worcester County, Massachusetts. *J Vet Intern Med.* (2012) 26:688–9. doi: 10.1111/j.1939-1676.2012.00900.x
14. Harkin KR, Roshto YM, Sullivan JT, Purvis TJ, Chengappa MM. Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. *J Am Vet Med Assoc.* (2003) 222:1230–3. doi: 10.2460/javma.2003.222.1230
15. Levett PN, Morey RE, Galloway RL, Turner DE. Detection of pathogenic leptospires by real-time quantitative PCR. *J Med Microbiol.* (2005) 54:45–9. doi: 10.1099/jmm.0.45860-0
16. Waggoner JJ, Pinsky BA. Molecular diagnostics for human leptospirosis. *Curr Opin Infect Dis.* (2016) 29:440–5. doi: 10.1097/QCO.0000000000000295
17. Abdoel TH, Houwers DJ, van Dongen AM, Adesiyun AA, Jiménez-Coelloe M, Cardoso L, et al. Rapid test for the serodiagnosis of acute canine leptospirosis. *Vet Microbiol.* (2011) 150:211–3. doi: 10.1016/j.vetmic.2011.01.015
18. Eric Klaasen HL, Adler B. Recent advances in canine leptospirosis: focus on vaccine development. *Vet Med (Auckl).* (2015) 19:245–60. doi: 10.2147/VMRR.S59521
19. Simner PJ, Miller S, Carroll KC. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. *Clin Infect Dis.* (2018) 66:778–88. doi: 10.1093/cid/cix881
20. Behjati S, Tarpey PS. What is next generation sequencing? *Arch Dis Child Educ Pract Ed.* (2013) 98:236–8. doi: 10.1136/archdischild-2013-304340
21. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol.* (2019) 14:319–38. doi: 10.1146/annurev-pathmechdis-012418-012751
22. Wilson MR, Naccache SN, Samayoa E, Biagtan M, Bashir H, Yu G, et al. Actionable diagnosis of neuroleptospirosis by next-generation sequencing. *N Engl J Med.* (2014) 370:2408–17. doi: 10.1056/NEJMoa1401268
23. Bleeker-Rovers CP, Vos FJ, de Kleijn E, Mudde AH, Dofferhoff TSM, Richter C, et al. A prospective multicenter study on fever of unknown origin: the yield of a structured diagnostic protocol. *Medicine.* (2007) 86:26–38. doi: 10.1097/MD.0b013e31802fe858
24. Hartskeerl RA, Collares-Pereira M, Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clin Microbiol Infect.* (2011) 17:494–501. doi: 10.1111/j.1469-0691.2011.03474.x
25. Vijayachari P, Sugunan AP, Shriram AN. Leptospirosis: an emerging global public health problem. *J Biosci.* (2008) 33:557–69. doi: 10.1007/s12038-008-0074-z
26. Dong WH, Chen Z. Leptospirosis with pulmonary haemorrhage and multiple organ failure: a case report and literature review. *Int Med Res.* (2021) 49:3000605211019665. doi: 10.1177/03000605211019665
27. Che R, Ding S, Zhang Q, Yang W, Yan J, Lin X. Haemolysin Sph2 of leptospira interrogans induces cell apoptosis via intracellular reactive oxygen species elevation and mitochondrial membrane injury. *Cell Microbiol.* (2019) 21:e12959. doi: 10.1111/cmi.12959
28. Narayanavari SA, Lourdault K, Sritharan M, Haake DA, Matsunaga J. Role of sph2 gene regulation in hemolytic and sphingomyelinase activities produced by leptospira interrogans. *PLoS Negl Trop Dis.* (2015) 9:e0003952. doi: 10.1371/journal.pntd.0003952
29. Matsunaga J, Haake DA. cis-Acting determinant limiting expression of sphingomyelinase gene sph2 in leptospira interrogans, identified with a gfp reporter plasmid. *Appl Environ Microbiol.* (2018) 84:e02068–18. doi: 10.1128/AEM.02068-18
30. Zavala-Alvarado C, Sismeiro O, Legendre R, Varet H, Bussotti G, Bayram J, et al. The transcriptional response of pathogenic leptospira to peroxide reveals new defenses against infection-related oxidative stress. *PLoS Pathog.* (2020) 16:e1008904. doi: 10.1371/journal.ppat.1008904
31. Fang JQ, Imran M, Hu WL, Ojcius DM, Li Y, Ge YM, et al. vWA proteins of leptospira interrogans induce hemorrhage in leptospirosis by competitive inhibition of vWF/GPIb-mediated platelet aggregation. *EBioMedicine.* (2018) 37:428–41. doi: 10.1016/j.ebiom.2018.10.033
32. Barthelemy A, Magnin M, Pouzot-Nevoret C, Bonnet-Garin JM, Hugonnard M, Goy-Thollot I. Hemorrhagic, hemostatic, and thromboelastometric disorders in 35 dogs with a clinical diagnosis of leptospirosis: a prospective study. *J Vet Intern Med.* (2017) 31:69–80. doi: 10.1111/jvim.14626
33. Passalia FJ, Heinemann MB, de Andrade SA, Nascimento A, Vieira ML. Leptospira interrogans bat proteins impair host hemostasis by fibrinogen cleavage and platelet aggregation inhibition. *Med Microbiol Immunol.* (2020) 209:201–13. doi: 10.1007/s00430-020-00664-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Lu, Hu, Yu and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 28 January 2022
doi: 10.3389/fmed.2022.819425

Case Report: Community-Acquired *Legionella gormanii* Pneumonia in an Immunocompetent Patient Detected by Metagenomic Next-Generation Sequencing

Cheng Lei[1,2,3†], *Xianglin Zhou*[1,2,3†], *Shuizi Ding*[1,2,3†], *Yingjie Xu*[1,2,3], *Binyi Yang*[1,2,3], *Wei Guo*[1,2,3], *Min Song*[1,2,3], *Min Yang*[1,2,3], *Yunan Jia*[1,2,3*] and *Hong Luo*[1,2,3*]

[1] Department of Pulmonary and Critical Care Medicine, The Second Xiangya Hospital, Central South University, Changsha, China, [2] Research Unit of Respiratory Disease, Central South University, Changsha, China, [3] Hunan Diagnosis and Treatment Center of Respiratory Disease, Changsha, China

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UCONN Health, United States

Reviewed by:
Ronald Balczon,
University of South Alabama, United States
Jason Sahl,
Northern Arizona University, United States

***Correspondence:**
Yunan Jia
jiayunan@csu.edu
Hong Luo
luohonghuxi@csu.edu.cn

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 21 November 2021
Accepted: 07 January 2022
Published: 28 January 2022

Citation:
Lei C, Zhou X, Ding S, Xu Y, Yang B, Guo W, Song M, Yang M, Jia Y and Luo H (2022) Case Report: Community-Acquired Legionella gormanii Pneumonia in an Immunocompetent Patient Detected by Metagenomic Next-Generation Sequencing. Front. Med. 9:819425. doi: 10.3389/fmed.2022.819425

Background: *Legionella* spp. has been well-recognized as an important cause of community-acquired pneumonia. Current community-acquired pneumonia guidelines recommended covering the treatment of *Legionella* because of the high mortality associated with inadequate antibiotic treatments. However, the symptom of *Legionella* pneumonia is non-specific, and routine diagnostic tests exhibit low sensitivity for *Legionella* spp., especially for non-*Legionella pneumophila* serogroup 1 strains.

Case Presentation: We report a 53-year-old man without underlying diseases admitted to respiratory intensive care unit because of severe community-acquired pneumonia and respiratory failure. Although, the results of routine culture of bronchoalveolar lavage fluid and the *Legionella* urinary antigen test were all negative, metagenomic next-generation sequencing (mNGS) identified a great amount of DNA and RNA sequences of *Legionella gormanii* in bronchoalveolar lavage fluid while negative in blood sample. The presence of *Legionella gormanii* in bronchoalveolar lavage fluid was further confirmed by polymerase-chain-reaction and Sanger sequencing.

Conclusion: *Legionella gormanii* has rarely been reported in patients with community-acquired pneumonia mainly due to lack of diagnostic test for non-*Legionella* pneumophila serogroup 1 strains. This is the first report of *Legionella gormanii* pneumonia in an immunocompetent patient detected by mNGS, which indicates that mNGS is a high-resolution and sensitive assay for the diagnosis and surveillance of *Legionella* infection.

Keywords: metagenomic next-generation sequencing (mNGS), *Legionella gormanii*, community-acquired pneumonia, pathogen diagnosis, bronchoalveolar lavage fluid

INTRODUCTION

Legionella is a Gram-negative fastidious bacterium and a common cause of atypical community-acquired pneumonia (1). The mortality rate of community-acquired *Legionella* pneumonia has decreased from 26% in 1985 to 10% in 1998, which may relate to the development of the sensitive urine antigen test or the changes in empirical antibiotic treatment strategies (2). However, the

genus *Legionella* comprises more than 65 different species (3), and the current widely used urine antigen test is only available to detect *Legionella pneumophila* serogroup 1 (4). The diagnosis of other serogroups and non-*Legionella* pneumophila species infection is difficult.

Metagenomic next-generation sequencing (mNGS) can directly identify the pathogens' sequences in clinical samples and has been applied in clinical infectious disease diagnostics including pneumonia (5–7). Here, we report a case of severe community-acquired pneumonia in a 53-year-old man, in which the conventional pathogen-detecting tests were all negative including legionella urinary antigen test, while a great amount of DNA (7,957 in a total of 21,977,576 sequences) and RNA (3,443 in a total of 12,763,560 sequences) sequences of *Legionella gormanii* in bronchoalveolar lavage fluid were identified by mNGS. After quinolones antibiotic therapy, the patient recovered.

Only five cases of *Legionella gormanii* pneumonia have been reported in PubMed, and all these reported patients were immunosuppressed (8–12). The present case represents the first case of community-acquired *Legionella gormanii* pneumonia in an immunocompetent patient detected by mNGS.

CASE DESCRIPTION

A previously healthy, 53-year-old male patient presented with a 7-day history of cough and chest pain, then a 4-day history of fever and dyspnea. He was referred to respiratory intensive care unit on September 28, 2021 (day 1). The patient had a smoking history of 40 years with a pack of cigarettes every day. He was a handyman but denied recent travel history, or any cooling systems or other special man-made water system exposure. A chest computed tomography (CT) taken on September 26 (day −2) showed consolidation in the left upper lobe and left lower lobe, left pleural effusion, and bullae in the posterior segment of the right upper lobe (**Figure 1**). Since he presented with hypoxia, high-flow nasal cannula oxygen therapy was used.

On admission, the laboratory examination results were as follows: white blood cell (WBC) count was 26.12×10^9/L, with a neutrophil percentage of 93.6%, the concentration of procalcitonin (PCT) was 5.39 ng/mL, the level of C-reactive protein was 167.21 mg/L, the CD4+ lymphocyte count was 245/μl, and the serum β-(1,3)-D-glucan and galactomannan were normal. The empiric antibiotic therapy included piperacillin-tazobactam and moxifloxacin (**Figure 2**). On day 2, his hypoxia progressed and he started to be supported by noninvasive ventilation with 90% FiO_2, inspiratory positive airway pressure of 12 cm H_2O, and expiratory positive airway pressure of 8 cm H_2O. To search the infectious etiology of pneumonia, bronchoalveolar lavage fluid (BALF) were harvested and sent with a blood sample to perform mNGS (KingMed Diagnostics, Changsha, China). The methods of BALF and blood mNGS were described in **Supplementary File**. The BALF culture for bacteria and fungi, galactomannan, polymerase-chain-reaction (PCR) for *Mycobacterium tuberculosis*, and *Legionella* urine antigen test (KingMed Diagnostics, Guangzhou, China) were negative. On day 3, the antibiotics were adjusted to meropenem and moxifloxacin, which were more broad-spectrum.

The BALF mNGS results reported *Legionella gormanii* infection on day 4 (**Figure 3**). DNA mNGS detected 7,957

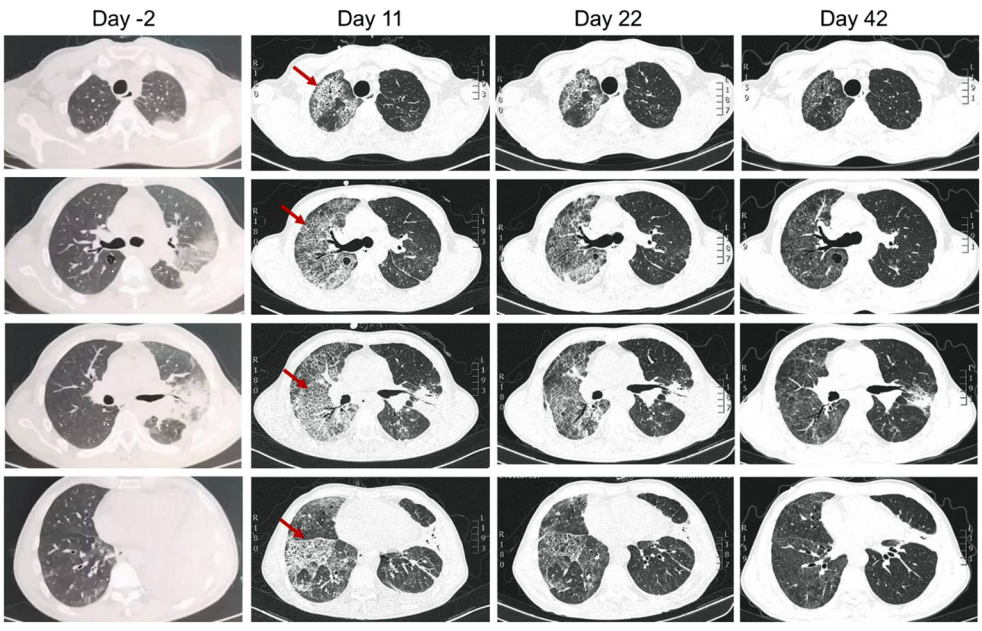


FIGURE 1 | Chest computed tomography of the patient on 2 days before admission (day −2), and day 11, 22, and 42 (20 days after discharge) after admitted to our hospital. Arrows indicated the right lung developed interstitial infiltrates on day 11.

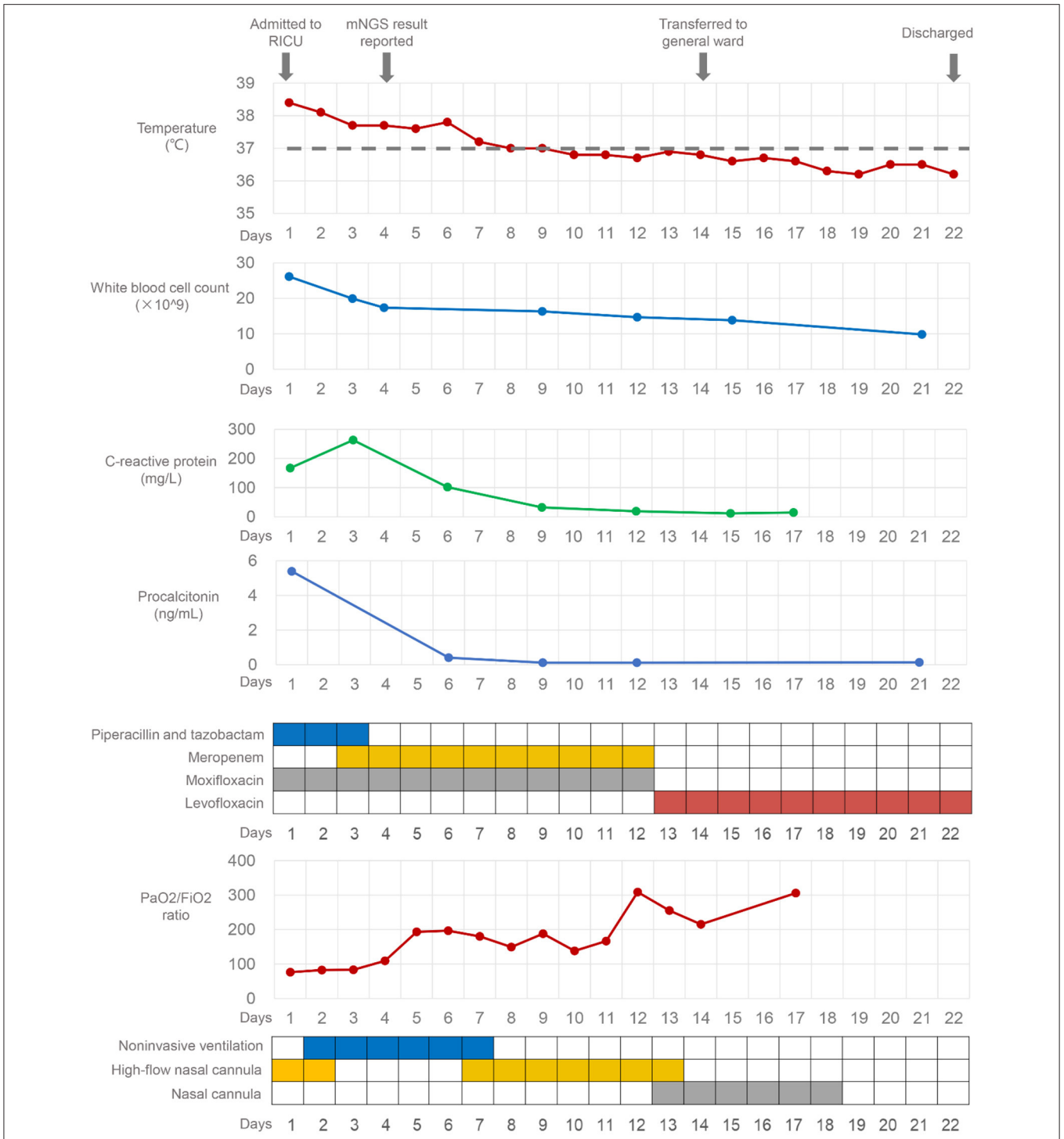


FIGURE 2 | The clinical course of the patient with *Legionella gormanii* infection.

sequences which can be mapped to *Legionella gormanii* in a total of 21,977,576 sequences, and the coverage was 13.52%, making up 95.76% of the total microbe sequences (**Figures 3A,B**). RNA mNGS detected 3,443 sequences which can be mapped to *Legionella gormanii* in a total of 12,763,560 sequences, and the coverage was 4.24%, making up 47.85% of the total microbe sequences (**Figures 3C,D**). However, the blood mNGS results for DNA and RNA were both negative. Targeted PCR of *Legionella gormanii* in BALF using two pairs of primers was applied: Primer1 forward 5′-CTCCGCCCACATCAATCGTA-3′,

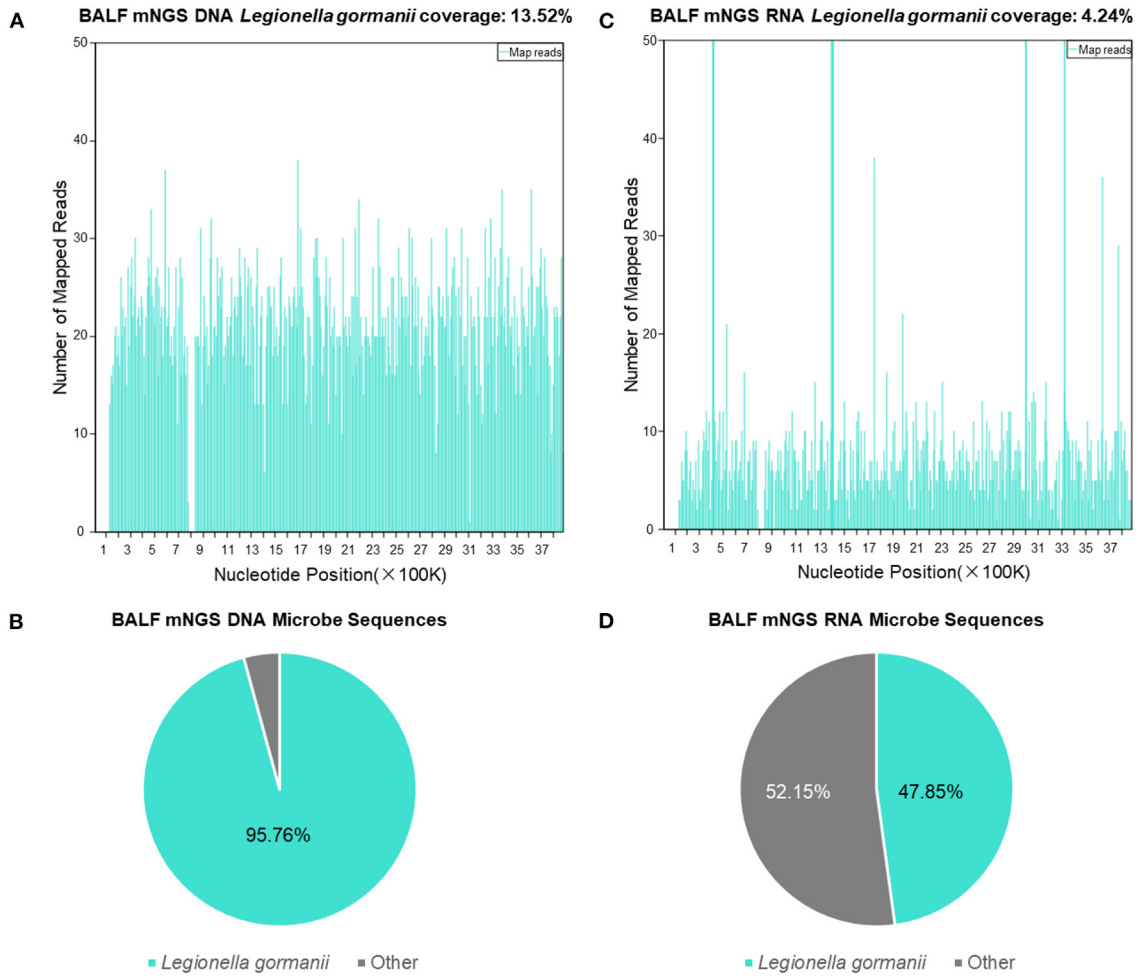


FIGURE 3 | The bronchoalveolar lavage fluid mNGS results of the patient. **(A)** The DNA mNGS results showed that the coverage of *Legionella gormanii* was 13.52%. **(B)** 95.76% of the microbe DNA sequences were *Legionella gormanii*. **(C)** The RNA mNGS results showed that the coverage of *Legionella gormanii* was 4.24%. **(D)** 47.85% of the microbe RNA sequences were *Legionella gormanii*.

reverse 5′-AAACCCGAACTCATGGCAGT-3′; Primer2 forward 5′-GAAAGTGGTATGGTGCGGGA-3′, reverse 5′-CTCTTCACGACTTGCACCCT-3′. The primers were designed and verified using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the reference genome sequence of *Legionella gormanii* in NCBI (https://www.ncbi.nlm.nih.gov/). The capillary electrophoresis technique (Qsep 100^{TM}; Bioptic) and Sanger sequencing confirmed the *Legionella gormanii* infection (**Figure 4**). The patient was finally diagnosed with community-acquired *Legionella gormanii* pneumonia.

After 1 week of therapy, the patient's body temperature, hypoxia, and inflammatory biomarkers improved (**Figure 2**). Chest CT on day 11 showed consolidation in the left upper and lower lobe of the lung improved, while the right lung developed interstitial infiltrates (**Figure 1**). On day 13, the oxygen therapy was changed to nasal cannula, and the antibiotics were de-escalated to levofloxacin monotherapy due to mild elevated liver enzyme (ALT 175.7 U/L, AST 82.8 U/L). The patient was transferred to the general ward on day 14. Chest CT on day 22 showed the infection had improved, and subsequently, the patient was discharged. He took oral levofloxacin therapy at home for 3 weeks, and the chest CT on day 42 (20 days after discharge) showed marked improvement in the lung (**Figure 1**). The patient recovered well without any complications, and he had return to work and normal activities on the last follow-up (2 months after discharge).

DISCUSSION AND CONCLUSIONS

Legionella spp. is an important etiology of community-acquired pneumonia, and *Legionella pneumophila* causes ∼90% of all reported cases, 79% of which are caused by *Legionella pneumophila* serogroup 1 (13). Non-*Legionella pneumophila* spp. infection occurred more commonly in patients with immunosuppression than immunocompetent patients, in which

the most common species are *Legionella micdadei*, *Legionella bozemanae*, and *Legionella dumoffii* (14). *Legionella gormanii*, previously known as *Fluoribacter gormanii*, was the first non-*Legionella pneumophila* species isolated in China (15), however, it has not been reported previously in Chinese patients, and this case is the first to report *Legionella gormanii* pneumonia in an immunocompetent patient.

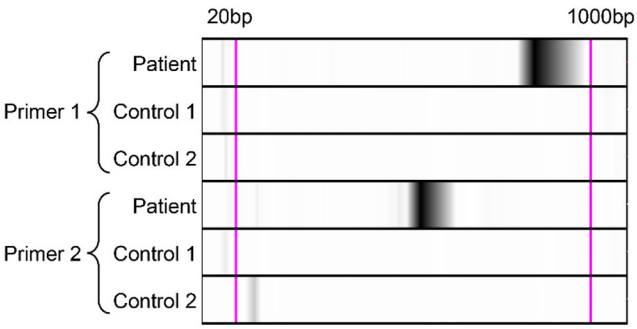


FIGURE 4 | Polymerase-chain-reaction and the capillary electrophoresis technique confirmed the *Legionella gormanii* infection in the patient. Lane Patient: the bronchoalveolar lavage fluid sample of the patient; Lane Control 1: the blank control; Lane Control 2: the negative control, the bronchoalveolar lavage fluid sample from a patient who was finally diagnosed without *Legionella* pneumonia.

Culture is the gold standard to diagnose *Legionella* infection, but only experienced laboratories can isolate *Legionella* successfully, and its sensitivity is highly variable ranging from <10 to 80% (4). Legionella urinary antigen test is the most widely used method to diagnose *Legionella* infection, and it has largely improved the diagnostic rate of *Legionella* infection. The sensitivity of the urinary antigen test ranged from 70 to 100%, and its specificity can approach 100% (16). However, the urinary antigen test is only reliable for the detection of *Legionella pneumophila* serogroup 1. Metagenomic next-generation sequencing may circumvent the disadvantages of culture and legionella urinary antigen test as it can profile the pathogens comprehensively. mNGS achieves higher taxonomy resolution compared to 16S rRNA gene sequencing and has been applied to pneumonia diagnosis, outbreak tracking, infection control surveillance, and pathogen discovery (5). The clinical manifestations of *Legionella pneumonia* are non-specific, and the diagnosis mainly relies on the laboratory pathogen examination. In our patient, the urinary antigen test was negative, and our laboratory was not able to conduct the *Legionella* culture. *Legionella gormanii* was detected by DNA and RNA BALF mNGS, and subsequently targeted PCR and Sanger sequencing validated *Legionella gormanii* infection. Although the current

TABLE 1 | Summary of cases with *Legionella gormanii* pneumonia.

Publication year	Gender	Age	Immunosuppressed status	Underlying diseases	Radiological features	Diagnostic test	Targeted antimicrobial therapy	Prognosis
1988 (8)	Female	64	High-dosage prednisone therapy	Systemic lupus erythematosus, adenocarcinoma	Right lower lobe pneumonia	Culture of bronchoalveolar lavage fluid	Erythromycin	Recovered
1989 (9)	Male	6	Primary immunodeficiency	Chronic granulomatous disease	Consolidation in the left lower and left upper lobes and a small left pleural effusion	Direct fluorescent antibody staining of the pleural exudate	Erythromycin and rifampin	Recovered
1994 (10)	Male	47	Oral prednisone (10 mg every other day), chronic lymphocytic leukemia	Chronic lymphocytic leukemia	Right middle and lower lobe infiltrate	Culture of bronchoalveolar lavage fluid	Erythromycin and rifampin	Recovered
2004 (11)	Male	75	Treatment with corticosteroids and methotrexate	Diabetes, dermatosis	Infiltrate in the right upper lobe, and subsequently developed bilateral basal infiltrates	Fluorescent in Situ Hybridization of the lung biopsy specimen	Erythromycin and rifampin	Died
2006 (12)	Male	54	Chronic lymphocytic leukemia	Chronic lymphocytic leukemia	Infiltration with nodular opacity at the left lower lobe	Culture of bronchoalveolar lavage fluid	Ofloxacine and rifampin	Recovered
This study	Male	53	No	No	Consolidation in the left upper and lower lobes and left pleural effusion, and subsequently developed infiltrates in both lungs	mNGS of the bronchoalveolar lavage fluid	Moxifloxacin, levofloxacin	Recovered

cost of mNGS (about 3500 Ren Min Bi per sample in China) is higher compared with any single traditional microbiological test, the benefit of its short turn-round time and the precise pathogenic diagnosis may have the potential to reduce the overall hospital costs through optimal patient management.

Only five previous case reports were identified in PubMed describing patients with *Legionella gormanii* pneumonia (**Table 1**). All these patients were immunosuppressed and had underlying diseases. Three of these patients were diagnosed by BALF culture, one by direct fluorescent antibody staining of the pleural exudate, and one by Fluorescent *in situ* Hybridization of the lung biopsy specimen. Although, the role of rifampin combination therapy was inconclusive and may increase adverse events (17), four of these patients used rifampin-based combination therapy, one of whom died. Our patient was the first immunocompetent patient diagnosed with *Legionella gormanii* pneumonia through mNGS and successfully recovered after fluoroquinolones monotherapy.

Multiple outbreaks of *Legionella* infections have been reported previously, but person-to-person spread was only reported in one case (18). Contaminated water or contaminated soil can be a source to transmit *Legionella* infection (19), however, the sources of infection remained unknown in all previously reported *Legionella gormanii* pneumonia. As a rapid and unbiased approach to detect microorganisms, mNGS also have the potential to identify pathogens directly from the environment. Further studies are warranted to explore the sources of *Legionella gormanii* infection using a metagenomic approach.

In summary, our study presented an immunocompetent patient with severe *Legionella gormanii* pneumonia diagnosed by mNGS while the *Legionella* urinary antigen test was negative, and the patient recovered after fluoroquinolones monotherapy. mNGS may be a high-resolution and sensitive assay for the diagnosis and surveillance of *Legionella* infection.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Review Board of the Second Xiangya Hospital of Central South University in China. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

CL, XZ, and SD collected the data and wrote the manuscript. YX, BY, WG, MS, MY, YJ, and HL analyzed, interpreted the data, and performed the clinical assessment. YJ and HL designed the study. All authors reviewed, edited, and approved the final manuscript.

FUNDING

This study was supported by National Natural Science Foundation of China (82070003 and 81770002 to HL), Natural Science Foundation of Hunan Province, China (2021JJ30943 to HL), the Science and Technology Program of Changsha, China (kq1901120 to HL), the Fundamental Research Funds for the Central Universities of Central South University (2021zzts0372 to SD), Xiangya Clinical Big Data System Construction Project in Pulmonary Inflammatory Disease of Central South University, and the National Key Clinical Specialty Construction Projects of China [(2012) No. 650].

ACKNOWLEDGMENTS

We thank Dr. Yan Yu, Dr. Hu Ge, and their colleagues from Changsha Kingmed diagnostics for technical support of this manuscript. The authors also want to thank Lin Wang for the assistance of data collection in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.819425/full#supplementary-material

REFERENCES

1. Cunha BA. Legionnaires' disease: clinical differentiation from typical and other atypical pneumonias. *Infect Dis Clin N Am.* (2010) 24:73–105. doi: 10.1016/j.idc.2009.10.014
2. Benin AL, Benson RF, Besser RE. Trends in legionnaires disease, 1980-1998: declining mortality and new patterns of diagnosis. *Clin Infect Dis.* (2002) 35:1039–46. doi: 10.1086/342903
3. Mondino S, Schmidt S, Rolando M, Escoll P, Gomez-Valero L, Buchrieser C. Legionnaires' disease: state of the art knowledge of pathogenesis mechanisms of Legionella. *Annu Rev Pathol.* (2020) 15:439–66. doi: 10.1146/annurev-pathmechdis-012419-032742
4. Mercante JW, Winchell JM. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. *Clin Microbiol Rev.* (2015) 28:95–133. doi: 10.1128/CMR.00029-14
5. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol.* (2019) 14:319–38. doi: 10.1146/annurev-pathmechdis-012418-012751
6. Chen X, Ding S, Lei C, Qin J, Guo T, Yang D, et al. Blood and bronchoalveolar lavage fluid metagenomic next-generation sequencing in pneumonia. *Can J Infect Dis Med Microbiol.* (2020) 2020:6839103.
7. Sun T, Wu X, Cai Y, Zhai T, Huang L, Zhang Y, et al. Metagenomic next-generation sequencing for pathogenic diagnosis and antibiotic management of severe community-acquired pneumonia in immunocompromised adults. *Front Cell Infect Microbiol.* (2021) 11:661589. doi: 10.3389/fcimb.2021.661589
8. Griffith ME, Lindquist DS, Benson RF, Thacker WL, Brenner DJ, Wilkinson HW. First isolation of *Legionella gormanii* from human disease. *J Clin Microbiol.* (1988) 26:380–1. doi: 10.1128/jcm.26.2.380-381.1988
9. Ephros M, Engelhard D, Maayan S, Bercovier H, Avital A, Yatsiv I. *Legionella gormanii* pneumonia in a child with chronic granulomatous disease. *Pediatr Infect Dis J.* (1989) 8:726–7. doi: 10.1097/00006454-198910000-00014

10. Towns ML, Fisher D, Moore J. Community-acquired pneumonia due to Legionella gormanii. *Clin Infect Dis.* (1994) 18:265–6. doi: 10.1093/clinids/18.2.265
11. Buchbinder S, Leitritz L, Trebesius K, Banas B, Heesemann J. Mixed lung infection by *Legionella pneumophila* and *Legionella gormanii* detected by fluorescent *in situ* hybridization. *Infection.* (2004) 32:242–5. doi: 10.1007/s15010-004-2127-z
12. David C, Bureau-Chalot F, Kolb B, Jarraud S, Delmer A, de Champs C, et al. Légionellose à *Legionella gormanii* de découverte fortuite et leucémie lymphoïde chronique: à propos d'un cas et revue de la littérature. *Méd Mal Infect.* (2006) 36:172–3. doi: 10.1016/j.medmal.2005.11.008
13. Fields BS, Benson RF, Besser RE. Legionella and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev.* (2002) 15:506–26. doi: 10.1128/CMR.15.3.506-526.2002
14. Cunha BA, Burillo A, Bouza E. Legionnaires' disease. *Lancet.* (2016) 387:376–85. doi: 10.1016/S0140-6736(15)60078-2
15. Yang RF, Huang LY, Gao SD. First isolation of *Legionella gormanii* in China. *Chin Med J (Engl).* (1990) 103:76–9.
16. Murdoch DR. Diagnosis of Legionella infection. *Clin Infect Dis.* (2003) 36:64–9. doi: 10.1086/345529
17. Varner TR, Bookstaver PB, Rudisill CN, Albrecht H. Role of rifampin-based combination therapy for severe community-acquired *Legionella pneumophila* pneumonia. *Ann Pharmacother.* (2011) 45:967–76. doi: 10.1345/aph.1Q074
18. Correia AM, Ferreira JS, Borges V, Nunes A, Gomes B, Capucho R, et al. Probable person-to-person transmission of Legionnaires' disease. *N Engl J Med.* (2016) 374:497–8. doi: 10.1056/NEJMc1505356
19. Phin N, Parry-Ford F, Harrison T, Stagg HR, Zhang N, Kumar K, et al. Epidemiology and clinical management of Legionnaires' disease. *Lancet Infect Dis.* (2014) 14:1011–21. doi: 10.1016/S1473-3099(14)70713-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Lei, Zhou, Ding, Xu, Yang, Guo, Song, Yang, Jia and Luo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 14 February 2022
doi: 10.3389/fmed.2021.759559

Autoimmune Encephalitis With Multiple Auto-Antibodies With Concomitant Human Herpesvirus-7 and Ovarian Teratoma: A Case Report

Jianhua Yang[1], *Pengcheng Wu*[1], *Xianghong Liu*[1], *Han Xia*[2] *and Zhaohui Lai*[1]*

[1] *Department of Neurology, The Affiliated Ganzhou Hospital of Nanchang University, Ganzhou, China,* [2] *Department of Scientific Affairs, Hugobiotech Co., Ltd., Beijing, China*

Infectious etiologies and tumors are common triggers of autoimmune encephalitis. We herein reported a rare case of autoimmune encephalitis with multiple autoantibodies in cerebrospinal fluid (CSF) and serum, with concomitant human herpesvirus 7 (HHV-7) infection and ovarian teratoma. A 36-year-old woman presented with mental and behavioral changes and gibberish for 13 days, followed by fever for 1 day. Her brain MRI indicated limbic encephalitis. Metagenomic next-generation sequencing (mNGS) of CSF revealed HHV-7. Antibody testing showed positive anti-N-methyl-D-aspartate receptor (NMDAR) and anti-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antibodies in CSF and serum. Ovarian teratoma was considered after pelvic MRI, which was then pathologically confirmed after laparoscopic ovariectomy. Her conditions improved after laparoscopic surgery, intravenous steroids, immunoglobulin, and rituximab therapy. Our findings suggested that the combination of multiple therapies including antiviral, immunotherapy, and resection of tumors were appropriate and improved the prognosis, when HHV-7 infection and ovarian teratoma were concomitant with multiple anti-neuronal antibodies of autoimmune encephalitis.

Keywords: human herpes virus 7, anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) encephalitis, autoimmune encephalitis, post viral encephalitis autoimmune encephalitis, paraneoplastic syndrome

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Suyue Pan,
Southern Medical University, China
Yang Zheng,
Zhejiang University, China
Kumaran Deiva,
Hôpitaux universitaires Paris-Sud (APHP), France

***Correspondence:**
Zhaohui Lai
13879729792@163.com

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *16 August 2021*
Accepted: *30 December 2021*
Published: *14 February 2022*

Citation:
Yang J, Wu P, Liu X, Xia H and Lai Z (2022) Autoimmune Encephalitis With Multiple Auto-Antibodies With Concomitant Human Herpesvirus-7 and Ovarian Teratoma: A Case Report. Front. Med. 8:759559. doi: 10.3389/fmed.2021.759559

INTRODUCTION

Human herpesvirus 7 (HHV-7) belongs to Betaherpesvirinae and is ubiquitous, like other human herpesviruses. Primary HHV-7 infection usually occurs during childhood, followed by a lifelong latent state with possible reactivation in case of immunodeficiency (1). The primary infections of HHV-7 can be asymptomatic, or with minor and nonspecific symptoms, including fever, rash, hepatitis, and pneumonitis. The HHV-7 rarely causes encephalitis, especially in immunocompetent patients, although it has a specific tropism for CD4+ lymphocytes and neurons (2). Clinical presentations of HHV-7 infection in the neurological system include a gradual loss of strength and weakness in the limbs, disorientation and confusion, flaccid paraplegia, localized anesthesia and hypoesthesia for pain and light touch, urinary retention, and constipation (3).

Post-viral autoimmune encephalitis has been established as a disease mechanism after herpes simplex virus (HSV) encephalitis. Other viruses including varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), cytomegalovirus (CMV), adenovirus, enterovirus, HIV, and hepatitis C virus can also cause this disease (4–9). However, there are few reports about this disease that is secondary to HHV-7 (10). The most frequent autoantibody, detected of autoimmune encephalitis is anti-NMDAR antibody, which can cause relapsing symptoms post herpes simplex virus encephalitis (HSE) in up to 28% of HSV encephalitis (11). Other autoantibodies such as anti-gamma-aminobutyric acid receptor, anti-dopamine 2 receptor, and anti-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antibodies are also common (8, 12). Coexisting antibodies may produce an overlap of clinical symptoms in patients with autoimmune encephalitis and raise the probability of an underlying malignancy. As is known, autoimmune encephalitis is paraneoplastic, and ovarian teratoma is one of the most common tumors associated with anti-NMDAR encephalitis. It is not common that viral infections and tumor can trigger autoimmune encephalitis simultaneously. We describe here a case of autoimmune encephalitis with anti-NMDAR and anti-AMPAR antibodies in the CSF and serum with concomitant HHV-7 infection and ovarian teratoma.

CASE PRESENTATION

A 36-year-old woman was admitted to the Department of Neurology at Affiliated Ganzhou Hospital of Nanchang University on May 13, 2021, due to mental and behavioral changes. She was gibberish for 13 days, followed by fever for 1 day (**Figure 1**). She had an unremarkable medical history with no prior medical conditions.

Neurological exams on admission showed delirium, aphasia, extensor toe response, and Kernig's sign with the right side. Brain MRI revealed a fluid attenuation inversion recover (FLAIR) high-intensity lesion involving the mesial temporal lobes and the hippocampus on May 13, 2021 (**Figure 2**). Electroencephalogram (EEG) revealed a diffused slow wave. A lumbar puncture was performed on May 14, and the cerebrospinal fluid (CSF) analysis revealed a white blood count of 5 × 10^6/L, protein of 28.6

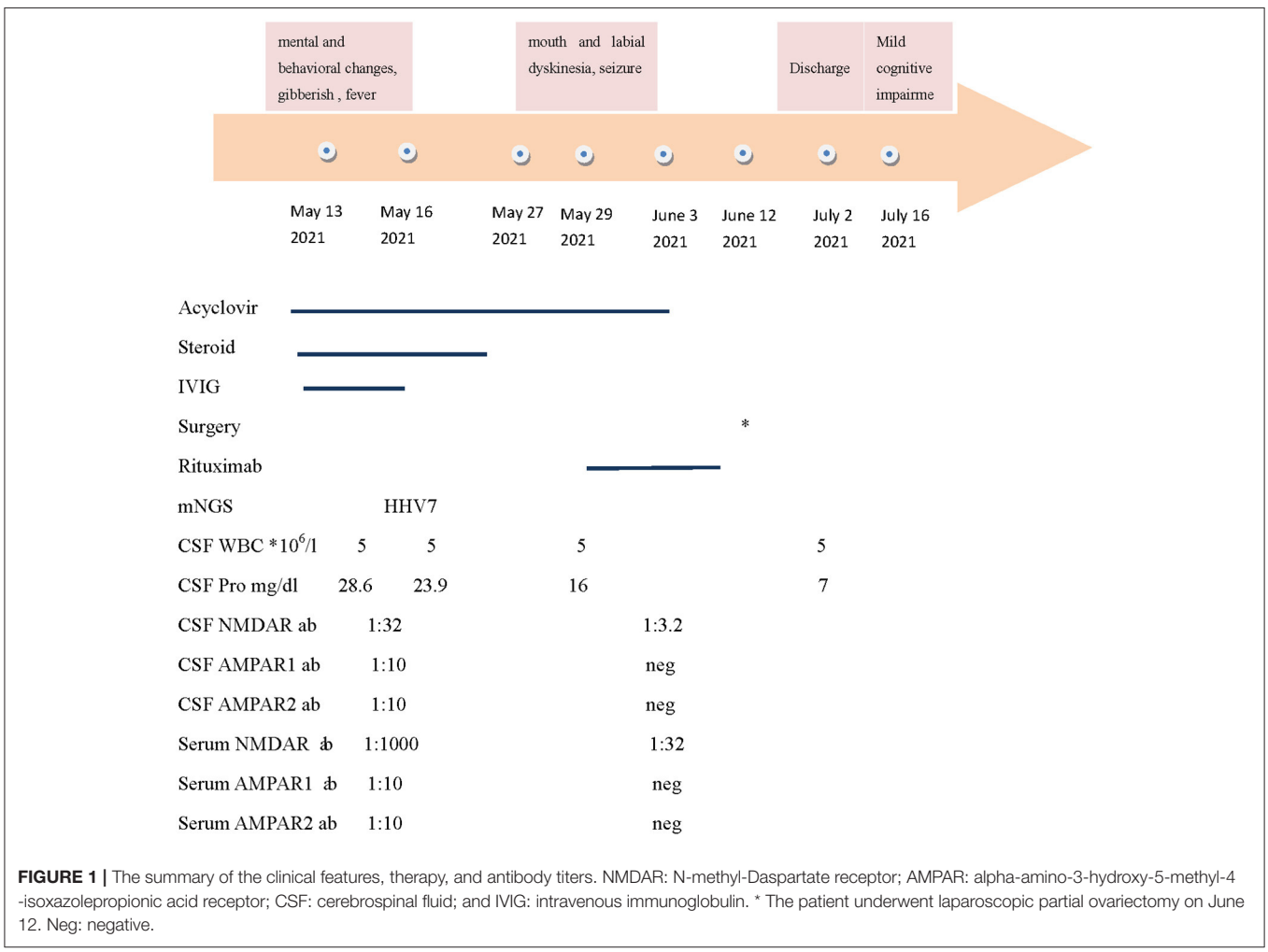


FIGURE 1 | The summary of the clinical features, therapy, and antibody titers. NMDAR: N-methyl-Daspartate receptor; AMPAR: alpha-amino-3-hydroxy-5-methyl-4 -isoxazolepropionic acid receptor; CSF: cerebrospinal fluid; and IVIG: intravenous immunoglobulin. * The patient underwent laparoscopic partial ovariectomy on June 12. Neg: negative.

mg/dl (normal range, < 40 mg/dL), and glucose of 3.01 mmol/L (normal range, 2.8–4.5 mmol/L). The PACEseq metagenomic next-generation sequencing (mNGS) (Hugobiotech, Beijing, China) of CSF was performed to identify the pathogens on May 16. A total of 7 unique reads of HHV-7 was detected. Considering the probability of autoimmune encephalitis, an antibody testing of CSF using a cell-based assay (Kindstar Global company) was also applied on May 15. The anti-NMDAR (titer of 1:32), anti-AMPAR1 (titer of 1:10), and anti-AMPAR2 (titer of 1:10) antibodies were detected as positive. Serum studies were also performed, demonstrating positive anti-NMDAR (titer of 1:1,000), anti-AMPAR1 (titer of 1:10), and anti-AMPAR2 (titer of 1:10) antibodies but negative anti-leucine-rich glioma-inactivated 1, anti-contactin-associated protein-like 2, and anti-GABA-B antibodies. Lymphocyte classification revealed the CD3 + CD4 + cells of 164.43/μl (normal range, 550–1,440/μl), CD3 + CD8 + cells of 190.47/μl (normal range, 320–1,250/μl), and CD19 + cells of 136.41/μl (normal range, 90–560/μl). So, autoimmune encephalitis was also considered. Antiviral treatment with acyclovir (0.5 g per 8 h, intravenously, for 21 days) was initiated on May 14. At the same time, high doses of corticosteroids (methylprednisolone, 1 g per day, intravenously, for 5 days, 500 mg per day for 3 days, 250 mg per day for 3 days) and intravenous immunoglobulin (0.4 g/kg body weight for 5 days) were also administered for the autoimmune encephalitis on May 16. However, the clinical condition of the patient deteriorated, with yelling and agitation. After the treatment of sedative drugs, the patient calmed down. However, her mental and behavioral symptoms did not disappear. On May 27, 2021, mouth and labial dyskinesia and seizure occurred. Levetiracetam and sodium valproate were administered for antileptic treatment. The condition of the patient is still impaired after the first-line therapy. On May 29, 2021, a second-line treatment of rituximab (375 mg/m^2 weekly for 3 weeks) was used for her autoimmune encephalitis. On June 2, the titer of anti-NMDAR antibody was decreased to 1:32 and 1:3.2 in serum and CSF, respectively, and the anti-AMPAR antibodies turned negative. The symptoms of the patient continued to improve.

To evaluate the tumors, malignancy screening including a computed tomography (CT) of the chest, the abdomen, and the pelvic was tested, which demonstrated a pelvic mass of 4.8 * 4.2 * 4.9 cm, indicating ovarian teratoma. A smaller mass was also found and considered as a cyst. Subsequent pelvic MRI indicated an ovarian teratoma on June 11. Laparoscopic partial ovariectomy was then performed with tracheal incubation and mechanical ventilation on June 12. The pathology report showed mature cystic teratoma in the right ovarian, consisting of choroid plexus, neuropil, sebaceous glands, hair follicles, and cartilage tissue (**Figure 3**). Although with mild memory impairment and gibberish sometimes, the patient can speak simple words. Lymphocyte classification showed CD3 + CD4 + cells of 780.76/μl, CD3 + CD8 + cells of 756.91/μl, and undetectable CD19 + cells on July 15. She was discharged on July 16. On 1-month follow-up, despite mild memory impairment, she can perform all the usual duties and activities.

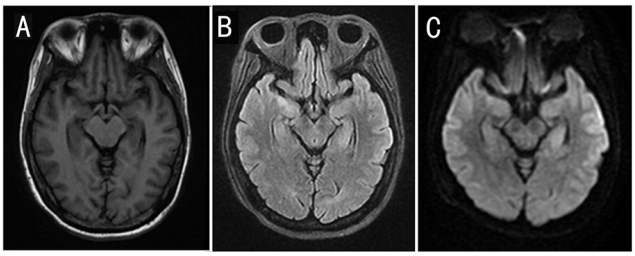


FIGURE 2 | Cranial MRI results of the patient. Cranial MRI showed an increased signal intensity in the mesial temporal lobes and the hippocampus on T2 FLAIR and diffusion-weighted images. **(A)** T1-weighted image. **(B)** T2 FLAIR image. **(C)** Diffusion-weighted image.

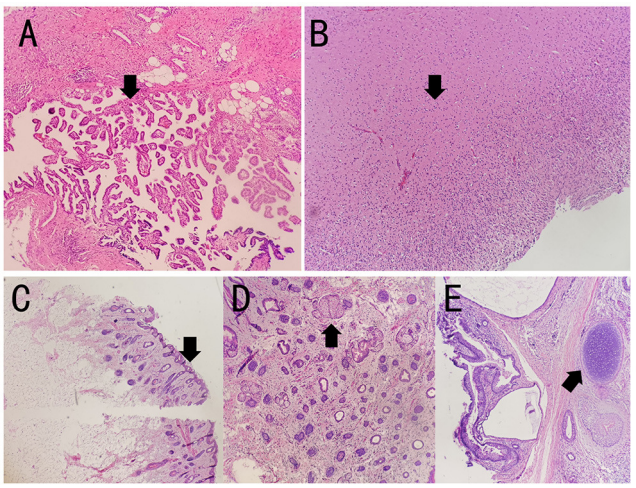


FIGURE 3 | The pathology results of the patient. The specimen showed mature cystic teratoma, consisting of choroid plexus, neuropil, sebaceous glands, hair follicles, and cartilage tissue by H&E staining. The arrows indicate lesions in **(A–E)**. **(A–E)** Representative histopathological features of mature teratoma. **(A)** Choroid plexus. **(B)** Neuropil. **(C)** Hair follicles. **(D)** Sebaceous glands. **(E)** Cartilage tissue.

DISCUSSION

To the best of our knowledge, this is the first reported case of autoimmune encephalitis with multiple autoantibodies with concomitant HHV-7 infection and ovarian teratoma. No standard treatment for HHV-7-associated neurological complications has been established, although ganciclovir, foscarnet, and cidofovir are commonly used for HHV-7 antiviral treatment (13). Acyclovir is also an antiviral agent, which has a marked activity against the herpesviruses. It is reported that acyclovir is one of the most potent compounds with the highest anti-HHV-7 and can be used in central nervous system infection (14, 15). So, we empirically used acyclovir for this case with human herpesvirus encephalitis. The common clinical manifestations of anti-NMDAR and anti-AMPAR encephalitis included cognitive impairment, psychiatric disorder, dyskinesia, speech disorder, insomnia, autonomic dysfunction, and

dysarthria (16). In this case, the patient's clinical manifestation of psychiatric disorder and cognitive impairment could be attributed to both anti-NMDAR antibody and anti-AMPAR antibody. Seizures and mouth and labial dyskinesia were associated with rgw anti-NMDAR antibody. After resection of ovarian teratoma and immunotherapy for autoimmune encephalitis, the related antibodies significantly decreased and even turned negative.

The HHV-7 rarely causes encephalitis, especially in immunocompetent patients. In a study conducted in Toronto, 57 (1.9%) out of 2,972 children were detected positive with HHV-7 DNA in their CSF, including 8 patients with meningitis and 7 patients with encephalitis (15). Clinical presentations of HHV-7 infection in the neurological system include a gradual loss of strength and weakness in the limbs, disorientation and confusion, flaccid paraplegia, localized anesthesia and hypoesthesia for pain and light touch, urinary retention, and constipation. These symptoms are similar to other encephalitides. So, it is hard to be successfully diagnosed by clinical practices. In addition, conventional diagnostic methods including culture and serology are not ideal for the detection of HHV-7. The mNGS is currently used in many infectious diseases and is performed with high sensitivity and specificity. It had a broad spectrum and can detect almost all pathogens. It has great advantages, for example, in identifying rare, novel, difficult-to-detect, and co-infected pathogens from clinical samples, especially for the detection of viruses (17). In this case report, HHV-7 was successfully identified by mNGS in CSF. The limitation of mNGS in this patient is that the HHV-7 DNA detection could not discriminate whether it is a primary infection or a latent reactivation, and it is complex to interpret the result. It has been reported that neurotropic viruses cause anti-NMDAR encephalitis by several mechanisms. One is that neurotropic viruses may lyse neurons, releasing antigens that stimulate antibodies to the NMDA receptor and triggering autoimmune encephalitis. Another proposed hypothesis is that anti-NMDAR encephalitis may stimulate latent virus reactivation, thus, leading to secondary encephalitis (8). To date, HHV-7 has not been documented in the setting of anti-NMDAR or anti-AMPAR encephalitis. The presentation is more of anti-NMDAR encephalitis than anti-AMPAR encephalitis, and AMPAR antibodies were at a low concentration (1:10 in CSF and serum). It may be a marker of increased auto-antibody production in response to a tumor cell breakdown, post-viral infection, and antigen release, or represent low-titer "false positive" results. Our patient completed the full first-line and second-line therapies for both anti-NMDAR and HHV-7 encephalitis, and her symptoms improved; it is unclear whether HHV-7 triggered the autoimmune condition of the patient, or it was present in the CSF as a result of latent viral reactivation. Her presenting symptoms were more consistent with anti-NMDAR encephalitis than HHV-7 encephalitis. Acyclovir treatment was used for 21 days because an immunosuppressive regimen for autoimmune encephalitis might worsen an underlying HHV-7 infection.

A recent study has reported that 4–7.5% of patients with anti-NMDAR encephalitis have concurrent glial antibodies or neuronal surface antibodies. Some of these antibodies have unique clinical features and may influence the prognosis (18). In that study, 6 patients had both anti-NMDAR and anti-AMPAR antibodies, most of whom were women (83%). About 67% of the patients had bilateral medial temporal lobe MRI abnormalities, and 83% had tumors (ovarian teratoma and breast cancer). All these patients had severe symptoms of anti-NMDAR encephalitis (4–5 groups of symptoms) with decreased levels of consciousness and required intensive care. In our case report, the patient was also a woman with severe symptoms of anti-NMDAR encephalitis (cognitive impairment, decreased level of consciousness, speech disorder, seizure, and dyskinesia), accompanied by a decreased level of consciousness and required admission to intensive care. Cranial MRI showed temporal lobe and hippocampal abnormality. Ovarian teratoma was found using cancer screening. Patients with co-existing anti-NMDAR antibody and neuronal surface antibody have a worse outcome than those with only anti-NMDAR antibody (19). Thus, early use of second-line immunotherapies is worth considering for these patients (18).

The limitation of the case is that HHV-7 DNA was only detected in the patient's CSF by mNGS. Serology and qPCR of HHV-7 in CSF and serum were not detected due to the limited amount of samples retained. The carrier frequency of HLA class II allele DRB1*16:02 was not detected in this paper either, which was reportedly associated with anti-NMDAR encephalitis (19).

In conclusion, a rare case of autoimmune encephalitis with multiple autoantibodies, concomitant HHV-7, and ovarian teratoma was found. These co-existing antibodies produced an overlap of clinical symptoms and lead to a worse prognosis of the patient. Thus, broad-spectrum etiological analyses, including multiple antibody panels, virus infections, and neoplastic risk factors, are needed for the evaluation of patients presenting with autoimmune encephalitis. Clinicians should consider concomitant therapy, especially in atypical cases. Additional pieces of research are needed to optimize the treatment regimen when the autoimmune encephalitis with multiple autoantibodies presents with concomitant viral detection.

DATA AVAILABILITY STATEMENT

The data of this manuscript is available at: http://ngdc.cncb.ac.cn, accession number: PRJCA007384.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Ganzhou People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PW was the physician of the patient and collected the data for case presentation. JY participated in collecting

data and drafted the manuscript. ZL reviewed the literature and participated in its design. XL reviewed the literature and performed infectious disease consultations. HX analyzed metagenomic next-generation sequencing data and contributed to language polishing. All the authors read and approved the final manuscript.

REFERENCES

1. Agut H, Bonnafous P, Gautheret-Dejean A. Human Herpesviruses 6A, 6B, and 7. *Microbiol Spect*. (2016) 4:4–3. doi: 10.1128/microbiolspec.DMIH2-0007-2015
2. Fule Robles JD, Cheuk DK, Ha SY, Chiang AK, Chan GC. Human herpesvirus types 6 and 7 infection in pediatric hematopoietic stem cell transplant recipients. *Ann Transplant.* (2014) 19:269–76. doi: 10.12659/AOT.889995
3. Ward KN, Andrews NJ, Verity CM, Miller E, Ross EM. Human herpesviruses-6 and−7 each cause significant neurological morbidity in Britain and Ireland. *Arch Dis Child.* (2005) 90:619–23. doi: 10.1136/adc.2004.062216
4. Schäbitz W-R, Rogalewski A, Hagemeister C, Bien CG, VZV. brainstem encephalitis triggers NMDA receptor immunoreaction. *Neurology.* (2014) 83:2309–11. doi: 10.1212/WNL.0000000000001072
5. Xu C-L, Liu L, Zhao W-Q, Li J-M, Wang R-J, Wang S-H, et al. Anti-N-methyl-D-aspartate receptor encephalitis with serum anti-thyroid antibodies and IgM antibodies against Epstein-Barr virus viral capsid antigen: a case report and one year follow-up. *BMC Neurol.* (2011) 11:1–7. doi: 10.1186/1471-2377-11-149
6. Hacohen Y, Wright S, Waters P, Agrawal S, Carr L, Cross H, et al. Paediatric autoimmune encephalopathies: clinical features, laboratory investigations and outcomes in patients with or without antibodies to known central nervous system autoantigens. *J Neurol Neurosurg Psychiatry.* (2013) 84:748–55. doi: 10.1136/jnnp-2012-303807
7. Arboleya S, Clemente A, Deng S, Bedmar M, Salvador I, Herbera P, et al. Anti-NMDAR antibodies in new-onset psychosis. Positive results in an HIV-infected patient. *Brain Behav Immunity*. (2016) 56:56–60. doi: 10.1016/j.bbi.2016.03.011
8. Linnoila JJ, Binnicker MJ, Majed M, Klein CJ, McKeon A. CSF herpes virus and autoantibody profiles in the evaluation of encephalitis. *Neurol Neuroimmunol Neuroinflamm*. (2016) 3:e245. doi: 10.1212/NXI.0000000000000245
9. Prüss H, Dalmau J, Harms L, Höltje M, Ahnert-Hilger G, Borowski K, et al. Retrospective analysis of NMDA receptor antibodies in encephalitis of unknown origin. *Neurology.* (2010) 75:1735–9. doi: 10.1212/WNL.0b013e3181fc2a06
10. Ward KN, Kalima P, MacLeod KM, Riordan T. Neuroinvasion during delayed primary HHV-7 infection in an immunocompetent adult with encephalitis and flaccid paralysis. *J Med Virol.* (2002) 67:538–41. doi: 10.1002/jmv.10135
11. Armangue T, Spatola M, Vlagea A, Mattozzi S, Cárceles-Cordon M, Martinez-Heras E, et al. Frequency, symptoms, risk factors, and outcomes of autoimmune encephalitis after herpes simplex encephalitis: a prospective observational study and retrospective analysis. *Lancet Neurol.* (2018) 17:760–72. doi: 10.1016/S1474-4422(18)30244-8
12. Armangue T, Moris G, Cantarín-Extremera V, Conde CE, Rostasy K, Erro ME, et al. Autoimmune post–herpes simplex encephalitis of adults and teenagers. *Neurology.* (2015) 85:1736–43. doi: 10.1212/WNL.0000000000002125
13. Riva N, Franconi I, Meschiari M, Franceschini E, Puzzolante C, Cuomo G, et al. Acute human herpes virus 7 (HHV-7) encephalitis in an immunocompetent adult patient: a case report and review of literature. *Infection.* (2017) 45:385–8. doi: 10.1007/s15010-017-1014-3
14. De Clercq E, Naesens L, De Bolle L, Schols D, Zhang Y, Neyts J. Antiviral agents active against human herpesviruses HHV-6, HHV-7 and HHV-8. *Rev Med Virol.* (2001) 11:381–95. doi: 10.1002/rmv.336
15. Schwartz KL, Richardson SE, Ward KN, Donaldson C, MacGregor D, Banwell B, et al. Delayed primary HHV-7 infection and neurologic disease. *Pediatrics.* (2014) 133:e1541–7. doi: 10.1542/peds.2013-3344
16. Lancaster E. The diagnosis and treatment of autoimmune encephalitis. *J Clin Neurol (Seoul, Korea).* (2016) 12:1–13. doi: 10.3988/jcn.2016.12.1.1
17. Han D, Li Z, Li R, Tan P, Zhang R, Li J. mNGS in clinical microbiology laboratories: on the road to maturity. *Crit Rev Microbiol.* (2019) 45:668–85. doi: 10.1080/1040841X.2019.1681933
18. Martinez-Hernandez E, Guasp M, García-Serra A, Maudes E, Ariño H, Sepulveda M, et al. Clinical significance of anti-NMDAR concurrent with glial or neuronal surface antibodies. *Neurology.* (2020) 94:e2302–e10. doi: 10.1212/WNL.0000000000009239
19. Zhang TY, Cai MT, Zheng Y, Lai QL, Shen CH, Qiao S, et al. Anti-alpha-amino-3-hydroxy-5-Methyl-4-isoxazolepropionic acid receptor encephalitis: a review. *Front Immunol.* (2021) 12:652820. doi: 10.3389/fimmu.2021.652820

Conflict of Interest: Author HX is employed by Hugobiotech Co., Ltd., Beijing, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be conducted as potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Yang, Wu, Liu, Xia and Lai. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

CASE REPORT
published: 16 February 2022
doi: 10.3389/fmed.2022.803554

Brain Abscess Caused by *Nocardia farcinica* and Diagnosed by Metagenomic Next-Generation Sequencing: A Case Report

Jianhua Yang[1†], *Shuhua Xie*[1†], *Junda Li*[1], *Han Xia*[2] *and Xianghong Liu*[1*]

[1] *Department of Neurology, The Affiliated Ganzhou Hospital of Nanchang University, Ganzhou, China,* [2] *Department of Scientific Affairs, Hugobiotech Co., Ltd., Beijing, China*

Background: Brain abscesses caused by *Nocardia farcinica* are rare and difficult to diagnose. Conventional methods for diagnosing Nocardia species include blood culture, microscopy, and tissue slice, but the performance is not satisfied. We report a case of brain abscess due to *N. farcinica* diagnosed by metagenomic next-generation sequencing (mNGS).

Case Presentation: We report a case of a 58-year-old man with brain abscess caused by *N. farcinica*. The patient had a history of pemphigus and required long-term methylprednisolone administration. No pathogen was detected in blood culture, cerebrospinal fluid (CSF) culture, and fast-acid staining. mNGS identified *N. farcinica* in the CSF. The symptoms and signs of the patient were significantly improved after changing the antibiotics accordingly to sensitive antibiotics.

Conclusion: Metagenomic next-generation sequencing (mNGS) is helpful for early diagnosis and subsequent treatment of *Nocardia*-associated meningitis and encephalitis, avoiding brain surgery. Early and accurate diagnosis and prompt antibiotic treatment reduced its mortality.

Keywords: *Nocardia farcinica*, central nervous system, infection, brain abscess, metagenomic next-generation sequencing

OPEN ACCESS

Edited by:
Yanjiao Zhou,
UConn Health, United States

Reviewed by:
Vania Aparecida Vicente Aparecida Vicente,
Federal University of Paraná, Brazil
Suresh Chand Bokoliya,
UConn Health, United States

***Correspondence:**
Xianghong Liu
lxh7176@126.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Infectious Diseases-Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *28 October 2021*
Accepted: *10 January 2022*
Published: *16 February 2022*

Citation:
Yang J, Xie S, Li J, Xia H and Liu X (2022) Brain Abscess Caused by Nocardia farcinica and Diagnosed by Metagenomic Next-Generation Sequencing: A Case Report. Front. Med. 9:803554. doi: 10.3389/fmed.2022.803554

BACKGROUND

Nocardia is a Gram-positive, branching aerobic bacterium and universal in soil, water, and decaying plant matters. The *Nocardia* bacterium is an opportunistic pathogen that infects through the respiratory tract or skin contact. Predisposing factors are immunosuppressive treatment (28%), premedication (26%), hematological neoplasm (13%), transplant recipients (13%), HIV infection (11%), chronic lung disease (9%), diabetes mellitus (8%), and renal disease (8%) (1). Clinical forms of *Nocardia farcinica* infection include pulmonary or pleural infections, brain abscesses and skin or soft tissue infections (2). Brain abscesses caused by *N. farcinica* are rare and account for 2% of all intracranial abscesses (3). However, the mortality rate of nocardial brain abscesses is as high as 30%, which is much higher than the 10% caused by other bacteria (4), even with the development of imaging technologies and new antibiotics.

The diagnosis of *N. farcinica* using conventional methods is difficult, and the misdiagnosis may lead to inappropriate treatments and delay the therapy, resulting in the high mortality of the disease. Routine diagnostic methods are blood,

cerebrospinal fluid, and brain aspirate culture, and craniotomy is generally performed to remove a lesion and obtain a specimen. According to the literature, 45.8% of patients underwent both medical and surgical management (5). Currently, metagenomic next-generation sequencing (mNGS) is being performed to identify pathogens in many infectious diseases, with high sensitivity and specificity (6). The introduction of this technique would contribute to early and accurate diagnosis of the disease, avoid surgery, and reduce mortality. Here, we report a case of *N. farcinica* brain abscess diagnosed by mNGS.

CASE PRESENTATION

A 58-year-old male with slurred speech for 11 days, accompanied by headache, fever, dizziness and vomiting for 1 day, was admitted to the Department of Neurology at the Affiliated Ganzhou Hospital of Nanchang University on November 15, 2018. He had a history of tuberculosis in 1978, pemphigus treated with oral methylprednisolone 8 mg per day for 1 year, and fungal pneumonia treated with voriconazole 200 mg twice daily. On examination, his body temperature was 37.1°C. Neurologic examination revealed nuchal rigidity, dysarthria, and impaired coordination (finger to nose test) of the right upper limb.

Laboratory findings included an initial leukocyte count of 9.85 $\times$ 10^9/L (with 88.1% polymorphonuclear cells, 5.7% monocytes, and 6.1% lymphocytes), ESR of 10 mm (normal range 0–20 mm per hour), C-reactive protein of 51 mg/L (normal range $<$ 10 mg/L), and albumin level of 41.3 g/L. Brain MRI showed cerebellar vermis and right cerebellar mass with peripheral contrast-enhancement and surrounding edema (**Figure 1**). Chest CT scan revealed chronic interstitial inflammation and localized emphysema of the left upper lung (**Figure 2A**).

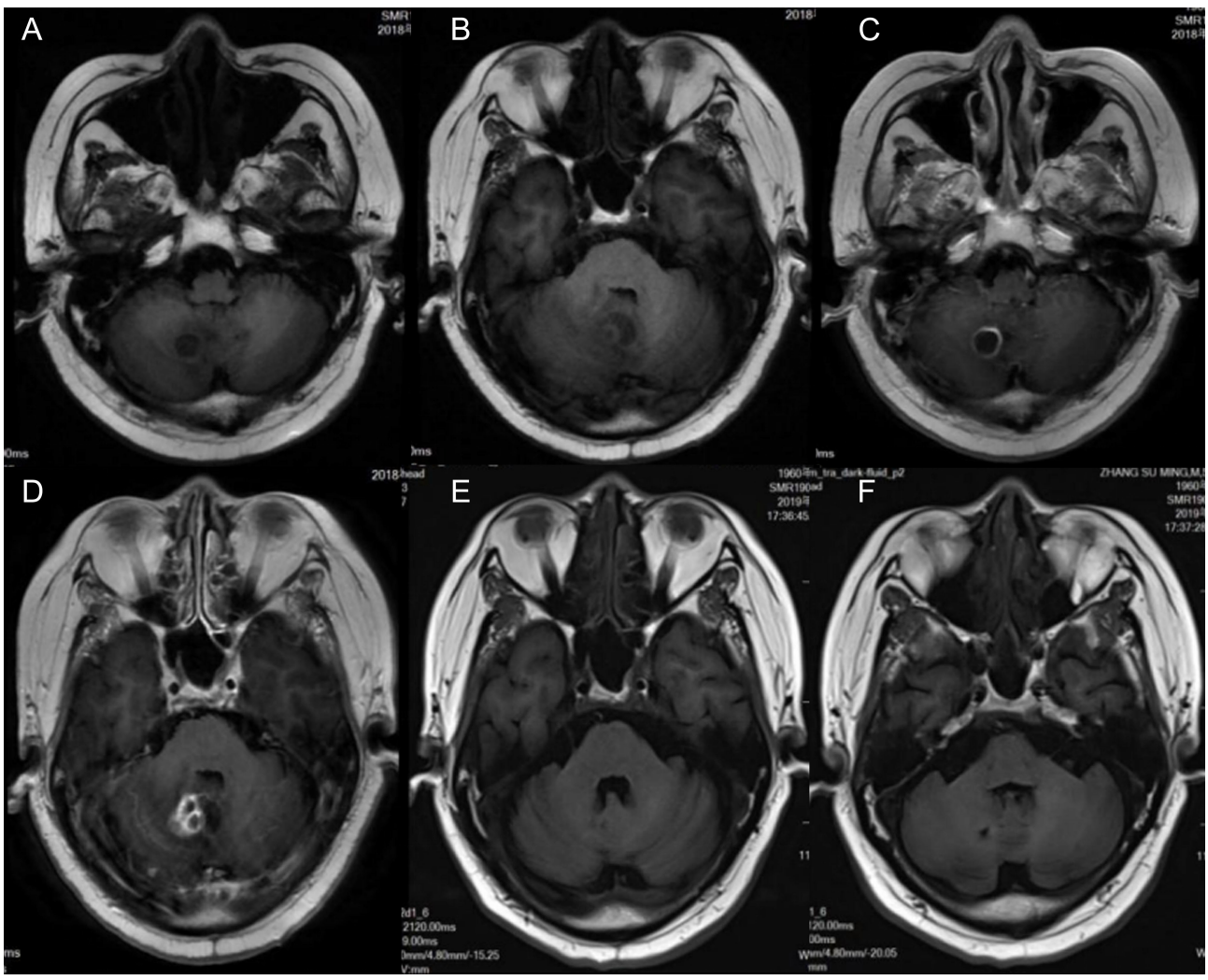


FIGURE 1 | Magnetic resonance imaging (MRI) of the patient shows cerebellar vermis and right cerebellar brain abscess with ring contrast enhancement and peripheral edema. **(A,B)** T1-weighted imaging. **(C,D)** Axial and sagittal T1 contrast enhancement imaging. **(E,F)** T1-weighted imaging 4 months after treatment; the lesion was reduced markedly.

FIGURE 2 | Thoracic computed tomography (CT) scan results and timeline of the treatment. **(A)** Thoracic CT scan showing chronic interstitial inflammation and localized emphysema of the left upper lung. **(B)** Timeline of the treatment of this case.

Antibacterial treatment with piperacillin/tazobactam (2.5 g q8h) was empirically started on November 15, 2018.

Lumbar puncture was then performed on November 17, 2018. The pressure was 210 mm H_2O (normal range 80–180 mmH_2O). The appearance of the CSF is colorless and clear. CSF analysis demonstrated leukocyte at 7,000 cells/ml, glucose levels at 3.2 mmol/L (normal range 2.8–4.5 mmol/L), and protein levels at 92.4 mg/dl (normal range 8–43 mg/dl). CSF staining including Gram stain, ink stain, and acid-fast stain were all negative. Blood culture and CSF culture were both negative.

PACEseq mNGS test (Hugobiotech, Beijing, China) using CSF sample was performed to identify the pathogens on November 23, 2018. QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany) was used for DNA extraction, and a library of total DNA was built with QIAseq^{TM} Ultralow Input Library Kit for Illumina (QIAGEN, Hilden, Germany). Qubit (Thermo Fisher, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) were used to assess the quality of the DNA library. The qualified library was finally sequenced on a Nextseq 550 platform (Illumina, San Diego, USA). After sequencing, adapters and short, low-quality, and low-complexity reads were removed from the raw data. Human DNA was also filtered out by mapping to the human reference database (hg38). The remaining reads were finally aligned to the Microbial Genome Database (ftp://ftp.ncbi.nlm.nih.gov/genomes/). *N. farcinica* (44 specific reads), human cytomegalovirus (2 specific reads), and human herpesvirus 4 (4 specific reads) were detected (**Figure 3**). The antibacterial regimen was changed to trimethoprim-sulfamethoxazole (TMP-SMZ) (160/800 mg q12h) and linezolid (600 mg q12h). Prednisone and voriconazole were continued to be used in the treatment of pemphigus and fungal pneumonia, respectively. The symptoms of the patient improved significantly, and he was discharged on November 25.

The antibiotic regimen was changed to oral TMP-SMZ, linezolid, and moxifloxacin, and maintained for 12 months. He returned to the hospital for follow-up on March 3, 2019. MRI showed a significant reduction of brain abscess (**Figure 1**).

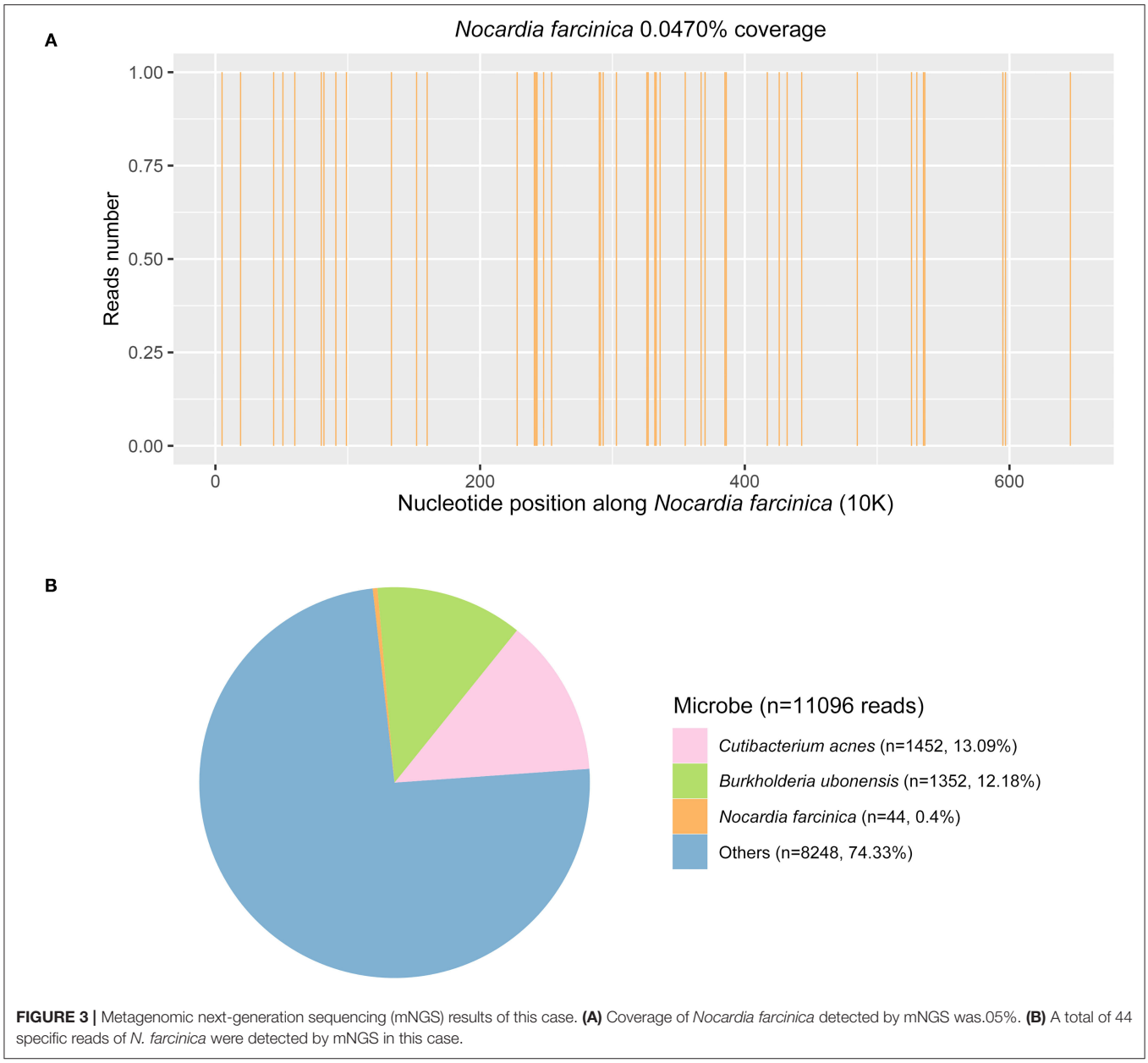


FIGURE 3 | Metagenomic next-generation sequencing (mNGS) results of this case. **(A)** Coverage of *Nocardia farcinica* detected by mNGS was.05%. **(B)** A total of 44 specific reads of *N. farcinica* were detected by mNGS in this case.

The patient was followed up for 28 months and was currently asymptomatic without neurological defects. The timeline of the treatment is shown in **Figure 2B**.

DISCUSSION AND CONCLUSIONS

To the best of our knowledge, this is the first case of nocardial brain abscess with pemphigus diagnosed by mNGS without surgical interference. Huang et al. (7) reported a case of brain abscess caused by *Nocardia asiatica* and detected with the combination of Ziehl-Neelsen stain and mNGS. After craniotomy, mNGS identified the pathogen in the purulent fluid from the core of abscess as *N. asiatica*. Another case reported by Zhou et al. (8) showed a idiopathic thrombocytopenic purpura patient with *N. farcinica* brain abscess diagnosed by mNGS using CSF. Unfortunately, the patient was transferred to a local hospital for treatment and lost contact.

The result of mNGS showed *N. farcinica* (44 specific reads), human cytomegalovirus (2 specific reads), and human herpesvirus 4 (4 specific reads). *Cutibacterium acnes* and *Burkholderia ubonensis* were detected as background bacteria. The role of human herpesvirus 4 (Epstein-Barr virus) in central nervous system infections is not fully resolved. EBV associated neurologic diseases include encephalitis, meningitis, cerebellitis, polyradiculomyelitis, transverse myelitis, cranial and peripheral

neuropathies, and psychiatric abnormalities. They are usually seen in immunocompromised patients. EBV is commonly found together with other CNS infections including varicella zoster virus, cytomegalovirus, *Cryptococcus*, and *Nocardia*. Immunosuppressive state and other infections in the CNS may trigger EBV reactivation (9). Imaging findings reported on EBV encephalitis are nonspecific. Brain magnetic resonance imaging (MRI) can show normal parenchyma, leptomeningeal enhancement, and multifocal areas of hyperintensity within the subcortical white matter and deep gray nuclei (10). Cytomegalovirus infection of CNS may cause encephalitis, ventriculitis, polyradiculitis, or polyradiculomyelitis (11). Neuroimaging abnormalities include nonspecific white matter changes, periventricular enhancement, and, occasionally, mass lesions with edema and enhancement that mimic tumors (11). In this case, the MRI of the patient showed cerebellar vermis and right cerebellar mass with peripheral contrast enhancement and surrounding edema, and the symptoms of the patient significantly improved after treatment with TMP-SMZ, linezolid, and moxifloxacin, not a combination of antiviral medicines, supporting the diagnosis of brain abscess caused by *Nocardia*.

Nocardia is an opportunistic microorganism that exists in nature. It can be inhaled into host. *Nocardia* affects the lungs more frequently than the central nervous system (12). Male sex has been reported to be a risk factor for nocardial infection (13). Brain abscess caused by *N. farcinica* usually occurs in immunocompromised individuals, such as those under long-term use of immunomodulatory therapy, infected with HIV, and those who underwent cancer chemotherapy. Chronic corticosteroid therapy, especially at higher doses and prolonged duration, is associated with increased risk of opportunistic infections such as pneumocystis pneumonia.

The diagnosis of *N. farcinica* brain abscess mainly depended on bacterial culture because of its lack of characteristic imaging and clinical features. Low positive rate of bacterial culture may lead to misdiagnosis and craniotomy. It can be misdiagnosed as ischemic or hemorrhagic stroke, glioma, primary brain tumor, or metastatic malignancy (8, 14).

In this case, the patient was male with a history of pemphigus and had impaired immune function after long-term use of methylprednisolone. The pathogen of CSF was diagnosed to be *N. farcinica* by mNGS. These results suggest that mNGS is a noninvasive and reliable method for pathogen identification. As a result, the patient received timely diagnosis and sensitive antibiotic treatment with good prognosis.

Case reports and small sample cohorts described the clinical features, treatment, and prognosis of nocardial brain abscess (5, 14). Nocardial brain abscess occurs in people aged 50–60 years (15). Our patient is 58 years old. Brain abscess caused by *N. farcinica* tends to have multiple lesions (16, 17). In this case, the right cerebellum and cerebellum vermis were involved simultaneously.

Although limited cases have been reported, in most cases, surgical excision and aspiration are considered necessary for diagnosis and treatment. Surgical aspiration is recommended in lesions larger than 2.5 cm in diameter or if the abscess does not decrease in size within 4 weeks (15). TMP-SMZ is the first line of therapy, and the duration is 6–12 months, or 1 year if CNS is involved. Daily TMP-SMZ prophylaxis may prevent nocardiosis and other opportunistic sensitive infections (17), while TMP-SMZ treatment may not be helpful in the differential diagnosis of nocardia brain abscess. Due to the high morbidity and mortality of nocardial brain abscesses, TMP-SMZ is usually used in combination with another highly bioavailable antibiotic with good penetration to central nervous system (CNS) for the therapy of nocardial brain abscess. Antibiotics with stronger potency, namely, imipenem, amikacin, and linezolid, are recommended for the initial therapy. Recent studies have shown that linezolid can be used in combination with TMP-SMZ for its good CNS penetration (18). In this case, the patient received a combination of TMP-SMZ and linezolid regimen during admission and it was changed to oral TMP-SMZ, linezolid, and moxifloxacin for 1 year.

In conclusion, the possibility of infection with *Nocardia* should be kept in mind in patients who are in immunocompromised condition and exhibiting CNS infection, especially those who lack response to conventional antibiotics. CSF mNGS detection accompanied by conventional blood and CSF culture and fast-acid staining are necessary for early and accurate diagnosis. Empirical therapy including sulfonamides in combination with other high blood-brain barrier penetration antibiotics may prevent high mortality.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://ngdc.cncb.ac.cn/, PRJCA007099.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Affiliated Ganzhou Hospital of Nanchang University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JY and SX participated in the collection of data and drafted the manuscript. JL collected the data for case presentation. XL reviewed the literature and participated in its design. All the authors read and approved the final version of the manuscript.

REFERENCES

1. Torres OH, Domingo P, Pericas R, Boiron P, Montiel JA, Vázquez G. Infection caused by Nocardia farcinica: case report and review. *Eur J Clin Microbiol Infectious Dis.* (2000) 19:205–12. doi: 10.1007/s100960050460
2. Corti ME, Villafañe-Fioti MF. Nocardiosis: a review. *Int J Infectious Dis.* (2003) 7:243–50. doi: 10.1016/S1201-9712(03)90102-0

3. Mamelak AN, Obana WG, Flaherty JF, Rosenblum ML. Nocardial brain abscess: treatment strategies and factors influencing outcome. *Neurosurgery.* (1994) 35:622–31. doi: 10.1227/00006123-199410000-00007
4. Cassir N, Million M, Noudel R, Drancourt M, Brouqui P. Sulfonamide resistance in a disseminated infection caused by Nocardia wallacei: a case report. *J Med Case Rep.* (2013) 7:103. doi: 10.1186/1752-1947-7-103
5. Corsini Campioli C, Castillo Almeida NE, O'Horo JC, Challener D, Go JR, DeSimone DC, et al. Clinical presentation, management, and outcomes of patients with brain abscess due to nocardia species. *Open Forum Infectious Dis.* (2021) 8:ofab067. doi: 10.1093/ofid/ofab067
6. Gu W, Deng X, Lee M, Sucu YD, Arevalo S, Stryke D, et al. Rapid pathogen detection by metagenomic next-generation sequencing of infected body fluids. *Nat Med.* (2021) 27:115–24. doi: 10.1038/s41591-020-1105-z
7. Huang T, Chen Y, Zhang J, He R, Qu D, Ye Q, et al. Rapid and accurate diagnosis of brain abscess caused by Nocardia asiatica with a combination of Ziehl-Neelsen staining and metagenomics next-generation sequencing. *Eur J Neurol.* (2021) 28:355–7. doi: 10.1111/ene.14533
8. Zhou C, Wang K, Li H, Zhang X. Idiopathic thrombocytopenic purpura with brain abscess caused by Nocardia farcinica diagnosed using metagenomics next-generation sequencing of the cerebrospinal fluid: a case report. *BMC Infectious Dis.* (2021) 21:380. doi: 10.1186/s12879-021-06071-1
9. Martelius T, Lappalainen M, Palomäki M, Anttila VJ. Clinical characteristics of patients with Epstein Barr virus in cerebrospinal fluid. *BMC Infectious Dis.* (2011) 11:281. doi: 10.1186/1471-2334-11-281
10. Tselis AC. Epstein-Barr virus infections of the nervous system. *Handbook Clin Neurol.* (2014) 123:285–305. doi: 10.1016/B978-0-444-53488-0.00013-4
11. Marra CM. Other central nervous system infections: cytomegalovirus, Mycobacterium tuberculosis, and Treponema pallidum. *Handbook Clin Neurol.* (2018) 152:151–66. doi: 10.1016/B978-0-444-63849-6.00012-8
12. Crozier JA, Andhavarapu S, Brumble LM, Sher T. First report of Nocardia beijingensis infection in an immunocompetent host in the United States. *J Clin Microbiol.* (2014) 52:2730–2. doi: 10.1128/JCM.00588-14
13. Gorevic PD, Katler EI, Agus B. Pulmonary nocardiosis. Occurrence in men with systemic lupus erythematosus. *Arch Internal Med.* (1980) 140:361–3. doi: 10.1001/archinte.1980.00330150075019
14. Hofmeister MG, Dao A, Young AM. Worsening Stroke Symptoms in an 80-Year-Old Man. *JAMA Neurol.* (2016) 73:1017–8. doi: 10.1001/jamaneurol.2016.0421
15. Nicolosi A, Hauser WA, Musicco M, Kurland LT. Incidence and prognosis of brain abscess in a defined population: Olmsted County, Minnesota, 1935-1981. *Neuroepidemiology.* (1991) 10:122–31. doi: 10.1159/000110257
16. Zhu JW, Zhou H, Jia WQ, You J, Xu RX. A clinical case report of brain abscess caused by Nocardia brasiliensis in a non-immunocompromised patient and a relevant literature review. *BMC Infectious Dis.* (2020) 20:328. doi: 10.1186/s12879-020-05052-0
17. Yang M, Xu M, Wei W, Gao H, Zhang X, Zhao H, et al. Clinical findings of 40 patients with nocardiosis: a retrospective analysis in a tertiary hospital. *Exp Therapeut Med.* (2014) 8:25–30. doi: 10.3892/etm.2014.1715
18. Rupprecht TA, Pfister HW. Clinical experience with linezolid for the treatment of central nervous system infections. *Eur J Neurol.* (2005) 12:536–42. doi: 10.1111/j.1468-1331.2005.01001.x

Conflict of Interest: HX is employed by Hugobiotech Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Yang, Xie, Li, Xia and Liu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

ORIGINAL RESEARCH
published: 03 March 2022
doi: 10.3389/fmed.2022.812698

Characteristics and Prognostic Factors of Non-HIV Immunocompromised Patients With Pneumocystis Pneumonia Diagnosed by Metagenomics Next-Generation Sequencing

*Jiali Duan, Jing Gao, Qiuhong Liu, Mengfei Sun, Yang Liu, Yingshuai Tan and Lihua Xing**

Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

Edited by:
Lei Chen,
Shanghai Jiao Tong University School of Medicine, China

Reviewed by:
Mark Poritz,
Idaho Molecular Inc., United States
Hossein Zarrinfar,
Mashhad University of Medical Sciences, Iran

***Correspondence:**
Lihua Xing
xinglihua95088@163.com

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *10 November 2021*
Accepted: *20 January 2022*
Published: *03 March 2022*

Citation:
Duan J, Gao J, Liu Q, Sun M, Liu Y, Tan Y and Xing L (2022) Characteristics and Prognostic Factors of Non-HIV Immunocompromised Patients With Pneumocystis Pneumonia Diagnosed by Metagenomics Next-Generation Sequencing. Front. Med. 9:812698. doi: 10.3389/fmed.2022.812698

Objective: The aim of this study was to evaluate the potential of metagenomic next-generation sequencing (mNGS) for the diagnosis of pneumocystis pneumonia (PCP) in patients with non-human immunodeficiency virus-infection and to discuss the clinical characteristics and identify prognostic factors associated with patients with non-HIV PCP.

Methods: Forty-six patients with PCP who were admitted in respiratory intensive care unit (ICU) between May 2018 and May 2020 were retrospectively reviewed. The subjects were divided into survivor and non-survivor groups according to the patients' outcome. Conventional methods and mNGS for detecting *Pneumocystis jirovecii (P. jirovecii)* were analyzed. The patients' demographics, comorbidities, laboratory parameters, and treatments were compared and evaluated in both groups to identify risk factors for mortality by using univariate and multivariate logistic regression.

Results: Metagenomic next-generation sequencing (mNGS) showed a satisfying diagnostic performance of 100% positive of detecting *P. jirovecii* from bronchoalveolar lavage (BAL) specimens in forty-six patients with non-HIV PCP, compared to only 15.2% for Gomori Methenamine silver (GMS) staining and 84.8% for Serum 1,3-beta-D-glucan (BDG). Among them, the mean age was 46.4-year-old (range 18–79-year-old) and mortality rate was 43.5%. The dominant underlying conditions were connective tissue diseases (34.8%), autoimmune kidney diseases (30.4%), followed by hematologic malignancies (10.9%), and solid organ transplantation (6.5%). A total of 38 cases (82.6%) received glucocorticoid and 19 cases (41.3%) used immunosuppressant within 3 months before diagnosed PCP. Multiple infections were very common, over two thirds' cases had mixed infections. Compared with survivors, non-survivors had a higher acute physiology and chronic health evaluation II (APACHE II) score (14.4 ± 4.8 vs. 10 ± 3.4), Procalcitonin (PCT) [ng/ml: 0.737 (0.122–1.6) vs. 0.23 (0.095–0.35)], lactic dehydrogenase (LDH) [U/L: 1372 (825.5–2150) vs. 739 (490.5–956)], and neutrophil-lymphocyte ratio (NLR) [21.6 (15.67–38.2) vs. 11.75 (5.1–15.52)], but had a lower PaO_2/FiO_2 ratio (mmHg:108.8 ± 42.4 vs. 150.5 ± 47.5),

lymphocytes [$\times 10^9$/L: 0.33 (0.135–0.615) vs. 0.69 (0.325–1.07)] and CD4+ T cells [cell/μl: 112 (53.5–264) vs. 255 (145–303.5)], all $P < 0.05$. Furthermore, we found non-survivors' PaO_2/FiO_2 ratio of day 3 and day 7 had not improved when compared with that of day one, and platelet level and NLR became worse. Multivariate analysis showed that other pathogens' co-infection (OR = 9.011, 95% CI was 1.052–77.161, $P = 0.045$) and NLR (OR = 1.283, 95% CI was 1.046–1.547, $P = 0.017$) were the independent risk factors of poor prognosis.

Conclusion: mNGS is a very sensitive diagnostic tool for identifying *P. jirovecii* in patients who are non-HIV immunocompromised. PCP in patients who are non-HIV infected is associated with a high rate of multiple infections and severe condition. Mixed infection and elevation of NLR were the independent risk factors of poor prognosis.

Keywords: immunocompromised, *Pneumocystis jirovecii*, pneumonia, non-HIV, mNGS

INTRODUCTION

Pneumocystis pneumonia (PCP) is an opportunistic pulmonary fungal infection caused by *Pneumocystis jirovecii* (*P. jirovecii*) in immunocompromised populations. And it is very common among patients with HIV/AIDS. Especially, patients with HIV/AIDS with a low CD4 count (CD4 counts < 200/μl) are at the highest risk of PCP (1, 2). With the widespread use of antiretroviral and PCP prophylaxis therapy, the incidence and mortality of PCP in patients with HIV/AIDS has gradually declined and it is around 10–20% nowadays (3, 4). However, the emerging number of conditions associated with immunosuppression has led to its continuing appearance in non-HIV patient population. The incidence of PCP is gradually increasing among patients who are non-HIV immunocompromised such as those receiving chronic corticosteroid therapies, with hematological or solid malignancies, transplant recipients and those who receive immunomodulatory or biological therapy (5, 6). Moreover, in the non-HIV-infected population, PCP is often associated with a higher rate of progressing to respiratory failure which occurs within a short time and causes a mortality rate of 30–60% (7). The growing incidence and high mortality of pneumocystis infection in the non-HIV patients suggest the need of more attention, including earlier diagnosis and treatment.

Despite the growing prevalence of PCP in immunocompromised individuals, establishing a microbiological diagnosis remains a challenge in this vulnerable population. Because the Inability to culture pneumocystis and non-specific clinical manifestations, together with relatively lower burden of organisms in HIV-uninfected patients (4, 8), conventional microbiological methods usually have an inadequate performance of finding *P. jirovecii*, and it may present false negative results of conventional tests (9). Metagenomics next-generation sequencing (mNGS) is a molecular technology of nucleic acid sequencing with high-throughput capacity and unbiased pathogen detection in a single assay, which has been considered a promising microbial identification technology in infectious diseases (10). Recently, several studies have shown its advantage in detecting a wide range of pathogens from different clinical specimens (11–13). However, the study about the utility of mNGS of bronchoalveolar lavage (BAL) fluid specimens for the diagnosis of PCP in non-HIV patients remains rare.

In the present study, we describe the use of mNGS of BAL fluids for detecting *P. jirovecii* and discuss the characteristics and risk factors of the outcome of non-HIV immunocompromised patients with PCP.

MATERIALS AND METHODS

Study Participants

We retrospectively collected a total of 46 immunocompromised patients diagnosed with PCP by mNGS test of BAL in our respiratory intensive care unit (ICU) from May 2018 to May 2020. This study was approved by the ethical committee of the first affiliated hospital of Zhengzhou University (The ethics approval number was 2020-KY-521), and informed consents were signed by patients or surrogates. Patients who met the following criteria were diagnosed with PCP: (1) Clinical symptoms (cough, fever, or shortness of breath) and laboratory parameters [1,3-beta-D-glucan (BDG) test and lactic dehydrogenase (LDH)] relevant to PCP; (2) Imaging findings compatible with PCP (present bilateral interstitial, ground-glass opacity and alveolar infiltrates in perihilar areas); (3) Identifying the genetic sequences of *P. jirovecii* by mNGS of BAL specimens. All patients enrolled were defined as HIV-negative, with one or more of the following immunosuppressive host conditions: receiving corticosteroid therapy within 3 months; active malignancy and/or receiving cancer chemotherapy; solid organ transplantation; hematological malignancy; receiving biologic immune modulators and/or immunosuppressive therapy. Patients with the following conditions were excluded: (1) Age < 18 years old; (2) HIV infection; (3) Length of stay in ICU < 24 h; (4) Incomplete medical record.

Microbiological Investigations

All patients had bronchoscopy performed, and BAL specimens were obtained. Each BAL specimen was divided into aliquots for both conventional microbiological test and mNGS test. Some specimens were sent to the Vision Medical Co., Ltd. (China) for

the mNGS analysis, and the processes of nucleic acid extraction, library construction, and high-throughput sequencing were performed, in turn. Finally, bioinformatics analysis and pathogen data interpretation were performed, all of which were already noted in detail in previous studies (14, 15).

At the same time, conventional tests were also conducted for microbiological analysis, including bacterial/fungal smear and culture, acid-fast staining, *P. jirovecii* smear [Gomori Methenamine silver (GMS) staining], and real-time PCR including *cytomegalovirus* (CMV), *influenza virus*. Real-time PCR was performed with a commercial CMV assay using MagNA Pure LC instrument (Roche Molecular Biochemicals, Indianapolis, IN). Positive control and negative control were included in all assays. The lower limit of detection of the assay was estimated to be <50 copies/ml (as per the instruction of the manufacturer). DNA integrity of the samples was confirmed by the presence of internal control DNA. The mixed infection was defined as the isolation of more than one pathogenic species. Furthermore, there was a consensus of clinically significant pathogens reached by physicians based on the comprehensive analysis of conventional and mNGS results, clinical features, and laboratory findings.

Clinical Data Collection

Clinical parameters of each patient were acquired through review of electronic medical records. We recorded patient data regarding demographics, underlying diseases, use of immunosuppressant, laboratory test results, acute physiology and chronic health evaluation II (APACHE II) score, the time of ICU stay, and the outcome of the patient.

Statistical Analysis

For continuous variables, normally distributed variables were reported as the mean and standard deviation and as the median and interquartile range (IQR) if they had a skewed distribution. Categorical variables were expressed as frequencies and percentages. Continuous variables were compared using the *t*-test or Mann-Whitney U test where suited. Categorical variables were compared using the chi-squared or Fisher's test, and repeated-measures analysis of variance was used when comparing differences between continuous variables over time. Logistic regression models were used to identify the risk factors of poor outcome. Covariates with a $P < 0.05$ in the univariate analysis were included in the final model of multivariable logistic regression. All statistical analyses were performed using the SPSS software version 23.0 (IBM, Armonk, NY, USA). $P < 0.05$ were considered significant, and all tests were 2-tailed.

RESULTS

Characteristics of Study Subjects and Detection of *P. jirovecii* in BAL Specimens by mNGS

Forty-six patients were enrolled during the study period. Among them, the mean age was 46.4 (18–79) years old and 22 (47.8%) of the patients were male. The mean age and gender compositions of survival and non-survival groups were similar. The underlying diseases of PCP patients were as follows: connect tissue diseases (34.8%), autoimmune kidney disease (30.4%), hematological malignancies (10.9%), and kidney transplant recipients (6.5%). As expected, PCP patients had various immunosuppressive conditions: Within 3 months prior the PCP diagnosis, 38 patients (82.6%) received steroids for inflammatory and/or autoimmune diseases, 19 cases (41.3%) took more than one immunomodulatory drug. However, none of them received PCP prophylaxis therapy (As shown in **Table 1**).

TABLE 1 | Underlying and co-infection conditions of non-HIV immunocompromised pneumocystis pneumonia (PCP) patients.

Item	Total ($n = 46$, %)	Survivors ($n = 26$, %)	Non-survivors ($n = 20$, %)
Underline conditions			
Connect tissue disease	16 (34.8)	10 (38.5)	6 (30)
Chronic kidney disease	14 (30.4)	7 (26.9)	7 (35)
Hematological malignancies	5 (10.9)	3 (11.5)	2 (10)
Solid organ transplantation	3 (6.5)	2 (7.7)	1 (5)
Solid tumors	2 (4.3)	1 (3.8)	1 (5)
Others	6 (13)	5 (19.2)	1 (5)
Use of corticosteroids	38 (82.6)	20 (76.9)	18 (90)
Use of immunosuppressive medications	19 (41.3)	10 (38.5)	9 (45)
Mixed infections			
No	15 (32.6)	14 (53.8)	1 (5)
Yes	31 (67.4)	12 (46.2)	19 (95)
Virus co-infection	23 (50)	11 (42.3)	12 (60)
Cytomegalovirus	19 (41.3)	9 (34.6)	10 (50)
Other viruses (*EB virus, Human adenovirus 7, and Human alpha herpesvirus 1*)	4 (8.7)	2 (7.7)	2 (10)
Bacteria co-infection	13 (28.3)	6 (23.1)	7 (35)
Klebsiella pneumoniae	4 (8.7)	2 (7.7)	2 (10)
Peudomonas aeruginosa	5 (10.9)	3 (11.5)	2 (10)
Acinetobacter baumannii	3 (6.5)	1 (3.8)	2 (10)
Staphylococcus aureus	1 (2.2)	0 (0)	1 (5)
Fungus co-infection	11 (23.9)	4 (15.4)	7 (35)
Candida albicans	6 (13)	2 (7.7)	4 (20)
Aspergillus fumigatus	3 (6.5)	1 (3.8)	2 (10)
Other fungi *(Candida tropicalis, Aspergillus flavus)*	2 (4.3)	1 (3.8)	1 (5)

Metagenomics next-generation sequencing (mNGS) and GMS staining of BAL specimens were performed in each patient. The mNGS test of forty-six patients' specimens all found *P. jirovecii* (sequence reads 12–214,898), and the result of GMS staining of BAL specimens showed that *P. jirovecii* was identified only in 7 cases (15.2%). Additionally, sputum specimens were obtained from 18 patients (39.1%); however, all showed negative results of *P. jirovecii*. BDG was widely used as a serologic biomarker of PCP. In this study, Serum BDG testing showed that 84.8% of patients had a positive result (Serum BDG > 100 ng/L).

Treatments and Outcome

All patients were treated with trimethoprim-sulfamethoxazole or combined with caspofungin. Meanwhile, 42 patients (93.5%)

received adjuvant corticosteroid therapy. As for oxygen support, 21 patients needed invasive mechanical ventilation, the remaining patients were oxygenated by non-invasive mechanical ventilation and high-flow nasal oxygen treatment. The non-survival group had a higher rate of receiving invasive mechanical ventilation when compared with the survival group (70% vs. 26.9%, $P = 0.004$).

Multi-infections were very common among PCP patients, and about 2/3 of patients had mixed infections. Among them, the most commonly detected co-infection pathogen was CMV. A total of 19 cases were co-infected with CMV, but there was no significant difference on the incidence between the two groups. Although patients with multi-infections were prescribed with relevant anti-bacterial or anti-fungal, antiviral agents, patients with multiple infections still had higher risk of poor outcomes than those without. Non-survivors had a significantly higher rate of mixed infection (95% vs. 46.2%, $P = 0.001$), which was also shown to be an independent risk factor of death in multivariate logistic analysis. The hospital mortality rate of the patients was 43.5%. The length of stay in the ICU of non-survivors was shorter than those of the survivors, but no statistical significance was observed (As shown in **Table 2**).

Prognostic Factors by Univariate Analysis

As shown in **Table 2**, compared with survivals, non-survivals appeared to have higher white blood cell count, neutrophil count and serum C-reactive protein, but no significance was observed between the two groups. All PCP patients showed lymphopenia with a median lymphocyte count of 0.55×10^9/L in peripheral blood and had significantly high LDH levels. Median serum levels of LDH (739 vs. 1,372 U/L) and neutrophil-to-lymphocyte ratio (NLR) (11.75 vs. 21.6) remarkably increased in both cohorts. Compared with survivors, non-survivors had a significantly higher APACHE II score within 24 h while admitted in the ICU, higher NLR ratio, PCT and LDH level, but had a lower PaO_2/FiO_2 ratio, lower lymphocytes, CD4+T cells and CD4+/CD8+ ratio; all $P < 0.05$. There was no significant difference in comparison of the cytokine' levels (IL-6, IL-10, and TNF-α) between the two groups.

Multivariate Analysis

Covariates with a $P < 0.05$ in the univariate analysis were included in the final model of multivariable logistic regression, and multivariate analysis showed that multiple infection and NLR were independent risk factors for non-HIV PCP patients that resulted in death (OR 1.283; 95% CI 1.046–1.574; OR 9.011; 95% CI 1.052–77.161; respectively) (Shown in **Table 3**).

Dynamic Changes of Laboratory Parameters Between Survival and Non-survival PCP Patients

Figure 1 showed the survivor and death groups' comparison on the dynamic changes of several laboratory parameters on day 1, day 3, and day 7 in ICU. Apparently, PaO_2/FiO_2 ratio of survival patients was improved gradually. PaO_2/FiO_2 ratios on day 3 and 7 were obviously higher than day 1. However, non-survivors hadn't gained PaO_2/FiO_2 ratio's elevation, and comparison of the tendency of PaO_2/FiO_2 ratio between the two groups also showed significant difference. In addition, the lymphocyte level had increased gradually and tended to be normal. C-reactive protein (CRP) and LDH levels decreased among survivors, all of which presented converse change in non-survivors. Furthermore, non-survivors had a significant decrement of platelets and an obvious rise of NLR ratio, and the repeated-measures analysis of these variance showed significant difference; all $P < 0.05$.

DISCUSSION

With a growing incidence among immunocompromised populations, PCP became a more common opportunistic infection which can be life-threatening. In this retrospective study of 46 immunocompromised patients in ICU settings, all patients were diagnosed with PCP infection based on mNGS of BAL fluids, and none of the subjects had received any PCP prophylaxis. Meanwhile, mixed infections were very common among PCP patients. The hospital mortality rate was 43.5%. Multivariate analysis showed that mixed infection and NLR were independent risk factors of poor prognosis.

Conventional method *via* direct staining and microscopy for the detection of *P. jirovecii* lacks sensitivity. Furthermore, the organism cannot be cultured in a laboratory (7, 16). In the present study, mNGS which is a burgeoning microbial detection method confirmed the diagnosis of PCP among these patients. Genetic sequences of *P. jirovecii* were founded by mNGS from the BAL specimen of each patient while GMS staining only showed 15.2% positive results. Compared with the inadequate sensitivity of conventional methods (9), we found that mNGS had an excellent capability for the diagnosis of PCP and also had advantages in identifying co-pathogens in PCP patients with mixed infections. Again, data from several recent studies supported its advantages in detecting opportunistic pathogens and mixed infections, especially in detecting uncultivable pathogens, such as *P. jirovecii* (17–19). More importantly, in a retrospective study of evaluating the utility of mNGS for the diagnosis of PCP, their research data revealed that the sensitivity of mNGS was 100% in non-HIV infected PCP patients, which was dramatically higher than GMS staining and serum BDG (20).

Previous studies showed that PaO_2/FiO_2 ratio and invasive mechanical ventilation were the influence factors of PCP prognosis (21, 22). Consistent with these studies, we also found that non-survivors had a higher rate of invasive mechanical ventilation and lower PaO_2/FiO_2 ratio level. Moreover, non-survivors had almost gained no PaO_2/FiO_2 ratio's elevation during the first 7 days in ICU while PaO_2/FiO_2 ratio of survivors was improved gradually and was obviously higher than those of the non-survivors.

The elevation of LDH was shown to be correlated with PCP patients' poor outcome (23) in the study of Schimdt et al. Meanwhile, Yoshida et al. found that hypoproteinemia also affects prognosis among PCP patients with inflammatory bowel disease (24). By comparing the dynamic change of laboratory parameters (PaO_2/FiO_2 ratio, LDH and NLR, and so on) on day 1, 3, and 7 after admission in the ICU, our study demonstrated a

TABLE 2 | Demographic and clinical characteristics of non-HIV PCP patients.

Variable	Total ($n = 46$)	Survivors ($n = 26$)	Non-survivors ($n = 20$)	*P*-value
Male, *n* (%)	22 (47.8)	12 (46.2)	10 (50)	0.796
Age, year	46.4 ± 15	45.8 ± 14.9	47.2 ± 15.5	0.78
APACHE II score	12.2 ± 3.9	10 ± 3.4	14.4 ± 4.8	0.042
PaO_2/FiO_2 ratio, mmHg	133.6 ± 49.4	150.5 ± 47.5	108.8 ± 42.4	0.006
Corticosteroid use, *n* (%)	38 (82.6)	20 (76.9)	18 (90)	0.246
Immunomodulatory medication use, *n* (%)	19 (41.3)	10 (38.5)	9 (45)	0.655
Laboratory tests				
White blood cells, $\times 10^9$/L	9.1 ± 4.2	8.4 ± 4.3	10.2 ± 3.8	0.185
Neutrophils, $\times 10^9$/L	7.85 (5.6–11.07)	6.9 (4.27–11)	9.05 (7.05–12.55)	0.075
Lymphocytes, $\times 10^9$/L	0.55 (0.25–0.82)	0.6 (0.325–1.07)	0.33 (0.135–0.615)	0.021
NLR	14.6 (7.85–21.65)	11.7 (5.1–15.52)	21.6 (15.67–38.2)	<0.001
Hemoglobin, g/L	109.6 ± 21.1	109.5 ± 22.5	109.8 ± 19.5	0.96
Platelet, $\times 10^9$/L	163.7 ± 71.2	176.6 ± 67.7	144.8 ± 73.9	0.158
C-reactive protein, mg/L	63.4 (30.5–117.4)	58.2 (30–83.7	112.7 (35.5–153.2)	0.056
Procalcitonin, ng/ml	0.238 (0.099–0.64)	0.2 (0.095–0.35)	0.737 (0.122–1.6)	0.035
Lactate dehydrogenase, U/L	891 (562–1701.5)	739 (490.5–956)	1,372 (825.5–2150)	0.003
Serum albumin, g/L	29.4 ± 4.3	29.9 ± 4.1	28.7 ± 4.6	0.39
Serum BDG, >100 ng/L (%)	39 (84.8)	21 (80.8)	18 (90)	0.388
IL-6, pg/ml	37.78 (13.1–166.3)	34.1 (13.1–166.3)	41.1 (13.25–190.33)	0.663
IL-10, pg/ml	12.5 (6.97–22.12)	12.77 (6.88–18.23)	12.42 (6.79–39.73)	0.758
TNF-α, pg/ml	2.44 (1.47–3.51)	2.40 (1.35–3.22)	2.47 (1.77–4.47)	0.377
CD4+T lymphocytes,/μL	179.5 (101.5–299.8)	255 (145–303.5)	112 (53.5–264)	0.046
CD8+T lymphocytes,/μL	179 (104–287)	200 (104–290)	124 (92–286)	0.311
CD4+T/CD8+ ratio	1.03 (0.69–1.5)	1.36 (0.78–1.73)	0.8 (0.63–1.05)	0.006
Multiple infections, *n* (%)	31 (67.4)	12 (46.2)	19 (95)	0.001
Treatment				
IMV, *n* (%)	21 (45.7)	7 (26.9)	14 (70)	0.004
Glucocorticoid, *n* (%)	42 (91.3)	23 (88.5)	19 (95)	0.435
ICU time, d	10.5 (8.5–15.3)	12 (9.5–16)	10 (6.5–12.5)	0.068

APACHE II, acute physiology and chronic health evaluation II; NLR, neutrophil-lymphocyte ratio; BDG, 1,3-beta-D-glucan; IMV, invasive mechanical ventilation; ICU, Intensive care unit.

TABLE 3 | Univariate and multivariate analysis of risk factors for poor outcome.

Variable	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
PaO_2/FiO_2, mmHg	0.979	0.963–0.995	0.012	0.985	0.960–1.010	0.227
APACHE II score	1.156	1.000–1.336	0.049	0.939	0.702–1.225	0.669
Lymphocytes, $\times 10^9$/L	0.13	0.02–0.857	0.034	0.436	0.031–6.113	0.538
NLR	1.227	1.067–1.412	0.004	1.283	1.046–1.574	0.017
CRP, mg/L	1.014	1001–1.026	0.03	1.008	0.985–1.031	0.492
LDH, U/L	1.001	1.000–1.002	0.03	1.001	1.000–1.003	0.076
CD4+T/CD8+T ratio	0.148	0.029–0.759	0.022	0.331	0.009–1.196	0.123
co-infection with other pathogens	8.545	1.791–50.882	0.008	9.011	1.052–77.161	0.045

APACHE II, acute physiology and chronic health evaluation II; NLR, Neutrophil-to-lymphocyte ratio; CRP, C-reactive protein; LDH, lactic dehydrogenase.

significant different trend between survivors and non-survivors. Briefly, the non-survival group showed an increase in NLR and a decrement in platelets gradually, but gained no improvement in PaO_2/FiO_2 ratio. At the same time, the increase of CRP and LDH, which would indicate the ineffective response to treatment and the high risk of poor prognosis, was also significantly higher than that of the survivors' group. Similarly, the research on risk factors of short-term outcome in PCP patients with HIV infection showed that over 90% of patients had an abnormal test of CRP, ESR, PaO_2, LDH, and KL-6 (25). In the present study, several

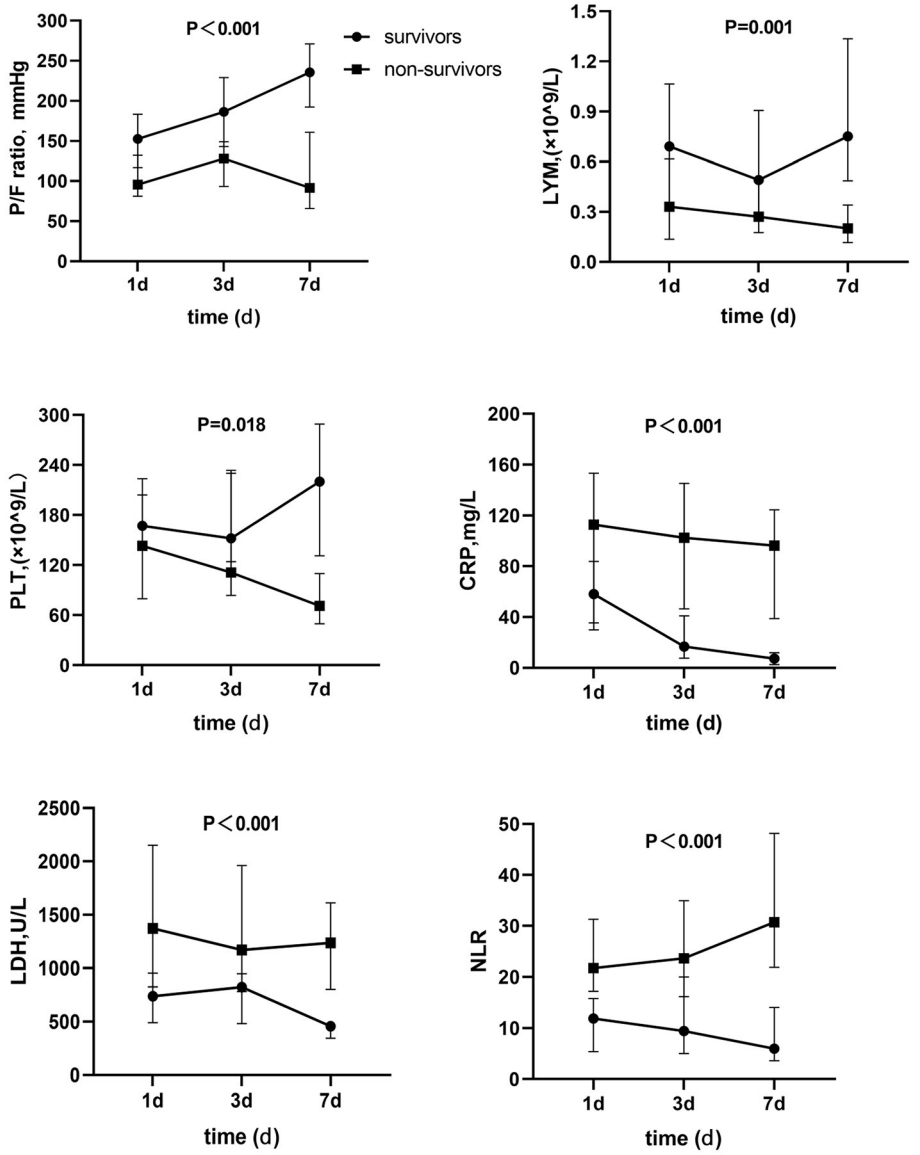


FIGURE 1 | Dynamic change of parameters between survivors and non-survivors. P/F ratio, PaO_2/PiO_2 ratio; LYM, Lymphocyte; PLT, Platelet; CRP, C reactive protein; LDH, Lactate dehydrogenase; NLR, Neutrophil-to-lymphocyte ratio.

laboratory parameters of PCP patients with poor prognosis got worse over the first 7 days in the ICU. Therefore, identifying the sickest patients early and taking the appropriate measures in a timely manner would improve the patient's outcome.

Because of depressive immune status, together with lower lymphocytes and T lymphocyte subsets than normal level, except PCP infection, these patients also had high risks of multiple infections occurring. The research of Huang et al. (26) of PCP patients with acute respiratory failure showed that occurrence of sepsis shock, ventilation related pneumonia, and CMV infection in PCP patients were 36.6, 43.9, and 58.5%, respectively. The result of another study about non-HIV PCP patients with CMV co-infection (27) indicated that fungus co-infection was a risk factor of CMV happening on PCP patients. Our study still found that mixed infections were very common in over 2/3 of patients with PCP, and the occurrence of mixed infection was also associated with poor prognosis. Multiple infections therefore need intense attention among immunocompromised PCP patients. Multilogistic regression analysis also showed NLR was a predictor of poor outcome. The elevation of NLR may be correlated with mixed infection and progress of the disease. The parameters of neutrophil and lymphocyte can be gained easily from regular daily blood test, so NLR would be a convenient and effective indicator which can be used to assess the patients' condition and prognosticate an outcome dynamically. However, there still need to be more prospective research to confirm its accuracy.

There is a need to mention that none of the patients in this study had received prophylaxis with sulfamethoxazole-trimethoprim, which would contribute to an occurrence of PCP in these patients. Almost similarly, the prophylaxis rates were all <20% in several previous studies on patients with PCP and non-HIV infection (21, 23, 28). Importantly, PCP prevention therapy can significantly reduce the morbidity and mortality of patients with PCP and HIV or patients with PCP but without HIV infection (29). There were already several clinical practice guidelines issued which suggest that the kidney transplant recipients and hematological malignancy patients should conduct PCP prophylaxis (30, 31). It is important that physicians should realize that non-HIV immunocompromised patients are also at risk of PCP and that rapid diagnosis and early initiation of treatment can lead to better prognosis. More attention is still needed to be paid on PCP prophylaxis among those non-HIV-infection populations with immunosuppression.

Our study had several limitations. First, the subjects were recruited from a single medical center, and the number of study participants was relatively small. Second, this was a retrospective study, some biases (such as selective bias) may influence the accuracy of our research outcome. Third, despite the combined use of clinical symptoms, radiographic findings, and mNGS test for PCP diagnosis, the possibility of including patients with *P. jirovecii* colonization cannot be completely eliminated. Finally, the study participants were all enrolled from ICU, so the patients' condition was critical and severely ill. Therefore, our research outcome may be inapplicable for mild patients. Further studies are still needed among patients with PCP.

In conclusion, mNGS is a very sensitive diagnostic tool for identifying *P. jirovecii* inpatients who are non-HIV immunocompromised. PCP in patients who are non-HIV-infected is associated with a high rate of multiple infections and severe condition. Mixed infection and NLR were the independent risk factors of poor prognosis.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI repository. Accession numbers, SRA: SRP356529, Bioproject: PRJNA782857.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of The First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JD and JG participated in the research design, data analysis, and writing of the paper. QL and MS participated in data analysis and revising of the paper. YL and YT participated in the improving and revising of the paper. LX provided substantial advice in designing the study and assisting in the division of labor, writing, and revising the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (82074212) and Key Medical Technologies R&D Program of Henan Province (SB201901036).

REFERENCES

1. Limper AH, Adenis A, Le T, Harrison TS. Fungal infections in HIV/AIDS. *Lancet Infect Dis.* (2017) 17:e334–43. doi: 10.1016/S1473-3099(17)30303-1
2. Izadi M, Jafari NJ, Sadraei J, Poornaki AM, Rezavand B, Zarrinfar H, et al. The prevalence of *Pneumocystis jiroveci* in bronchoalveolar lavage specimens of lung transplant recipients examined by the nested PCR. *Jundishapur J Microbiol.* (2014) 7:e13518. doi: 10.5812/jjm.13518
3. Thomas CF Jr., Limper AH. Pneumocystis pneumonia. *N Engl J Med.* (2004) 350:2487–98. doi: 10.1056/NEJMra032588
4. Fishman JA. *Pneumocystis jiroveci. Semin Respir Crit Care Med.* (2020) 41:141–57. doi: 10.1055/s-0039-3399559
5. Pegorie M, Denning DW, Welfare W. Estimating the burden of invasive and serious fungal disease in the United Kingdom. *J Infect.* (2017) 74:60–71. doi: 10.1016/j.jinf.2016.10.005
6. Reid AB, Chen SC, Worth LJ. Pneumocystis jirovecii pneumonia in non-HIV-infected patients: new risks and diagnostic tools. *Curr Opin Infect Dis.* (2011) 24:534–44. doi: 10.1097/QCO.0b013e32834cac17
7. Tasaka S. Recent advances in the diagnosis and management of pneumocystis pneumonia. *Tuberc Respir Dis.* (2020) 83:132–40. doi: 10.4046/trd.2020.0015
8. Limper AH, Offord KP, Smith TF, Martin WJ II. Pneumocystis carinii pneumonia. Differences in lung parasite number inflammation in patients with without AIDS. *Am Rev Respir Dis.* (1989) 140:1204–9. doi: 10.1164/ajrccm/140.5.1204
9. Procop GW, Haddad S, Quinn J, Wilson ML, Henshaw NG, Reller LB, et al. Detection of *Pneumocystis jiroveci* in respiratory specimens by four staining methods. *J Clin Microbiol.* (2004) 42:3333–5. doi: 10.1128/JCM.42.7.3333-3335.2004
10. Goldberg B, Sichtig H, Geyer C, Ledeboer N, Weinstock GM. Making the leap from research laboratory to clinic: challenges and opportunities for next-generation sequencing in infectious disease diagnostics. *mBio.* (2015) 6:e01888–15. doi: 10.1128/mBio.01888-15
11. Chiu CY, Miller SA. Clinical metagenomics. *Nat Rev Genet.* (2019) 20:341–55. doi: 10.1038/s41576-019-0113-7
12. Han D, Li R, Shi J, Tan P, Zhang R, Li J. Liquid biopsy for infectious diseases: a focus on microbial cell-free DNA sequencing. *Theranostics.* (2020) 10:5501–13. doi: 10.7150/thno.45554
13. Wu X, Li Y, Zhang M, Li M, Zhang R, Lu X, et al. Etiology of severe community-acquired pneumonia in adults based on metagenomic next-generation sequencing: a prospective multicenter study. *Infect Dis Ther.* (2020) 9:1003–15. doi: 10.1007/s40121-020-00353-y
14. Zhan Y, Xu T, He F, Guan WJ, Li Z, Li S, et al. Clinical evaluation of a metagenomics-based assay for pneumonia management. *Front Microbiol.* (2021) 12:751073. doi: 10.3389/fmicb.2021.751073
15. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis.* (2018) 67:S231–40. doi: 10.1093/cid/ciy693
16. Chen J, He T, Li X, Wang X, Peng L, Ma L. Metagenomic next-generation sequencing in diagnosis of a case of *Pneumocystis jirovecii* pneumonia in a

kidney transplant recipient and literature review. *Infect Drug Resist.* (2020) 13:2829–36. doi: 10.2147/IDR.S257587
17. Xie Y, Du J, Jin W, Teng X, Cheng R, Huang P, et al. Next generation sequencing for diagnosis of severe pneumonia: China, 2010-2018. *J Infect.* (2019) 78:158–69. doi: 10.1016/j.jinf.2018.09.004
18. Wilson MR, Sample HA, Zorn KC, Arevalo S, Yu G, Neuhaus J, et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. *N Engl J Med.* (2019) 380:2327–40. doi: 10.1056/NEJMoa1803396
19. Pan T, Tan R, Qu H, Weng X, Liu Z, Li M, et al. Next-generation sequencing of the BALF in the diagnosis of community-acquired pneumonia in immunocompromised patients. *J Infect.* (2019) 79:61–74. doi: 10.1016/j.jinf.2018.11.005
20. Jiang J, Bai L, Yang W, Peng W, An J, Wu Y, et al. Metagenomic next-generation sequencing for the diagnosis of *Pneumocystis jirovecii* pneumonia in non-HIV-infected patients: a retrospective study. *Infect Dis Ther.* (2021) 10:1733–45. doi: 10.1007/s40121-021-00482-y
21. Gaborit BJ, Tessoulin B, Lavergne RA, Morio F, Sagan C, Canet E, et al. Outcome and prognostic factors of *Pneumocystis jirovecii* pneumonia in immunocompromised adults: a prospective observational study. *Ann Intensive Care.* (2019) 9:131. doi: 10.1186/s13613-019-0604-x
22. Choi JS, Lee SH, Leem AY, Song JH, Kim SY, Chung KS, et al. Pneumocystis jirovecii pneumonia (PCP) PCR-negative conversion predicts prognosis of HIV-negative patients with PCP and acute respiratory failure. *PLoS ONE.* (2018) 13:e0206231. doi: 10.1371/journal.pone.0206231
23. Schmidt JJ, Lueck C, Ziesing S, Stoll M, Haller H, Gottlieb J, et al. Clinical course, treatment and outcome of Pneumocystis pneumonia in immunocompromised adults: a retrospective analysis over 17 years. *Crit Care.* (2018) 22:307. doi: 10.1186/s13054-018-2221-8
24. Yoshida A, Kamata N, Yamada A, Yokoyama Y, Omori T, Fujii T, et al. Risk factors for mortality in *Pneumocystis jirovecii* pneumonia in patients with inflammatory Bowel Disease. *Inflamm Intest Dis.* (2019) 3:167–72. doi: 10.1159/000495035
25. Li AX, Huang CY, Zhang HW, Zhang T, Wu H, Wang W. Research on risk factors of short-term outcome in AIDS patients with pneumocystis pneumonia. *Natl Med J China.* (2017) 97:833–7. doi: 10.3760/cma.j.issn.0376-2491.2017.11.008
26. Huang X, Weng L, Yi L, Li M, Feng Y, Tian Y, et al. Acute respiratory failure due to Pneumocystis pneumonia in connective tissue disease patients: clinical manifestation and prognostic factors related to hospital mortality. *Zhonghua Jie He He Hu Xi Za Zhi.* (2018) 41:196–200. doi: 10.3760/cma.j.issn.1001-0939.2018.03.010
27. Jiang H, Zhang QB, Zhu HD. Analysis of risk factors of non HIV *Pneumocystis jirovecii* pneumonia patients complicated with cytomegaloviremia. *Chin J Emerg Med.* (2018) 27:1004–9. doi: 10.3760/cma.j.issn.1671-0282.2018.09.010
28. Dunbar A, Schauwvlieghe A, Algoe S, Hellemond J, Reynders M, Vandecasteele S, et al. Epidemiology of *Pneumocystis jirovecii* pneumonia and (non-)use of prophylaxis. *Front Cell Infect Microbiol.* (2020) 10:224. doi: 10.3389/fcimb.2020.00224
29. Lim PL, Zhou JL, Ditangco RA, Law MG, Sirisanthana T, Kumarasamy N, et al. Failure to prescribe Pneumocystis prophylaxis is associated with increased mortality, even in the cART era: results from the Treat Asia HIV observational database. *J Int AIDS Soc.* (2012) 15:1–9. doi: 10.1186/1758-2652-15-1
30. Kasiske BL, Zeier MG, Chapman JR, Craig JC, Ekberg H, Garvey CA, et al. KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. *Kidney Int.* (2010) 77:299–311. doi: 10.1038/ki.2009.377
31. Maertens J, Marchetti O, Herbrecht R, Cornely OA, Flückiger U, Frêre P, et al. Third European Conference on Infections in Leukemia. European guidelines for antifungal management in leukemia and hematopoietic stem cell transplant recipients: summary of the ECIL 3–2009 update. *Bone Marrow Transplant.* (2011) 46:709–18. doi: 10.1038/bmt.2010.175

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Duan, Gao, Liu, Sun, Liu, Tan and Xing. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Medicine

ORIGINAL RESEARCH
published: 24 March 2022
doi: 10.3389/fmed.2022.751617

Invasive Pulmonary Aspergillosis Diagnosis *via* Peripheral Blood Metagenomic Next-Generation Sequencing

*Xiaoxu Ma[1], Suping Zhang[2], Haizhou Xing[2], Huiling Li[1], Jiajun Chen[3], Haijun Li[4], Mengfan Jiao[5,6], Qingmiao Shi[5,6], Aiguo Xu[1], Lihua Xing[1] and Weijie Cao[2]**

[1] Department of Respiration, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Department of Hematology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] College of Public Health, Zhengzhou University, Zhengzhou, China, [4] Department of Clinical Laboratory, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [5] Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [6] Gene Hospital of Henan , Precision Medicine Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Invasive pulmonary aspergillosis (IPA) is one of the major causes of morbidity and mortality in immunocompromised patients such as hematological malignancies, hematopoietic stem cell transplantation, and solid organ transplantation. The diagnosis of IPA in these patients is still difficult because it has no obvious specificity in clinical symptoms, signs and imaging, and test sensitivity of blood 1,3-β-d-glucan test, galactomannan are low. Therefore, we still need to explore more diagnostic methods. In our study, *via* peripheral blood metagenomic next-generation sequencing (mNGS), five patients were tested positive for *Aspergillus* DNA and then quickly diagnosed as IPA. Out of the 5 cases, 1 was proven and 4 were probable IPA. The underlying diseases of the 5 patients were myelodysplastic syndrome (2 cases), acute myeloid leukemia (2 cases), and renal transplantation (1 case). Then they were diagnosed as IPA using other methods such as lung histopathology, bronchoalveolar lavage fluid (BALF) mNGS, and sputum culture or sputum mNGS. In case 1, sputum culture suggested *Aspergillus flavus*. In case 2, both Grocott methenamine silver (GMS) stain of lung histopathology and lung tissue mNGS suggested *Aspergillus* infection. In cases 3 and 4, BALF-mNGS suggested *Aspergillus* infection. In case 5, sputum mNGS suggested *Aspergillus* infection. In conclusion, detecting the cfDNA of *Aspergillus via* peripheral blood mNGS can be used to diagnose IPA and is a rapid and non-invasive diagnosis method.

Keywords: invasive pulmonary aspergillosis, diagnosis, peripheral blood, metagenomic next generation sequencing, neutropenia

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Beiwen Zheng,
Zhejiang University, China
Hossein Zarrinfar,
Mashhad University of Medical Sciences, Iran
Timothy Kudinha,
Charles Sturt University, Australia

***Correspondence:**
Weijie Cao
caoweijie2003@126.com

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *01 August 2021*
Accepted: *17 February 2022*
Published: *24 March 2022*

Citation:
Ma X, Zhang S, Xing H, Li H, Chen J, Li H, Jiao M, Shi Q, Xu A, Xing L and Cao W (2022) Invasive Pulmonary Aspergillosis Diagnosis via Peripheral Blood Metagenomic Next-Generation Sequencing. Front. Med. 9:751617. doi: 10.3389/fmed.2022.751617

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is the most serious type of *Aspergillus*-related infection with the worst prognosis (1, 2). Risk factors for IPA include neutropenia, hematopoietic stem cell transplantation (HSCT), solid organ transplantation (SOT), and long-term use of corticosteroids (3, 4). A study on autopsy results showed that the incidence of IPA is increasing, and in spite of the improvement of diagnostic techniques, 75% of invasive fungal

diseases still cannot be diagnosed before death (5). The mortality rate of IPA is high. The 90-day mortality rate of hematology patients combined with IPA who received and did not receive voriconazole prophylaxis was 46 and 59.3%, respectively (6). Delayed diagnosis and treatment were important causes of the high mortality of IPA (7).

At present, the commonly used clinical methods to diagnose IPA include chest computed tomography (CT), galactomannan test (GM test), direct microscopic examination, fungal culture, plasma, serum, whole blood or bronchoalveolar lavage fluid (BALF) polymerase chain reaction (PCR), and histopathological evaluation. The chest CT images of IPA are often atypical, and it is difficult to distinguish it from other infections using imaging. The 1,3-β-D-glucan test (G test), GM test, direct examination, fungal culture, and other tests also have disadvantages such as low sensitivity and poor specificity (8, 9). Although histopathology is the gold standard, patients with hematological diseases, HSCT, and SOT are often in poor physical condition and blood coagulation function, making invasive procedures to obtain tissue specimen often difficult to tolerate. The sensitivity and specificity of the GM test of BALF are higher than those of blood GM test and culture (8), but it can be easily affected by the patient's disease state, which makes its clinical application quite limited. Therefore, there is an urgent need for a rapid and non-invasive method to accurately diagnose IPA.

Metagenomic next-generation sequencing (mNGS) is increasingly used in the clinical diagnosis of infectious diseases. As a new technology that does not require culture, it can directly identify non-cultivable, fastidious, and non-bacterial (viral and fungal) pathogens from blood samples, with a higher sensitivity than conventional culture methods (10). Although it is difficult for *Aspergillus* to grow in peripheral blood, hyphae growing in the alveoli can penetrate the air and blood barrier, erode capillary endothelial cells, and invade small arteries and lung parenchyma. In that process, DNA fragments may enter the blood, so the PCR detection of peripheral blood can be used to diagnose IPA. PCR detection improves the possibility of early diagnosis of *Aspergillus*; however, the limitation of primer design and incomplete coverage may cause false negative results and delay the diagnosis. It has been confirmed that plasma mNGS can identify circulating DNA of respiratory pathogens in critically ill patients with bacterial pneumonia (11), but it is still unclear whether plasma mNGS can be used to diagnose IPA.

In this study, IPA was identified *via* peripheral blood mNGS, demonstrating its important role in the clinical diagnosis of IPA. Now we are reporting demographic information, clinical symptoms, diagnosis and treatment, and prognosis of the 5 patients. To our knowledge, this is the first report of IPA diagnosis *via* peripheral blood mNGS in China.

MATERIALS AND METHODS

We collected 5 patients whose peripheral blood mNGS test was positive for *Aspergillus* between September 9, 2020, and December 31, 2020, and recorded the demographic information, clinical symptoms, imaging examination results, laboratory examination results, treatment medication, and prognosis. We categorized IPA as proven, probable, and possible according to the 2019 European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) criteria (12). The galactomannan (GM) test was performed at least 3 times in each case, and we recorded the highest detection value. The positive threshold of GM test is 1.0 μg/L (12). This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The ethics approval number is 2021-KY-0439-002.

Peripheral Blood MNGS

Peripheral venous blood specimens were collected using blood collection tube (BCT) tubes, centrifuged at 1,600 g for 10 min, and the plasma supernatant was used for cell-free DNA (cfDNA) extraction. Experiment quality was controlled by internal controls (IC) and external controls. To ensure that there were no mistakes in specimen tracking, 24 different ICs were designed. Each mNGS assay was run along with an external negative batch control consisting of 20 ng human cell line DNA (ATCC, CCL-243) in 600 μl 1M Tris-HCl buffer. DNA from all specimens, including the negative controls, was extracted using the TIANamp Micro DNA Kit (TIANGEN) and quantified using fluorometry (ThermoFisher Scientific). DNA libraries were constructed using an NGS library construction kit (Enzymatics) with unit dual index adapters. Libraries were pooled to be sequenced on Illumina NextSeq sequencers using a 75-cycle single-end sequencing strategy.

The primary sequencing output was demultiplexed with bcl2fastq version 2.20.0.422 with default parameters. Reads for quality trimming, low-complexity sequences, and adapter removal were performed using fastp version 0.19.5 (13). Specimens with fewer than 10 million reads after quality control (QC) were excluded in this study. The QC information of specimens is shown in online **Supplementary Table 1**. Reads mapped to human reference assembly GRCh38 were removed using bowtie2 version 2.3.4.3 (14). A set of criteria similar to the National Center for Biotechnology Information (NCBI) criteria was used for selecting representative genomes for microorganisms from the NCBI Nucleotide and Genome databases. The final database consisted of about 12,000 genomes. All putative microbial reads were aligned to our microorganism database with SNAP version 1.0beta.18 (15). The laboratory of our hospital adopts the mNGS process of Professor Wang Hui of Peking University People's Hospital (16).

RESULTS

We identified 5 patients who tested positive for *Aspergillus via* peripheral blood mNGS. Pathological or other pathogenic results confirmed that all the 5 patients had IPA. The flow diagram of included patients is shown in **Figure 1**. Demographic and clinical characteristics are shown in **Table 1**. The detailed medical records of those 5 patients are as follows.

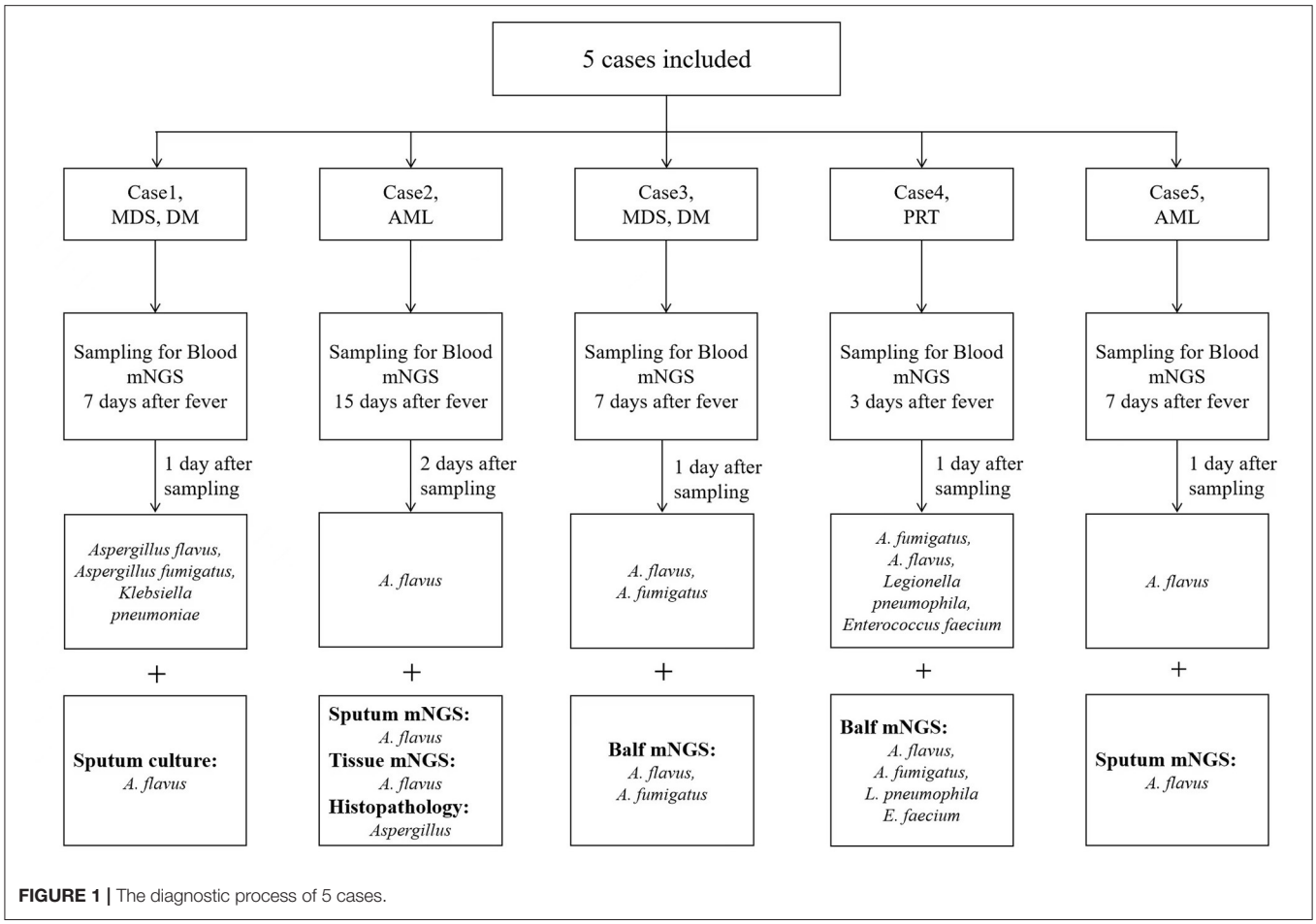


FIGURE 1 | The diagnostic process of 5 cases.

TABLE 1 | Demographic and clinical characteristics.

	Case 1	Case 2	Case 3	Case 4	Case 5
Age	55	21	56	55	39
Gender	Male	Female	Female	Female	Male
Underlying conditions	MDS, DM	AML	MDS, DM	PRT	AML
Fever	Yes	Yes	Yes	Yes	Yes
Cough	Yes	Yes	NA	Yes	NA
Sputum	White sticky sputum	Thin sputum	NA	Blood-stained sputum	NA
Chest pain	NA	Yes	NA	NA	NA
Neutropenia	Yes	Yes	Yes	Yes	Yes
Prognosis	Death	Improved	Death	Death	Improved

MDS, myelodysplastic syndrome; DM, diabetes mellitus; NA, not available; AML, acute myeloid leukemia; PRT, post renal transplantation.

Case Descriptions

Case 1, a male patient aged 55 years, was admitted to the hospital on September 11, 2020, with "fever and cough for 3 days." The patient was diagnosed with myelodysplastic syndrome (MDS) in July 2019. With type II diabetes, the patient's serum glucose was controlled well. On August 20, 2020, "decitabine + CAG (cytarabine + acramomycin + granulocyte colony-stimulating factor) chemotherapy regimen" was performed, and there was mild bone marrow suppression after chemotherapy. On September 8, 2020, the patient developed fever, with a maximum body temperature of 39.6°C, accompanied by cough, white sticky sputum, chest tightness, and asthma. Laboratory examinations on admission are as follows (**Table 2**): white blood cell (WBC) count, 0.32×10^9 cells/L; red blood cell (RBC) count, 2.41×10^{12} cells/L; hemoglobin (Hb), 79.3 g/L; platelet (PLT), 6×10^9 cells/L; neutrophile (NE), 0.03×10^9 cells/L; lymphocyte (Ly), 0.28×10^9 cells/L; procalcitonin (PCT), 3.45 ng/ml; C-reactive protein (CRP), 131 mg/L; G test, <10 pg/ml; GM test, <0.25 μg/L; and chest CT on September 14, 2020: multiple small nodules could be seen in both lungs (**Figure 2A**). After admission, blood culture and sputum culture were conducted, and the patient was given anti-infective treatment with biapenem, voriconazole, and teicoplanin, but body temperature did not improve significantly. On September 15, 2020, peripheral blood was collected for mNGS. On September 16, 2020, the results of peripheral blood mNGS suggested *Aspergillus flavus* and *Aspergillus fumigatus* infection (**Table 3**). On September 18, 2020,

TABLE 2 | Laboratory examination and treatment.

	Case 1	Case 2	Case 3	Case 4	Case 5
Laboratory examination					
WBC (10∧9/L)	0.32	0.02	0.4	0.21	0.22
RBC (10∧12/L)	2.41	3	3.4	2.97	1.7
Lymphocyte (10∧^{9}/L)	0.28	0.02	0.34	0.06	0.14
Monocyte (10∧^{9}/L)	0.01	0.08	0	0	0.01
Neutrophile (10∧9/L)	0.03	0.17	0.03	0.15	0.06
Platelet (10∧9/L)	6	26	267	22	11
Hemoglobin (g/L)	79.3	95	96.5	86	57.1
Albumin (g/L)	29.1	32.7	21.9	29.2	32.1
LDH (U/L)	234	86	73	840	88
CRP(mg/L)	131	108	143	234.3	99.4
PCT (ng/mL)	3.45	0.747	6.07	13.4	68.3
G test	Negative	Negative	Positive	Negative	Negative
GM test	Negative	Negative	Positive	Negative	Negative
Methods of diagnosis					
Histopathology	NA	*Aspergillus*	NA	NA	NA
Blood mNGS	*Aspergillus flavus, Aspergillus fumigatus*	*A. flavus*	*A. flavus, A. fumigatus*	*A. fumigatus, A. flavus*	*A. flavus*
Blood culture	Negative	Negative	Negative	Negative	Negative
Sputum culture	*A. flavus*	Negative	Negative	Negative	Negative
BALF culture	NA	Negative	Negative	Negative	NA
BALF mNGS	NA	NA	*A. flavus, A. fumigatus*	*Legionella pneumophila, Enterococcus faecium, A. fumigatus, A. flavus*	NA
Sputum mNGS	NA	*A. flavus*	NA	NA	*A. flavus*
Tissue mNGS	NA	*A. flavus*	NA	NA	NA
Treatment	Voriconazole	Posaconazole Voriconazole, AMB, LMB	Voriconazole, Caspofungin	Voriconazole, Caspofungin	Posaconazole

WBC, white blood cell; RBC, red blood cell; LDH, lactic dehydrogenase; CRP, C-reactive protein; PCT, procalcitonin; G test, 1,3-β-D-glucan test; GM test, galactomannan test; mNGS, metagenomic next-generation sequencing; BALF, bronchoalveolar lavage fluid; NA, not available; AMB, amphotericin B; LMB, amphotericin B liposome.

sputum culture suggested *A. flavus* infection. After continued voriconazole treatment for 1 week, the patient died of secondary mucor infection.

Case 2, a female patient aged 21 years, was admitted to the hospital on November 26, 2020, with "acute myeloid leukemia (AML) diagnosed for more than 11 months and fever for 6 days." On November 5, 2020, the patient received "decitabine 10 mg × 10 d" chemotherapy, severe bone marrow transplantation after chemotherapy, and oral posaconazole to prevent fungal infection. On September 20, 2020, the patient developed fever, with a maximum body temperature of 39°C, accompanied by cough and chest pain. Laboratory examinations on admission are as follows (**Table 2**): WBC count, 0.02×10^9 cells/L; RBC count, 3×10^{12} cells/L; Hb, 95 g/L; PLT, 26×10^9 cells/L; NE, 0.17×10^9 cells/L; Ly, 0.02×10^9 cells/L; PCT, 0.747 ng/ml; CRP, 108 mg/L; G test, <10 pg/ml; and GM test, <0.25 μg/L. Under treatment with cefoperazone and sulbactam + levofloxacin + voriconazole anti-infection, fever did not improve. Chest CT scan on December 3, 2020, showed multiple nodules in both lungs and halo signs around the nodules (**Figure 2B**). On December 5, 2020, peripheral blood was collected for mNGS. On December 7, 2020, the results of peripheral blood mNGS suggested *A. flavus* infection, combined with intravenous infusion of amphotericin B liposome and aerosol inhalation of amphotericin B, then body temperature returned to normal 12 days later. Reexamination of chest CT on January 3, 2021, showed no obvious absorption of lung nodules. At this time, the patient's blood platelets reached 85×10^9 cells/L. To further confirm the diagnosis,

CT-guided percutaneous lung puncture was conducted for pathology and mNGS. Grocott methenamine silver (GMS) stain of lung histopathology revealed septal hyphae with 45 degree branches. and lung tissue mNGS suggested *A. flavus* infection (**Table 3**). The patient is still being treated with amphotericin B antifungal therapy.

Case 3, a female patient aged 56 years, was admitted to the Department of Endocrinology on August 3, 2020, with "thirsty, excessive drinking for more than 6 years and sore throat for 4 days." Laboratory examinations on admission are as follows (**Table 2**): WBC count, 0.4×10^9 cells/L; RBC count, 3.4×10^{12} cells/L; Hb, 96.5 g/L; PLT, 267×10^9 cells/L; NE, 0.03×10^9 cells/L; Ly, 0.34×10^9 cells/L; PCT, 6.07 ng/ml; CRP, 143 mg/L; G test, 333.56 pg/ml; and GM test, 1.01 μg/L. Chest CT on admission was normal. The patient was diagnosed with type II diabetes and hyperthyroidism. Later, to further identify the cause of agranulocytosis, the patient was transferred to the Department of Hematology for further treatment. After the transfer, the patient was diagnosed as MDS using bone marrow aspiration, bone marrow biopsy, and flow cytometry. During the diagnosis, the patient developed a high fever and had a negative blood culture. On September 6, 2020, reexamination of chest CT revealed multiple small nodules in both lungs (**Figure 2C**). She was treated with voriconazole first, but she still had high fever. On September 8, 2020, peripheral blood was collected for mNGS. On September 9, 2020, the results of peripheral blood mNGS suggested *A. flavus* and *A. fumigatus* infection (**Table 3**). Although voriconazole has been used for the treatment of *Aspergillus*, the patient still had high fever. To further identify the pathogen, on September 12, 2020, a fiberoptic bronchoscope was made for her, and the BALF was taken for bacterial culture, fungal culture, immunofluorescence staining, and mNGS. BALF-mNGS suggested *A. flavus* and *A. fumigatus* infection, but bacterial culture, fungal culture, and immunofluorescence staining were negative. The patient gave up medical treatment and died on September 16, 2020.

Case 4, a female patient aged 55 years, was admitted to the hospital on November 4, 2020, with "abnormal renal function for 3 years and regular dialysis for 3 years." On November 5, 2020, the patient was given an allograft renal transplantation under general anesthesia. After operation, intravenous injection of Cefminox, ganciclovir, caspofungin, ganciclovir, and oral sulfonamide were used to prevent infection and oral tacrolimus and mycophenolate mofetil to prevent graft-vs.-host disease. Fever developed on the 20th day after the operation, and the highest body temperature was 39°C. Laboratory examinations on admission are as follows (**Table 2**): WBC count, 0.21×10^9 cells/L; RBC count, 2.97×10^{12} cells/L; Hb, 86 g/L; PLT, 22×10^9 cells/L; NE, 0.15×10^9 cells/L; Ly, 0.06×10^9 cells/L; PCT, 13.4 ng/ml; CRP, 234.3 mg/L; G test, <10 pg/ml; and GM test, <0.25 μg/L. Chest CT showed multiple irregular nodules in both lungs and a little pleural effusion on the right side (**Figure 2D**). With switching to meropenem, voriconazole, and doxycycline for anti-infective therapy, body temperature did not improve significantly. On November 28, 2020, blood and BALF were collected for mNGS. The test results both suggested *Legionella pneumophila*, *Enterococcus faecium*, *A. fumigatus*, and *A. flavus* infection (**Table 3**). On December 6, 2020, medical treatment failed and the patient died.

Case 5, a male patient aged 39 years, was admitted to the hospital with a diagnosis of AML for more than 6 months and fever for 5 h. On October 12, 2020, the patient was given "decitabine + CAG" chemotherapy for 14 days. After chemotherapy, the patient suffered severe bone marrow suppression. On October 30, 2020, the patient developed a high fever, with a body temperature of 39.7°C, and the body temperature could not return to normal on its own. Laboratory examinations on admission are as follows (**Table 2**): WBC count, 0.22×10^9 cells/L; RBC count, 1.7×10^{12} cells/L; Hb, 57.1 g/L; PLT, 11×10^9 cells/L; NE, 0.06×10^9 cells/L; Ly, 0.14×10^9 cells/L; PCT, 68.3 ng/ml; CRP, 99.4 mg/L; G test, <10 pg/ml; and GM test, <0.25 μg/L. The fever was still high after treatment with imipenem and cilastatin, teicoplanin, posaconazole suspension. On November 3, 2020, chest CT scan showed multiple nodules and masses in both lungs (**Figures 2E,F**). On November 7, 2020, blood and sputum mNGS both suggested *A. flavus* (**Table 3**). The anti-infective treatment plan was adjusted to cefoperazone and sulbactam + tigecycline antibacterial treatment, and the plasma concentration of posaconazole was 2.21 μg/ml. The body temperature returned to normal on November 18, 2020. The patient is still taking oral posaconazole suspension and continued regular chemotherapy since December 10, 2020, to treat AML. The patient is generally in good condition and is still being followed up.

DISCUSSION

In recent years, the incidence of invasive aspergillosis (IA) has increased rapidly. The estimated number of patients worldwide has risen from 200,000 to more than 300,000 cases per year. The average annual incidence is 4.1 cases per 100,000, of which IPA accounts for more than 90%. The mortality rate is still very high (17). Currently, the diagnosis of IPA is difficult, and early and accurate diagnosis is one of the key steps to effectively treat infections and reduce high mortality (18). Non-specific clinical and radiological manifestations and routine diagnostic methods may delay the correct diagnosis. Of the 5 patients included in this study, 2 had MDS as the underlying disease, 2 had AML, and 1 had renal transplantation, and all of them were at high risk of IPA. After treatment, 3 patients died, giving a mortality rate of 60%. The clinical symptoms of those 5 patients were fever and cough. PCT and CRP were higher than normal, which could not be distinguished from bacterial infections by clinical symptoms and laboratory tests. The chest imaging showed multiple nodules in both lungs, and only 1 case had a halo sign, lacking the typical imaging findings of IPA. Although the 5 patients were all in high-risk groups of IPA, none of them met the diagnostic criteria of EORTC/MSGERC IPA before mNGS. In our study, peripheral blood mNGS rapidly diagnosed *Aspergillus* infection, and then combined with the patient's chest CT, the pulmonary infection was considered as IPA.

These 5 patients were not only diagnosed with *Aspergillus* infection *via* peripheral blood mNGS, but 3 of them were

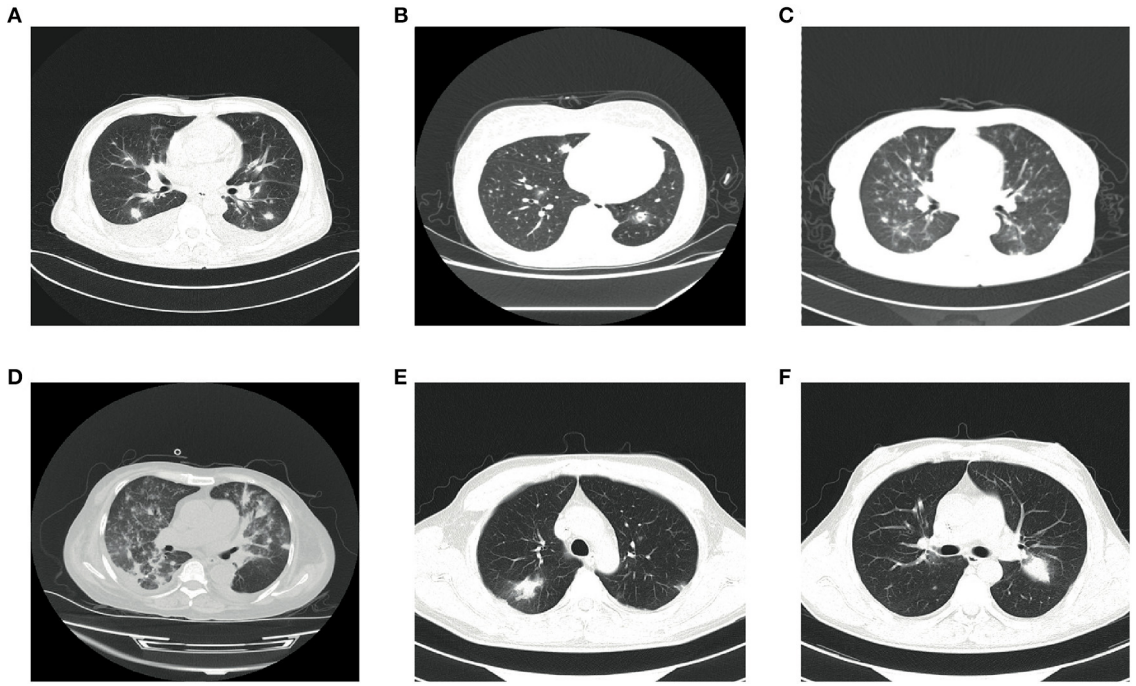


FIGURE 2 | Findings of chest CT scan. **(A)** Chest CT of case 1 showing multiple small nodules in both lungs and a little pleural effusion on both sides. **(B)** Chest CT of case 2 showing multiple small nodules in both lungs and halo signs around some nodules. **(C)** Chest CT of case 3 showing multiple small nodules along the vascular bundles in both lungs and a little pleural effusion on both sides. **(D)** Chest CT of case 4 showing multiple irregular nodules in both lungs and a little pleural effusion on the right side. **(E,F)** Chest CT of case 5 showing multiple nodules and masses in both lungs.

TABLE 3 | Results of peripheral blood mNGS.

Patient NO.	Sample	mNGS testing results	mNGS sequence number
1	peripheral blood	*Aspergillus flavus*	2,067
		Aspergillus fumigatus	568
		Klebsiella pneumoniae	38
2	peripheral blood	*A. flavus*	9
3	peripheral blood	*A. flavus*	502
		A. fumigatus	1
4	peripheral blood	*A. fumigatus*	2,680
		A. flavus	2
		Legionella pneumophila	174
		Enterococcus faecium	77
5	peripheral blood	*A. flavus*	44

mNGS, metagenomic next-generation sequencing.

also tested for combined bacterial infection, and blood cultures collected during their diagnosis and treatment were all negative. Previous studies had shown that the positive rate of peripheral blood culture in infectious diseases was only 1%, and the positive rate of peripheral blood mNGS could reach 24%, suggesting that peripheral blood mNGS has great diagnostic value in infectious diseases (19). Our study shows that peripheral blood mNGS can simultaneously detect bacterial and fungal infections that cause lung infections, which helps to rapidly clarify the condition and adjust the treatment plan.

The current methods for the diagnosis of IPA mainly include histopathology, direct examination method, fungal culture, fungal antigen and antibody detection, and molecular biology detection. All guidelines of *Aspergillus* diagnosis and treatment clearly point out that histopathology and sterile body fluid culture are the gold standards for the diagnosis of IPA. However, the proportion of proven IPA is extremely low because most patients in clinic cannot tolerate invasive procedures required by histopathology, and the positive rate of fungal culture is also extremely low. The G and GM tests are the two most used

biomarkers of *Aspergillus*. But both the blood G and BALF-G tests have relatively low sensitivity and poor specificity for the diagnosis of IPA (20). The GM test, especially the BALF-GM test, has high sensitivity and specificity in patients with agranulocytosis and IPA and is currently the most widely used IA biomarker in clinical practice. However, the problem that puzzles clinicians is that the cutoff value of the BALF-GM test is still not uniform, and the sensitivity and specificity are different under different cutoff values (21). Infectious Diseases Society of America (IDSA) strongly recommends that patients with suspected IPA undergo bronchoscopy and obtain BALF for routine culture, cytology, and GM tests. However, in clinical practice, severely infected patients may be complicated by severe hypoxemia, bleeding, or thrombocytopenia, and invasive operations such as bronchoscopy will be difficult to implement, so relying on the BALF-GM test to diagnose IPA is not feasible (22). According to the diagnostic criteria of EORTC/MSGERC for IPA, we reported 5 patients, of whom 1 case was proven and 4 cases were probable. However, only 1 patient had a positive blood GM test, and 3 cases of BALF-GM test were all negative. Therefore, clinical attention should also be paid to the issue of false negative blood GM test and BALF-GM test.

Due to the high morbidity and mortality of IPA, a non-invasive detection method is urgently needed for the rapid and accurate diagnosis of IPA in clinical practice. It was found that DNA fragments of pathogens that can cause different infection sites in human body can be found in purified plasma. Sequencing the cfDNA of this microorganism can improve the possibility of non-invasive detection of multiple infections (17, 23, 24). PCR is recommended by the EORTC/MSGERC as a method for detecting *Aspergillus*, but research shows that the sensitivity and specificity of *Aspergillus* PCR analysis methods from different vendors are quite different. The sensitivity of Artus Aspergillus RG PCR assay was only 47.6%, while the sensitivity of MycAssay Aspergillus PCR was 61.9% (25). Another study showed that the sensitivity of GM and *Aspergillus* PCR in blood was even lower, only 31 and 0% (21). Due to the lack of uniform standards in different studies and environments and the huge differences in diagnostic performance, the exact role of PCR in the diagnosis of IPA remains controversial. mNGS is another molecular diagnostic technique used in the diagnosis of infectious diseases in recent years (26). Hong et al. used plasma NGS technology for the first time in 2018 to identify the pathogens of confirmed invasive fungal infections, including *Aspergillus* and non-*Aspergillus* species. Among the 9 patients, 7 cases of plasma NGS detected the same fungus found in the biopsy at the genus level (24). Our research has also confirmed that peripheral blood mNGS can be used to diagnose IPA, but the detection efficiency of peripheral blood mNGS still needs to be confirmed by large-scale clinical trials. Peripheral blood specimens of the 5 patients in this study were all collected after the onset of fever. No more peripheral blood mNGS test was performed during the course of the disease. Therefore, it is still unknown when *Aspergillus* cfDNA would exist in the peripheral blood and when to collect peripheral blood. The application of IPA diagnosis *via* peripheral blood mNGS is a subject that needs to be further explored. Studies have confirmed that antifungal treatment can reduce the sensitivity of PCR detection of peripheral blood *Aspergillus* (27), so collecting peripheral blood for mNGS early in the course of the disease may increase the diagnostic rate of IPA.

Our study has some limitations. First, this is a retrospective study that may be biased. The 5 cases included in this study cannot tolerate CT guided percutaneous lung puncture or tracheal puncture under tracheoscopy due to low platelet or poor physical condition, and only case 2 had pathological findings for diagnosis. Although cases 1, 3, 4, and 5 had microbiological evidence, they were not the gold standard for the diagnosis of IPA after all, so there is a slight chance that they are not *Aspergillus* infection. Second, the subjects are all patients with neutropenia, and the results of the study may not be applicable to patients with non-neutropenia IPA. Third, the sample size is small. During the diagnosis and treatment of these patients, the peripheral blood mNGS test was performed only once. It is impossible to confirm the existence time of *Aspergillus* cfDNA in the peripheral blood. The time to collect peripheral blood for the mNGS test is not yet clear.

In conclusion, our study confirmed that detecting the cfDNA of *Aspergillus via* peripheral blood mNGS can be used to diagnose IPA and is a rapid and non-invasive diagnosis method.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject/, PRJEB43573.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XM, WC, AX, and LX conceived the project. JC, MJ, QS, and HaL performed the experiments. SZ, WC, and HX collected cases. XM and HuL analyzed and interpreted patient data. XM wrote the manuscript. All authors have read and approved the final manuscript.

FUNDING

This research was supported by the Beijing Medical and Health Public Welfare Foundation (YWJKJJHKYJJ-B182121-1).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.751617/full#supplementary-material

REFERENCES

1. Kashefi E, Seyedi SJ, Zomorodian K, Zare Shahrabadi Z, Zarrinfar H. Successful treatment of pulmonary aspergillosis due to aspergillus fumigatus in a child affected by systemic lupus erythematosus: a case report from Northeastern Iran. *Clin Case Rep.* (2021) 9:e04248. doi: 10.1002/ccr3.4248
2. Zanganeh E, Zarrinfar H, Rezaeetalab F, Fata A, Tohidi M, Najafzadeh MJ, et al. Predominance of non-fumigatus aspergillus species among patients suspected to pulmonary aspergillosis in a tropical and subtropical region of the Middle East. *Microb Pathog.* (2018) 116:296–300. doi: 10.1016/j.micpath.2018.01.047
3. Zarrinfar H, Makimura K, Satoh K, Khodadadi H, Mirhendi H. Incidence of pulmonary aspergillosis and correlation of conventional diagnostic methods with nested PCR and real-time PCR assay using BAL fluid in intensive care unit patients. *J Clin Lab Anal.* (2013) 27:181–5. doi: 10.1002/jcla.21580
4. Zarrinfar H, Mirhendi H, Fata A, Khodadadi H, Kordbacheh P. Detection of Aspergillus flavus and A. fumigatus in bronchoalveolar lavage specimens of hematopoietic stem cell transplants and hematological malignancies patients by real-time polymerase chain reaction, nested pcr and mycological assays. *Jundishapur. J Microbiol.* (2015) 8:e13744. doi: 10.5812/jjm.13744
5. Chamilos G, Luna M, Lewis RE, Bodey GP, Chemaly R, Tarrand JJ, et al. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989-2003). *Haematologica.* (2006) 91:986–9.
6. Budin S, Salmanton-García J, Koehler P, Stemler J, Cornely OA, Mellinghoff SC. Validation of the EQUAL Aspergillosis Score by analysing guideline-adherent management of invasive pulmonary aspergillosis. *J Antimicrob Chemotherap*. (2021) 21:518. doi: 10.1093/jac/dkaa518
7. Ardi P, Daie-Ghazvini R, Hashemi SJ, Salehi MR, Bakhshi H, Rafat Z, et al. Study on invasive aspergillosis using galactomannan enzyme immunoassay and determining antifungal drug susceptibility among hospitalized patients with hematologic malignancies or candidates for organ transplantation. *Microb Pathog.* (2020) 147:104382. doi: 10.1016/j.micpath.2020.104382
8. Boch T, Spiess B, Cornely OA, Vehreschild JJ, Rath PM, Steinmann J, et al. Diagnosis of invasive fungal infections in haematological patients by combined use of galactomannan, 1,3-β-D-glucan, Aspergillus PCR, multifungal DNA-microarray, and Aspergillus azole resistance PCRs in blood and bronchoalveolar lavage samples: results of a prospective multicentre study. *Clinic Microbiol Infect Offic Publicat Europ Soc Clinic Microbiol Infect Dis.* (2016) 22:862–8. doi: 10.1016/j.cmi.2016.06.021
9. Linder KA, Kauffman CA, Zhou S, Richards BJ, Kleiboeker S, Miceli MH. Performance of the (1,3)-Beta-D-Glucan Assay on Bronchoalveolar Lavage Fluid for the Diagnosis of Invasive Pulmonary Aspergillosis. *Mycopathologia.* (2020) 185:925–9. doi: 10.1007/s11046-020-00479-0
10. Duan H, Li X, Mei A, Li P, Liu Y, Li X, et al. The diagnostic value of metagenomic next?generation sequencing in infectious diseases. *BMC Infect Dis.* (2021) 21:62. doi: 10.1186/s12879-020-05746-5
11. Langelier C, Fung M, Caldera S, Deiss T, Lyden A, Prince BC, et al. Detection of Pneumonia Pathogens from Plasma Cell-Free DNA. *Am J Respir Crit Care Med.* (2020) 201:491–5. doi: 10.1164/rccm.201904-0905LE
12. Donnelly JP, Chen SC, Kauffman CA, Steinbach WJ, Baddley JW, Verweij PE, et al. Revision and update of the consensus definitions of invasive fungal disease from the european organization for research and treatment of cancer and the mycoses study group education and research consortium. *Clinic Infect Dis Offic Publicat Infect Dis Soc Am.* (2020) 71:1367–76. doi: 10.1093/cid/ciz1008
13. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics.* (2018) 34:i884–90. doi: 10.1093/bioinformatics/bty560
14. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* (2012) 9:357–9. doi: 10.1038/nmeth.1923
15. Zaharia, M., Bolosky, W. J., Curtis, K., Fox, A., Patterson, D., Shenker, S., et al. (2011). Faster and More Accurate Sequence Alignment with SNA.P. arXiv. https://arxiv.org/abs/1111.5572
16. Jing C, Chen H, Liang Y, Zhong Y, Wang Q, Li L, et al. Clinical Evaluation of an Improved Metagenomic Next-Generation Sequencing Test for the Diagnosis of Bloodstream Infections. *Clin Chem.* (2021) 67:1133–43. doi: 10.1093/clinchem/hvab061
17. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases-estimate precision. *J Fungi.* (2017) 3:57. doi: 10.3390/jof3040057
18. Ledoux MP, Guffroy B, Nivoix Y, Simand C, Herbrecht R. Invasive Pulmonary Aspergillosis. *Semin Respir Crit Care Med.* (2020) 41:80–98. doi: 10.1055/s-0039-3401990
19. Blauwkamp TA, Thair S, Rosen MJ, Blair L, Lindner MS, Vilfan ID, et al. Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease. *Nat Microbiol.* (2019) 4:663–74. doi: 10.1038/s41564-018-0349-6
20. Taghizadeh-Armaki M, Hedayati MT, Moqarabzadeh V, Ansari S, Mahdavi Omran S, Zarrinfar H, et al. Effect of involved Aspergillus species on galactomannan in bronchoalveolar lavage of patients with invasive aspergillosis. *J Med Microbiol.* (2017) 66:898–904. doi: 10.1099/jmm.0.000512
21. Eigl S, Hoenigl M, Spiess B, Heldt S, Prattes J, Neumeister P, et al. Galactomannan testing and Aspergillus PCR in same-day bronchoalveolar lavage and blood samples for diagnosis of invasive aspergillosis. *Medical mycology.* (2017) 55:528–34. doi: 10.1093/mmy/myw102
22. Patterson TF, Thompson GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the infectious diseases society of America. *Clinic Infect Dis Offic Publicat Infect Dis Soc Am*. (2016) 63:e1–e60. doi: 10.1093/cid/ciw326
23. Abril MK, Barnett AS, Wegermann K, Fountain E, Strand A, Heyman BM, et al. Diagnosis of Capnocytophaga canimorsus sepsis by whole-genome next-generation sequencing. *Open Forum Infect Dis.* (2016) 3:ofw144. doi: 10.1093/ofid/ofw144
24. Hong DK, Blauwkamp TA, Kertesz M, Bercovici S, Truong C, Banaei N. Liquid biopsy for infectious diseases: sequencing of cell-free plasma to detect pathogen DNA in patients with invasive fungal disease. *Diagn Microbiol Infect Dis.* (2018) 92:210–3. doi: 10.1016/j.diagmicrobio.2018.06.009
25. Erman-Daloglu A, Ozhak B, Salim O, Turhan O, Ongut G, Gunseren F, et al. Evaluation of Commercially available real-time polymerase chain reaction assays for the diagnosis of invasive aspergillosis in patients with haematological malignancies. *Mycopathologia.* (2020) 185:269–77. doi: 10.1007/s11046-020-00424-1
26. Egger M, Jenks JD, Hoenigl M, Prattes J. Blood Aspergillus PCR: The Good, the Bad, and the Ugly. *J Fungi.* (2020) 6:10018. doi: 10.3390/jof6010018

27. Cruciani M, White PL, Mengoli C, Löffler J, Morton CO, Klingspor L, et al. The impact of anti-mold prophylaxis on Aspergillus PCR blood testing for the diagnosis of invasive aspergillosis. *J Antimicrob Chemother.* (2020) 21:498. doi: 10.1093/jac/dkaa498

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Ma, Zhang, Xing, Li, Chen, Li, Jiao, Shi, Xu, Xing and Cao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Medicine

CASE REPORT
published: 08 April 2022
doi: 10.3389/fmed.2022.755308

Recurrent Pneumonia With Tuberculosis and *Candida* Co-infection Diagnosed by Metagenomic Next-Generation Sequencing: A Case Report and Literature Review

Ning Ma[1†], Mei Chen[1†], Jingyi Ding[2†], Fang Wang[3], Jingbo Jin[1], Sitong Fan[1] and Jiajia Chen[2]*

[1] Department of Infectious Diseases, Beilun District People's Hospital, Ningbo, China, [2] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [3] Department of Hospital-Acquired Infection Control, Beilun District People's Hospital, Ningbo, China

An 82-year-old male patient was hospitalized in the Respiratory Department for "repeated cough and shortness of breath for 10 years, recurrence worsened for 1 month." Later, he was transferred for further diagnosis and treatment, to the Infectious Disease Department for further hospitalization. Previously, the patient had repeatedly undergone tuberculosis-related examinations including bronchoscopy examinations. However, no evidence of *Mycobacterium tuberculosis* (*MTB*) infection was found. Early anti-infection treatments failed. Due to repeated symptoms, we performed bronchoscopy again and sent alveolar lavage fluid for the metagenomic next-generation sequencing (mNGS) test. Subsequently, *MTB* and *Candida albicans* were detected by mNGS. After antituberculosis and antifungal treatments, the symptoms were significantly relieved, and the chest CT showed resolution of the lung lesions. Therefore, we successfully diagnosed and treated a case of recurrent pneumonia with tuberculosis and *Candida* co-infection diagnosed by mNGS.

Keywords: tuberculosis, *Candida* pneumonia, case report, literature review, mNGS

OPEN ACCESS

Edited by:
Li Ang,
The First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Tracy H. Erkkila,
Los Alamos National Laboratory (DOE), United States
Andrey Kechin,
Institute of Chemical Biology and Fundamental Medicine (RAS), Russia
Claudia Colabella,
Experimental Institute of Zooprophylaxis of Umbria and Marche (IZSUM), Italy
Amit Rawat,
Post Graduate Institute of Medical Education and Research (PGIMER), India
Yongguo Li,
The First Affiliated Hospital of Harbin Medical University, China

***Correspondence:**
Jiajia Chen
jiajiatale0@zju.edu.cn

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *08 August 2021*
Accepted: *23 February 2022*
Published: *08 April 2022*

Citation:
Ma N, Chen M, Ding J, Wang F, Jin J, Fan S and Chen J (2022) Recurrent Pneumonia With Tuberculosis and Candida Co-infection Diagnosed by Metagenomic Next-Generation Sequencing: A Case Report and Literature Review. Front. Med. 9:755308. doi: 10.3389/fmed.2022.755308

INTRODUCTION

Tuberculosis is one of the most important infectious diseases monitored in China, which seriously affects human health. *Mycobacterium tuberculosis* (*MTB*) can invade multiple organs, but tuberculosis is the most common disease they cause. In 2019, approximately 10 million people (range: 8.9–11 million) suffered from tuberculosis (1). Its worldwide prevalence has had a huge impact on the healthcare system both in economic and health terms, which has prompted the World Health Organization (WHO) to include it among the top-priority infectious diseases (2). This study analyzes the clinical data of a patient who suffered from recurrent pneumonia with tuberculosis and *Candida* co-infection, admitted to our department. A review of the available literature on the clinical application of metagenomic next-generation sequencing (mNGS) has also been performed.

CASE PRESENTATION

An 82-year-old male patient was hospitalized with recurrent cough and shortness of breath for 1 month. Prior to the appearance of these symptoms, he had recurrent cough and shortness of breath for 10 years. He was previously diagnosed with chronic bronchitis. Although his symptoms were slightly relieved after repeated anti-infection (*Piperacillin Sodium/Tazobactam Sodium and Cefoperazone Sodium/Sulbactam Sodium*), anti-asthmatic (*Doxofylline*), and expectorant (*Bromhexine Hydrochloride*) treatments, his cough and shortness of breath were still recurring. At the same time, the patient suffered from weight loss and repeated low-grade fever. A detailed physical examination revealed moist rales in the left lung and diminished respiration in the right lung. Thus, given his poor immunity, it was necessary to consider the infection of pathogens such as *MTB* and fungi. However, repeated examinations showed no pathogenic evidence. The chest CT scan after admission is depicted in **Figure 1**. Bilateral bronchial disease, bilateral pleural effusion, and lung infection in the right upper lung and left lower lung can be observed on the images. Part of the examination results on admission is presented in **Table 1**. Due to a large amount of pleural effusion, ultrasound-guided thoracentesis was performed to clarify the nature of the pleural effusion and the adjuvant treatment needed. Meanwhile, the pleural fluid routine, biochemical test, examination of exfoliated cells, fluid culture, and fluid acid-fast bacilli stain were performed, the results of the pleural effusion analysis are listed in **Table 2**. More than 500 pcs/μl white blood cells were found in the pleural effusion, and the Rivalta test was positive, and exudative pleural effusion needed to be considered. Since the lymphocyte proportion was relatively high, and the purified protein derivative (PPD) was positive, tuberculous pleurisy could not be excluded. However, the Xpert test and γ-interferon release test (T-spot) were negative. In summary, evidence for *MTB* infection was still insufficient. Except for the carbohydrate antigen 125, the tumor markers of the patient were within the normal reference range, and thus, the possibility of tumor involvement was low. However, after the application of conventional antibacterial treatment for 14 days, the patient's symptoms, signs, and reexamination indicators (**Table 1**) did not significantly improve. Therefore, bronchoscopy, sputum culture, and fungal examination were performed again. The alveolar lavage fluid was sent for mNGS. Additionally, multiple *MTB* culture identification examinations were performed. The mNGS results revealed the presence of *MTB* (3 reads) and fungi (1,761 reads). The results are displayed in **Table 3**. *Candida* was isolated in the sputum culture.

At present, the diagnosis of *Candida* infection was clear, but the reads of nucleotide sequences of *MTB* were less. The diagnosis of tuberculosis was still in doubt. However, based on the patient's chest CT scan and pleural effusion tests, *MTB* infection could not be ruled out. Also considering the adverse effects of missing diagnosis and treatment of tuberculosis on the prognosis of patients, we diagnosed the patient with *MTB* and *Candida* co-infection. Then, considering the patient's advanced age and poor physical condition, we implemented triple antituberculosis (Isoniazid: 300 mg one time a day, Rifampin: 450 mg one time a day, and Levofloxacin: 500 mg one time a day) and antifungal (Fluconazole: 400 mg one time a day) treatments after informed consent was received from the patient. Subsequently, the patient's laboratory results had improved (**Table 1**), and Fluconazole was discontinued after 2 weeks. Later, *MTB* was isolated from the patient's sputum culture (performed on June 14) and alveolar lavage fluid culture (performed on June 15). Hence, the results were reconfirmed. Fortunately, this patient had good adherence, and we followed him up for half a year. Transaminase had been slightly elevated briefly in antituberculosis treatment, and the transaminase was within the normal range after the addition of hepatoprotective drugs. In the subsequent follow-up, we learned that his cough, shortness of breath, and other symptoms were considerably relieved. The lung lesions and pleural effusion were obviously resolved (**Figure 1**). At present, the drug is orderly reduced to *Isoniazid* and *Rifampin*.

MATERIALS AND METHODS

Sample Processing and DNA Extraction

Notably, a 1.5–3 ml *BALF* sample from the patient was collected according to standard procedures and inactivated in a water bath at 65°C for 30 min; 1.5-ml microcentrifuge tube with 0.6 ml sample and 250 μl 0.5-mm glass bead (weight: 1 g, the concentration was 1,176 mg/ml) were attached to a horizontal platform on a vortex mixer using FastPrep-24^{TM} 5G Instrument and agitated vigorously at 2,800–3,200 rpm for 30 min. Then, 7.2 μl lysozyme (RT410-TA, TIANGEN BIOTECH, Beijing, China) was added for wall-breaking reaction. Of note, 0.3 ml sample was separated into a new 1.5-ml microcentrifuge tube, and DNA was extracted using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH, Beijing, China) according to the manufacturer's recommendation; concentration was measured by Qubit dsDNA HS Assay Kit 3.0 Fluorometer; samples were taken according to the measured concentration; and the nucleic acid fragment was performed using the nucleic acid enzyme digestion reaction kit produced by Huada Biotechnology (Wuhan) Co., Ltd. (3).

Construction of DNA Libraries and Sequencing

The PMseq^{TM} High-throughput Detection Kit for infectious pathogens produced by Huada Biotechnology (Wuhan) Co., Ltd. (i.e., combined probe-anchored polymerase sequencing) was used to prepare the DNA libraries. DNA fragmentation, end-repair, adapter-ligation, and unbiased PCR amplification were performed strictly in accordance with the instructions. Agilent 2,100 was used for quality control of the DNA libraries. Quality qualified libraries were pooled, and DNA nanoball was made and sequenced by the BGISEQ-50/MGISEQ-2000 platform (4).

Bioinformatic Analysis

High-quality sequencing data were generated by removing low-quality reads, followed by computational subtraction of

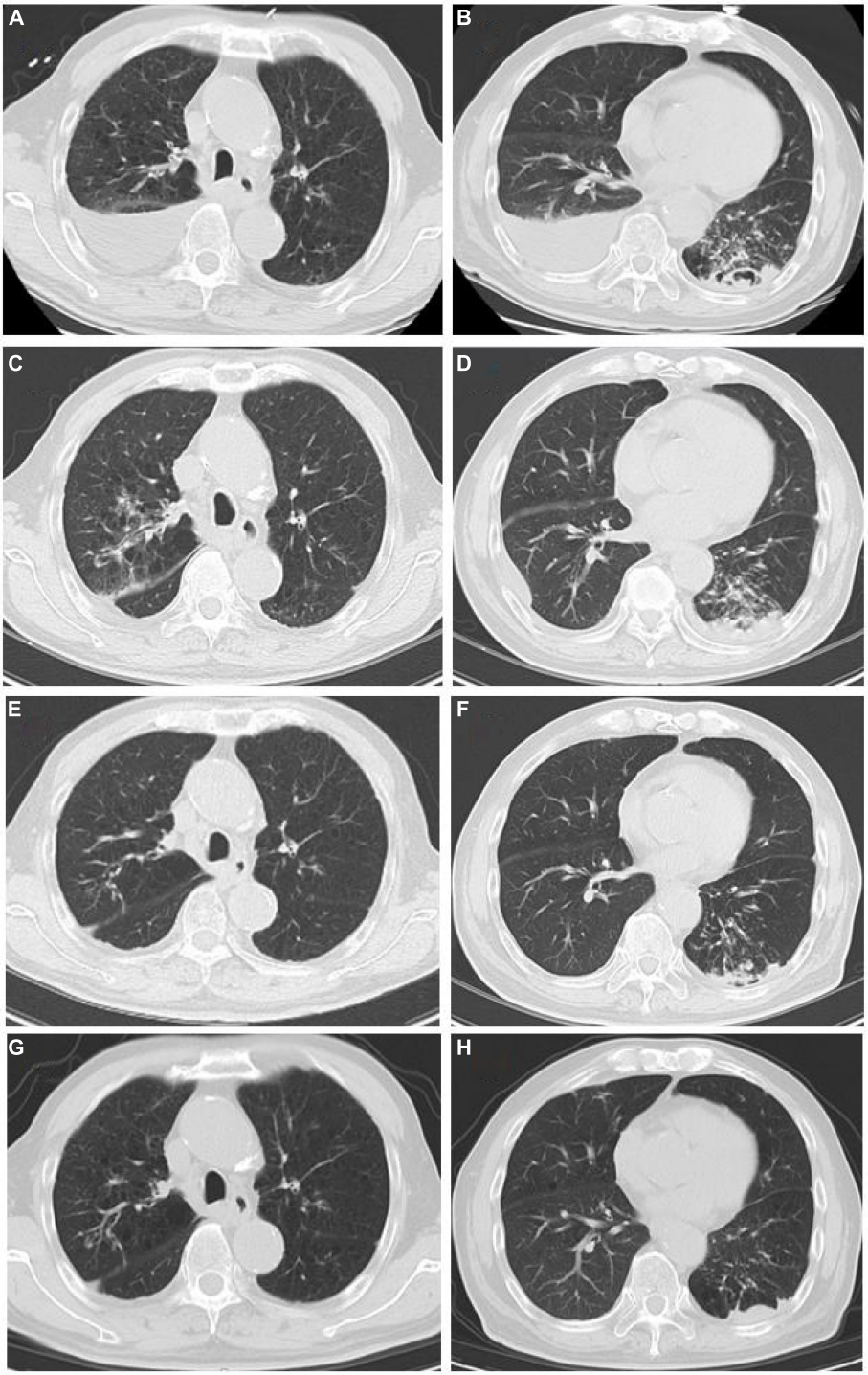


FIGURE 1 | (A,B) Chest CT scan at the first admission (June 8). **(A)** Both lungs had increased field permeability; the density of the fluid in the right pleural cavity was affected, and emphysema was considered; the right pleural effusion was accompanied by local atelectasis. **(B)** Bilateral pleural fluid density shadows, multiple patchy high-density shadows in the left lower lung, and a local cavity formation led to suspected bilateral pleural effusion and infectious lesion in the left lung. **(C,D)** Chest CT scan after conventional antibacterial treatments (June 21): Infectious lesions in both lungs increased, left pleural effusion increased, and right pleural effusion decreased as compared to **(A,B)**. **(E,F)** Chest CT scan after the antituberculosis and antifungal treatments (September 2): The scope of infectious lesions in both lungs was the resolution, the left lower lung was more obvious, and the pleural effusions on both sides were significantly reduced as compared to **(C,D)**. **(G,H)** Chest CT at follow-up (December 21): The infectious lesions in both lungs were obviously resolved as compared to **(E,F)**.

TABLE 1 | Laboratory examination results during the diagnosis and treatment of patients.

Item (unit)	On admission	Ordinary antibacterial treatment for 14 days	Antituberculosis and antifungal treatment for 14 days	Latest follow–up (antituberculosis treatment for half one year)
High-sensitivity C-reactive protein (mg/L)	50.90	99.60	20.20	< 0.5
White blood cell count (×109/L)	3.90	3.50	3.40	-
Neutrophil count (×109/L)	2.56	2.45	2.11	-
Lymphocyte count (×109/L)	0.69	0.49	0.75	-
Albumin (g/L)	31.60	29.3	29.10	35.7
Procalcitonin (ng/ml)	0.05	0.076	0.05	-
Erythrocyte sedimentation rate analysis(mm/h)	78	-	-	26
Sputum culture	**Candida**	-	***Mycobacterium tuberculosis***	-
Acid-fast bacilli stain in sputum	Negative	Negative	Negative	Negative
1,3-β-D-glucan detection	Negative	-	-	-
Carbohydrate antigen 125 (U/ml)	129.70	-	-	-
Carbohydrate antigen 19–9 (U/ml)	3.72	-	-	-
Alpha fetoprotein (ng/ml)	1.48	-	-	-
Carcinoembryonic antigen (ng/ml)	1.74	-	-	-

-, absence of performance.

human host sequences mapped to the human reference genome (hg19) using Burrows-Wheeler Alignment (5). The remaining data by removal of low-complexity reads were classified by simultaneously aligning to the pathogen metagenomics database. The classification reference databases were downloaded from NCBI.[1] RefSeq contains 4,945 whole-genome sequences of viral taxa, 6,350 bacterial genomes or scaffolds, 1,064 fungi related to human infection, and 234 parasites associated with human diseases. We used single-end sequencing, the selected read length was 50 bp, the number of total reads obtained was 50,900,630, and Q30 reached 95.34%.

A positive mNGS was given to bacterial or viral cases when the number of reads mapping to a microbe was 10-fold greater than that of any other microbes, and when the coverage rate for a fungus (species level) was 5-fold greater than that of another fungus, a positive mNGS was given for the fungus. When mNGS detected at least one sequence of the *MTB complex* at the genus level, a positive mNGS was given for *MTB*.

The sputum culture of the patient on admission was *Candida* which we considered as contamination, but his condition had not improved after 14 days of ordinary anti-infective treatment. Referring to the mNGS results, we started antituberculosis and antifungal treatments; later, the sputum culture for *MTB* was positive (performed on June 15), which medium was BD BACTEC^{TM}MGIT^{TM} 960 Supplement Kit, which again supported our diagnosis.

[1]http://ftp.ncbi.nlm.nih.gov/genomes/

TABLE 2 | Results of the patient's pleural fluid examinations on admission.

Item	Result
Color	Yellow
Transparency	Turbid
Rivalta test	**3+**
Microscopic examination of red blood cell count (pcs/μl)	12,773
Microscopic examination of white blood cell count (pcs/μ l)	**2,701**
Neutrophil classification (%)	8
Lymphocyte classification (%)	**80**
Percentage of microscopically examined monocytes (%)	12
Total protein (g/L)	11.9
Glucose (mmol/L)	1.33
Lactate dehydrogenase (U/L)	249
Adenosine deaminase (U/L)	13
Pleural fluid culture	Negative
Acid-fast bacilli in pleural fluid	Negative

Bold values emphasize that these results suggest that pleural effusion may be caused by tuberculosis.

DISCUSSION

Tuberculosis is a disease to be conquered worldwide. Once the diagnosis is clear, 80% of patients can be cured or relieved; at present, the main difficulties are the difficulty of diagnosis and the treatment of multidrug-resistant tuberculosis (6, 7). In this study, a case of recurrent pneumonia with tuberculosis and *Candida* co-infection is reported. The patient had repeatedly suffered from cough and shortness of breath for many years. Multiple bronchoscopies, Xpert, T-spot, and sputum tests for acid-fast bacilli and sputum culture did not detect evidence of *MTB* infection. The efficacy of anti-infection treatment was not effective; therefore, common pathogen infections, tumors, etc., could not explain the patient's disease characteristics. In such cases, special bacterial infections need to be considered. Nevertheless, the complex infection by *Candida* and *MTB* was detected by the mNGS test. Considering the patient's age, underlying diseases, and poor immunity, *Candida* and *MTB* were determined to be the infection-causative agents reconfirmed by the results of his sputum culture, excluding the possibility of bacteria colonization and contamination.

TABLE 3 | Patient's next-generation sequencing (NGS) analysis results (https://www.ncbi.nlm.nih.gov/sra/PRJNA773989).

Genus			Species		
Name	**NGS reads[a]**	**Relative abundance[b]**	**Name**	**NGS reads**	**Relative abundance**
Candida	1,758	88.28%	*Candida albicans*	1,607	86.70%
Mycobacterium tuberculosis complex	3[c]	0.00	-	-	-

[a] *The NGS reads of the microorganism detected at the genus/species level in rigid alignment.*
[b] *The proportion of the microorganism in all the microorganisms detected.*
[c] *We analyzed them with the BLAST NCBI tool and found that they could be matched to the MTB genome in the NCBI library.*

Finally, after adjustments in the treatment plan and the implementation of antituberculosis and antifungal therapy, the patient's general condition and reexamination indicators improved. Chest CT reexamination showed slight attenuation in the lung lesions (**Figure 1**).

Tuberculosis is a chronic contagious disease that seriously endangers human health. The increase in morbidity and mortality from tuberculosis is a consequence of the complex disease process caused by the pathogen *MTB*, which brings a huge burden to society and individuals (8). *MTB* infectious occurs in the lung tissue, trachea, bronchi, and pleura. It can be diagnosed based on the patient's epidemiological history, clinical symptoms and signs, chest imaging, and laboratory examinations (including bacteriological examination, molecular biological examination, pathological examination, immunological examination and culture, tuberculin skin tests, and tuberculosis antibody tests), bronchoscopy, and other examination methods and tests. The new γ-interferon release test has high sensitivity and specificity. It is not affected by bacillus Calmette-Guérin (BCG) vaccination and body immunity (9). A meta-analysis compared the accuracy of Gene Xpert Assay, Microscopic Observation of Drug Sensitivity (MODS), and WHO 2007 algorithm in the diagnosis of tuberculosis in smear-negative patients and found that the sensitivity rates were 67, 73, and 61%, respectively (10). Based on the different inspection methods and results, cases can be classified as confirmed, clinically diagnosed, or suspected. Despite the progress in diagnostic techniques, a considerable number of clinically diagnosed tuberculosis cases but not bacteriologically confirmed were reported to the WHO. The WHO's 2020 target for ending tuberculosis aims at the reduction of the incidence of tuberculosis by 20% and the number of deaths from tuberculosis by 35% as compared to 2015 figures (11). Conventional treatment methods cannot meet the requirements of rapid clinical diagnosis. Therefore, new methods need to be developed in China for more effective diagnosis.

With technology advancement, NGS technology, which is high-throughput and can thus perform large-scale gene analysis, has been widely applied in various fields (12). NGS can directly and non-specifically determine all nucleic acid fragments and can obtain the genomic information of all microorganisms in the samples, substantially reducing missed detection (13). mNGS obtains the nucleotide sequence in the sample by means of the NGS platform and further maps it with the genome sequence of each species, so as to know the type and proportion of microorganisms in the sample. Current literature reports that mNGS has been successfully applied to a variety of specimen types such as cerebrospinal fluid, respiratory secretions, and blood (14, 15). Successful diagnosis of rare and difficult-to-diagnose diseases has been gradually achieved over recent years. For example, NGS can reportedly guide the diagnosis and treatment of Mendelian susceptibility to mycobacterial disease (MSMD), *Listeria monocytogenes* infection, aseptic meningitis related to varicella-zoster virus infection, and fungal infection (16–19). A cohort study found that NGS had the highest sensitivity (58.8%) in the diagnosis of tuberculous meningitis as compared to those of Xpert (38.2%) and conventional methods (29.4%) (20). Currently, NGS research on tuberculosis is used mainly for epidemiological investigation, diagnosis, gene polymorphism, and drug resistance detection (13).

A large number of studies have confirmed that NGS can quickly detect multiple genes, facilitating the diagnosis of various pathogens. However, we cannot directly identify them as pathogenic bacteria. For example, if a variety of bacteria and fungi are detected, they must be judged as colonizing bacteria, contaminating bacteria, or pathogenic bacteria. Candidiasis tends to occur in immunocompromised subjects. The prevalence rate is (2.1–21.0)/100,000, and the fatality rate is 40–60%. Its clinical manifestations are diverse, and it is thus difficult to diagnose. *Candida albicans*, smooth *Candida*, and *Candida tropicalis* are the most common pathogens, among which *Candida albicans* accounts for 65–70% (21). Clinically, several methods such as mannan antigen/antibody detection, NGS, 1-3-β-D-glucan test, *Candida* score, and colonization index can be used to assist in determining colonization and infection. But, *Candida* colonization is an important prerequisite for the occurrence of invasive candidiasis. For this reason, it is necessary to combine clinical manifestations and laboratory examinations to make a conclusion to guide the treatment (22, 23). Compared with the huge amount of medical costs, targeted medication can significantly reduce the cost of treatment through defining the etiology using mNGS.

CONCLUSION

In case of ineffective therapy of patients with lung infection by conventional anti-infective treatments and persistent lesions, the possibility of infection with *MTB*, fungi, viruses, and specific flora species is to be considered. In cases of suspected *MTB* infection with no evidence of infection obtained by repeated traditional testing methods, novel examination approaches such as mNGS are worthy of consideration to assist in the detection process, which may contribute to facilitating the diagnosis of unspecified infections.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Beilun District People's Hospital of Ningbo. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NM, MC, and JD wrote the manuscript. JJ and SF carried out the design and corrections of the chart. FW reviewed the manuscript. JC designed the study and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Harding E. WHO global progress report on tuberculosis elimination. *Lancet Respir Med.* (2020) 8:19. doi: 10.1016/S2213-2600(19)30418-7
2. Al-Zanbagi Adnan B, Shariff MK. Gastrointestinal tuberculosis: a systematic review of epidemiology, presentation, diagnosis and treatment. *Saudi J Gastroenterol.* (2021) 27:261–74. doi: 10.4103/sjg.sjg.148.21
3. Long Y, Zhang YX, Gong YP, Sun RX, Su LX, Lin X, et al. Diagnosis of sepsis with cell-free DNA by next- generation sequencing technology in ICU patients. *Arch Med Res.* (2016) 47:365–71. doi: 10.1016/j.arcmed.2016.08.004
4. Jeon YJ, Zhou Y, Li Y, Guo Q, Chen J, Quan S, et al. The feasibility study of non-invasive fetal trisomy 18 and 21 detection with semiconductor sequencing platform. *PLoS One.* (2014) 9:e110240. doi: 10.1371/journal.pone.0110240
5. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* (2009) 25:1754–60. doi: 10.1093/bioinformatics/btp324
6. Gadoev J, Asadov D, Harries AD, Kumar AMV, Boeree MJ, Hovhannesyan A, et al. Factors associated with unfavourable treatment outcomes in patients with tuberculosis: a 16-year Cohort Study (2005-2020), Republic of Karakalpakstan, Uzbekistan. *Int J Environ Res Public Health.* (2021) 18:12827. doi: 10.3390/ijerph182312827
7. World Health Organization [WHO]. *Global Tuberculosis Report 2020*. Geneva: World Health Organization (2020).
8. Dorhoi A, Kaufmann SH. Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. *Semin Immunopathol.* (2016) 38:153–66. doi: 10.1007/s00281-015-0531-3
9. Wan DY, Huang ZG, He J. The clinical value of anti-tuberculosis antibody and r-interferon release test in the diagnosis of tuberculosis. *Int J Lab Med.* (2016) 37:2912–3. doi: 10.3969/j.issn.1673-4130.2016.20.047
10. Walusimbi S, Bwanga F, De CA, Haile M, Joloba M, Hoffner S. Meta-analysis to compare the accuracy of GeneXpert, MODS and the WHO 2007 algorithm for diagnosis of smear-negative pulmonary tuberculosis. *BMC Infect Dis.* (2013) 13:507. doi: 10.1186/1471-2334-13-507
11. Fukunaga R, Glaziou P, Harris JB, Date A, Floyd K, Kasaeva T. Epidemiology of tuberculosis and progress toward meeting global targets-worldwide, 2019. *MMWR Morb Mortal Wkly Rep.* (2021) 70:427–30. doi: 10.15585/mmwr.mm7012a4
12. Peng YH, Qiu XJ, Zhang J. Application of NGS in etiology of lower respiratory tract infection. *J Hebei Med.* (2020) 42:1572–6.
13. Wu HL, Wei JH, Yu DJ. Application of NGS in diagnosis of tuberculous pleurisy with multiple negative tests: a case report. *Infect Drug Resist.* (2020) 13:3543–50. doi: 10.2147/IDR.S269779
14. Han DS, Li ZY, Li R, Tan P, Zhang R, Li JM. mNGS in clinical microbiology laboratories: on the road to maturity. *Crit Rev Microbiol.* (2019) 45:668–85. doi: 10.1080/1040841X.2019.1681933
15. Yan LP, Sun WW, Lu ZH, Fan L. Metagenomic next-generation sequencing (mNGS) in cerebrospinal fluid for rapid diagnosis of Tuberculosis meningitis in HIV-negative population. *Int J Infect Dis.* (2020) 96:270–5. doi: 10.1016/j.ijid.2020.04.048
16. Taur PD, Gowri V, Pandrowala AA, Iyengar VV, Chougule A, Golwala Z. Clinical and molecular findings in mendelian susceptibility to mycobacterial diseases: experience from India. *Front Immunol.* (2021) 12:631298. doi: 10.3389/fimmu.2021.631298
17. Lan ZW, Xiao MJ, Guan YL, Zhan YJ, Tang XQ. Detection of Listeria monocytogenes in a patient with meningoencephalitis using next-generation sequencing: a case report. *BMC Infect Dis.* (2020) 20:721. doi: 10.1186/s12879-020-05447-z
18. Chen LL, Xu Y, Liu CF, Huang H, Zhong XX, Ma CC, et al. Clinical features of aseptic meningitis with varicella zoster virus infection diagnosed by next-generation sequencing: case reports. *BMC Infect Dis.* (2020) 20:435. doi: 10.1186/s12879-020-05155-8
19. Consortium OPATHY, Gabaldón T. Recent trends in molecular diagnostics of yeast infections: from PCR to NGS. *FEMS Microbiol Rev.* (2019) 43:517–47. doi: 10.1093/femsre/fuz015
20. Lin AQ, Cheng BT, Han XC, Han XC, Zhang H, Liu XL, et al. Value of next-generation sequencing in early diagnosis of patients with tuberculous meningitis. *J Neurol Sci.* (2021) 422:117310. doi: 10.1016/j.jns.2021.117310
21. Chinese Adult Candidiasis Diagnosis and Management Expert Consensus Group. Chinese consensus on the diagnosis and management of adult candidiasis. *Chin J Intern Med.* (2020) 59:5–17. doi: 10.3760/cma.j.issn.0578-1426.2020.01.002
22. Matthijs G, Souche E, Alders M, Corveleyn A, Eck S, Feenstra I, et al. Guidelines for diagnostic next-generation sequencing. *Eur J Hum Genet.* (2016) 24:2–5. doi: 10.1038/ejhg.2015.226
23. Pezoulas VC, Hazapis O, Lagopati N, Exarchos TP, Goules AV, Tzioufas AG, et al. Machine learning approaches on high throughput NGS data to unveil mechanisms of function in biology and disease. *Cancer Genomics Proteomics.* (2021) 18:605–26. doi: 10.21873/cgp.20284

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Ma, Chen, Ding, Wang, Jin, Fan and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Medicine

CASE REPORT
published: 12 April 2022
doi: 10.3389/fmed.2022.808205

Multisystem Involvement Induced by Human Parvovirus B19 Infection in a Non-immunosuppressed Adult: A Case Report

Qihang Zou[1,2†], *Peisong Chen*[3†], *Jiaxin Chen*[1,2], *Dingbang Chen*[1,2], *Han Xia*[4], *Ling Chen*[1,2], *Huiyu Feng*[1,2*] and *Li Feng*[1,2*]

[1] Department of Neurology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China, [2] Guangdong Provincial Key Laboratory of Diagnosis and Treatment of Major Neurological Diseases, National Key Clinical Department and Key Discipline of Neurology, Guangzhou, China, [3] Department of Laboratory Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China, [4] Department of Scientific Affairs, Hugobiotech Co., Ltd., Beijing, China

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Priscila Giavedoni,
Hospital Clínic de Barcelona, Spain
Maria Söderlund-Venermo,
University of Helsinki, Finland
Jianming Qiu,
University of Kansas Medical Center, United States

***Correspondence:**
Li Feng
fengl7@mail.sysu.edu.cn
Huiyu Feng
fenghuiy@mail.sysu.edu.cn

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: 03 November 2021
Accepted: 16 March 2022
Published: 12 April 2022

Citation:
Zou Q, Chen P, Chen J, Chen D, Xia H, Chen L, Feng H and Feng L (2022) Multisystem Involvement Induced by Human Parvovirus B19 Infection in a Non-immunosuppressed Adult: A Case Report. Front. Med. 9:808205. doi: 10.3389/fmed.2022.808205

Background: Human parvovirus B19 (B19V) infection is usually symptomless and occurs in the childhood. While in immunocompromised adults, B19V infection also presents various clinical symptoms due to the host's immune status. The classic symptoms include erythema, anemia, arthropathy, and edema, but neurological involvement is rare.

Case Presentation: In this report, we present a case of B19V infection caused multiple organ dysfunction in a non-immunosuppressed adult. Metagenomic next-generation sequencing (mNGS) was used and successfully detected the pathogen in multiple types of samples, including blood, cerebrospinal fluid (CSF), and bronchoalveolar lavage fluid (BALF). The diagnosis was subsequently confirmed by polymerase chain reaction (PCR). He was treated with intravenous gamma globulin, resulting in a significant resolution of symptoms after 1 month.

Conclusion: Multisystem involvement induced by B19V infection was found in this case report. mNGS performed great advantages in rapidly and accurately diagnosing B19V infection in multiple types of samples, which helps the timely adjustment of treatment and improves the prognosis.

Keywords: human parvovirus B19, immunosuppressed, multiple organ dysfunction, case report, mNGS

INTRODUCTION

Human parvovirus B19 (B19V) was first detected in 1974 (1). The most common symptom is erythema infectiosum. It is a mild exanthematous disease commonly on the face and is named "slapped cheek syndrome" due to the shape and location of the rash. In addition, the trunk and skin rashes may appear as lace-like, reticular, and spontaneously resolving macular rashes (2). Its transmission route is mainly through the respiratory tract, but it can also be transmitted by blood or from mother-to-child vertical transmission (3). Children are the main target of B19V

infection. Notably, in infants, children, and adolescents with unexplained meningoencephalitis, the detection rate of B19V can be as high as 4.3% (4, 5), and the possibility of B19V pathogenesis in these cases cannot be ruled out. Adults infected by B19V can present a variety of clinical symptoms, which depend mainly on the host's immune status. In immunosuppressed adults, it can cause severe or chronic anemia due to persistent infection (6–8). The virus mainly attacks red blood cells in the hematologic system and manifests, resulting in clinical manifestations, such as rapidly worsening anemia and peripheral reticulocytopenia. Still, there are also cases with leukopenia or thrombocytopenia (37.5 and 21%, respectively) (9). In immunocompetent adults, B19V can lead to acute arthropathy (10, 11) and various neurological diseases such as encephalitis, meningoencephalitis, and Guillain-Barre syndrome (12). Alternatively, B19V has also been associated with myocarditis (13, 14) and hepatitis (15), as well as fetal hydrops and fetal abortion during pregnancy (16). In sickle cell anemia patients, B19V infection can cause transient aplastic crises.

Although B19V has previously been reported to cause various clinical symptoms in non-immunosuppressed patients, cases of B19V infection involving more than two systems are infrequent. Here we report a case who was hospitalized due to multiple organ dysfunction (MODS) caused by unexplained sepsis, without immunosuppressive background and clear focus of infection. B19V was detected in multiple samples throughout the body, including blood, cerebrospinal fluid (CSF), pleural fluid, and bronchoalveolar lavage fluid (BALF). The condition improved after blood transfusion and gamma globulin pulse therapy.

CASE REPORT

A 54-year-old man was admitted to the Department of Intensive Care Medicine of the First Affiliated Hospital, Sun Yat-sen University, on June 30th, 2021, due to headache and fatigue for 2 days.

The patient developed a headache on June 28th, 2021, without obvious inducement, and gradually had drowsiness, accompanied by fatigue, anorexia, and occasional cough, which could not be relieved after rest. No other abnormal symptoms were detected. The patient had a history of hypertension and gout for more than 10 years. He since had been diagnosed with sepsis and multiple organ failure in the ICU of a local hospital due to fever with upper abdominal pain. Metagenomic next-generation sequencing (mNGS) of blood on May 8th showed negative. After treatment of mepem 1 g every 6 h (Q6H), the patient was cured and was discharged 2 months ago. He did not take immunosuppressive drugs regularly for a long time and had no history of exposure to poisons, chemical agents, or animals. Personal and family histories were unremarkable.

Physical examination on admission showed body temperature of 36.0°C, pulse at 94 beats/min, respiratory rate of 14 breaths/min, blood pressure at 69/37 mmHg, drowsiness, chemosis, coarse breath sounds, and a small number of moist rales heard in both lower lungs. Cardio abdominal physical examination was unremarkable. Extremities were moderate edema. The movement of both upper limbs was good, and the movement of both lower limbs was limited. Auxiliary examination was also performed. Blood gas analysis showed metabolic acidosis, pH of 7.25, BE −16 mmol/L, and oxygenation >300. Elevated infection indicators analysis showed CRP of 234.75 mg/L, PCT of 5.06 ng/mL, and WBC of 41.84 × 10^9/L. The patient was detected with mild anemia (RBC of 3.26 × 10^{12}/L and Hb 93 g/L), prolonged coagulation time (APTT of 58.2 s), renal insufficiency (urea of 19.5 mmol/L and CREA 357 μmol/L), and acute heart failure (TnT-T of 0.130 ng/mL and NT-proBNP of 20813.0 pg/mL). In addition, the patient had elevated liver metabolic total bilirubin (TBIL of 79.6 μmol/L) and predominantly elevated direct bilirubin (DBIL of 55.9 μmol/L). Immunosuppressive status revealed a lower immunoglobulin M (0.49 g/L) and immunoglobulin G (7.37 g/L), B lymphocytes (CD3-CD19 +) % of 0.48%, B lymphocytes (CD3-CD19 +) count of 2.41 cells/μl, regulatory T-cells (Treg) of 18.75%, interleukin 6 of 1508.65 pg/mL, and interleukin of 10 5.57 pg/mL. Bacteriology, mycology, and tuberculosis tests of blood, sputum, urine, and stool showed no pathogens. HIV, syphilis, and Hepatitis virus tests were negative. Radiography of the pulmonary showed worsening infiltrate (**Figure 1**). Septic shock was considered. The patient was given Sulperazone 3 g, Q8H for anti-infection, fluid infusion, correction of acidosis, acid suppression, stomach protection, and prothrombin complex supplement on June 30th. After 7-day treatment, the infection indicators were significantly lower than before, with PCT of 1.19 ng/mL, CRP of 49.41 mg/L, WBC of 8.08 × 109/L, NEUT of 6.94 × 109/L. The conditions were under control.

On July 7th, the patient had a sudden light coma with repeated generalized tonic-clonic seizures, with the most extended duration lasting for about 90 s. He was transferred to the neurological ICU for treatment with a GCS score of 5. Twenty-four-hour bedside quantitative EEG monitoring was immediately performed, which showed simultaneous epileptic discharges in both cerebral hemispheres during convulsive seizures. Sodium valproate 1,200 mg QD (from July 7th to July 10th), and Oxcarbazepine 300 mg Q12H combined Levetiracetam 1 g Q12H (from July 9th to August 16th) were then used. The epilepsy did not recur.

On July 8th, a lumbar puncture was performed to measure the initial pressure of 210 cmH_2O and the final pressure of 135 cmH_2O. CSF was collected and submitted for examination. Biochemical combination of CSF revealed glucose of 3.0 mmol/L, CSF chloride (Cl) of 131 mmol/L, CSF protein (PROT) of 734.7 mg/L, synchronous blood glucose of 5.9 mmol/L, and blood chloride of 117 mmol/L.

Samples were also sent for PACEseq mNGS (Hugobiotech, Beijing, China). QIAamp DNA Micro Kit (QIAGEN, Germany) was used for DNA extraction, and the libraries were then constructed using QIAseq™ Ultralow Input Library Kit for Illumina (QIAGEN, Germany). The quality of libraries was assessed by Qubit (Thermo Fisher) and Agilent 2100 Bioanalyzer (Agilent Technologies). The qualified DNA libraries were finally sequenced on Nextseq 550 platform (Illumina). Adapter, short, low-quality, and low-complexity reads were removed from the raw data. The human DNA was filtered out by mapping to

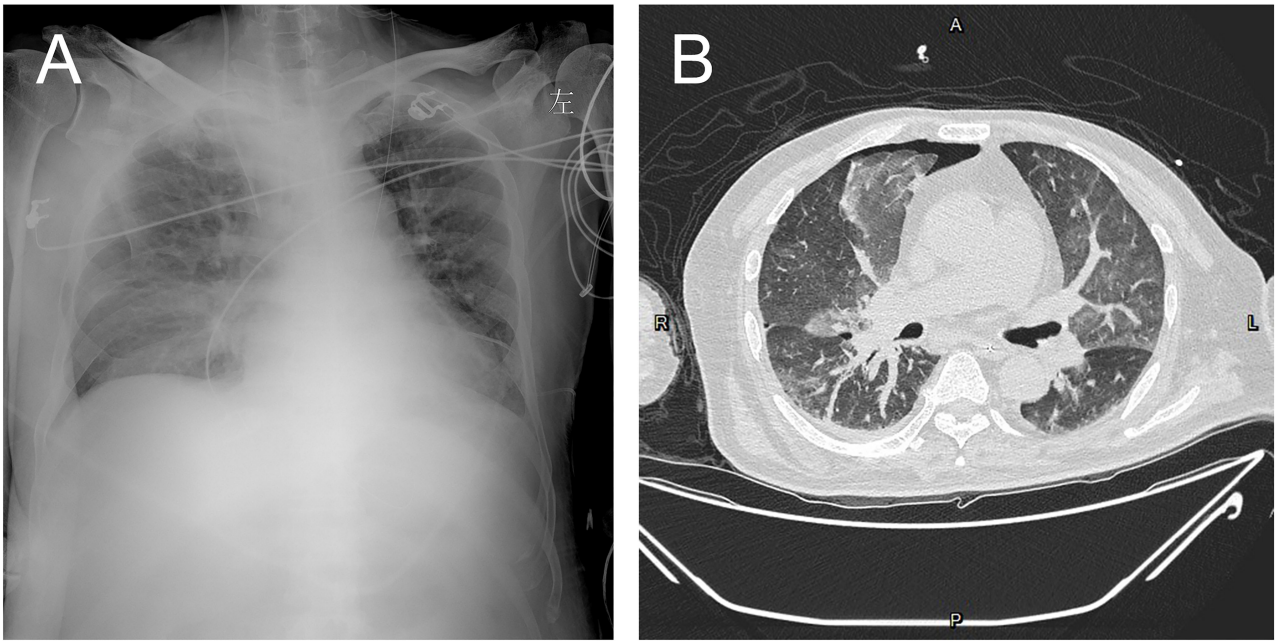


FIGURE 1 | The patients' chest X-ray on July 1st **(A)** and CT on July 16th **(B)**.

human reference database (hg38). The remaining reads were finally aligned to the Microbial Genome Databases (http://ftp.ncbi.nlm.nih.gov/genomes/). B19V DNA (2 specific reads) in CSF was detected. Polymerase chain reaction (PCR) was then performed using B19V Q-PCR Detection Kit (ZJ Bio-Tech, Shanghai, China) and confirmed the mNGS results. CSF cytology showed a lymphocyte-monocyte reaction pattern. CSF routine, bacterial culture, autoimmune encephalitis antibody detection, and oligoclonal bands (OB) were unremarkable. Determination of CSF B19V DNA on July 9th showed 1.0 × 10^ copies/mL. B19V caused encephalitis was considered.

After transfer, the patient's pneumonia was gradually aggravated, with type I respiratory failure and oxygenation index <200 on July 11th. Chest radiography showed inflammatory infiltration in bilateral lungs with bilateral pleural effusion. Brain plain MRI + enhancement MRI + DWI showed only scattered ischemic lesions in bilateral cerebral hemispheres. Echocardiography and color Doppler ultrasound of liver, gallbladder, pancreas, and spleen were unremarkable. Determination of B19V DNA in blood on July 11st showed 2.96 × 10^5 copies/mL; B19V DNA and *Enterococcus faecium* was detected by PACEseq mNGS on July 13th, with the specific reads number of 1,430 and 22, respectively. Blood B19V IgM was negative, and IgG was positive. The reexamination of EEG on July 14th showed no epileptic discharges. Determination of B19V DNA in pleural fluid on July 15th showed the DNA determination of 1.36 × 10^5 copies/mL; mNGS detected B19V in BALF on July 15th, with the specific reads number of 11, *Corynebacterium striatum*, with the particular reads number of 250, *Acinetobacter baumannii*, with the particular reads number of 17, *Enterococcus faecium*, with the specific reads number of 13, *Staphylococcus aureus*, with the particular reads number of 11, *Pseudomonas aeruginosa*, with the particular reads number of 11. In addition to the symptoms of the central nervous system, the patient's anemia was progressively aggravated. After multiple blood transfusions and platelet therapy, the lowest hemoglobin was 58 g/L on August 8th, the proportion of reticulocytes was 0.0026, the reticulocyte count was 0.0089 × 10^{12}/L, the lowest platelet count was 60 × 10^9/L, red blood cells and platelets were inhibited, and red blood cell morphology examination showed shrinkage and acanthocytes. The patient was finally diagnosed with encephalitis caused by B19V, secondary epilepsy, severe anemia, and bilateral pneumonia.

The patient was given mepem 1 g, Q8H on July 10th, and then switched to vancomycin 1 g, Q12H on July 18th, intravenous gamma globulin 0.4 g/Kg body weight for 5 days from July 15th for symptomatic treatment of heart failure, renal failure, and other symptoms of organ damage. The patient did not have any more seizures. He gradually became conscious, opened and closed his eyes when instructed, made eye contact when called, GCS score increased to 10 points, oxygenation index was more significant than 300, and anemia was improved. On July 20th, reexamination showed the proportion of reticulocytes was 0.0702, and reticulocyte count was 0.2078 × 10^{12}/L. On July 27th, reexamination showed that blood B19V DNA was <1,000 copies/ml. The number of mNGS specific reads and viral DNA quantification in the different specimens are showed

TABLE 1 | Test results of PV B19 in various body fluid samples of the patient.

Item / Specimen	Number of mNGS specific reads (time of collection)	Viral DNA quantification (copies/ml) (time of collection)
CSF	+, 2 (2021-7-8)	1.0 × 10^5 (2021-7-8)
Serum 1	+, 1,430 (2021-7-13)	2.96 × 10^5 (2021-7-11)
Hydrothorax	–	1.36 × 10^5 (2021-7-15)
BALF	+, 11 (2021-7-15)	/
Serum 2	Not reexamined	<1,000 (2021-7-27)

in **Table 1**, while mNGS outcomes belong to the patient are showed in **Figure 2**. Afterment treatment, the patient had clear consciousness and could simply communicate with his family. He was finally discharged on August 16th. There was no sequelae or relapse of this patient after 4-month follow-up. The patient could take care of himself and communicate with his family normally. The timeline of this case was shown in **Figure 3**.

DISCUSSION AND CONCLUSION

Human parvovirus infection mainly affects the skin, respiratory system, joints, and hematologic system, characterized by rash, flu-like symptoms, self-limiting arthritis, and transient erythroid hematopoietic disorders (17–19), the clinical manifestations of which lack specificity. In addition, it is difficult to isolate the virus by conventional cell culture, hence not feasible in clinical practice (20). Therefore, its diagnosis relies on serology and DNA detection (21). At the same time, real-time qPCR has been proven to be more reliable, especially in the diagnosis of recurrent or persistent infections, as antibody production may be lacking or minimal in these individuals (21): B19V IgM appears 1 week after infection (22) and persists for 2–3 months (21, 22). Due to tremendous differences between individuals, parvovirus B19V IgM may not be detected. IgG can persist after infection. A 4-fold increase of titer in 2 weeks is usually used to diagnose B19V infection. However, we did not test the titer after 2 weeks due to the use of gamma globulin treatment.

In this case, blood IgM test results were negative. Still, B19V DNA was detected in multiple serum samples, CSF, pleural fluid, and BALF. Though the detected DNA in blood might also be non-infectious naked DNA sipping out from the injured tissues, the high titer indicated that the DNA detected by real-time PCR is more likely from replicating virus. In addition, the discovery of B19V DNA and various bacteria in BALF can confirm that it is at least partly directly related to pneumonia (23–25). Due to convulsions during the course of the disease, lumbar puncture, and CSF mNGS detection also revealed B19V. The patient's CSF routine, biochemistry, protein, and cytology tests showed viral encephalitis changes, consistent with the diagnosis of B19V encephalitis (12).

On the other hand, after admission, the patient had a progressive decrease in hemoglobin and severe anemia, which was not improved significantly after multiple transfusion therapy. Reticulocyte level was extremely low, along with thrombocytopenia and leukopenia. Although B19V can inhibit erythroid proliferation, but severe anemia rarely occurs in immunocompetent patients with B19V infection. Immunocompromised patients with B19V infection are more likely to have decreased levels of erythrocytes and platelets or even whole blood cells (26). In this case, the blood routine showed severe immunosuppression, explaining the suppression of red blood cells and platelets.

Unexplained liver function impairment with B19V infection may be associated with the direct action of the virus, with elevated trans-aminases as the primary manifestation, which may be clinically asymptomatic or accompanied by acute hepatitis, primarily seen in children (27–30). Although the patient had liver function impairment mainly shown by elevated bilirubin, his liver enzymes were normal, so it could not rule out that drugs were the cause.

The majority of existing reports of B19V infection occur in children, pregnant women, and immunosuppressed populations. In contrast, infection accounts in immunocompetent individuals are relatively rare, and the symptoms are mild or asymptomatic. This case is a middle-aged male with acute onset and severe condition, not the ordinary population of previous B19V cases. He had no long-term immunosuppression, history of exposure to chemicals, poisons, animals, or other underlying conditions that can cause a state of immunosuppression. Meanwhile, there are few B19V involving multiple systems and accompanied by multiple organ failure (12, 23). The possibility of B19V infection is rarely considered in routine pathogen screening.

However, when evaluating the immune function of patients, it should also be taken into account that, in addition to organ transplantation, AIDS, long-term use of immunosuppressive drugs or drug abuse, severe infection might also cause suppressed humoral and cellular immune status and lead to immunosuppression, especially in clinical settings. The immune-related indicators of the patient suggested severe immunosuppression, with low lymphocyte count and proportion, mainly B lymphocytes, which might be due to latent viral infection after the last sepsis-induced immunosuppressive state of more than 2 months ago.

The presence of high-titer B19V was eventually identified with the aid of mNGS technology and subsequently confirmed in multiple clinical samples. The manifestation of multisystem involvement due to B19V infection was resolved with the addition of targeted therapy, so it was considered a multi-organ infection caused by B19V. The outbreak of B19V infection might be due to the severe immunosuppression caused by sepsis 2 months before.

The second-generation sequencing technology is significant for detecting rare or unexpected pathogens. It is an essential auxiliary means for the etiological diagnosis of patients with severe infections, which should be timely applied in the diagnosis and treatment process. However, it should be noted that the interpretation of mNGS results should be

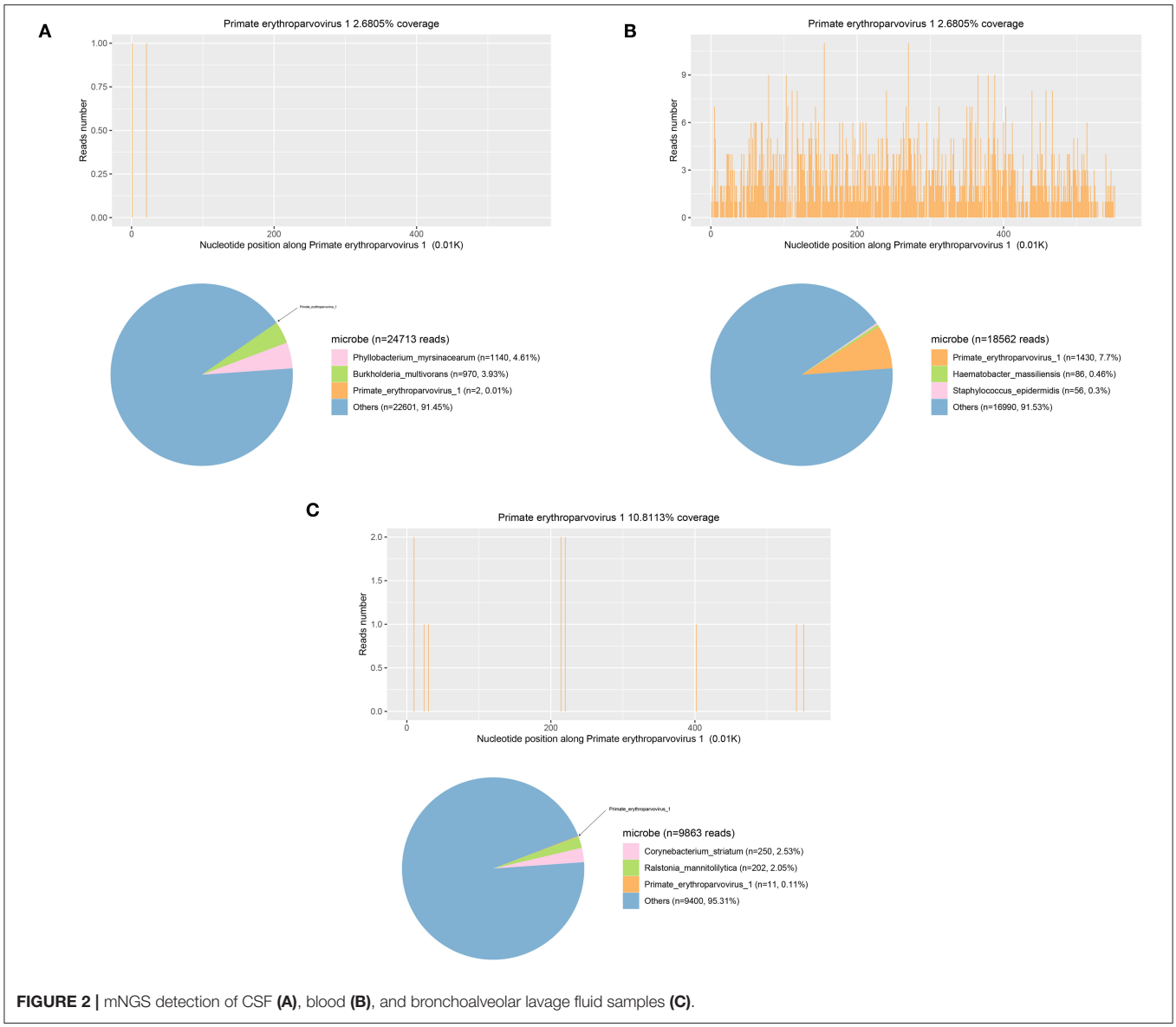


FIGURE 2 | mNGS detection of CSF **(A)**, blood **(B)**, and bronchoalveolar lavage fluid samples **(C)**.

comprehensive and careful. This case shows that although mNGS has a more substantial pathogen detection capability than conventional detection, the number of virus-specific reads does not match the DNA quantification, resulting in a small number of mNGS reads. While the viral load is large and the clinical symptoms are severe. Therefore, the etiological hints of mNGS should be used rationally, and its results should not be based solely on its reads number, nor should mNGS be a substitute for quantitative pathogen detection.

Currently, there are no specific antiviral drugs for B19V infection. Treatment options depend on host factors such as immune status, potential underlying diseases, and manifestations of infection (31). Due to the short duration of symptoms, most infections in immunocompetent hosts do not require treatment. Intravenous gamma globulin (IVIG) 0.4 g/Kg bodyweight for 5 consecutive days can effectively treat a variety of clinical symptoms caused by B19V that could not recover spontaneously (32–35). However, the treatment is not once and for all, and in individuals with severe anemia treated with IVIG, the anemia may recur once the passive antibody effect is diminished (36, 37). If the hematocrit begins to decline, the B19V viral load should be measured again to identify the cause. The next IVIG course can be performed depending on the degree of anemia (20, 38, 39).

It should be noted that in the absence of symptoms, even after IVIG treatment, B19V DNA in serum can be detected by polymerase chain reaction (PCR) at low levels over several months to years (21, 40–43). Therefore, the

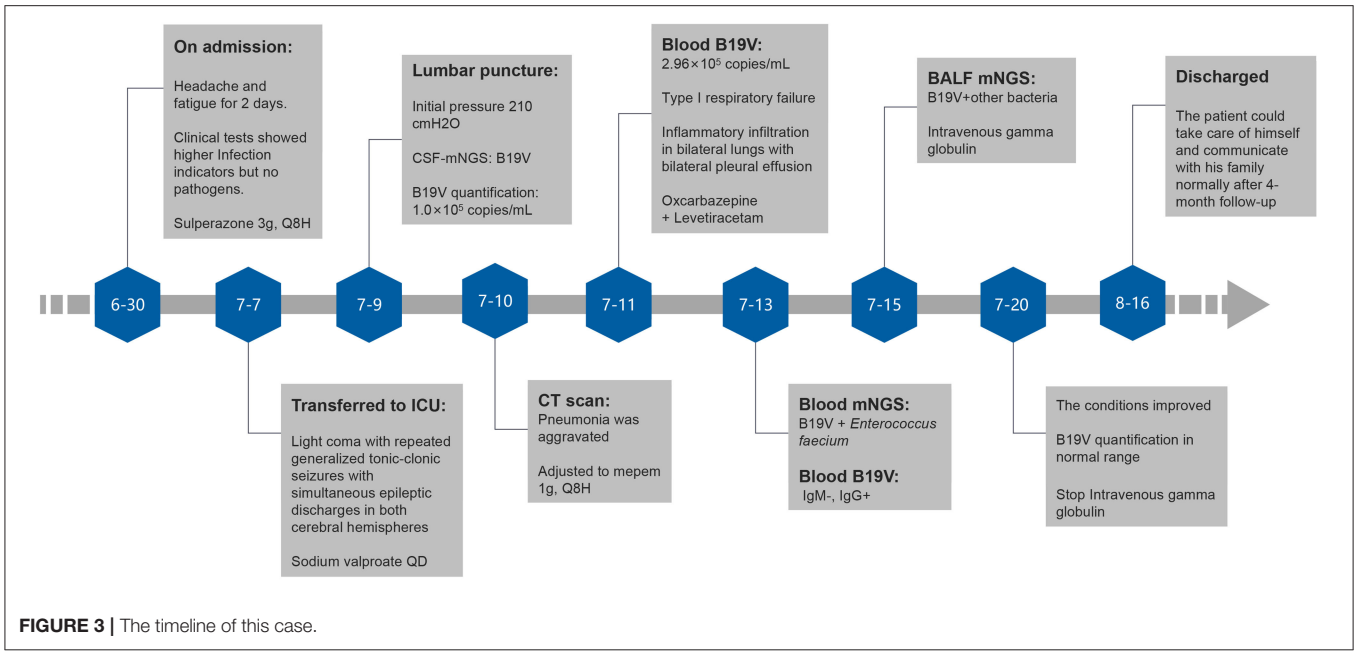


FIGURE 3 | The timeline of this case.

evaluation of the therapeutic effect should be combined with clinical practice. PCR positive results alone should not be an indication for treatment, nor should the elimination of B19V DNA from the blood be the goal of therapy. After treatment with gamma globulin, the reticulocyte count increased, hemoglobin level increased, and serum viral nucleic acid load reduced significantly. The function of various organs was improved, and the patient was discharged, confirming the effectiveness of the treatment regimen. *Enterococcus faecium* was detected by mNGS in both blood and BALF samples. Though multiple anti-bacteria drugs were used for treatment, the conditions did not improve. However, the bacteria infection especially in the lung could not be ruled out.

In addition, corticosteroids can be used for central nervous system symptoms caused by B19V infection. However, there is still a lack of objective evaluation in the efficacy of IVIG and corticosteroids or a combination of the two (12). In addition, although cyclosporine has been reported as the treatment of B19V (44), it is usually recommended to reduce the use of immunosuppressive agents to facilitate the body's immune system to be activated and neutralize the virus (45). For other symptoms caused by B19V, such as rash and arthritis, most of them subside spontaneously, and non-steroidal anti-inflammatory drugs may be helpful for arthropathy (46). Patients with a transient aplastic crisis may require transfusion support (47).

This case's diagnosis and treatment experience have contributed to our understanding of the etiological detection of infectious diseases. The clinical manifestations caused by B19V infection are diverse. Still, few reports involve multiple organ or even concurrent central nervous system infection (23), and the rarity of B19V infection brings difficulties in diagnosis. Clinicians should pay attention to the immune status of patients in the disease state and rationally use mNGS technology to assist clinical diagnosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QZ, PC, and LF participated in collecting data and drafted the manuscript. JC and DC collected the data for case presentation. HX analyzed the mNGS data. LC participated in clinical management. HF and LF reviewed the literature and participated in its design. All authors read and approved the final manuscript.

FUNDING

This study was supported by grants from Guangdong Provincial Key Laboratory of Diagnosis and Treatment of Major Neurological Diseases (2020B1212060017), Guangdong Provincial Clinical Research Center for Neurological Diseases

(2020B1111170002), the Southern China International Cooperation Base for Early Intervention and Functional Rehabilitation of Neurological Diseases (2015B050501003 and 2020A0505020004), Guangdong Provincial Engineering Center For Major Neurological Disease Treatment, Guangdong Provincial Translational Medicine Innovation Platform for Diagnosis and Treatment of Major Neurological Disease, and the Clinical Research Supporting Programe for Lingnan Junior Doctors of Guangdong Province Medical Association (2021067).

REFERENCES

1. Cossart YE, Field AM, Cant B, Widdows D. Parvovirus-like particles in human sera. *Lancet.* (1975) 1:72–3. doi: 10.1016/S0140-6736(75)91074-0
2. Anderson MJ, Jones SE, Fisher-Hoch SP, Lewis E, Hall SM, Bartlett CL, et al. Human parvovirus, the cause of erythema infectiosum (fifth disease)? *Lancet.* (1983) 1:1378. doi: 10.1016/S0140-6736(83)92152-9
3. Servey JT, Reamy BV, Hodge J. Clinical presentations of parvovirus B19 infection. *Am Fam Physician.* (2007) 75:373–6.
4. von Landenberg P, Lehmann HW, Modrow S. Human parvovirus B19 infection and antiphospholipid antibodies. *Autoimmun Rev.* (2007) 6:278–85. doi: 10.1016/j.autrev.2006.09.006
5. Barah F, Vallely PJ, Chiswick ML, Cleator GM, Kerr JR. Association of human parvovirus B19 infection with acute meningoencephalitis. *Lancet.* (2001) 358:729–30. doi: 10.1016/S0140-6736(01)05905-0
6. Van Horn DK, Mortimer PP, Young N, Hanson GR. Human parvovirus-associated red cell aplasia in the absence of underlying hemolytic anemia. *Am J Pediatr Hematol Oncol.* (1986) 8:235–9. doi: 10.1097/00043426-198623000-00011
7. Morinet F, Perol Y. B19 chronic bone marrow failure: a persistent parvovirus infection of humans. *Nouv Rev Fr Hematol.* (1990) 32:91–4.
8. Kurtzman GJ, Ozawa K, Cohen B, Hanson G, Oseas R, Young NS. Chronic bone marrow failure due to persistent B19 parvovirus infection. *N Engl J Med.* (1987) 317:287–94. doi: 10.1056/NEJM198707303170506
9. Brodin-Sartorius A, Mekki Y, Bloquel B, Rabant M, Legendre C. Infection par le Parvovirus B19 après transplantation rénale [Parvovirus B19 infection after kidney transplantation]. *Nephrol Ther.* (2012) 8:5–12. doi: 10.1016/j.nephro.2011.06.001
10. Reid DM, Reid TM, Brown T, Rennie JA, Eastmond CJ. Human parvovirus-associated arthritis: a clinical and laboratory description. *Lancet.* (1985) 1:422–5. doi: 10.1016/S0140-6736(85)91146-8
11. White DG, Woolf AD, Mortimer PP, Cohen BJ, Blake DR, Bacon PA. Human parvovirus arthropathy. *Lancet.* (1985) 1:419–21. doi: 10.1016/S0140-6736(85)91145-6
12. Barah F, Whiteside S, Batista S, Morris J. Neurological aspects of human parvovirus B19 infection: a systematic review. *Rev Med Virol.* (2014) 24:154–68. doi: 10.1002/rmv.1782
13. Enders G, Dötsch J, Bauer J, Nützenadel W, Hengel H, Haffner D, et al. Life-threatening parvovirus B19-associated myocarditis and cardiac transplantation as possible therapy: two case reports. *Clin Infect Dis.* (1998) 26:355–8. doi: 10.1086/516295
14. Simpson KE, Storch GA, Lee CK, Ward KE, Danon S, Simon CM, et al. High frequency of detection by PCR of viral nucleic acid in the blood of infants presenting with clinical myocarditis. *Pediatr Cardiol.* (2016) 37:399–404. doi: 10.1007/s00246-015-1290-6
15. Yoto Y, Kudoh T, Haseyama K, Suzuki N, Chiba S. Human parvovirus B19 infection associated with acute hepatitis. *Lancet.* (1996) 347:868–9. doi: 10.1016/S0140-6736(96)91348-3
16. Brown T, Anand A, Ritchie LD, Clewley JP, Reid TM. Intrauterine parvovirus infection associated with hydrops fetalis. *Lancet.* (1984) 2:1033–4. doi: 10.1016/S0140-6736(84)91126-7
17. Sim JY, Chang LY, Chen JM, Lee PI, Huang LM, Lu CY. Human parvovirus B19 infection in patients with or without underlying diseases. *J Microbiol Immunol Infect.* (2019) 52:534–41. doi: 10.1016/j.jmii.2019.05.009
18. Skaff PT, Labiner DM. Status epilepticus due to human parvovirus B19 encephalitis in an immunocompetent adult. *Neurology.* (2001) 57:1336–7. doi: 10.1212/WNL.57.7.1336
19. Bonvicini F, Marinacci G, Pajno MC, Gallinella G, Musiani M, Zerbini M. Meningoencephalitis with persistent parvovirus B19 infection in an apparently healthy woman. *Clin Infect Dis.* (2008) 47:385–7. doi: 10.1086/589863
20. Landry ML. Parvovirus B19. *Microbiol Spectr.* (2016) 4:0008. doi: 10.1128/microbiolspec.DMIH2-0008-2015
21. Young NS, Brown KE. Parvovirus B19. *N Engl J Med.* (2004) 350:586–97. doi: 10.1056/NEJMra030840
22. Makhlouf MM, Elwakil SG, Ibrahim NS. Molecular and serological assessment of parvovirus B-19 infection in Egyptian children with sickle cell disease. *J Microbiol Immunol Infect.* (2017) 50:565–9. doi: 10.1016/j.jmii.2015.10.016
23. Katoh D, Ochi Y, Hiramoto N, Morita M, Yabushita T, Shimomura Y, et al. Parvovirus B19 infection in adult patients after allogeneic stem cell transplantation: our experience of five cases and literature review. *Bone Marrow Transplant.* (2020) 55:653–6. doi: 10.1038/s41409-019-0533-1
24. Wardeh A, Marik P. Acute lung injury due to parvovirus pneumonia. *J Intern Med.* (1998) 244:257–60. doi: 10.1046/j.1365-2796.1998.00364.x
25. Beske F, Modrow S, Sörensen J, Schmidt H, Kriener S, Allwinn R, et al. Parvovirus B19 pneumonia in a child undergoing allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* (2007) 40:89–91. doi: 10.1038/sj.bmt.1705693
26. Wolfromm A, Rodriguez C, Michel M, Habibi A, Audard V, Benayoun E, et al. Spectrum of adult Parvovirus B19 infection according to the underlying predisposing condition and proposals for clinical practice. *Br J Haematol.* (2015) 170:192–9. doi: 10.1111/bjh.13421
27. Das P, Chatterjee K, Chattopadhyay NR, Choudhuri T. Evolutionary aspects of Parvovirus B-19V associated diseases and their pathogenesis patterns with an emphasis on vaccine development. *Virusdisease.* (2019) 30:32–42. doi: 10.1007/s13337-019-00525-6
28. Bihari C, Rastogi A, Saxena P, Rangegowda D, Chowdhury A, Gupta N, et al. Parvovirus b19 associated hepatitis. *Hepat Res Treat.* (2013) 2013:472027. doi: 10.1155/2013/472027
29. Bültmann BD, Klingel K, Sotlar K, Bock CT, Kandolf R. Parvovirus B19: a pathogen responsible for more than hematologic disorders. *Virchows Arch.* (2003) 442:8–17. doi: 10.1007/s00428-002-0732-8
30. Krygier DS, Steinbrecher UP, Petric M, Erb SR, Chung SW, Scudamore CH, et al. Parvovirus B19 induced hepatic failure in an adult requiring liver transplantation. *World J Gastroenterol.* (2009) 15:4067–9. doi: 10.3748/wjg.15.4067
31. Broliden K. Parvovirus B19 infection in pediatric solid-organ and bone marrow transplantation. *Pediatr Transplant.* (2001) 5:320–30. doi: 10.1034/j.1399-3046.2001.00035.x
32. Morey AL, Ferguson DJ, Fleming KA. Combined immunocytochemistry and non-isotopic *in situ* hybridization for the ultrastructural investigation of human parvovirus B19 infection. *Histochem J.* (1995) 27:46–53. doi: 10.1007/BF00164171
33. Moudgil A, Shidban H, Nast CC, Bagga A, Aswad S, Graham SL, et al. Parvovirus B19 infection-related complications in renal transplant recipients: treatment with intravenous immunoglobulin. *Transplantation.* (1997) 64:1847–50. doi: 10.1097/00007890-199712270-00037
34. Murer L, Zacchello G, Bianchi D, Dall'amico R, Montini G, Andreetta B, et al. Thrombotic microangiopathy associated with parvovirus B19 infection after renal transplantation. *J Am Soc Nephrol.* (2000) 11:1132–37. doi: 10.1681/ASN.V1161132
35. Egbuna O, Zand MS, Arbini A, Menegus M, Taylor J. A cluster of parvovirus B19 infections in renal transplant recipients: a prospective case series and review of the literature. *Am J Transplant.* (2006) 6:225–31. doi: 10.1111/j.1600-6143.2005.01139.x
36. Gosset C, Viglietti D, Hue K, Antoine C, Glotz D, Pillebout E. How many times can parvovirus B19-related anemia recur in solid organ transplant recipients? *Transpl Infect Dis.* (2012) 14:E64–70. doi: 10.1111/j.1399-3062.2012.00773.x

37. Rodríguez-Espinosa D, Esforzado N, Hermida E, Cuadrado E, Broseta JJ, Diekmann F, et al. A case of recurrent anemia due to chronic parvovirus B19 infection in a kidney transplant recipient. Can everolimus make a difference? *CEN Case Rep.* (2021) 10:388–92. doi: 10.1007/s13730-021-00575-0
38. Brown KE. Detection and quantitation of parvovirus B19. *J Clin Virol.* (2004) 31:1–4. doi: 10.1016/j.jcv.2004.05.001
39. Harder TC, Hufnagel M, Zahn K, Beutel K, Schmitt HJ, Ullmann U, et al. New LightCycler PCR for rapid and sensitive quantification of parvovirus B19 DNA guides therapeutic decision-making in relapsing infections. *J Clin Microbiol.* (2001) 39:4413–9. doi: 10.1128/JCM.39.12.4413-4419.2001
40. Flunker G, Peters A, Wiersbitzky S, Modrow S, Seidel W. Persistent parvovirus B19 infections in immunocompromised children. *Med Microbiol Immunol.* (1998) 186:189–94. doi: 10.1007/s004300050063
41. Calvet A, Pujol MO, Bertocchi M, Bastien O, Boissonnat P, Mornex JF. Parvovirus B19 infection in thoracic organ transplant recipients. *J Clin Virol.* (1999) 13:37–42. doi: 10.1016/S1386-6532(99)00012-8
42. Cassinotti P, Burtonboy G, Fopp M, Siegl G. Evidence for persistence of human parvovirus B19 DNA in bone marrow. *J Med Virol.* (1997) 53:229-32.
43. Cassinotti P, Siegl G. Quantitative evidence for persistence of human parvovirus B19 DNA in an immunocompetent individual. *Eur J Clin Microbiol Infect Dis.* (2000) 19:886–7. doi: 10.1007/s100960000384
44. Pamidi S, Friedman K, Kampalath B, Eshoa C, Hariharan S. Human parvovirus B19 infection presenting as persistent anemia in renal transplant recipients. *Transplantation.* (2000) 69:2666–9. doi: 10.1097/00007890-200006270-00030
45. Lamont RF, Sobel JD, Vaisbuch E, Kusanovic JP, Mazaki-Tovi S, Kim SK, et al. Parvovirus B19 infection in human pregnancy. *BJOG.* (2011) 118:175–86. doi: 10.1111/j.1471-0528.2010.02749.x
46. Rogo LD, Mokhtari-Azad T, Kabir MH, Rezaei F. Human parvovirus B19: a review. *Acta Virol.* (2014) 58:199–213. doi: 10.4149/av_2014_03_199
47. Bonvicini F, Mirasoli M, Gallinella G, Zerbini M, Musiani M, Roda A. PNA-based probe for quantitative chemiluminescent *in situ* hybridisation imaging of cellular parvovirus B19 replication kinetics. *Analyst.* (2007) 132:519–23. doi: 10.1039/b701664f

Conflict of Interest: HX was employed by Hugobiotech Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be conducted as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Zou, Chen, Chen, Chen, Xia, Chen, Feng and Feng. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Medicine

CASE REPORT
published: 18 April 2022
doi: 10.3389/fmed.2022.801086

Case Report: An Ulceration With a Stalactite Appearance on the Index Finger

Liangliang Zhang[1†], Yalan Dai[2†], Mengting Lin[1], Qiuyun Xu[1], Tingting Lin[1], Ting Gong[3], Bo Cheng[1], Chao Ji[1] and Donghua Cai[2*]*

[1] Department of Dermatology, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China, [2] Department of Dermatology, Shishi Municipal General Hospital, Quanzhou, China, [3] Central Laboratory, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China

Proteus mirabilis, the most widespread species of all *Proteus* spp. bacteria, is proven to be one of the most universal pathogens in chronic wounds. In this case, a woman in her 40s consulted a physician about an asymptomatic ulceration with a stalactite appearance at the distal end of the index finger after she was exposed to a needle when vaccinating chickens. The patient did not response to ceftazidime. Physical examination revealed a well-demarcated violescent ulceration with a stalactite appearance at the distal end of the index finger. A biopsy of the lesion showed dense infiltration of multinucleated giant cells, histiocytes, and lymphocytes in the dermis. The result of metagenomics next-generation sequencing (NGS) showed 306 unique sequence reads of *P. mirabilis*, covering 33.49% of the nucleotide sequences. The pathogen was identified as *P. mirabilis,* which was resistant to ceftazidime. The patient was treated with ciprofloxacin hydrochloride and improved considerably. This case reported a distinctive cutaneous lesion of *P. mirabilis* on human infection and showed a successful use of NGS in *P. mirabilis*.

Keywords: *Proteus mirabilis*, cutaneous exanthem, next generation sequencing, *Proteus* spp. bacteria, human infection

OPEN ACCESS

Edited by:
Li Ang,
First Affiliated Hospital of Zhengzhou University, China

Reviewed by:
Ammar Husami,
Cincinnati Children's Hospital Medical Center, United States
Reham Wasfi,
October University for Modern Sciences and Arts, Egypt

***Correspondence:**
Chao Ji
jichaofy@fjmu.edu.cn
Donghua Cai
caidhdoctor@126.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *24 October 2021*
Accepted: *09 March 2022*
Published: *18 April 2022*

Citation:
Zhang L, Dai Y, Lin M, Xu Q, Lin T, Gong T, Cheng B, Ji C and Cai D (2022) Case Report: An Ulceration With a Stalactite Appearance on the Index Finger. Front. Med. 9:801086. doi: 10.3389/fmed.2022.801086

INTRODUCTION

Proteus mirabilis is the most widespread species of *Proteus* spp. bacteria (1). Culture, biochemical tests, and multiplex PCR can be used for diagnosis of *P. mirabilis* (2–4). Metagenomics next-generation sequencing (NGS) has the potential to identify and quantify new or unexpected pathogens in challenging situations such as culture- and PCR-negative results (5–8).

CASE REPORT

A 47-year-old woman presented with an asymptomatic ulceration with a stalactite appearance at the distal of end of the index finger for 30 days. She reported no dyspnea, fever, arthralgias, or other systemic symptoms. Prior to admission to our department, the patient received ceftazidime 2 mg twice daily, but her lesion worsened. One month earlier, the patient had a needle-stick injury while vaccinating chickens. However, she did not report any exposure to the vaccine. Physical examination revealed a well-demarcated violescent ulceration with a stalactite appearance at the distal end of the index finger (**Figure 1**). Biopsy of the lesion showed a dense infiltration

of multinucleated giant cells, histiocytes, and lymphocytes in the dermis (**Figure 2**). A culture performed on the cutaneous lesion was negative in this case. Metagenomics NGS of the specimen was performed (**Supplementary Methods**). Four negative and positive control samples were subjected to the same procedures. NGS result showed that *P. mirabilis* had 306 unique sequence reads, covering 33.49% of the nucleotide sequences (**Figure 3**). The pathogen was identified as *P. mirabilis,* which was resistant to ceftazidime. The patient was treated with ciprofloxacin hydrochloride (at a dose of 1.5 g three times daily for 20 days), and her lesion showed substantial improvement (**Supplementary Figure 1**). After 2 weeks, dermatology life quality index (DLQI) decreased rapidly from 15 to 2.

DISCUSSION

Proteus mirabilis is one of the most universal pathogens in chronic trauma, accounting for 8.0% (9). Humans were infected with *P. mirabilis* from consumption and slaughter of wild and domestic animals (mammals, birds, reptiles, amphibians, insects, and seafood) (10). The phenotypic and virulence gene characteristics in human and chicken isolates of *P. mirabilis* are similar (11). Cellulitis has been reported in broiler chickens (12). However, features of skin lesions in human *P. mirabilis* infection are rarely described. Culture is an important diagnostic tool for *P. mirabilis,* which has a characteristic swarming motility and distinct fishy smell (2). There are also both traditional and kit-based biochemical tests in addition to molecular-based multiplex PCR, which can be used for diagnosis of *P. mirabilis* (3).

In this case, the patient had a history of a needle-stick injury and a specific exanthem of asymptomatic ulceration with stalactite appearance. The antibiotic (ceftazidime) was ineffective, and the culture was negative. *P. mirabilis* may be in a viable but non-culturable (VBNC) state. It was a state of bacteria where bacteria under certain conditions of starvation or stress could enter a state of no division and, therefore, become non-culturable but in the same time keep their viability and sometimes their ability to cause infection (13). Thus, VBNC of *P. mirabilis* was a possible reason for non-culturability of the bacteria and may also explain the failure of ceftazidime treatment, which required actively dividing bacteria to performed their antimicrobial effect (14, 15).

We chose to perform mNGS to blindly detect the causative agent, as PCR required multiple tests. Besides, NGS has the potential to predict antibiotic resistance. Many resistant genes coding beta-lactamases were detected in *P. mirabilis*, including TEM genes, VEB-1 genes, OXA genes, CTX-M genes and CIT genes, with the TEM genes being the most common resistant gene (16). In conclusion, this case reported a characteristic cutaneous lesion of *P. mirabilis* after human infection and showed the successful use of NGS in *P. mirabilis*. Further studies need to enlarge the sample size to support the clinical utility of NGS metagenomic testing.

FIGURE 1 | Ulceration with a stalactite appearance at the distal end of the index finger.

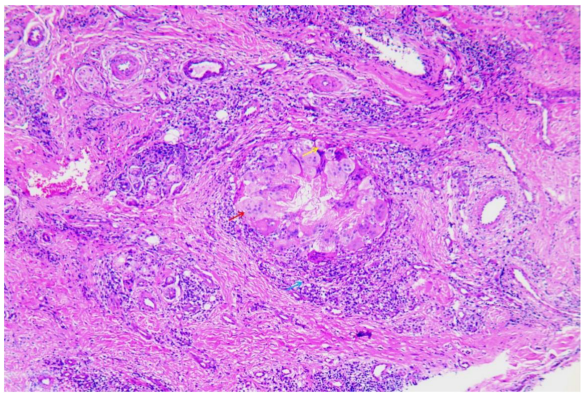


FIGURE 2 | Biopsy of the lesion showed dense infiltration of multinuclear giant cells (red arrow), histiocytes (yellow arrow), and lymphocytes (blue arrow) in the dermis (hematoxylin and eosin stain, ×400).

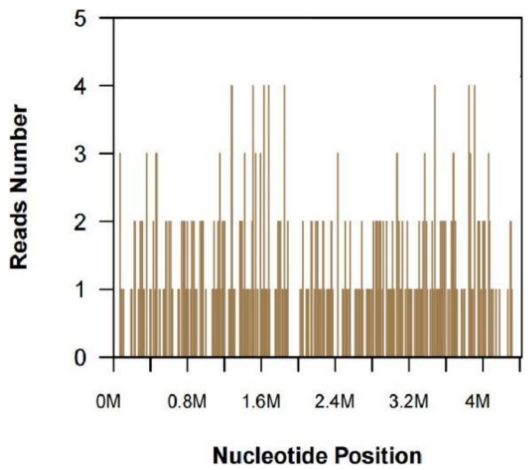


FIGURE 3 | Next-generation sequencing and metagenomics analysis. *Proteus mirabilis* specific reads and its nucleotide position along the *Proteus mirabilis* genome.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the online repository at: https://www.ncbi.nlm.nih.gov/guide under accession number PRJNA813626.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Technology Clinical Application Ethics Committee of The First Affiliated Hospital of Fujian Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LZ, YD, CJ, and DC conceptualized and designed the study. LZ and YD wrote the manuscript. TG, BC, CJ, and DC revised the manuscript critically for important intellectual content. ML, QX, and TL collected clinical pictures and analyzed the data. All authors have read and approved the final manuscript.

FUNDING

This work was supported by grants from the Natural Science Foundation of Fujian Province (No. 2020J02053).

ACKNOWLEDGMENTS

We thank all study participants involved in facilitating and running this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.801086/full#supplementary-material

Supplementary Figure 1 | Data of therapeutic interventions.

REFERENCES

1. Lin MF, Liou ML, Kuo CH, Lin YY, Chen JY, Kuo HY. Antimicrobial susceptibility and molecular epidemiology of *Proteus mirabilis* isolates from three hospitals in Northern Taiwan. *Microb Drug Resist.* (2019) 25:1338–46. doi: 10.1089/mdr.2019.0066
2. Jamil RT, Foris LA, Snowden J. *Proteus Mirabilis Infections. StatPearls*. Treasure Island, FL: StatPearls Publishing. Copyright © 2022, StatPearls Publishing LLC (2022).
3. Yang R, Xu G, Wang X, Qing Z, Fu L. Establishment and application of a dual TaqMan Real-Time PCR method for *Proteus mirabilis* and proteus vulgaris. *Pol J Microbiol.* (2020) 69:293–300. doi: 10.33073/pjm-2020-032
4. Girlich D, Bonnin RA, Bogaerts P, De Laveleye M, Huang DT, Dortet L, et al. Chromosomal amplification of the blaOXA-58 carbapenemase gene in a *Proteus mirabilis* clinical isolate. *Antimicrob Agents Chemother.* (2017) 61:e1697–1616. doi: 10.1128/aac.01697-16
5. Michel C, Raimo M, Lazarevic V, Gaïa N, Leduc N, Knoop C, et al. Case report: about a case of hyperammonemia syndrome following lung transplantation: could metagenomic next-generation sequencing improve the clinical management? *Front Med (Lausanne).* (2021) 8:684040. doi: 10.3389/fmed.2021.684040
6. Kolb M, Lazarevic V, Emonet S, Calmy A, Girard M, Gaïa N, et al. Next-generation sequencing for the diagnosis of challenging culture-negative endocarditis. *Front Med (Lausanne).* (2019) 6:203. doi: 10.3389/fmed.2019.00203
7. Imai A, Gotoh K, Asano Y, Yamada N, Motooka D, Fukushima M, et al. Comprehensive metagenomic approach for detecting causative microorganisms in culture-negative infective endocarditis. *Int J Cardiol.* (2014) 172:e288–9. doi: 10.1016/j.ijcard.2013.12.197
8. Fukui Y, Aoki K, Okuma S, Sato T, Ishii Y, Tateda K. Metagenomic analysis for detecting pathogens in culture-negative infective endocarditis. *J Infect Chemother.* (2015) 21:882–4. doi: 10.1016/j.jiac.2015.08.007
9. Guan H, Dong W, Lu Y, Jiang M, Zhang D, Aobuliaximu Y, et al. Distribution and antibiotic resistance patterns of pathogenic bacteria in patients with chronic cutaneous wounds in China. *Front Med (Lausanne).* (2021) 8:609584. doi: 10.3389/fmed.2021.609584
10. Drzewiecka D. Significance and roles of proteus spp. Bacteria in natural environments. *Microb Ecol.* (2016) 72:741–58. doi: 10.1007/s00248-015-0720-6
11. Barbour EK, Hajj ZG, Hamadeh S, Shaib HA, Farran MT, Araj G, et al. Comparison of phenotypic and virulence genes characteristics in human and chicken isolates of *Proteus mirabilis*. *Pathog Glob Health.* (2012) 106:352–7. doi: 10.1179/2047773212y.0000000042
12. Sanches MS, Baptista AAS, de Souza M, Menck-Costa MF, Justino L, Nishio EK, et al. *Proteus mirabilis* causing cellulitis in broiler chickens. *Braz J Microbiol.* (2020) 51:1353–62. doi: 10.1007/s42770-020-00240-1
13. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol.* (2014) 5:258. doi: 10.3389/fmicb.2014.00258
14. Wilks SA, Koerfer VV, Prieto JA, Fader M, Keevil CW. Biofilm development on urinary catheters promotes the appearance of viable but nonculturable bacteria. *mBio.* (2021) 12:e3584–3520. doi: 10.1128/mBio.03584-20
15. Wasfi R, Abdellatif GR, Elshishtawy HM, Ashour HM. First-time characterization of viable but non-culturable *Proteus mirabilis*: induction and resuscitation. *J Cell Mol Med.* (2020) 24:2791–801. doi: 10.1111/jcmm.15031
16. Girlich D, Bonnin RA, Dortet L, Naas T. Genetics of acquired antibiotic resistance genes in proteus spp. *Front Microbiol.* (2020) 11:256. doi: 10.3389/fmicb.2020.00256

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Zhang, Dai, Lin, Xu, Lin, Gong, Cheng, Ji and Cai. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Cellular and Infection Microbiology

REVIEW
published: 13 July 2022
doi: 10.3389/fcimb.2022.757844

Potential Opportunities and Challenges of Deploying Next Generation Sequencing and CRISPR-Cas Systems to Support Diagnostics and Surveillance Towards Malaria Control and Elimination in Africa

Beatus M. Lyimo[1,2†], Zachary R. Popkin-Hall[3†], David J. Giesbrecht[4], Celine I. Mandara[1], Rashid A. Madebe[1], Catherine Bakari[1], Dativa Pereus[1], Misago D. Seth[1], Ramadhan M. Ngamba[1], Ruth B. Mbwambo[1], Bronwyn MacInnis[5,6], Daniel Mbwambo[7], Issa Garimo[7], Frank Chacky[7], Sijenunu Aaron[7], Abdallah Lusasi[7], Fabrizio Molteni[8], Ritha Njau[9], Jane A. Cunningham[10], Samwel Lazaro[7], Ally Mohamed[7], Jonathan J. Juliano[3], Jeffrey A. Bailey[4] and Deus S. Ishengoma[1,5,11]*

[1] National Institute for Medical Research, Dar es Salaam, Tanzania, [2] School of Life Sciences and Bio-Engineering, Nelson Mandela African Institution of Science and Technology, Arusha, Tanzania, [3] School of Medicine, University of North Carolina, Chapel Hill, NC, United States, [4] Pathology and Laboratory Medicine, Center for International Health Research, Brown University, Providence, RI, United States, [5] Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, United States, [6] Infectious Disease and Microbiome Program, Broad Institute, Boston, MA, United States, [7] National Malaria Control Programme, Dodoma, Tanzania, [8] Swiss Tropical Public Health Institute, Dar es Salaam, Tanzania, [9] World Health Organization, Country Office, Dar es Salaam, Tanzania, [10] Global Malaria Programme, World Health Organization, Headquarters, Geneva, Switzerland, [11] Faculty of Pharmaceutical Sciences, Monash University, Melbourne, VIC, Australia

Recent developments in molecular biology and genomics have revolutionized biology and medicine mainly in the developed world. The application of next generation sequencing (NGS) and CRISPR-Cas tools is now poised to support endemic countries in the detection, monitoring and control of endemic diseases and future epidemics, as well as with emerging and re-emerging pathogens. Most low and middle income countries (LMICs) with the highest burden of infectious diseases still largely lack the capacity to generate and perform bioinformatic analysis of genomic data. These countries have also not deployed tools based on CRISPR-Cas technologies. For LMICs including Tanzania, it is critical to focus not only on the process of generation and analysis of data generated using such tools, but also on the utilization of the findings for policy and decision making. Here we discuss the promise and challenges of NGS and CRISPR-Cas in the context of malaria as Africa moves towards malaria elimination. These innovative tools are urgently needed to strengthen the current diagnostic and surveillance systems. We discuss ongoing efforts to deploy these tools for malaria detection and molecular surveillance highlighting potential opportunities presented by these innovative technologies as well as

OPEN ACCESS

Edited by:
Yanfei Chen,
Zhejiang University, China

Reviewed by:
Changchun Liu,
University of Connecticut Health Center, United States
Daniel Neafsey,
Harvard University, United States

***Correspondence:**
Deus S. Ishengoma
deusishe@yahoo.com

†These authors share first authorship

Specialty section:
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *12 August 2021*
Accepted: *17 March 2022*
Published: *13 July 2022*

Citation:
Lyimo BM, Popkin-Hall ZR, Giesbrecht DJ, Mandara CI, Madebe RA, Bakari C, Pereus D, Seth MD, Ngamba RM, Mbwambo RB, MacInnis B, Mbwambo D, Garimo I, Chacky F, Aaron S, Lusasi A, Molteni F, Njau R, Cunningham JA, Lazaro S, Mohamed A, Juliano JJ, Bailey JA and Ishengoma DS (2022) Potential Opportunities and Challenges of Deploying Next Generation Sequencing and CRISPR-Cas Systems to Support Diagnostics and Surveillance Towards Malaria Control and Elimination in Africa. Front. Cell. Infect. Microbiol. 12:757844. doi: 10.3389/fcimb.2022.757844

challenges in adopting them. Their deployment will also offer an opportunity to broadly build in-country capacity in pathogen genomics and bioinformatics, and to effectively engage with multiple stakeholders as well as policy makers, overcoming current workforce and infrastructure challenges. Overall, these ongoing initiatives will build the malaria molecular surveillance capacity of African researchers and their institutions, and allow them to generate genomics data and perform bioinformatics analysis in-country in order to provide critical information that will be used for real-time policy and decision-making to support malaria elimination on the continent.

Keywords: next generation sequencing, CRISPR-Cas systems, pathogen genomics, malaria diagnosis, malaria molecular surveillance, Africa, Tanzania

OVERVIEW

Significant progress was made between 2005 and 2015 in malaria control globally but progress has stalled in recent years (World Health Organization, 2020). Changes in malaria burden were attributed to different interventions that target both mosquito vectors and parasites, which have been scaled-up in the past two decades. Recent evidence shows an increase in malaria cases globally from 2017 to 2019 compared to previous years, but the number of deaths remained relatively unchanged from 2017 to 2019. However, in 2020, malaria deaths increased from 2019 levels, with the majority of both cases and deaths involving children and pregnant women from sub-Saharan Africa (SSA) (World Health Organization, 2021). The increase in malaria cases can be partially explained by the emergence and spread of insecticide resistance in *Anopheles* populations (Kisinza et al., 2017 and antimalarial resistance in parasites (Dondorp et al., 2009; Haldar et al., 2018; Ippolito et al., 2021) both of which threaten the effectiveness of the major malaria interventions, long-lasting insecticide treated bed-nets (LLINs), indoor residual spraying (IRS) and antimalarial drugs (Tawe et al., 2018; Apinjoh et al., 2019; Morgan et al., 2020). Increased malaria cases in 2020 were also attributed to the COVID-19 pandemic, which was believed to have disrupted malaria control activities (World Health Organization, 2021). However, other potential factors such as transmission from asymptomatic reservoirs (Andolina et al., 2021, climate change, and other environmental changes caused by human activities are thought to be responsible for the resurgence of malaria (Upatham et al., 1988; Chaumeau et al., 2019; Mitchell et al., 2022).

In 2020, 29 countries in Africa contributed about 96% of all reported malaria deaths globally, despite consistent implementation of the major malaria interventions since 2002 (World Health Organization, 2021). Malaria control in Africa relies mainly on the use of LLINs and effective antimalarial drugs, predominantly artemisinin-based combination therapy (ACTs) for routine case management and sulphadoxine/pyrimethamine (SP) for intermittent preventive therapy in pregnant women (iPTp) (World Health Organization, 2021). Despite the detection of insecticide resistance and occurrence of mutations in *PfKelch13* gene (associated with resistance to artemisinins) in Rwanda, Uganda and Eritrea, treatment failure rates remain below 10%, hence both ACT and vector control measures are still considered highly effective (World Health Organization, 2021). However, a robust surveillance and response strategy is urgently needed to detect any early failures in the current interventions and facilitate effective response to ensure malaria elimination by 2030.

At present, malaria case management and routine surveillance rely on traditional diagnostic methods based on microscopy and rapid diagnostic tests (RDTs). These methods are widely deployed by the National Malaria Control Programmes (NMCPs) in most malaria endemic countries in Africa (Tawe et al., 2018; Abbas et al., 2019; Tessema et al., 2019b; Morgan et al., 2020). Despite their short turnaround time, these methods have major limitations (Apinjoh et al., 2019 (**Table 1**). In areas of low transmission and those closer to malaria elimination, microscopy and RDTs are less sensitive and unable to detect transmissible but very low parasite densities (Nsanzabana, 2019; Andolina et al., 2021). Since most of the widely used RDTs are based on the detection of histidine rich protein 2 (HRP2), the situation is further exacerbated by the emergence and spread of *Plasmodium falciparum* with histidine rich protein 2 and 3 (*pfhrp2* and *pfhrp3)* gene deletions (Thomson et al., 2019; Feleke et al., 2021). To strengthen the current surveillance system, innovative methods of malaria diagnosis and population surveillance based on advanced molecular techniques are urgently needed to support effective case management (Ishengoma et al., 2019.

Two key technologies that are slated to have a large impact on surveillance and diagnosis of malaria are Next Generation Sequencing (NGS) and clustered regularly interspaced short palindromic repeats (CRISPR). NGS, which is also termed high-throughput or massive parallel sequencing, allows for thousands to billions of DNA or RNA fragments to be sequenced and analyzed in either a targeted or whole genome approach (Deurenberg et al., 2017; Talundzic et al., 2018). Routine malaria surveillance programs could benefit from deployment of NGS technologies, as they provide a scalable, cost-effective means of surveillance in large numbers of samples (Juliano et al., 2010; Van Tyne et al., 2011; Manske et al., 2012; Lerch et al., 2017; Ngondi et al., 2017; Tawe et al., 2018; ; Moser et al., 2021).

CRISPR and CRISPR associated protein (Cas) derived from type II CRISPR bacterial immune systems, provide the basis for a new family of assays that detect nucleic acid targets with high sensitivity and specificity. While CRISPR-Cas is mostly known to enable easy gene editing, it also has enormous diagnostic potential (Jiang et al., 2013. Indeed, CRISPR-Cas systems have

TABLE 1 | Various methods used for diagnostic and surveillance of malaria.

Method	Target	Sensitivity (percentage of true positives detected)	Specificity (percentage of true negatives detected)	Limit of detection	Cost per sample (USD)	Time	Advantages	Limitations	Reference
Microscopy	N/A	95%	98%	50–200 parasites/ µL of blood	$0.12–$0.40	60 min	Identification of parasite morphologies, species and stage	Requires trained personnel and microscopes	(Hopkins et al., 2013; Pham et al., 2018; Mbanefo and Kumar, 2020)
Rapid Diagnostic Test (RDT)	PfHRP2, *Pf*LDH	85% to 94.8%	95.2% to 99%	50–200 parasites/ µL of blood	$0.60-$2.50	15–30 min	Fast and easy to use	Mutation in *pfhrp-2* leading to false negatives, Unable to quantify parasitaemia, can produce false-positive results well after resolution of infection	(Cordray and Richards-Kortum, 2012; Hopkins et al., 2013; Pham et al., 2018; Mbanefo and Kumar, 2020)
Polymerase Chain Reaction (PCR)	18S rRNA, *cox3*, TARE-2, *varATS* and *Pfs25*	98% to 100%	88% to 94%	0.5–5 parasites/ µL of blood	$0.35–$5.00	1-2 h	Low limit of detection makes it easier to detect low parasitaemia, High throughput, detects drug-resistant parasites, mixed infections	Requires expensive instruments and reagents and is not able to quantify parasitaemia	(Tangpukdee et al., 2009; Cordray and Richards-Kortum, 2012; Hopkins et al., 2013; Pham et al., 2018Mbanefo and Kumar, 2020)
High-volume quantitative PCR (qPCR)	*Plasmodium* sp. 18S RNA	100%	99.75%.	0.1 parasite/µl of blood	$0.50	45 min-2h	Low limit of detection makes it easier to detect low parasitaemia	Requires expensive instruments and reagents, Requires trained personnel	(Kamau et al., 2013; Imwong et al., 2014; Haanshuus et al., 2019)
Nucleic Acid Sequence-Based Amplification (NASBA)	18S mRNA	97.4–100%	80.9–94%	0.01–0.1 parasites/ µL of blood	$5-$20	1–2 h	No thermocycler needed	Requires highly trained personnel, expensive	(Cordray and Richards-Kortum, 2012; Pham et al., 2018; Mbanefo and Kumar, 2020)
Loop-mediated Isothermal Amplification (LAMP)	18S rRNA, mDNA	98.3% to 100%	94.3% to 100%	1–5 parasites/ µL of blood	$0.28-$5.31	30–60 min	Low limit of detection, faster reaction time than PCR, no thermocycler needed, high throughput	Easily susceptible to contamination	(Cordray and Richards-Kortum, 2012; Pham et al., 2018; Mbanefo and Kumar, 2020)
Serological test	Detection of antibodies against parasites	69.9%	100%	50–200 parasites/ µL of blood	$0.50-$5.50	30-60 min	Useful for epidemiologic surveys, Relatively cheap, Provides retrospective confirmation of malaria infection	Not suitable for the diagnosis of acute malaria, Cannot discriminate species	(She et al., 2007; Tangpukdee et al., 2009; Tusting et al., 2014; Goh et al., 2021)

cox3, Mitochondrial cytochrome c oxidase III; TARE-2,Telomere associated repetitive element; varATS, Var. gene acidic terminal sequence; PfLDH, P. falciparum lactate dehydrogenase; PfHRP2; Plasmodium falciparum histidine-rich protein 2.

**Sensitivity and specificity estimates come from (Boonma et al., 2007; Han, 2013) cost estimates come from (Han, 2013) except NASBA (Mbanefo and Kumar, 2020) and serology (Tusting et al., 2014; Goh et al., 2021).*

been utilized to detect and differentiate arbovirus strains (Gootenberg et al., 2017, to diagnose SARS-CoV-2 infections (Guo et al., 2020, to distinguish pathogenic bacteria (Strich and Chertow, 2019, to diagnose cancers (Yin et al., 2019, and indeed to diagnose malaria (Lee et al., 2020; Cunningham et al., 2021).

This review discusses ongoing efforts to deploy advanced molecular tools for the diagnosis and surveillance of malaria, developing the capacity for pathogen genomics in Africa, and highlights both potential opportunities presented by these innovative technologies as well as challenges facing these efforts.

Epidemiological Applications of NGS

Our ability to detect and track the spread of malaria is potentially improved by advances in malaria genomics (particularly those

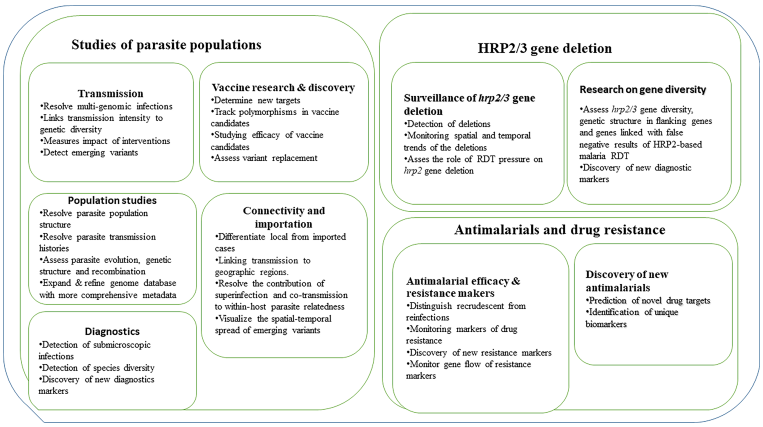


FIGURE 1 | Current and potential application of NGS in studies of parasite populations, drug resistance and surveillance of hrp2/3 gene deletions.

based on NGS) and associated bioinformatics tools in combination with epidemiological data (Escalante et al., 2015; Kwiatkowski, 2015; Watson et al., 2021). These tools can facilitate different studies of parasite populations, antimalarial drug resistance, surveillance of hrp2/3 gene deletions, and monitoring the impact of current and future interventions (**Figure 1**). Studies utilizing these methods are urgently needed and if scaled-up, can be critical for monitoring transmission to achieve proper control and the ultimate goal of malaria elimination.

Monitoring Malaria Transmission

Historically, transmission intensity and dynamics of malaria burden were measured using tools like entomological inoculation rates (EIR), case incidence and prevalence. These tools have helped to generate maps of mortality rates, prevalence and incidence as well as the overall global burden of malaria (Weiss et al., 2019; Neafsey et al., 2021), but recently have become less sensitive especially in areas of very low transmission. Due to their inherent limitations, they are unable to detect genetic signatures and parasite population structure indicative of modified transmission in response to interventions. For example, higher incidence could indicate a decline in the effectiveness of malaria interventions, even though the increase might actually be due to increased transmission rates from a source which was not addressed in the initial interventions (Mohd Abd Razak et al., 2016). Furthermore, decreased transmission limits outcrossing, influencing high inbreeding rates within the population and triggering population fragmentation. As such, conventional surveillance methods may result in misinterpreting the underlying processes shaping malaria epidemiology. Advances in NGS technologies supported by bioinformatic tools and increased availability of epidemiological data have made it possible to generate high quality and sensitive data which may help detect changes in transmission intensity, identify multi-genomic infections, evaluate intervention effectiveness, and identify potential deficiencies in malaria control programmes in both high and low transmission areas (Watson et al., 2021). This improved understanding can be achieved by determining the number of parasite strains, genetic diversity (e.g. within-host diversity index (F_{ws}) as well as relatedness and genetic structure of parasites in an infection.

Monitoring Parasite Populations

Polymorphic genes such as merozoite surface protein (*msp1*), merozoite surface protein 2 (*msp2*) and glutamate rich protein (*glurp*) have been used extensively to study parasite genetic diversity and population structure, mostly to detect complexity/multiplicity of infection (COI/MOI), wherein multiple distinct genotypes co-occur in a single host (Lalremruata et al., 2017). Studies using these methods have demonstrated associations between MOI and such factors as host age, clinical severity, and transmission intensity (Mayengue et al., 2009; Lalremruata et al., 2017). However, these PCR-based genotyping methods have limited inter-laboratory reproducibility and also lack sensitivity, hence may underestimate allelic diversity in co-infected hosts (Assefa et al., 2014). Highly sensitive NGS tools, by contrast, can illuminate not only MOI but also parasite transmission history, genetic structure, recombination, and evolutionary history.

The use of these tools to study evolutionary history can improve our understanding of the parasite-host interactions involved in the emergence of new parasites in the region (Su et al., 2020, as well as evolutionary responses to selective pressures caused by intensified malaria elimination interventions, such as antimalarial drugs (Neafsey and Volkman, 2017). It is even possible to estimate recombination events that may lead to evasion of control and detection efforts (Miles et al., 2016). In addition, NGS data facilitates population genetic and evolutionary metric calculations, such as linkage disequilibrium (LD), effective population size (N_e), site frequency spectrum (SFS), identity by descent (IBD), identity by state (IBS), haplotype diversity (H),

principal component analysis (PCA), F_{WS}, and F_{ST}. The extent of parasite genomic data now available provides opportunities to expand and refine the global genome database and public resources, which in turn will greatly expand our understanding of parasite biology and accelerate the discovery of new interventions.

Parasite Connectivity and Importation

Traditionally, case incidence was used in combination with human migration data to link parasite populations across geographical and transmission gradients, which would inform intervention strategies. However, this method lacks the precision of NGS-based methods.

Genetic relatedness inferred from genomic data provides much higher geographic resolution of transmission (Tessema et al., 2019a) and can be quantified between 0 (completely unrelated parasites) and 1 (identical clones). Genetic relatedness between parasite samples is positively correlated with reduced transmission, and therefore can be used as a proxy measure of transmission level (Daniels et al., 2015; Cerqueira et al., 2017). Using advanced NGS tools, it is also possible to infer malaria transmission networks over a given timescale in a population (Ajibola et al., 2021). Importation of malaria parasites from higher to lower transmission areas, particularly those in the elimination phase, remains a major challenge to malaria elimination, and is difficult to detect using traditional methods. However, NGS methods are able to do so, as was demonstrated in Zanzibar (Morgan et al., 2020). Strengthening malaria molecular surveillance (MMS) using advanced genomic tools will reveal the number of secondary cases arising from each imported infection, allowing NMCPs to determine what interventions are required to prevent re-establishment of transmission. The data derived from effective MMS systems will also help to resolve the contribution of superinfection and co-transmission (Nair et al., 2014; Nkhoma et al., 2018).

Using NGS to Address Challenges in Malaria Control and Elimination

Antimalarials and Drug Resistance

Antimalarial resistance is a major obstacle to the effective control and elimination of malaria. Malaria parasites have evolved resistance to all widely deployed antimalarial drugs, leading to withdrawal of some of the drugs such as chloroquine and SP (Ishengoma et al., 2019). Antimalarial drug resistance has spread globally, although it remains rare and localized in some areas with lower transmission and therefore lower selective pressure (Haldar et al., 2018). We can clearly use these methods to retrospectively understand how and why resistance emerged and spread. However, their prospective use by NMCPs through enhancing genomic surveillance infrastructure can identify signatures of emerging drug resistance both when resistance mutations are known and unknown (Neafsey et al., 2021.

Antimalarial efficacy monitoring is usually done in therapeutic efficacy studies (TES) which enroll infected patients and genotype those with recurrent infection after treatment to distinguish between recrudescence (drug failure) and reinfection (World Health Organization, 2009). Traditional methods rely on the polymorphic markers mentioned above (World Health Organization, 2007) or microsatellites (Greenhouse et al., 2006; Malvy et al., 2018). While these methods are widespread, they are limited by their low throughput, poor resolution, and technical challenges regarding analysis, scoring and interpretation of results. Specifically, these methods under-estimate MOI or misclassify infections as either recrudescent or reinfection (Shaukat et al., 2012). However, new NGS methods using amplicon sequencing (Rao et al., 2016 or SNP barcodes are being optimized and may potentially replace the current methods, which would improve estimates of antimalarial efficacy especially in low transmission areas with limited diversity and polymorphisms in parasite populations.

NGS provides a robust, high throughput tool for identifying and tracking molecular markers of drug resistance (Ishengoma et al., 2019). Currently available markers can be used for spatial and temporal monitoring of resistance to different antimalarials, including both current drugs and those which were used in the past (Rao et al., 2016; Levitt et al., 2017; Nag et al., 2017; Talundzic et al., 2018). This monitoring is especially critical in the context of partial artemisinin resistance, which has already been reported in South-East Asia (SEA) (Leang et al., 2015; Takala-Harrison and Laufer, 2015). Artemisinin resistant parasites may be introduced from SEA (as drug resistance genes are known to spread between populations) (Roper et al., 2004; Dwivedi et al., 2016) or could potentially emerge independently in SSA, underscoring the necessity of this continuous surveillance.

It is important to understand the origin and flow of resistance genes in order to anticipate the diffusion of antimalarial resistance and interrupt its spread (Dwivedi et al., 2016). Current methods for doing this are expensive and less robust. For example, Sanger sequencing is considered to be useful in identification of major alleles but has low accuracy in identifying alleles with frequency less than 50% (minor alleles) (Rohlin et al., 2009). NGS based methods are promising as they are high throughput, less labor intensive, have high sensitivity in identifying all variants, even those with minor allele frequencies, and facilitate the discovery of novel markers (Tessema et al, 2019a).

Surveillance of *pfhrp2/3* Gene Deletions

Malaria rapid diagnostic tests (mRDTs) based on lateral flow detection of *P. falciparum* Histidine-Rich Protein 2 (*Pf*HRP2) have supported prompt and effective identification of *P. falciparum* malaria infections, particularly in resource poor regions. However, recent studies in various countries have reported some cases where pfHRP2 based mRDTs failed to detect malaria parasites in patients with positive results by either microscopy or PCR (Gamboa et al., 2010; Thomson et al., 2019; Bakari et al., 2020). While other factors may be involved (Murray et al., 2008, deletion of the *pfhrp2* gene (as well as the related *pfhrp3* that cross-reacts in RDTs) has also been reported to contribute to false-negative test results (Luchavez et

al., 2011; Cheng et al., 2014; Thomson et al., 2019), which has significantly compromised the performance of *Pf*HRP2-based RDTs. Thus, WHO currently recommends for all malaria endemic countries to undertake surveys to identify the prevalence and trends of the deletion in space and time (World Health Organization, 2019).

NGS data enables both the detection of emergence and monitoring of *pfhrp2*/3 gene deletion frequencies in all settings (low and high transmission) in relation to the pressure caused by the use of HRP2-based RDTs. With intensified surveillance, it will therefore be possible to identify areas which may need to change diagnostic strategies for effective malaria control. The deletion is currently detected based on initial clinical evidence (e.g. a microscopy-positive result for *P.falciparum* but negative RDT) and confirmed with molecular approaches such as PCR and antigen detection assays (Cheng et al., 2014). However, some samples can be RDT-negative but have intact *pfhrp2/3* genes (Nsobya et al., 2021). Indeed, a study from the Democratic Republic of Congo that performed WGS on samples with suspected deletions showed that the genes were actually intact (Parr et al., 2021). However, recent data from the Horn of Africa, which utilized complementary molecular, immunological and sequencing assays, demonstrates that there have been multiple independent deletions in *pfhrp3*, which have been accompanied by strong selection for a single *pfhrp2* deletion due to pressure from RDTs (Feleke et al., 2021). As such, the MalariaGEN network has highlighted the utility of widely deployed NGS as a critical tool to detect the true *pfhrp2/3* gene deletion prevalence (MalariaGEN et al., 2021.

Gene diversity can potentially explain the variability in the sensitivity of *Pf*HRP2-based RDTs (Baker et al., 2005; ; Wurtz et al., 2013; Li et al., 2015; Gendrot et al., 2019). Indeed, studies conducted in different geographical settings have shown that gene diversity in the *pfhrp*2 gene plays a role in the sensitivity of RDTs especially among infections with low parasitaemia (≥ 200 asexual parasites/μl) (Baker et al., 2010). Given that countries such as Eritrea (Berhane et al., 2018 and Ethiopia (Golassa et al., 2020 have reported abnormally high prevalence of these deletions, NGS methods will likely be necessary for further characterization and monitoring of the diversity in these genes.

NGS and CRISPR as Novel Surveillance and Diagnostic Tools

Timely and accurate diagnosis is a key element of effective malaria management. Traditional microscopy and RDT-based diagnostic methods involve detection of malaria parasites or products e.g. antigens in the blood of infected patients (Kasetsirikul et al., 2016). In other laboratories, malaria diagnosis involves molecular methods such as PCR (**Table 1**). Both microscopy and RDTs have been deployed as diagnostic tools in many malaria endemic areas, despite low sensitivity due to their high detection limits (≥ 50 parasites/μL) and limited ability to detect non-falciparum species. These deficiencies cause under-reporting of infections, specifically in areas with low transmission and for individuals with asymptomatic malaria or low density infections (Slater et al., 2019). By contrast, PCR has high sensitivity and specificity which make it capable of detecting low parasite density of less than 5 parasites/μl. This increased sensitivity will improve our ability to diagnose very low density infections, and detect asymptomatic individuals who can unknowingly contribute to disease transmission. Furthermore, the NGS-enabled ability to scan hundreds of *Plasmodium* genomes to better understand parasite biology and for novel diagnostic markers facilitates the development of tools that are species-specific, highly sensitive and agreeable to PCR or other nucleic acid amplification techniques (Lucchi et al., 2013.

In addition to the shortcomings outlined above, currently widespread robust malaria diagnostics require substantial financial investment and equipment, along with extensive personnel training. CRISPR-based diagnostics are an exciting development, as they have similar sensitivity and specificity to PCR assays, but require fewer resources, and are fast, cost-effective, and easy to use (Kostyusheva et al., 2021). The simplicity of the CRISPR-Cas based detection system allows the rapid development of diagnostic methods of a wide array of infectious diseases (Jiang et al., 2013; Gootenberg et al., 2017; Kostyusheva et al. 2021). Four CRISPR-based diagnostic methods have particular potential to replace PCR-based methods in low-resource settings: DETECTR, SHERLOCK, CARMEN, and CRISPR-Chip (Kaminski et al., 2021; Kostyusheva et al., 2021). These methods work reliably in isothermal conditions (negating the need for an expensive thermocycler) and results can be determined by lateral flow strips at the point of care, or by fluorescence read by a fluorimeter, or in some cases, with the naked eye. The use of lateral flow strips or visual inspection of fluorescence avoids the use of more technically challenging gel electrophoresis or quantification using a qPCR machine (Kaminski et al., 2021; Kostyusheva et al., 2021). CRISPR-based methods that have the potential to be developed for malaria diagnosis are described in **Table 2**.

The deployment of CRISPR-based diagnostic methods for *P. falciparum* could revolutionize the field of malaria surveillance in Africa. The ability of this method to detect as little as one copy of target DNA per reaction will play a major role in the detection of both symptomatic and asymptomatic infections and any associated resistance mutations (Kwiatkowski, 2015. CRISPR has already been used to detect malaria strain variants in mixed infections, an important challenge for malaria control and elimination efforts and a modality that could be extended to many areas of epidemiology (Kwiatkowski, 2015. One-pot systems that are suitable for field deployment due to their minimal technological and logistical prerequisites, high diagnostic reliability, and rapid turnaround time have been developed for SARS-CoV-2 and show additional promise for the expansion of these tools in malaria-endemic countries (Nguyen et al., 2021). The development of isothermal reactions performed with lyophilized reagents and with straightforward visualization at the point-of-care are particularly revolutionary as a means to quickly, cheaply, and accurately diagnose malaria infections in low-resource and/or remote locations (Cunningham et al., 2021). While these tools are still in early

TABLE 2 | CRISPR-based diagnostics used in the context of malaria.

End Point	Method (Cas enzyme, amplification)	Advantages	Limitations	Reference
Lateral Flow	SHERLOCK Version 2 (Cas13 RPA)	Similar sensitivity to RT-PCR methods but without the need for expensive thermocyclers; highly specific species delineation capabilities; can perform drug-resistance genotyping; potential for use in mosquitoes as well as in clinical samples	Not yet ready for wide-scale field use; requires higher crRNA concentrations than SHERLOCK in other pathogens; "one-pot" approach not yet achievable; assay design costs are high	(Gootenberg et al., 2018; Kellner et al., 2019)
	SHINE Cas13 RPA)	Single step tool with high sensitivity compared to RT-qPCR. Detects virus from unextracted samples. Reduced contamination risk as amplification reaction tubes remain sealed.	Not yet validated with field samples	(Arizti-Sanz et al., 2020
	STOPCovid (Cas12 LAMP)	Sensitivity of this tool is similar to RT-qPCR Appropriate for low-complexity clinical laboratories.	Not yet validated with field samples	(Joung et al., 2020
Fluorescence/ Colorimetry	SHERLOCK VERSION 1 (Cas9, RPA)	Similar sensitivity to RT-PCR methods but without the need for expensive thermocyclers; highly specific species delineation capabilities; can perform drug-resistance genotyping; potential for use in mosquitoes as well as in clinical samples	Not yet ready for wide-scale field use; requires higher crRNA concentrations than SHERLOCK in other pathogens; "one-pot" approach not yet achievable; assay design costs are high	(Cunningham et al., 2021
	SHERLOCK version 2 (Cas13/RPA)	Multiplexable, portable, rapid, and quantitative detection platform of nucleic acids.	Not yet validated with field samples	(Gootenberg et al., 2018; Kellner et al., 2019)
	CARMEN (Cas13, PCR/ RPA)	Detects all human-associated viruses with high sensitivity. Enables comprehensive subtyping of some viruses e.g. influenza A strains and multiplexed identification of dozens of HIV drug-resistance mutations	Not yet validated with field samples	(Ackerman et al., 2020
	NASBACC (Cas9/NASBA)	Can discriminate between viral strains with single base resolution	Long turnaround time Not yet validated with field samples Challenge in the sample preparation	(Pardee et al., 2016
	DETECTR	Higher sensitivity than SHERLOCK; functions in a single tube ("one-pot" approach); isothermal like SHERLOCK; all reaction components can be lyophilized (no need for refrigeration)	Not yet validated with field samples	(Lee et al., 2020

development, their accuracy, ease-of-use, cost-effectiveness, and rapidity give them great potential to complement currently available tools.

APPLICATION OF NGS AND CRISPR-CAS SYSTEMS IN AFRICA: CURRENT STATUS AND FUTURE PERSPECTIVES

In Africa, few studies have been undertaken to determine the diversity and other features of parasite populations using NGS methods. Previous studies utilized PCR genotyping of polymorphic genes (*msp1*, *msp2* and *glurp*) to determine parasite diversity with little focus on population structures and other population genetics metrics (Carlsson et al., 2011; Yavo et al., 2016; Metoh et al., 2020; Amoah et al., 2021). Recently, some regional studies have been conducted in Africa using genomic tools and have provided initial data on parasite population structure (Manske et al., 2012; Amambua-Ngwa et al., 2019; Abera et al., 2021; Moser et al., 2021). However, other areas, particularly those with low transmission, remain largely unexplored (Morgan et al., 2020). In addition, the metrics that are routinely used in these studies [e.g. F_{WS}, F_{ST} (the fixation index), COI/MOI, IBD, inbreeding coefficient (F), and coefficient of uniqueness (COU)] have not been fully evaluated to be reliably informative for monitoring intervention-modulated changes in malaria transmission (Abera et al., 2021; Onyango et al., 2021). Therefore, further intensive study and metric development is required. The integration of traditional epidemiological data with NGS data will likely improve malaria surveillance in service of elimination strategies. While CRISPR-based diagnostics could revolutionize the field of malaria surveillance in malaria endemic countries in Africa, they have not yet been deployed or tailored to the specific needs of NMCPs.

Meanwhile, there are ongoing collaborative projects between African researchers, NMCPs, and partners in the USA and Europe to establish local molecular, genetic, and genomic laboratory and analytic capacity to support MMS. These projects are located in several countries in SSA including Senegal, Mozambique, Tanzania, Mali, The Gambia, Ghana, Gabon, Madagascar, Cameroon, Ivory Coast, Burkina Faso, Ethiopia and Uganda (Amambua-Ngwa et al., 2019; MalariaGEN et al., 2021). In Tanzania, previous efforts were

made with the support of different funding agencies and partners and set-up the stage for the current initiatives (Amambua-Ngwa et al., 2019; MalariaGEN et al., 2021). Our current collaboration (Molecular Surveillance of Malaria in Tanzania, MSMT) is funded by the Bill and Melinda Gates Foundation and focuses on six key aims to attain the bold and ambitious goal of establishing local capacity for in-country generation and analysis of genomics data to support policy and decision making by the NMCP and its stakeholders. To that end, Tanzanian stakeholders identified three scientific aims for MSMT: 1) unraveling parasite population genetics, 2) country-wide drug resistance monitoring, and 3) country-wide surveys of *pfhrp2/3* gene deletion. In addition, the project will address three supportive aims with a focus on training for capacity building, career development, and science leadership for sustainability of MMS. The project will also focus on developing a reliable model for effective engagement with NMCP and other stakeholders, and developing use cases for MMS in Tanzania. Lastly, the project will develop and implement a framework and platforms for storage, management, analysis, and sharing of the genomic data and associated metadata.To successfully adopt and transfer NGS and bioinformatics tools to Africa and build local capacity, a robust strategy of capacity building is critical. The MSMT team has developed a "counterpart model" of capacity building. This model involves pairing Tanzanian researchers with US-based expert (counterparts) collaborators with the role ensuring successful technology transfer.

To ensure key local stakeholders are effectively engaged, the MMS projects should bring on board all local stakeholders and ensure they are fully engaged in the project. In Tanzania, the MSMT project established an advisory committee which is under the Chief Medical Officer of the Tanzanian Ministry of Health with the role of providing oversight and guiding the implementation and sustainability of the project. The project is run by the joint implementation committee, which includes the study team, NIMR, NMCP and the Ministry in charge of the local governments (which owns and runs all health facilities in Tanzania). The technical implementation committee involves NIMR researchers, NMCP and collaborating partners who are advised by a committee of experts with experience and track records in MMS and capacity building particularly in Africa.

Thus far, a genomics laboratory has been established at NIMR in Dar es Salaam and will soon be equipped with state-of-the-art equipment to ensure genomics data are generated in-country and analysed by local experts from the second year of the project in 2022. The Tanzanian researchers have sequenced over 8,500 field collected samples in laboratories in the US as part of their training, and future analysis will take place in the NIMR laboratory with further training and coaching from the US-based collaborators. It is anticipated that our training and capacity building model and the laboratory capacity to be built in Tanzania will pave a way to a sustainable MMS for supporting not only malaria but other pathogens as part of the broader pathogen genomics initiative.

CHALLENGES ASSOCIATED WITH NGS APPLICATION IN AFRICA

African researchers seeking to answer biological questions using NGS face many challenges, ranging from funding to procurement to lack of trained personnel (**Table 3**). The long-standing imbalance between collaborators in the global North and South has resulted in unsustainable patterns of research practice that must be broken if African researchers are to be free to initiate and execute NGS-dependent research studies. For the wide application of NGS technology in malaria surveillance, African countries need substantial investment to establish genomic capacity (Haanshuus et al., 2019 including human capacity, ancillary equipment for library preparation, quality control and sequencing (Escalante et al., 2015.

STUDY DESIGN AND SAMPLE COLLECTION

Historical imbalances are visible beginning with the funding and design of research programs. Too often, minimal intellectual input is sought from African researchers during this phase, and donors must critique their funding calls to address this

TABLE 3 | Challenges and opportunities for NGS application in a resource constrained country such as Tanzania.

	Challenges	Potential impacts of NGS	Successes
	Obstacles to implementation of NGS	Potential impacts of NGS	Lessons from other African countries
Procurement and sample collection	•Study design impacted by tensions between global North and South collaborators. •Field and clinic work requires multi-level approval and buy-in •Logistics for sample collection hampered by transportation network, lack of expertise in rural areas. •Equipment for sample collection depends on supply chains in global North. •Sampling equipment and reagents are overpriced in-country. •Collaborations often lack training component	•Training a new cohort of experts will enable local researchers to drive projects from concept to implementation. •Multiple iterations of stakeholder engagement by Tanzanian led investigators will build trust in the approval process. •Local purchasing will encourage suppliers to stock key supplies and reagents at rational prices. •Long-term North-South collaborations with productive training of all staff will build expertise at multiple levels, including logistics and procurement.	•Sars-CoV2 sequencing in centers of excellence in Africa benefited from simple study design and rapid approvals (Tessema et a., 2020; Happi et al., 2021; Tegally et al., 2021). •MalariaGEN collaboration has facilitated a large number of research studies across Africa.
Sequencing	•Staff require training to complete library preparation and sequencing. •Supply and service contracts for sequencing machines is based in the North. •Electricity must be continuous or battery backup is required.	•Portable devices (iSeq, minION) allow for sequencing at a clinic or community site. •Computers to run sequencers have benefitted from rapidly improving GPU capabilities. •A growing number of lyophilized sequencing reagents simplifies shipping.	•The rapid deployment of sequencing reagents and machines for Sars-CoV2 at centers of excellence in Africa (Tessema et al., 2020; Happi et al., 2021; Tegally et al., 2021). •Both Illumina and Oxford Nanopore are improving their supply chains in Africa.
Bioinformatics	•High performance computing (HPC) centers require long-term. Institution needs regular funding •No local universities are currently offering a bioinformatics degree. •Loss of bioinformatics expertise to other countries with higher research and private sector salaries	•A centre of excellence with long-term funding allows research to continue. The wider research community benefits from this analysis capability. •Training a cohort of bioinformatics experts will enable local mentoring and serve sequencing needs beyond malaria research. •Local institutions with up-to-date equipment may attract African researchers working abroad.	•Ghana: Noguchi Memorial Institute for Medical Research benefits from long-term funding and a growing bioinformatics team (Karikari, 2015). •Nigerian bioinformaticians at multiple universities are actively training PhD level researchers (Fatumo et al., 2014) •South African universities offer bioinformatics degrees and many find employment within the local biomedical research community.
Storage	•Human NGS data requires secure storage. •Regulators may be unfamiliar with rapidly changing technologies. Hardware needed to store data requires significant capital investment. Cloud-based solutions face regulatory challenges.	•Archiving allows for long term studies. •Storage on local servers empowers researchers in country to initiate analyses using archived data. •Building a strong regulatory framework will streamline ethical reviews for future studies.	• Sars-CoV2 sequence data has been stored and shared globally (Tessema et al., 2020; Happi et al., 2021; Tegally et al., 2021). • Human NGS data storage at the National Biotechnology Development Agency in Nigeria (Fatumo et al., 2014).

imbalance. For large-scale studies where samples are collected from many districts within a country, buy-in from a large number of authorities is needed. The need to meet funding deadlines for an overseas collaborator may create tension during this phase. African procurement services and scientific suppliers to furnish basic sample collection necessities are often limited or unavailable, creating further imbalance between collaborators. To overcome this challenge, long-term partnerships that emphasize training are needed. Funding for NGS projects must be balanced, and local supply chains must be developed to support sample collection and sequencing without reliance on procurement abroad. One of the best recent examples of the rapid implementation of NGS for infectious disease research was seen in the centres of excellence for SARS-CoV-2 sequencing (Tessema et al., 2020; Happi et al., 2021; Tegally et al., 2021). To monitor the emergence of novel viral strains, several African laboratories rapidly pivoted to sequencing, taking advantage of publicly shared protocols, using kits and consumables tailored to this problem, and laboratories in Botswana and South Africa were able to quickly detect and raise awareness of the omicron variant of concern (Viana et al., 2022).

SEQUENCING

The sequencing phase of an NGS study faces many related challenges. Until sequencing becomes routine, skilled staff are difficult to find and retain. Sequencing machines, consumables and quality control reagents are challenging to procure, and are priced above the budgets of many African laboratories. Both Illumina and Oxford Nanopore Technologies have taken steps to address procurement challenges by supplying flow cells and consumables directly to several African agencies, but gaps in procurement continue to be filled by collaborating Northern institutions. Equipment and flow cells were rapidly distributed to several laboratories early in the spread of SARS-CoV-2. Robust supply chains and investment from the major suppliers of sequencing technology are needed to expand similar supply chains.

BIOINFORMATICS AND DATA STORAGE INFRASTRUCTURE

NGS studies produce large amounts of data that is analyzed with an ever-evolving suite of bioinformatics tools. Adequate data storage is a significant cost and requires in-house expertise. Few African institutions are training bioinformaticians, and those that are trained may leave for better paying jobs in the North-centered biotechnology industry. Building high performance computing clusters will require sustained investment, as will building up a cohort of bioinformaticians to work on African infectious disease sequencing projects. There is also a need to develop local expertise for equipment installation and maintenance to prevent long delays and additional costs accrued from overseas outsourcing (Apinjoh et al., 2019). Examples of institutions that have successfully built bioinformatics systems include the Noguchi institute in Ghana (Karikari, 2015, Redeemer's University in Nigeria (Fatumo et al., 2014, and several South African universities.

LONG-TERM DATA STORAGE AND REGULATORY FRAMEWORKS

Human and pathogen sequence data is sensitive, requiring adequate long-term storage, and a regulatory framework to protect patients' information. Relatively low-cost storage exists in cloud storage systems, but privacy concerns must be addressed for this to be allowed by regulators. Hardware for long-term storage must be budgeted for in research proposals, and efficient storage formats are needed.

Skilled Workforce

Despite their potential, the initial investments in infrastructure for NGS are high, the methods require highly skilled personnel to perform sample processing, bioinformaticians, molecular epidemiologists for data analysis and interpretation, field epidemiologists, disease specialists for interpretation of the generated data, and public health specialists for adoption of the findings into policy (Escalante et al., 2015; Ishengoma et al., 2019). However, there are limited initiatives to train, retain, mentor and maintain highly skilled experts in Africa. The ongoing capacity building initiatives such as those under the African Academy of Sciences (https://www.aasciences.africa/), The human heredity and Health (H3Africa Initiative, https://h3africa.org/) and others need to be strongly supported by African countries for sustainability.

CONCLUSION

The application of advanced NGS tools and CRISPR-Cas technologies to malaria diagnosis and surveillance has great potential for malaria elimination in Africa. These tools will improve our understanding of malaria transmission dynamics, including our ability to track the spread of parasites and their associated clinically relevant mutations. With the wide scale distribution of genomics facilities and expertise, as well as state-of-the-art CRISPR-based diagnostics that substantially reduce equipment and training needs, our understanding of many aspects of malaria biology will dramatically expand. In addition to wider reaching and more accurate mobile and point-of-care diagnostics, African capacity for malaria control and ultimately elimination will result from this technological expansion. Furthermore, the deployment of these technologies and associated spread of expertise is not limited to malaria, but can also facilitate the better understanding and control of other

infectious diseases as part of the ongoing initiative of building the capacity for pathogen genomics in Africa.

Nevertheless, significant challenges remain, which need to be addressed to facilitate smooth implementation of these tools necessary for malaria surveillance and elimination. Simulation studies which consider surveillance tools as an intervention strategy can also be performed to quantitatively evaluate deployment of these tools. Available simulation models such as openmalaria can be used to optimize these tools in terms of cost-effectiveness and public health. These challenges range from technological hurdles to a lack of funding investment and sufficiently skilled workforce in the areas where malaria interventions are most desperately needed. However, should these hurdles be overcome, the potential public health implications of deploying NGS and CRISPR-Cas tools in Tanzania and elsewhere are invaluable.

AUTHORS CONTRIBUTIONS

DI- Formulated the original idea, supervised and edited the manuscript; BL, ZPH, DG, CB, DP, MS, JJ, JB, and DI - wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported, in whole, by the Bill & Melinda Gates Foundation [grant number 02202]. Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission. JJ also received funding from NIH K24AI134990.

REFERENCES

Abbas, N., Saba, T., Rehman, A., Mehmood, Z., Javaid, N., Tahir, M., et al. (2019). *Plasmodium* Species Aware Based Quantification of Malaria Parasitemia in Light Microscopy Thin Blood Smear. *Microsc. Res. Tech.* 82, 1198–1214. doi: 10.1002/jemt.23269

Abera, D., Kibet, C. K., Degefa, T., Amenga-Etego, L., Bargul, J. L., and Golassa, L. (2021). Genomic Analysis Reveals Independent Evolution of *Plasmodium Falciparum* Populations in Ethiopia. *Malar. J.* 20, 129. doi: 10.1186/s12936-021-03660-y

Ackerman, C. M., Myhrvold, C., Thakku, S. G., Freije, C. A., Metsky, H. C., Yang, D. K., et al. (2020). Massively Multiplexed Nucleic Acid Detection With Cas13. *Nature* 582, 277–282. doi: 10.1038/s41586-020-2279-8

Ajibola, O., Diop, M. F., Ghansah, A., Amenga-Etego, L., Golassa, L., Apinjoh, T., et al. (2021). In Silico Characterisation of Putative *Plasmodium Falciparum* Vaccine Candidates in African Malaria Populations. *Sci. Rep.* 11, 16215. doi: 10.1038/s41598-021-95442-4

Amambua-Ngwa, A., Amenga-Etego, L., Kamau, E., Amato, R., Ghansah, A., Golassa, L., et al. (2019). Major Subpopulations of *Plasmodium Falciparum* in Sub-Saharan Africa. *Science* 365, 813–816. doi: 10.1126/science.aav5427

Amoah, L. E., Abukari, Z., Dawson-Amoah, M. E., Dieng, C. C., Lo, E., and Afrane, Y. A. (2021). Population Structure and Diversity of *Plasmodium Falciparum* in Children With Asymptomatic Malaria Living in Different Ecological Zones of Ghana. *BMC Infect. Dis.* 21, 439. doi: 10.1186/s12879-021-06120-9

Andolina, C., Rek, J. C., Briggs, J., Okoth, J., Musiime, A., Ramjith, J., et al. (2021). Sources of Persistent Malaria Transmission in a Setting With Effective Malaria Control in Eastern Uganda: A Longitudinal, Observational Cohort Study. *Lancet Infect. Dis.* 21, 1568–1578. doi: 10.1016/S1473-3099(21)00072-4

Apinjoh, T. O., Ouattara, A., Titanji, V. P. K., Djimde, A., and Amambua-Ngwa, A. (2019). Genetic Diversity and Drug Resistance Surveillance of *Plasmodium Falciparum* for Malaria Elimination: Is There an Ideal Tool for Resource-Limited Sub-Saharan Africa? *Malar. J.* 18, 217. doi: 10.1186/s12936-019-2844-5

Arizti-Sanz, J., Freije, C. A., Stanton, A. C., Petros, B. A., Boehm, C. K., Siddiqui, S., et al. (2020). Streamlined Inactivation, Amplification, and Cas13-Based Detection of SARS-CoV-2. *Nat. Commun.* 11, 5921. doi: 10.1038/s41467-020-19097-x

Assefa, S. A., Preston, M. D., Campino, S., Ocholla, H., Sutherland, C. J., and Clark, T. G. (2014). estMOI: Estimating Multiplicity of Infection Using Parasite Deep Sequencing Data. *Bioinformatics* 30, 1292–1294. doi: 10.1093/bioinformatics/btu005

Bakari, C., Jones, S., Subramaniam, G., Mandara, C. I., Chiduo, M. G., Rumisha, S., et al. (2020). Community-Based Surveys for *Plasmodium Falciparum Pfhrp2* and *Pfhrp3* Gene Deletions in Selected Regions of Mainland Tanzania. *Malar. J.* 19, 391. doi: 10.1186/s12936-020-03459-3

Baker, J., Ho, M.-F., Pelecanos, A., Gatton, M., Chen, N., Abdullah, S., et al. (2010). Global Sequence Variation in the Histidine-Rich Proteins 2 and 3 of *Plasmodium Falciparum*: Implications for the Performance of Malaria Rapid Diagnostic Tests. *Malar. J.* 9, 129. doi: 10.1186/1475-2875-9-129

Baker, J., McCarthy, J., Gatton, M., Kyle, D. E., Belizario, V., Luchavez, J., et al. (2005). Genetic Diversity of *Plasmodium Falciparum* Histidine-Rich Protein 2 (PfHRP2) and its Effect on the Performance of PfHRP2-Based Rapid Diagnostic Tests. *J. Infect. Dis.* 192, 870–877. doi: 10.1086/432010

Berhane, A., Anderson, K., Mihreteab, S., Gresty, K., Rogier, E., Mohamed, S., et al. (2018). Major Threat to Malaria Control Programs by *Plasmodium Falciparum* Lacking Histidine-Rich Protein 2, Eritrea. *Emerging Infect. Dis.* 24, 462–470. doi: 10.3201/eid2403.171723

Boonma, P., Christensen, P. R., Suwanarusk, R., Price, R. N., Russell, B., and Lek-Uthai, U. (2007). Comparison of Three Molecular Methods for the Detection and Speciation of *Plasmodium Vivax* and *Plasmodium Falciparum*. *Malar. J.* 6, 124. doi: 10.1186/1475-2875-6-124

Carlsson, A. M., Ngasala, B. E., Dahlström, S., Membi, C., Veiga, I. M., Rombo, L., et al. (2011). *Plasmodium Falciparum* Population Dynamics During the Early Phase of Anti-Malarial Drug Treatment in Tanzanian Children With Acute Uncomplicated Malaria. *Malar. J.* 10, 380. doi: 10.1186/1475-2875-10-380

Cerqueira, G. C., Cheeseman, I. H., Schaffner, S. F., Nair, S., McDew-White, M., Phyo, A. P., et al. (2017). Longitudinal Genomic Surveillance of *Plasmodium Falciparum* Malaria Parasites Reveals Complex Genomic Architecture of Emerging Artemisinin Resistance. *Genome Biol.* 18, 78. doi: 10.1186/s13059-017-1204-4

Chaumeau, V., Kajeechiwa, L., Fustec, B., Landier, J., Naw Nyo, S., Nay Hsel, S., et al. (2019). Contribution of Asymptomatic *Plasmodium* Infections to the Transmission of Malaria in Kayin State, Myanmar. *J. Infect. Dis.* 219, 1499–1509. doi: 10.1093/infdis/jiy686

Cheng, Q., Gatton, M. L., Barnwell, J., Chiodini, P., McCarthy, J., Bell, D., et al. (2014). *Plasmodium Falciparum* Parasites Lacking Histidine-Rich Protein 2 and 3: A Review and Recommendations for Accurate Reporting. *Malar. J.* 13, 283. doi: 10.1186/1475-2875-13-283

Cordray, M. S., and Richards-Kortum, R. R. (2012). Emerging Nucleic Acid-Based Tests for Point-of-Care Detection of Malaria. *Am. J. Trop. Med. Hyg.* 87, 223–230. doi: 10.4269/ajtmh.2012.11-0685

Cunningham, C. H., Hennelly, C. M., Lin, J. T., Ubalee, R., Boyce, R. M., Mulogo, E. M., et al. (2021). A Novel CRISPR-Based Malaria Diagnostic Capable of *Plasmodium* Detection, Species Differentiation, and Drug-Resistance Genotyping. *EBioMedicine* 68, 103415. doi: 10.1016/j.ebiom.2021.103415

Daniels, R. F., Schaffner, S. F., Wenger, E. A., Proctor, J. L., Chang, H.-H., Wong, W., et al. (2015). Modeling Malaria Genomics Reveals Transmission Decline and Rebound in Senegal. *Proc. Natl. Acad. Sci. U.S.A.* 112, 7067–7072. doi: 10.1073/pnas.1505691112

Deurenberg, R. H., Bathoorn, E., Chlebowicz, M. A., Couto, N., Ferdous, M., García-Cobos, S., et al. (2017). Application of Next Generation Sequencing in Clinical Microbiology and Infection Prevention. *J. Biotechnol.* 243, 16–24. doi: 10.1016/j.jbiotec.2016.12.022

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., et al. (2009). Artemisinin Resistance in *Plasmodium Falciparum* Malaria. *N. Engl. J. Med.* 361, 455–467. doi: 10.1056/NEJMoa0808859

Dwivedi, A., Khim, N., Reynes, C., Ravel, P., Ma, L., Tichit, M., et al. (2016). *Plasmodium Falciparum* Parasite Population Structure and Gene Flow Associated to Anti-Malarial Drugs Resistance in Cambodia. *Malar. J.* 15, 319. doi: 10.1186/s12936-016-1370-y

Escalante, A. A., Ferreira, M. U., Vinetz, J. M., Volkman, S. K., Cui, L., Gamboa, D., et al. (2015). Malaria Molecular Epidemiology: Lessons From the International Centers of Excellence for Malaria Research Network. *Am. J. Trop. Med. Hyg.* 93, 79–86. doi: 10.4269/ajtmh.15-0005

Fatumo, S. A., Adoga, M. P., Ojo, O. O., Oluwagbemi, O., Adeoye, T., Ewejobi, I., et al. (2014). Computational Biology and Bioinformatics in Nigeria. *PloS Comput. Biol.* 10, e1003516. doi: 10.1371/journal.pcbi.1003516

Feleke, S. M., Reichert, E. N., Mohammed, H., Brhane, B. G., Mekete, K., Mamo, H., et al. (2021). *Plasmodium Falciparum* is Evolving to Escape Malaria Rapid Diagnostic Tests in Ethiopia. *Nat. Microbiol.* 6, 1289–1299. doi: 10.1038/s41564-021-00962-4

Gamboa, D., Ho, M.-F., Bendezu, J., Torres, K., Chiodini, P. L., Barnwell, J. W., et al. (2010). A Large Proportion of *P. Falciparum* Isolates in the Amazon Region of Peru Lack *Pfhrp2* and *Pfhrp3*: Implications for Malaria Rapid Diagnostic Tests. *PloS One* 5, e8091. doi: 10.1371/journal.pone.0008091

Gendrot, M., Fawaz, R., Dormoi, J., Madamet, M., and Pradines, B. (2019). Genetic Diversity and Deletion of *Plasmodium Falciparum* Histidine-Rich Protein 2 and 3: A Threat to Diagnosis of *P. Falciparum* Malaria. *Clin. Microbiol. Infect.* 25, 580–585. doi: 10.1016/j.cmi.2018.09.009

Goh, B., Ching, K., Soares Magalhães, R. J., Ciocchetta, S., Edstein, M. D., Maciel-de-Freitas, R., et al. (2021). The Application of Spectroscopy Techniques for Diagnosis of Malaria Parasites and Arboviruses and Surveillance of Mosquito Vectors: A Systematic Review and Critical Appraisal of Evidence. *PloS Negl. Trop. Dis.* 15, e0009218. doi: 10.1371/journal.pntd.0009218

Golassa, L., Messele, A., Amambua-Ngwa, A., and Swedberg, G. (2020). High Prevalence and Extended Deletions in *Plasmodium Falciparum Hrp2/3* Genomic Loci in Ethiopia. *PloS One* 15, e0241807. doi: 10.1371/journal.pone.0241807

Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., and Zhang, F. (2018). Multiplexed and Portable Nucleic Acid Detection Platform With Cas13, Cas12a, and Csm6. *Science* 360, 439–444. doi: 10.1126/science.aaq0179

Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., et al. (2017). Nucleic Acid Detection With CRISPR-Cas13a/C2c2. *Science* 356, 438–442. doi: 10.1126/science.aam9321

Greenhouse, B., Myrick, A., Dokomajilar, C., Woo, J. M., Carlson, E. J., Rosenthal, P. J., et al. (2006). Validation of Microsatellite Markers for Use in Genotyping Polyclonal *Plasmodium Falciparum* Infections. *Am. J. Trop. Med. Hyg.* 75, 836–842. doi: 10.4269/ajtmh.2006.75.836

Guo, L., Sun, X., Wang, X., Liang, C., Jiang, H., Gao, Q., et al. (2020). SARS-CoV-2 Detection With CRISPR Diagnostics. *Cell Discovery* 6, 34. doi: 10.1038/s41421-020-0174-y

Haanshuus, C. G., Mørch, K., Blomberg, B., Strøm, G. E. A., Langeland, N., Hanevik, K., et al. (2019). Assessment of Malaria Real-Time PCR Methods and Application With Focus on Low-Level Parasitaemia. *PloS One* 14, e0218982. doi: 10.1371/journal.pone.0218982

Haldar, K., Bhattacharjee, S., and Safeukui, I. (2018). Drug Resistance in *Plasmodium*. *Nat. Rev. Microbiol.* 16, 156–170. doi: 10.1038/nrmicro.2017.161

Han, E.-T. (2013). Loop-Mediated Isothermal Amplification Test for the Molecular Diagnosis of Malaria. *Expert Rev. Mol. Diagn.* 13, 205–218. doi: 10.1586/erm.12.144

Happi, A. N., Ugwu, C. A., and Happi, C. T. (2021). Tracking the Emergence of New SARS-CoV-2 Variants in South Africa. *Nat. Med.* 27, 372–373. doi: 10.1038/s41591-021-01265-1

Hopkins, H., González, I. J., Polley, S. D., Angutoko, P., Ategeka, J., Asiimwe, C., et al. (2013). Highly Sensitive Detection of Malaria Parasitemia in a Malaria-Endemic Setting: Performance of a New Loop-Mediated Isothermal Amplification Kit in a Remote Clinic in Uganda. *J. Infect. Dis.* 208, 645–652. doi: 10.1093/infdis/jit184

Imwong, M., Hanchana, S., Malleret, B., Rénia, L., Day, N. P. J., Dondorp, A., et al. (2014). High-Throughput Ultrasensitive Molecular Techniques for Quantifying Low-Density Malaria Parasitemias. *J. Clin. Microbiol.* 52, 3303–3309. doi: 10.1128/JCM.01057-14

Ippolito, M. M., Moser, K. A., Kabuya, J.-B. B., Cunningham, C., and Juliano, J. J. (2021). Antimalarial Drug Resistance and Implications for the WHO Global Technical Strategy. *Curr. Epidemiol. Rep.* 8, 46–62. doi: 10.1007/s40471-021-00266-5

Ishengoma, D. S., Saidi, Q., Sibley, C. H., Roper, C., and Alifrangis, M. (2019). Deployment and Utilization of Next-Generation Sequencing of *Plasmodium Falciparum* to Guide Anti-Malarial Drug Policy Decisions in Sub-Saharan Africa: Opportunities and Challenges. *Malar. J.* 18, 267. doi: 10.1186/s12936-019-2853-4

Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L. A. (2013). RNA-Guided Editing of Bacterial Genomes Using CRISPR-Cas Systems. *Nat. Biotechnol.* 31, 233–239. doi: 10.1038/nbt.2508

Joung, J., Ladha, A., Saito, M., Kim, N.-G., Woolley, A. E., Segel, M., et al. (2020). Detection of SARS-CoV-2 With SHERLOCK One-Pot Testing. *N. Engl. J. Med.* 383, 1492–1494. doi: 10.1056/NEJMc2026172

Juliano, J. J., Ariey, F., Sem, R., Tangpukdee, N., Krudsood, S., Olson, C., et al. (2009). Misclassification of Drug Failure in *Plasmodium Falciparum* Clinical Trials in Southeast Asia. *J. Infect. Dis.* 200, 624–628. doi: 10.1086/600892

Juliano, J. J., Porter, K., Mwapasa, V., Sem, R., Rogers, W. O., Ariey, F., et al. (2010). Exposing Malaria in-Host Diversity and Estimating Population Diversity by Capture-Recapture Using Massively Parallel Pyrosequencing. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20138–20143. doi: 10.1073/pnas.1007068107

Kamau, E., Alemayehu, S., Feghali, K. C., Saunders, D., and Ockenhouse, C. F. (2013). Multiplex qPCR for Detection and Absolute Quantification of Malaria. *PloS One* 8, e71539. doi: 10.1371/journal.pone.0071539

Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F., and Collins, J. J. (2021). CRISPR-Based Diagnostics. *Nat. Biomed. Eng.* 5, 643–656. doi: 10.1038/s41551-021-00760-7

Karikari, T. K. (2015). Bioinformatics in Africa: The Rise of Ghana? *PloS Comput. Biol.* 11, e1004308. doi: 10.1371/journal.pcbi.1004308

Kasetsirikul, S., Buranapong, J., Srituravanich, W., Kaewthamasorn, M., and Pimpin, A. (2016). The Development of Malaria Diagnostic Techniques: A Review of the Approaches With Focus on Dielectrophoretic and Magnetophoretic Methods. *Malar. J.* 15, 358. doi: 10.1186/s12936-016-1400-9

Kellner, M. J., Koob, J. G., Gootenberg, J. S., Abudayyeh, O. O., and Zhang, F. (2019). SHERLOCK: Nucleic Acid Detection With CRISPR Nucleases. *Nat. Protoc.* 14, 2986–3012. doi: 10.1038/s41596-019-0210-2

Kisinza, W. N., Nkya, T. E., Kabula, B., Overgaard, H. J., Massue, D. J., Mageni, Z., et al. (2017). Multiple Insecticide Resistance in *Anopheles Gambiae* From Tanzania: A Major Concern for Malaria Vector Control. *Malar. J.* 16, 439. doi: 10.1186/s12936-017-2087-2

Kostyusheva, A., Brezgin, S., Babin, Y., Vasilyeva, I., Glebe, D., Kostyushev, D., et al (2021). CRISPR-Cas Systems for Diagnosing Infectious Diseases. *Methods.* 203, 431–446. doi: 10.1016/j.ymeth.2021.04.007

Kwiatkowski, D. (2015). Malaria Genomics: Tracking a Diverse and Evolving Parasite Population. *Int. Health* 7, 82–84. doi: 10.1093/inthealth/ihv007

Lalremruata, A., Jeyaraj, S., Engleitner, T., Joanny, F., Lang, A., Bélard, S., et al. (2017). Species and Genotype Diversity of *Plasmodium* in Malaria Patients From Gabon Analysed by Next Generation Sequencing. *Malar. J.* 16, 398. doi: 10.1186/s12936-017-2044-0

Leang, R., Taylor, W. R. J., Bouth, D. M., Song, L., Tarning, J., Char, M. C., et al. (2015). Evidence of *Plasmodium Falciparum* Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment. *Antimicrob. Agents Chemother.* 59, 4719–4726. doi: 10.1128/AAC.00835-15

Lee, R. A., Puig, H. D., Nguyen, P. Q., Angenent-Mari, N. M., Donghia, N. M., McGee, J. P., et al. (2020). Ultrasensitive CRISPR-Based Diagnostic for Field-Applicable Detection of *Plasmodium* Species in Symptomatic and Asymptomatic Malaria. *Proc. Natl. Acad. Sci. U.S.A.* 117, 25722–25731. doi: 10.1073/pnas.2010196117

Lerch, A., Koepfli, C., Hofmann, N. E., Messerli, C., Wilcox, S., Kattenberg, J. H., et al. (2017). Development of Amplicon Deep Sequencing Markers and Data Analysis Pipeline for Genotyping Multi-Clonal Malaria Infections. *BMC Genomics* 18, 864. doi: 10.1186/s12864-017-4260-y

Levitt, B., Obala, A., Langdon, S., Corcoran, D., O'Meara, W. P., and Taylor, S. M. (2017). Overlap Extension Barcoding for the Next Generation Sequencing and Genotyping of *Plasmodium Falciparum* in Individual Patients in Western Kenya. *Sci. Rep.* 7, 41108. doi: 10.1038/srep41108

Li, P., Xing, H., Zhao, Z., Yang, Z., Cao, Y., Li, W., et al. (2015). Genetic Diversity of *Plasmodium Falciparum* Histidine-Rich Protein 2 in the China-Myanmar Border Area. *Acta Trop.* 152, 26–31. doi: 10.1016/j.actatropica.2015.08.003

Lucchi, N. W., Oberstaller, J., Kissinger, J. C., and Udhayakumar, V. (2013). Malaria Diagnostics and Surveillance in the Post-Genomic Era. *Public Health Genomics* 16, 37–43. doi: 10.1159/000345607

Luchavez, J., Baker, J., Alcantara, S., Belizario, V., Cheng, Q., McCarthy, J. S., et al. (2011). Laboratory Demonstration of a Prozone-Like Effect in HRP2-Detecting Malaria Rapid Diagnostic Tests: Implications for Clinical Management. *Malar. J.* 10, 286. doi: 10.1186/1475-2875-10-286

MalariaGEN, Ahouidi, A., Ali, M., Almagro-Garcia, J., Amambua-Ngwa, A., Amaratunga, C., et al. (2021). An Open Dataset of *Plasmodium Falciparum* Genome Variation in 7,000 Worldwide Samples. *Wellcome Open Res.* 6, 42. doi: 10.12688/wellcomeopenres.16168.2

Malvy, D., Torrentino-Madamet, M., L'Ollivier, C., Receveur, M.-C., Jeddi, F., Delhaes, L., et al. (2018). *Plasmodium Falciparum* Recrudescence Two Years After Treatment of an Uncomplicated Infection Without Return to an Area Where Malaria Is Endemic. *Antimicrob. Agents Chemother.* 62, e01892-17. doi: 10.1128/AAC.01892-17

Manske, M., Miotto, O., Campino, S., Auburn, S., Almagro-Garcia, J., Maslen, G., et al. (2012). Analysis of *Plasmodium Falciparum* Diversity in Natural Infections by Deep Sequencing. *Nature* 487, 375–379. doi: 10.1038/nature11174

Matiya, D. J., Philbert, A. B., Kidima, W., and Matowo, J. J. (2019). Dynamics and Monitoring of Insecticide Resistance in Malaria Vectors Across Mainland Tanzania From 1997 to 2017: A Systematic Review. *Malar. J.* 18, 102. doi: 10.1186/s12936-019-2738-6

Mayengue, P. I., Luty, A. J. F., Rogier, C., Baragatti, M., Kremsner, P. G., and Ntoumi, F. (2009). The Multiplicity of *Plasmodium Falciparum* Infections is Associated With Acquired Immunity to Asexual Blood Stage Antigens. *Microbes Infect.* 11, 108–114. doi: 10.1016/j.micinf.2008.10.012

Mbanefo, A., and Kumar, N. (2020). Evaluation of Malaria Diagnostic Methods as a Key for Successful Control and Elimination Programs. *Trop. Med. Infect. Dis.* 5 (2), 102. doi: 10.3390/tropicalmed5020102

Metoh, T. N., Chen, J.-H., Fon-Gah, P., Zhou, X., Moyou-Somo, R., and Zhou, X.-N. (2020). Genetic Diversity of *Plasmodium Falciparum* and Genetic Profile in Children Affected by Uncomplicated Malaria in Cameroon. *Malar. J.* 19, 115. doi: 10.1186/s12936-020-03161-4

Miles, A., Iqbal, Z., Vauterin, P., Pearson, R., Campino, S., Theron, M., et al. (2016). Indels, Structural Variation, and Recombination Drive Genomic Diversity in *Plasmodium Falciparum*. *Genome Res.* 26, 1288–1299. doi: 10.1101/gr.203711.115

Mitchell, C. L., Janko, M. M., Mwandagalirwa, M. K., Tshefu, A. K., Edwards, J. K., Pence, B. W., et al. (2022). Impact of Extractive Industries on Malaria Prevalence in the Democratic Republic of the Congo: A Population-Based Cross-Sectional Study. *Sci. Rep.* 12, 1737. doi: 10.1038/s41598-022-05777-9

Mohd Abd Razak, M. R., Sastu, U. R., Norahmad, N. A., Abdul-Karim, A., Muhammad, A., Muniandy, P. K., et al. (2016). Genetic Diversity of *Plasmodium Falciparum* Populations in Malaria Declining Areas of Sabah, East Malaysia. *PloS One* 11, e0152415. doi: 10.1371/journal.pone.0152415

Morgan, A. P., Brazeau, N. F., Ngasala, B., Mhamilawa, L. E., Denton, M., Msellem, M., et al. (2020). Falciparum Malaria From Coastal Tanzania and Zanzibar Remains Highly Connected Despite Effective Control Efforts on the Archipelago. *Malar. J.* 19, 47. doi: 10.1186/s12936-020-3137-8

Moser, K. A., Madebe, R. A., Aydemir, O., Chiduo, M. G., Mandara, C. I., Rumisha, S. F., et al. (2021). Describing the Current Status of *Plasmodium Falciparum* Population Structure and Drug Resistance Within Mainland Tanzania Using Molecular Inversion Probes. *Mol. Ecol.* 30, 100–113. doi: 10.1111/mec.15706

Murray, C. K., Gasser, R. A., Magill, A. J., and Miller, R. S. (2008). Update on Rapid Diagnostic Testing for Malaria. *Clin. Microbiol. Rev.* 21, 97–110. doi: 10.1128/CMR.00035-07

Nag, S., Dalgaard, M. D., Kofoed, P.-E., Ursing, J., Crespo, M., Andersen, L. O., et al. (2017). High Throughput Resistance Profiling of *Plasmodium Falciparum* Infections Based on Custom Dual Indexing and Illumina Next Generation Sequencing-Technology. *Sci. Rep.* 7, 2398. doi: 10.1038/s41598-017-02724-x

Nair, S., Nkhoma, S. C., Serre, D., Zimmerman, P. A., Gorena, K., Daniel, B. J., et al. (2014). Single-Cell Genomics for Dissection of Complex Malaria Infections. *Genome Res.* 24, 1028–1038. doi: 10.1101/gr.168286.113

Neafsey, D. E., Taylor, A. R., and MacInnis, B. L. (2021). Advances and Opportunities in Malaria Population Genomics. *Nat. Rev. Genet.* 22, 502–517. doi: 10.1038/s41576-021-00349-5

Neafsey, D. E., and Volkman, S. K. (2017). Malaria Genomics in the Era of Eradication. *Cold Spring Harb. Perspect. Med.* 7, a025544. doi: 10.1101/cshperspect.a025544

Ngondi, J. M., Ishengoma, D. S., Doctor, S. M., Thwai, K. L., Keeler, C., Mkude, S., et al. (2017). Surveillance for Sulfadoxine-Pyrimethamine Resistant Malaria Parasites in the Lake and Southern Zones, Tanzania, Using Pooling and Next-Generation Sequencing. *Malar. J.* 16, 236. doi: 10.1186/s12936-017-1886-9

Nguyen, L. T., Macaluso, N. C., Pizzano, B. L. M., Cash, M. N., Spacek, J., Karasek, J., et al. (2021). A Thermostable Cas12b From *Brevibacillus* Leverages One-Pot Detection of SARS-CoV-2 Variants of Concern. *eBioMedicine*, 77,103926. doi: 10.1016/j.ebiom.2022.103926

Nkhoma, S. C., Banda, R. L., Khoswe, S., Dzoole-Mwale, T. J., and Ward, S. A. (2018). Intra-Host Dynamics of Co-Infecting Parasite Genotypes in Asymptomatic Malaria Patients. *Infect. Genet. Evol.* 65, 414–424. doi: 10.1016/j.meegid.2018.08.018

Nsanzabana, C. (2019). Strengthening Surveillance Systems for Malaria Elimination by Integrating Molecular and Genomic Data. *Trop. Med. Infect. Dis.* 4 (4), 139. doi: 10.3390/tropicalmed4040139

Nsobya, S. L., Walakira, A., Namirembe, E., Kiggundu, M., Nankabirwa, J. I., Ruhamyankaka, E., et al. (2021). Deletions of *Pfhrp2* and *Pfhrp3* Genes Were Uncommon in Rapid Diagnostic Test-Negative *Plasmodium Falciparum* Isolates From Uganda. *Malar. J.* 20, 4. doi: 10.1186/s12936-020-03547-4

Onyango, S. A., Ochwedo, K. O., Machani, M. G., Omondi, C. J., Debrah, I., Ogolla, S. O., et al. (2021). Genetic Diversity and Population Structure of the Human Malaria Parasite *Plasmodium Falciparum* Surface Protein Pfs47 in Isolates From the Lowlands in Western Kenya. *PloS One* 16, e0260434. doi: 10.1371/journal.pone.0260434

Pardee, K., Green, A. A., Takahashi, M. K., Braff, D., Lambert, G., Lee, J. W., et al. (2016). Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* 165, 1255–1266. doi: 10.1016/j.cell.2016.04.059

Parr, J. B., Kieto, E., Phanzu, F., Mansiangi, P., Mwandagalirwa, K., Mvuama, N., et al. (2021). Analysis of False-Negative Rapid Diagnostic Tests for Symptomatic Malaria in the Democratic Republic of the Congo. *Sci. Rep.* 11, 6495. doi: 10.1038/s41598-021-85913-z

Pham, N. M., Karlen, W., Beck, H.-P., and Delamarche, E. (2018). Malaria and the "Last" Parasite: How can Technology Help? *Malar. J.* 17, 260. doi: 10.1186/s12936-018-2408-0

Rao, P. N., Uplekar, S., Kayal, S., Mallick, P. K., Bandyopadhyay, N., Kale, S., et al. (2016). A Method for Amplicon Deep Sequencing of Drug Resistance Genes in *Plasmodium Falciparum* Clinical Isolates From India. *J. Clin. Microbiol.* 54, 1500–1511. doi: 10.1128/JCM.00235-16

Rohlin, A., Wernersson, J., Engwall, Y., Wiklund, L., Björk, J., and Nordling, M. (2009). Parallel Sequencing Used in Detection of Mosaic Mutations: Comparison With Four Diagnostic DNA Screening Techniques. *Hum. Mutat.* 30, 1012–1020. doi: 10.1002/humu.20980

Roper, C., Pearce, R., Nair, S., Sharp, B., Nosten, F., and Anderson, T. (2004). Intercontinental Spread of Pyrimethamine-Resistant Malaria. *Science* 305 (5687), 1124. doi: 10.1126/science.1098876

Shaukat, A. M., Gilliams, E. A., Kenefic, L. J., Laurens, M. B., Dzinjalamala, F. K., Nyirenda, O. M., et al. (2012). Clinical Manifestations of New Versus Recrudescent Malaria Infections Following Anti-Malarial Drug Treatment. *Malar. J.* 11, 207. doi: 10.1186/1475-2875-11-207

She, R. C., Rawlins, M. L., Mohl, R., Perkins, S. L., Hill, H. R., and Litwin, C. M. (2007). Comparison of Immunofluorescence Antibody Testing and Two Enzyme Immunoassays in the Serologic Diagnosis of Malaria. *J. Travel Med.* 14, 105–111. doi: 10.1111/j.1708-8305.2006.00087.x

Slater, H. C., Ross, A., Felger, I., Hofmann, N. E., Robinson, L., Cook, J., et al. (2019). The Temporal Dynamics and Infectiousness of Subpatent *Plasmodium Falciparum* Infections in Relation to Parasite Density. *Nat. Commun.* 10, 1433. doi: 10.1038/s41467-019-09441-1

Strich, J. R., and Chertow, D. S. (2019). CRISPR-Cas Biology and Its Application to Infectious Diseases. *J. Clin. Microbiol.* 57, e01307-18. doi: 10.1128/JCM.01307-18

Su, X.-Z., Zhang, C., and Joy, D. A. (2020). Host-Malaria Parasite Interactions and Impacts on Mutual Evolution. *Front. Cell. Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.587933

Takala-Harrison, S., and Laufer, M. K. (2015). Antimalarial Drug Resistance in Africa: Key Lessons for the Future. *Ann. N. Y. Acad. Sci.* 1342, 62–67. doi: 10.1111/nyas.12766

Talundzic, E., Ravishankar, S., Kelley, J., Patel, D., Plucinski, M., Schmedes, S., et al. (2018). Next-Generation Sequencing and Bioinformatics Protocol for Malaria Drug Resistance Marker Surveillance. *Antimicrob. Agents Chemother.* 62, e02474-17. doi: 10.1128/AAC.02474-17

Tangpukdee, N., Duangdee, C., Wilairatana, P., and Krudsood, S. (2009). Malaria Diagnosis: A Brief Review. *Korean J. Parasitol.* 47, 93–102. doi: 10.3347/kjp.2009.47.2.93

Tawe, L., Menegon, M., Ramatlho, P., Muthoga, C. W., Mutukwa, N., Vurayai, M., et al. (2018). Molecular Surveillance of *Plasmodium Falciparum* Drug Resistance Markers in Clinical Samples From Botswana. *Am. J. Trop. Med. Hyg.* 99, 1499–1503. doi: 10.4269/ajtmh.18-0440

Tegally, H., Wilkinson, E., Lessells, R. J., Giandhari, J., Pillay, S., Msomi, N., et al. (2021). Sixteen Novel Lineages of SARS-CoV-2 in South Africa. *Nat. Med.* 27, 440–446. doi: 10.1038/s41591-021-01255-3

Tessema, S. K., Inzaule, S. C., Christoffels, A., Kebede, Y., de Oliveira, T., Ouma, A. E. O., et al. (2020). Accelerating Genomics-Based Surveillance for COVID-19 Response in Africa. *Lancet Microbe* 1, e227–e228. doi: 10.1016/S2666-5247(20)30117-8

Tessema, S. K., Raman, J., Duffy, C. W., Ishengoma, D. S., Amambua-Ngwa, A., and Greenhouse, B. (2019b). Applying Next-Generation Sequencing to Track *Falciparum* Malaria in Sub-Saharan Africa. *Malar. J.* 18, 268. doi: 10.1186/s12936-019-2880-1

Tessema, S., Wesolowski, A., Chen, A., Murphy, M., Wilheim, J., Mupiri, A.-R., et al. (2019a). Using Parasite Genetic and Human Mobility Data to Infer Local and Cross-Border Malaria Connectivity in Southern Africa. *eLife* 8, e43510. doi: 10.7554/eLife.43510

Thomson, R., Beshir, K. B., Cunningham, J., Baiden, F., Bharmal, J., Bruxvoort, K. J., et al. (2019). *Pfhrp2* and *Pfhrp3* Gene Deletions That Affect Malaria Rapid Diagnostic Tests for *Plasmodium Falciparum*: Analysis of Archived Blood Samples From 3 African Countries. *J. Infect. Dis.* 220, 1444–1452. doi: 10.1093/infdis/jiz335

Tusting, L. S., Bousema, T., Smith, D. L., and Drakeley, C. (2014). Measuring Changes in *Plasmodium Falciparum* Transmission: Precision, Accuracy and Costs of Metrics. *Adv. Parasitol.* 84, 151–208. doi: 10.1016/B978-0-12-800099-1.00003-X

Upatham, E. S., Prasittisuk, C., Ratanatham, S., Green, C. A., Rojanasunan, W., Setakana, P., et al. (1988). Bionomics of *Anopheles Maculatus* Complex and Their Role in Malaria Transmission in Thailand. *Southeast Asian J. Trop. Med. Public Health* 19, 259–269.

Van Tyne, D., Park, D. J., Schaffner, S. F., Neafsey, D. E., Angelino, E., Cortese, J. F., et al. (2011). Identification and Functional Validation of the Novel Antimalarial Resistance Locus *PF10_0355* in *Plasmodium Falciparum*. *PloS Genet.* 7, e1001383. doi: 10.1371/journal.pgen.1001383

Viana, R., Moyo, S., Amoako, D. G., Tegally, H., Scheepers, C., Althaus, C. L., et al. (2022). Rapid Epidemic Expansion of the SARS-CoV-2 Omicron Variant in Southern Africa. *Nature* 603, 679–686. doi: 10.1038/s41586-022-04411-y

Watson, O. J., Okell, L. C., Hellewell, J., Slater, H. C., Unwin, H. J. T., Omedo, I., et al. (2021). Evaluating the Performance of Malaria Genetics for Inferring Changes in Transmission Intensity Using Transmission Modeling. *Mol. Biol. Evol.* 38, 274–289. doi: 10.1093/molbev/msaa225

Weiss, D. J., Lucas, T. C. D., Nguyen, M., Nandi, A. K., Bisanzio, D., Battle, K. E., et al. (2019). Mapping the Global Prevalence, Incidence, and Mortality of Plasmodium Falciparum 2000-17: A Spatial and Temporal Modelling Study. *Lancet*. 394 (10195), 322–331. doi: 10.1016/S0140-6736(19)31097-9

World Health Organization (2007) *Methods and Techniques for Clinical Trials on Antimalarial Drug Efficacy: Genotyping to Identify Parasite Populations* (World Health Organization). Available at: http://www.who.int/malaria/publications/atoz/9789241596305/en/ (Accessed October 8, 2020).

World Health Organization (2009). *Methods for Surveillance of Antimalarial Drug Efficacy* (Geneva: World Health Organization).

World Health Organization (2019). *Response Plan to Pfhrp2 Gene Deletions* (World Health Organization).

World Health Organization (2020). *Master Protocol for Surveillance of Pfhrp2/3 Deletions and Biobanking to Support Future Research* (World Health Organization).

World Health Organization (2021). *World Malaria Report 2021* (Geneva: World Health Organization).

Wurtz, N., Fall, B., Bui, K., Pascual, A., Fall, M., Camara, C., et al. (2013). *Pfhrp2* and *Pfhrp3* Polymorphisms in *Plasmodium Falciparum* Isolates From Dakar, Senegal: Impact on Rapid Malaria Diagnostic Tests. *Malar. J.* 12, 34. doi: 10.1186/1475-2875-12-34

Yavo, W., Konaté, A., Mawili-Mboumba, D. P., Kassi, F. K., Tshibola Mbuyi, M. L., Angora, E. K., et al. (2016). Genetic Polymorphism of *Msp1* and *Msp2* in *Plasmodium Falciparum* Isolates From Côte D'Ivoire Versus Gabon. *J. Parasitol. Res.* 2016, 3074803. doi: 10.1155/2016/3074803

Yin, H., Xue, W., and Anderson, D. G. (2019). CRISPR-Cas: A Tool for Cancer Research and Therapeutics. *Nat. Rev. Clin. Oncol.* 16, 281–295. doi: 10.1038/s41571-019-0166-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer DN declared a shared affiliation with the author BM to the handling editor at the time of review.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Lyimo, Popkin-Hall, Giesbrecht, Mandara, Madebe, Bakari, Pereus, Seth, Ngamba, Mbwambo, MacInnis, Mbwambo, Garimo, Chacky, Aaron, Lusasi, Molteni, Njau, Cunningham, Lazaro, Mohamed, Juliano, Bailey and Ishengoma. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Frontiers in Medicine

Translating medical research and innovation into improved patient care

A multidisciplinary journal which advances our medical knowledge. It supports the translation of scientific advances into new therapies and diagnostic tools that will improve patient care.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact