frontiers | Research Topics

"One health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II

Edited by
Jean-Marc Rolain and Ziad Daoud

Published in
Frontiers in Microbiology

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 978-2-83251-747-5
DOI 10.3389/978-2-83251-747-5

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

"One health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II

Topic editors

Jean-Marc Rolain — INSERM U1095 Unité de Recherche sur les Maladies infectieuses et Tropicales Emergentes, France

Ziad Daoud — Central Michigan University, United States

Citation

Rolain, J.-M., Daoud, Z., eds. (2023). *"One health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-747-5

Table of contents

06 Editorial: "One Health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II
Ziad Daoud and Jean-Marc Rolain

10 High Prevalence of Antimicrobial Resistance and Integron Gene Cassettes in Multi-Drug-Resistant *Klebsiella pneumoniae* Isolates From Captive Giant Pandas (*Ailuropoda melanoleuca*)
Xia Yan, Xiaoyan Su, Zhihua Ren, Xueyang Fan, Yunli Li, Chanjuan Yue, Mei Yang, Huidan Deng, Youtian Deng, Zhiwen Xu, Dongsheng Zhang, Lin Li, Rong Hou, Songrui Liu and Junliang Deng

22 Population Diversity of Antibiotic Resistant *Enterobacterales* in Samples From Wildlife Origin in Senegal: Identification of a Multidrug Resistance Transposon Carrying $bla_{CTX-M-15}$ in *Escherichia coli*
Rim Abdallah, Edmond Kuete Yimagou, Linda Hadjadj, Oleg Mediannikov, Ahmad Ibrahim, Bernard Davoust, Amanda Barciela, R. Adriana Hernandez-Aguilar, Georges Diatta, Cheikh Sokhna, Didier Raoult, Jean-Marc Rolain and Sophie Alexandra Baron

34 Molecular Epidemiology and Colistin-Resistant Mechanism of *mcr*-Positive and *mcr*-Negative *Escherichia coli* Isolated From Animal in Sichuan Province, China
Fulei Li, Ping Cheng, Xiaoting Li, Ruimeng Liu, Haibin Liu and Xiuying Zhang

45 The Dynamics of the Antimicrobial Resistance Mobilome of *Salmonella enterica* and Related Enteric Bacteria
Suad Algarni, Steven C. Ricke, Steven L. Foley and Jing Han

64 Identifying the Sources of Intestinal Colonization With Extended-Spectrum β-Lactamase-Producing *Escherichia coli* in Healthy Infants in the Community
Mohammed Badrul Amin, Kazi Injamamul Hoque, Subarna Roy, Sumita Rani Saha, Md. Rayhanul Islam, Timothy R. Julian and Mohammad Aminul Islam

76 Wild Boars as an Indicator of Environmental Spread of ESβL-Producing *Escherichia coli*
Alessandra Mercato, Claudia Cortimiglia, Aseel Abualsha'ar, Aurora Piazza, Federica Marchesini, Giovanni Milani, Silvia Bonardi, Pier Sandro Cocconcelli and Roberta Migliavacca

86 A 6-Year Update on the Diversity of Methicillin-Resistant *Staphylococcus aureus* Clones in Africa: A Systematic Review
Opeyemi Uwangbaoje Lawal, Olaniyi Ayobami, Alaa Abouelfetouh, Nadira Mourabit, Mamadou Kaba, Beverly Egyir, Shima M. Abdulgader and Adebayo Osagie Shittu

103 High Prevalence of *bla*$_{CTXM-1}$/IncI1-Iγ/ST3 Plasmids in Extended-Spectrum β-Lactamase-Producing *Escherichia coli* Isolates Collected From Domestic Animals in Guadeloupe (French West Indies)
Gaëlle Gruel, David Couvin, Stéphanie Guyomard-Rabenirina, Guillaume Arlet, Jean-Christophe Bambou, Matthieu Pot, Xavier Roy, Antoine Talarmin, Benoit Tressieres, Séverine Ferdinand and Sébastien Breurec

114 High-Frequency Detection of *fosA3* and *bla*$_{CTX-M-55}$ Genes in *Escherichia coli* From Longitudinal Monitoring in Broiler Chicken Farms
Maísa Fabiana Menck-Costa, Ana Angelita Sampaio Baptista, Luiz Eduardo de Souza Gazal, Larissa Justino, Matheus Silva Sanches, Marielen de Souza, Erick Kenji Nishio, Beatriz Queiroz dos Santos, Victor Dellevedove Cruz, João Vitor Monteiro Berbert, Bruna Carolina Gonçalves, Galdino Andrade, Eliana Carolina Vespero, Gerson Nakazato and Renata Katsuko Takayama Kobayashi

127 Assessment of Antibacterial and Anti-biofilm Effects of Vitamin C Against *Pseudomonas aeruginosa* Clinical Isolates
Wedad M. Abdelraheem, Marwa M. M. Refaie, Rehab Kamal Mohamed Yousef, Aliaa S. Abd El Fatah, Yosra M. Mousa and Rabab Rashwan

136 Genomic Analysis of a Highly Virulent NDM-1-Producing *Escherichia coli* ST162 Infecting a Pygmy Sperm Whale (*Kogia breviceps*) in South America
Fábio P. Sellera, Brenda Cardoso, Danny Fuentes-Castillo, Fernanda Esposito, Elder Sano, Herrison Fontana, Bruna Fuga, Daphne W. Goldberg, Lourdes A. V. Seabra, Marzia Antonelli, Sandro Sandri, Cristiane K. M. Kolesnikovas and Nilton Lincopan

146 Prevalence of Livestock-Associated MRSA ST398 in a Swine Slaughterhouse in Guangzhou, China
Xiaoshen Li, Longfei Xie, Honghao Huang, Zhi Li, Guihua Li, Peng Liu, Danyu Xiao, Xucai Zhang, Wenguang Xiong and Zhenling Zeng

158 Genomic Characterization of *Enterococcus hirae* From Beef Cattle Feedlots and Associated Environmental Continuum
Sani-e-Zehra Zaidi, Rahat Zaheer, Ruth Barbieri, Shaun R. Cook, Sherry J. Hannon, Calvin W. Booker, Deirdre Church, Gary Van Domselaar, Athanasios Zovoilis and Tim A. McAllister

172 Resistome Analysis of Global Livestock and Soil Microbiomes
Katie Lawther, Fernanda Godoy Santos, Linda Boniface Oyama, Francesco Rubino, Steven Morrison, Chris J. Creevey, John W. McGrath and Sharon Ann Huws

185 Antimicrobial resistance surveillance of *Escherichia coli* from chickens in the Qinghai Plateau of China
Biao Tang, Jingge Wang, Xue Zheng, Jiang Chang, Jiangang Ma, Juan Wang, Xiaofeng Ji, Hua Yang and Baoan Ding

199 Within-farm dynamics of ESBL-producing *Escherichia coli* in dairy cattle: Resistance profiles and molecular characterization by long-read whole-genome sequencing
Maitane Tello, Medelin Ocejo, Beatriz Oporto, José Luis Lavín and Ana Hurtado

212 A clinical *Pseudomonas juntendi* strain with bla_{IMP-1} carried by an integrative and conjugative element in China
Lin Zheng, Xinfang Zhang, Lingwei Zhu, Gejin Lu, Jiayao Guan, Mingwei Liu, Jie Jing, Shiwen Sun, Ying Wang, Yang Sun, Xue Ji, Bowen Jiang, Jun Chen, Jun Liu, Ping Chen and Xuejun Guo

223 Mobile Tigecycline Resistance: An Emerging Health Catastrophe Requiring Urgent One Health Global Intervention
Madubuike Umunna Anyanwu, Obichukwu Chisom Nwobi, Charles Odilichukwu R. Okpala and Ifeoma M. Ezeonu

241 Dose-dependent impact of enrofloxacin on broiler chicken gut resistome is mitigated by synbiotic application
Robin Temmerman, Mahdi Ghanbari, Gunther Antonissen, Gerd Schatzmayr, Luc Duchateau, Freddy Haesebrouck, An Garmyn and Mathias Devreese

255 Distribution and genetic diversity of multi-drug-resistant *Klebsiella pneumoniae* at the human–animal–environment interface in Pakistan
Bilal Aslam, Tamoor Hamid Chaudhry, Muhammad Imran Arshad, Saima Muzammil, Abu Baker Siddique, Nafeesa Yasmeen, Mohsin Khurshid, Afreenish Amir, Muhammad Salman, Muhammad Hidayat Rasool, Xueshan Xia and Zulqarnain Baloch

266 Livestock-associated methicillin-resistant *Staphylococcus aureus* epidemiology, genetic diversity, and clinical characteristics in an urban region
Maria M. Konstantinovski, Leo M. Schouls, Sandra Witteveen, Eric C. J. Claas, Margriet E. Kraakman, Jayant Kalpoe, Eva Mattson, David J. Hetem, Erika P. M. van Elzakker, Jos Kerremans, Vishal Hira, Thijs Bosch and Jairo Gooskens

frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 06 March 2023
DOI 10.3389/fmicb.2023.1170407

Check for updates

OPEN ACCESS

EDITED AND REVIEWED BY
Rustam Aminov,
University of Aberdeen, United Kingdom

*CORRESPONDENCE
Ziad Daoud
✉ zdaoud@mihealthclinic.com

SPECIALTY SECTION
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

RECEIVED 20 February 2023
ACCEPTED 23 February 2023
PUBLISHED 06 March 2023

CITATION
Daoud Z and Rolain JM (2023) Editorial: "One Health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II. *Front. Microbiol.* 14:1170407. doi: 10.3389/fmicb.2023.1170407

COPYRIGHT
© 2023 Daoud and Rolain. 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.

Editorial: "One Health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II

Ziad Daoud[1,2]* and Jean-Marc Rolain[3]

[1]College of Medicine, Central Michigan University, Mount Pleasant, MI, United States, [2]Department of Clinical Microbiology & Infection Prevention, Michigan Health Clinics, Saginaw, MI, United States, [3]IHU Mediterranee Infection, Marseille, France

KEYWORDS

MDR, One Health (OH)—approach, economic impact, global change, bacterial resis

Editorial on the Research Topic
"One Health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II

One Health emphasizes the interdependence of human, animal, and environmental health, and highlights the need for collaboration and coordinated approaches to promote the health and wellbeing of all. Published literature collectively demonstrate the importance of a One Health approach in addressing complex global health challenges, including emerging infectious diseases, zoonotic disease control, climate change, and economic benefits (Behravesh, 2016; Cunningham et al., 2017).

This Research Topic, "*"One Health" approach for revealing reservoirs and transmission of antimicrobial resistance, volume II*," encompasses 21 manuscripts exploring antimicrobial resistance (AMR) from a One Health perspective, and investigating AMR prevalence and characteristics in different contexts, including pets, wildlife, and poultry production. The studies highlighted the potential role of animals in the transmission of AMR and emphasized the importance of surveillance and preventative measures to combat the spread of resistant bacteria. The findings underscored the interdependence of human, animal, and environmental health, and the need for a One Health approach to combat the global threat of AMR. These studies emphasized the importance of basic hygiene measures, preventative measures, and action to mitigate the spread of AMR.

In this volume II, several studies addressed the "One Health" issue in animal farms, highlighting the need for surveillance to address the public health importance of antimicrobial-resistant pathogens and their resistance mechanisms. These studies revealed the varying levels of resistant bacteria in livestock and emphasized the urgent need for AMR surveillance in various regions to identify primary risk points and the importance of monitoring antimicrobial resistance in poultry production.

A longitudinal study by Tello et al. was conducted on five dairy cattle farms to assess the dynamics of ESBL-/AmpC-/carbapenemase-producing *Escherichia coli* and their resistance profiles, along with genes responsible for resistance. Cefotaxime-resistant *E. coli* was detected in all farms, but their isolation frequency and resistance profiles varied among farms and age groups. Whole-genome sequencing of a selection of isolates recovered from two farms showed the predominance of a few genomic subtypes in one farm, while great variability of strains was observed in the other. The study highlighted the need for One Health surveillance

due to the public health importance of ESBL-producing *E. coli* as pathogens and vectors for resistance mechanisms. Sellera et al. found a carbapenem-resistant NDM-1-positive *E. coli* strain (BA01) in a stranded pygmy sperm whale on the southern coast of Brazil during COVID-19 lockdown. BA01 strain belonged to the global sequence type (ST) 162 and carried blaNDM-1, in addition to other antimicrobial resistance genes and genes associated with resistance to heavy metals, biocides, and glyphosate. The strain also exhibited halophilic behavior, and *in silico* analysis confirmed the presence of halotolerance-associated genes katE and nhaA. The phylogenomics analysis clustered BA01 with poultry- and human-associated ST162 lineages circulating in European and Asian countries, and important virulence genes were detected. A study from China by Xiaoshen Li, Xie, et al. reports that livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is present in high frequencies in samples from swine, with spa type t571 accounting for a higher proportion. MRSA ST398 strains may spread among swine, humans, and the environment, and there is potential transmission among different countries. The MRSA isolates showed multidrug-resistant phenotypes, and the poxtA-carrying segment and the coexistence of cfr and optrA in a plasmid were detected in MRSA ST398 for the first time. The study highlighted the urgent need for LA-MRSA surveillance, given the potential transmission among humans, animals, and the environment. Another study from China by Tang et al. looked into the prevalence of antimicrobial resistance (AMR) and the *mcr-1* gene in poultry-derived *E. coli* in Qinghai Plateau is investigated. Three hundred forty-six *E. coli* strains were isolated, with eight carrying *mcr-1*, and multidrug-resistant strains accounted for 95.66% of the total isolates. Colistin resistance was identified in 12 *E. coli* strains. Whole-genome sequencing revealed 46 AMR genes and 36 virulence factors. The *mcr-1* gene was located on various plasmids and its spread through the food chain is a concern. The study emphasized the need for strengthened AMR surveillance in various regions under the background of one health.

Temmerman et al. investigated the impact of fluoroquinolone antibiotics on the microbiome and resistome of broiler chickens receiving an optimized dose of enrofloxacin with or without synbiotic supplementation. Results showed that the optimized dose caused significant perturbations in the microbiota and increased the antibiotic resistance gene (ARG) resistome diversity. Symbiotic treatment reduced the diversity and number of enriched ARGs, suggesting a positive impact on the gut resistome. Proteobacteria were significantly increased in the cecal metagenome of chickens receiving enrofloxacin, and the optimized dosage application resulted in a two-fold higher number of affected ARG compared to high dosage application. These findings highlight the need to understand the impact of antibiotic use on the gut resistome of poultry and to develop strategies to mitigate potential resistance gene spread. In another study by Zaidi et al. study examined *Enterococcus hirae* isolates, primarily from beef production systems, for antimicrobial resistance and comparative genomics. The isolates showed high resistance to tetracycline and erythromycin, with multi-drug resistance present in over 50% of the beef production isolates. Genes for tetracycline and macrolide resistance were frequently found in these isolates. The resistance profiles appeared to reflect the kind of antimicrobial usage in beef and human sectors. Comparative genomic analysis showed *E. hirae* has unique genes associated with vitamin production and cellulose and pectin degradation, which may support its adaptation to the bovine digestive tract. *Enterococcus faecium* and *Enterococcus faecalis* more frequently harbored virulence genes, suggesting niche specificity within these species. Menck-Costa et al. monitored antimicrobial resistance in broiler chicken farms over a 2-year period in northern Paraná, Brazil, with a focus on evaluating resistance to fosfomycin and β-lactams. *Escherichia coli* was isolated from various sources, including cloacal swabs, meconium, poultry feed, water, poultry litter, and *Alphitobius diaperinus*. The results showed a high frequency of multidrug-resistant *E. coli*, with 51% of strains being ESBL-positive, and the *blaCTX-M-1* group being the most common. The fosA3 gene was also prevalent, particularly in day-old chickens. The study highlights the need for longitudinal monitoring of antimicrobial resistance in poultry production to identify primary risk points.

Other four studies in this volume explored the prevalence and characteristics of antibiotic-resistant bacteria in wildlife in different regions. The studies found high resistance rates to various antibiotics, including colistin and third-generation-cephalosporin, as well as the presence of new sequence types and mobile genetic elements carrying AMR genes. These studies highlight the potential role of wildlife in the transmission of AMR and emphasize the need for further research, effective surveillance, and strict biosecurity strategies to prevent the spread of antibiotic-resistant bacteria and monitor the emergence of mobile genetic elements and integrons. These findings underscore the importance of the One Health approach to address the issue of AMR, recognizing the interdependence of human, animal, and environmental health. Abdallah et al. investigated the prevalence and dynamics of antibiotic-resistant Enterobacterales in wildlife in antibiotic-limited areas of Senegal. Fecal samples were collected from monkeys and apes, and non-fecal samples were collected in 2015 and 2019. The study found a high resistance rate to colistin and third-generation-cephalosporin, and 46% of isolates belong to 34 new sequence types. The presence of a transposon that carries AMR genes is intriguing since no antibiotics are used in the non-human primates studied. The results highlight the potential role of wildlife in the transmission of AMR and the need for further research to better understand this risk. Mercato et al. investigated the prevalence and characteristics of Extended Spectrum Beta-Lactamases (ESβLs) producing *E. coli* strains in wild boars in Northern Italy. The analysis of 60 animals revealed a prevalence of 23.3% for ESβLs-producing *E. coli* strains, which were found to be multi-drug resistant and carried different types of plasmid replicons. Genome analysis showed the presence of two pandemic sequence types, ST131 and ST10, with different collections of virulence factors, and suggested the potential for AMR gene exchange. This highlights the importance of including wild boars in surveillance programs and understanding their role in the spread of AM resistance in the "One Health" framework. Li, Cheng, et al. investigated the mechanisms of chromosome-mediated and plasmid-mediated colistin resistance in *E. coli* isolates from animals in Sichuan Province, China. Out of 101 colistin-resistant isolates, the MCR gene was detected in 58 of them. The study found that the *MCR-1* gene was primarily localized on an *IncX4*-type and

IncI2 plasmid. Furthermore, colistin resistance in MCR-negative isolates was related to chromosomal point mutations including the two-component systems PhoP/PhoQ and PmrA/PmrB and their regulators MgrB. The study highlights the high prevalence of colistin resistance in farms in Sichuan, China, and the importance of monitoring and controlling the spread of antibiotic resistance. A study conducted by Yan et al. on giant pandas found a high prevalence of multi-drug-resistant (MDR) *Klebsiella pneumoniae* (*K. pneumoniae*) in their feces. Antimicrobial susceptibility testing results showed that 16.5% of *K. pneumoniae* isolates were MDR, mainly resistant to AMX, DOX, CHL, SXT, and TMP. The study also found 50 different types of ARGs and 13 mobile genetic elements. Furthermore, the investigation of integrons revealed the emergence of class 1 integrons in 96.67% of the isolates, while class 2 and class 3 integrons were not screened. The study suggests the need for effective surveillance and strict biosecurity strategies to prevent the spread of MDR bacteria and monitor the emergence of mobile genetic elements and integrons.

As per the wide spread of resistance genes, four articles covered different aspects of antimicrobial resistance (AMR) from a One Health perspective, including identifying drug resistance mechanisms, exploring the role of mobile genetic elements in spreading AMR genes, investigating potential treatments, and highlighting the threat of mobile tigecycline resistance. They emphasize the urgent need for action and One Health approaches to combat this global threat. The study by Zeng et al. aimed to identify the species of a carbapenem-resistant *Pseudomonas* strain and analyze its integrative and conjugative element formation mechanism. Single molecule real-time sequencing was used to identify the species as *Pseudomonas juntendi* and determine its drug resistance mechanism. The study found that the capture of the accA4' gene cassette by the Tn402-like type 1 integron (IntI1-blaIMP-1) forms In1886 before being captured by the ΔTn4662a-carrying ICE 1276, which confers carbapenem resistance to *P. juntendi* 18091276. In this same context, a review by Algarni et al. discussed the role of mobile genetic elements (MGEs) in the spread of antimicrobial resistance (AMR) genes in *Salmonella enterica* and related enteric bacteria. The mobilome, which includes all mobile genetic elements that can spread genes, played a key role in the rapid spread of AMR genes in *S. enterica*. The review highlights the importance of studying the dynamics of AMR genes in the mobilome for the identification and verification of emerging multidrug resistance. Abdelraheem et al. investigated the antibacterial and anti-biofilm effects of vitamin C (ascorbic acid) on *Pseudomonas aeruginosa*. The minimal inhibitory concentration of ascorbic acid and antibiotics was determined, and the effect of ascorbic acid on biofilm-forming isolates was assessed. Real-time PCR was used to test the effect of ascorbic acid on antibiotic-resistant and biofilm encoding genes. *In vivo*, rats were infected with *P. aeruginosa* and treated with ascorbic acid alone or combined with an antibiotic. Ascorbic acid had a 100% biofilm inhibitory effect at sub-inhibitory concentrations and downregulated genes related to biofilm formation and antibiotic resistance. *In vivo*, combining ascorbic acid and ceftazidime had a synergistic effect. The study suggests that vitamin C could be prescribed with antibiotics for the treatment of bacterial infections in clinical settings. The threat of mobile tigecycline resistance (MTR) to the clinical efficacy of the antibiotic tigecycline, which is used to treat deadly infections caused by superbugs was discussed by Anyanwu et al. MTR is caused by plasmid-mediated transmissible genes that confer high-level resistance to tigecycline. The article stressed the need for urgent global intervention to mitigate the spread of MTR, which has significant health and economic impacts due to limited options for therapy. The article covered the antimicrobial activity of tigecycline, mechanism of tigecycline resistance, distribution, reservoirs, and traits of MTR gene-harboring isolates, causes of MTR development, possible MTR gene transfer modes and One Health implications, and MTR spread and mitigating strategies.

Three studies revealed the potential role of pets as a reservoir of antimicrobial-resistant bacteria or genes for humans, the inter-human transmission of LA-MRSA, and the high prevalence of fecal colonization with ESBL-Ec among children <1-year-old in rural households in Bangladesh. The studies emphasized the importance of basic hygiene measures, surveillance, and preventative measures to combat the spread of antimicrobial resistance. Gruel et al. found that 7.6% of household and shelter pets carry extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E), with the only risk factor being a stay in a shelter. The study identified the presence of a *blaCTX-M-1*/IncI1-Iγ/sequence type (ST3) plasmid in 11 of the ESBL-producing *E. coli* isolates, and noted the potential role of pets as a reservoir of antimicrobial-resistant bacteria or genes for humans, highlighting the importance of basic hygiene measures by owners of companion animals. Similarly, Konstantinovski et al. assessed the genetic epidemiology and clinical characteristics of LA-MRSA in an urban area with limited livestock. LA-MRSA strains were cultured from 81 patients, and most patients had no livestock link. Contact tracing led to the identification of two hospital transmissions and five additional clusters without a known epidemiological link. The study confirms that LA-MRSA may cause a relevant burden of disease in urban areas, suggesting inter-human transmission. The presence of virulence genes warrants future surveillance and preventative measures. A study by Badrul Amin et al. conducted in rural households in Bangladesh found a high prevalence of fecal colonization with extended spectrum β-lactamase-producing *E. coli* (ESBL-Ec) among children <1-year-old. The study aimed to identify the sources of ESBL-Ec colonization in children by analyzing *E. coli* isolates from child stool, child's mother stool, and point-of-use drinking water. The study found that ESBL-Ec colonization in children is linked to the colonization status of mothers and exposure to household environments contaminated with ESBL-Ec. Interventions such as improved hygiene practices and safe drinking water supply may help reduce the transmission of ESBL-Ec at the household level.

The last three studies explore the issue of antimicrobial resistance (AMR) from a One Health perspective. The studies investigate the prevalence and mechanisms of AMR in different contexts, such as household pets, urban areas, rural households, and environmental sources. The study by Aslam et al. collected 775 samples from human, animal, and environmental sources and found *K. pneumoniae* in 15.7% of them, with the highest prevalence in humans. The isolates showed significant resistance to antibiotics, except colistin. The study identified various antibiotic resistance

genes, with the highest prevalence of *blaCTX-M*. Multi-locus sequence typing analysis revealed 21 distinct sequence types and 13 clonal complexes. The distribution of multi-drug resistant *K. pneumoniae* clones in the community and associated environment is alarming for public and animal health, requiring attention from health policymakers. Lawther et al. used the ResFinder database to identify resistance genes in over 5,800 metagenomes. Antimicrobial resistance genes were diverse and widespread, with tetracycline resistance genes being the most prevalent in livestock microbiomes. The oleandomycin resistance gene was the most abundant gene in soil. Fifty-five resistance genes were shared by the four microbiomes, with 11 actively expressed in two or more microbiomes. The study provides a global insight into the diverse and abundant antimicrobial resistance gene reservoirs in both livestock and soil microbiomes, posing a serious threat to public health. Finally, Lawal et al. conducted a systematic review to provide an update on the clonal distribution of MRSA in Africa. Genotyping data was based primarily on multilocus sequence types (STs) and Staphylococcal Cassette Chromosome mec (SCCmec) types. MRSA with diverse spa and SCCmec types in CC5 and CC8 were reported across the continent. The study identified an increase in the distribution of ST1, ST22, and ST152, but a decline of ST239/241 in Africa. There is a need to strengthen genomic surveillance capacity based on a "One-Health" strategy to prevent and control MRSA in Africa.

To conclude, many studies are currently looking into the antimicrobial resistance in the One Health context, this is providing more data knowledge. It is very important for future studies to look at Climate change and One Health and the economics of One Health to highlight the importance of this approach in addressing the interdependent health of humans, animals, and the environment (Machalaba et al., 2017; Zinsstag et al., 2018).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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.

References

Behravesh, C. B. (2016). One Health: people, animals, and the environment. *Emerg. Infect. Dis.* 22, 766–7. doi: 10.3201/eid2204.151887

Cunningham, A. A., Scoones, I., and Wood, J. L. N. (2017). One Health for a changing world: new perspectives from Africa. *Philos. Trans. R Soc. Lond. B Biol. Sci.* 372, 20160162. doi: 10.1098/rstb.2016.0162

Machalaba, C., Smith, K. M., Awada, L., Berry, K., Berthe, F., Bouley, T. A., et al. (2017). One Health economics to confront disease threats. *Trans. R Soc. Trop. Med. Hyg.* 111, 235–7. doi: 10.1093/trstmh/trx039

Zinsstag, J., Crump, L., Schelling, E., Hattendorf, J., Maidane, Y. O., Ali, K. O., et al. (2018). Climate change and One Health. *FEMS Microbiol. Lett.* 365, fny085. doi: 10.1093./femsle/fny085

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 February 2022
doi: 10.3389/fmicb.2021.801292

High Prevalence of Antimicrobial Resistance and Integron Gene Cassettes in Multi-Drug-Resistant *Klebsiella pneumoniae* Isolates From Captive Giant Pandas (*Ailuropoda melanoleuca*)

Xia Yan[1,2†], Xiaoyan Su[2†], Zhihua Ren[1†], Xueyang Fan[2], Yunli Li[2], Chanjuan Yue[2], Mei Yang[2], Huidan Deng[1], Youtian Deng[1], Zhiwen Xu[1], Dongsheng Zhang[2], Lin Li[2], Rong Hou[2], Songrui Liu[2] and Junliang Deng[1]**

[1] College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China, [2] Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, Chengdu Research Base of Giant Panda Breeding, Sichuan Academy of Giant Panda, Chenghua, China

OPEN ACCESS

Edited by:
Jean-Marc Rolain,
URMITE CNRS-IRD UMR 6236,
France

Reviewed by:
Justin Joseph Donato,
University of St. Thomas,
United States
Chengming Wang,
Auburn University, United States

***Correspondence:**
Songrui Liu
srui_liu@163.com
Junliang Deng
dengjl213@126.com

† These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 25 October 2021
Accepted: 29 December 2021
Published: 03 February 2022

Citation:
Yan X, Su X, Ren Z, Fan X, Li Y, Yue C, Yang M, Deng H, Deng Y, Xu Z, Zhang D, Li L, Hou R, Liu S and Deng J (2022) High Prevalence of Antimicrobial Resistance and Integron Gene Cassettes in Multi-Drug-Resistant Klebsiella pneumoniae Isolates From Captive Giant Pandas (Ailuropoda melanoleuca). Front. Microbiol. 12:801292. doi: 10.3389/fmicb.2021.801292

Multi-drug-resistant *Klebsiella pneumoniae* (MDR *K. pneumonia*) is increasingly being reported with corresponding increase in morbidity and mortality all over the world. However, limited information is available concerning MDR *K. pneumonia* in giant pandas. The objective of this study was to grasp the drug resistance profile of MDR *K. pneumonia* isolated from giant pandas. A total of 182 *K. pneumoniae* isolates were collected from fresh feces of 94 captive giant pandas of different ages and sex and separated by season. We performed a standard disk diffusion antimicrobial susceptibility test with the isolates and further evaluated the antibiotic resistance genes (ARGs) of multi-drug-resistant strains by high-throughput quantitative PCR. In addition, we then analyzed mobile genetic elements (MGEs), integron gene cassettes, and the multi-locus sequence typing of multi-drug-resistant strains by PCR. Antimicrobial susceptibility testing results demonstrated that a total of 30 (16.5%) *K. pneumoniae* isolates showed multiple drug resistances. The thirty MDR *K. pneumonia* isolates were mainly resistant to amoxicillin (100.0%), doxycycline (86.7%), chloramphenicol (60.0%), compound trimethoprim (60.0%) and trimethoprim (56.7%). Fifty different types of antibiotic resistance genes were found, which included a total of 671 antibiotic resistance genes, in the 30 multi-drug-resistant isolates. The top ten resistance genes were: vanTC-02, aacC, blaCTX-M-04, blaSHV-01, blaSHV-02, ampC-04, blaOXY, tetD, blaTEM and tetA-02. Thirteen mobile genetic elements were detected, of which IS26 (96.67%) and intI1 (96.67%) had the highest frequency. The thirty MDR K. pneumonia isolates were negative for the traA, traF, tnsA, IS1133, ISpa7, ISkpn6, intI2 and intI3 genes. Moreover, a further investigation of integrons revealed that two types of specific gene cassettes (dfrA12 + orfF + aadA2 and dfrA12 + orfF) were identified in class 1 integrons. Multi-locus sequence typing results showed that 22 STs in the thirty MDR

K. pneumonia isolates were identified, the main type was ST37 (5/30). Our results illustrate that effective surveillance and strict biosecurity strategies should be taken to prevent the spread of multi-drug-resistant bacteria, and monitor the emergence of mobile genetic elements and integrons.

Keywords: giant panda, MDR *K. pneumonia*, ARGS, MGEs, integron

INTRODUCTION

Klebsiella Pneumonia is a gram-negative *Enterobacteria*, and it is also an opportunistic pathogen responsible for an important proportion of nosocomial infections in human. Multi-drug-resistant *Klebsiella pneumoniae* (MDR *K. pneumonia*) was defined as being resistant to three or more antimicrobials (Dolejska et al., 2007; Magiorakos et al., 2012), and is increasingly being reported with corresponding increase in morbidity and mortality. MDR *K. pneumoniae* was more and more prevalent in the tertiary teaching hospital in western Kenya from 2002 to 2013 (Apondi et al., 2016), and MDR *K. pneumoniae* was an increasing cause of neonatal infections in India and other developing countries, with 60.86% of MDR *K. pneumoniae* infections leading to death (Jeena et al., 2001). Furthermore, researchers have detected MDR *K. pneumoniae* isolated from raw milk from dairy farms in Jiangsu and Shandong provinces in China recent years, which was a potential threat to food safety and public health (Yang et al., 2021).

Antimicrobial abuse is a serious risk for the emergence of MDR *K. pneumoniae*. In addition, horizontal gene transfer (HGT) *via* mobile genetic elements (MGEs) such as integrons, transposons, integrative conjugative elements, genomic islands, and plasmids are another important reason for the spread of antibiotic resistance genes (ARGs) carried by MDR *K. pneumonia* (Partridge et al., 2009; Chen et al., 2016). Integrons can capture, convert and adapt one or more resistance gene cassettes into functionally expressed genes *via* a self-efficient gene expression system. Meanwhile it can also simply transfer these genes between diverse bacterial species due to their linkage with plasmids (Gillings, 2014). Class 1, 2, and 3 integrons are the three main types of MGEs associated with antimicrobial resistance (Kaushik et al., 2018).

The giant panda (*Ailuropoda melanoleuca*) is one of the world's most recognized and rarest animals and is now only distributed in the mountainous areas of Sichuan, Gansu, and Shanxi provinces in China (Wang et al., 2018). Giant pandas infected with *K. pneumoniae* have developed hemorrhagic enteritis, and died from hemorrhagic sepsis and subsequent cases of genital hematuria, and sepsis. Infection of *K. pneumoniae* has increased within the captive population of giant pandas (Wang et al., 2006; Sun et al., 2013; Lu et al., 2015), generally causes mixed infection with other bacteria, and has become one of the most important pathogens of captive pandas (Peng and Xiong, 2000). According to our investigation, for the past several decades, antibiotics were widely applied to prevent and treat bacterial infectious diseases in giant pandas (Guo L. et al., 2015; Yang et al., 2017), which may have led to the emergence of antibiotic resistant bacteria (ARB) and even multi-drug-resistant (MDR) bacteria. Our previous studies have confirmed the prevalence of drug-resistance in *K. pneumoniae* in captive giant pandas (Su et al., 2021). However, MDR *K. pneumoniae* isolates from giant pandas have gained little attention and characterization so far. To the best of our knowledge, this is the first report showing the baseline characteristics of antimicrobial resistance and integron gene cassettes in *K. pneumoniae* isolates from captive giant pandas. The study aims to characterize the antimicrobial resistance of 182 *K. pneumoniae* isolates collected from the feces of giant pandas of different ages and sex separated by season, and to study the ARGs and integron gene cassettes and other MGEs in 30 MDR *K. pneumonia*, and further analyze the impact of age, sex and season on the antibiotic resistance of 30 MDR *K. pneumonia*. This study will help improve the clinical treatment and *ex situ* conservation of giant pandas.

MATERIALS AND METHODS

Samples

To investigate the prevalence of the antimicrobial resistance of *K. pneumoniae* isolates, 376 fresh fecal samples were collected from 94 captive giant pandas (female: $n = 47$, male: $n = 47$) housed at the Chengdu Research Base of Giant Panda Breeding (CRBGP) in Sichuan, China during 2020. Four fecal samples were collected from each individual giant panda during each season: spring (March to May), summer (June to August), autumn (September to November) and winter (December to February). All animals were considered healthy at the time and were managed under routine CRBGP husbandry practices. A total of 182 *K. pneumonia* isolates were identified from the fecal samples by Gram staining, 16s rDNA, and bacterial biochemical identification. Isolates were separated and analyzed by the sex of the panda (female samples: $n = 107$, male samples: $n = 75$), the season of sampling (spring: $n = 62$, summer: $n = 80$, autumn: $n = 29$ and winter: $n = 11$), and divided into three age groups [sub-adult (aged 1.5–5 years): $n = 67$, adult (aged 5–19 years): $n = 92$, and geriatric (aged 20 years or older): $n = 23$]. Confirmed *K. pneumonia* strains were stored in Luria-Bertani (LB) broth containing 20% glycerol at −80°C for further analyses.

Antimicrobial Susceptibility Test of the *Klebsiella pneumonia* Isolates

The susceptibilities of all isolates to 24 antimicrobials were tested using the standard disk diffusion method recommended by the (Clinical and Laboratory Standards Institute [CLSI], 2020). The following seven classes of antibiotics of antimicrobial

disks were used (Hangzhou Microbial Reagent Co., Ltd., China): β-lactams: piperacillin (PIC, 100 μg), amoxicillin (AML, 20 μg), cefotaxime (CTX, 30 μg), cefazolin (CEZ, 30 μg), ceftriaxone (CRO, 30 μg), cefuroxim (CXM, 30 μg), cefaclor (CEC, 30 μg), ampicillin/sulbactam 1:1 (SAM, 10/10 μg), aztreonam (AZM, 30 μg), meropenem (MEM, 10 μg), imipenem (IPM, 10 μg); Aminoglycosides: kanamycin (KAN, 30 μg), gentamicin (GM, 10 μg), streptomycin (STR, 10 μg); Quinolones: ofloxacin (OFX, 5 μg), norfloxacin (NOR, 10 μg), levofloxacin (LEV, 5 μg), ciprofloxacin (CIP, 5 μg); Chloramphenicol: chloramphenicol (CHL, 30 μg); Macrolides: azithromycin (AZM, 15 μg); Tetracyclines: doxycycline (DOX, 30 μg), minocycline (MH, 30 μg); Sulfonamides: compound trimethoprim (SXT, 23.75/1.25), trimethoprim (TMP, 5 μg). Results were interpreted in accordance with (Clinical and Laboratory Standards Institute [CLSI], 2020) criteria. *Escherichia coli* ATCC25922 was used as a control. All the antibiotics selected in this study were based on the information collected from CRBGP veterinarians and recent studies on the resistance of giant pandas (Zhang et al., 2009; Guo L. et al., 2015; Chen et al., 2018; Zou et al., 2018; Zhu et al., 2020) and antibiotics commonly resistant to *K. pneumonia* in clinics (Gao L. et al., 2020; Gao Q. et al., 2020). MDR was defined as being resistant to three or more classes of antibiotics (Magiorakos et al., 2012; Zhu et al., 2020).

TABLE 1 | Primers of MGEs used in the study.

Primers	Sequence (5′–3′)	Product (bp)	References
TraA	F:AAGTGTTCAGGGTGCTTCTGCGC R:GTCATGTACATGATGACCATTT	272	Zhu et al., 2020
TbrC	F:CGGYATWCCGSCSACRCTGCG R:GCCACCTGYSBGCAGTCMCC	255	Zhu et al., 2020
TraF	F:CGGTGATGATTTGCGAACGA R:AGCATTCCGGTCGGCCTGTA	400	Zhu et al., 2020
TnpU	F:CCAACTGATGGCGGTGCCTT R:GGTATGGTGGCTTTCGC	403	Zhu et al., 2020
tnpA/Tn21	F:ATGCCACGTCGTTCCATCCTGTCC R:CGGGTCTGCTCCCGCTGGCC	300	Zhu et al., 2020
TnsA	F:GCAGCAGCCTTACAAGACGAG R:GCCACATAGCGCAACTCCTCC	406	Zhu et al., 2020
MerA	F:GACCAGCCGCAGTTCGTCTA R:GCAGCASGAAAGCTGCTTCA	462	Zhu et al., 2020
tnp513	F:ATGTCGCTGGCAAGGAACGC R:GGGTTCGCTGCGAGGGATTGT	240	Zhu et al., 2020
ISCR1	F:ATGTCGCTGGCAAGGAACGC R:GGGTTCGCTGCGAGGATTGT	240	Zhu et al., 2020
ISCR3/14	F:GGACTTCATTGCACGGATCGAAGC R:TGCACGATGCCCAGCACCGGGCCA	339	Zhu et al., 2020
IS1133	F:AGTACAAAAGCTGTGAGATTTCAG R:GATATTCATGAGCGCAATATTGGC	622	Zhu et al., 2020
ISEcp1	F:CTTCATTGGCATTGATAAGTTAG R:TGTAGCATCGGTTTCCCAGTTTC	299	Zhu et al., 2020
ISpa7	F:TCAGGCCTTCATCGCTGCCATCAGG R:TAGGCGTACAGTGCTCTTTCAACGCA	300	Zhu et al., 2020
IS26	F:ATGAAGCCATTCAAAGGCCGGCAT R:TATGCAGCTTTGCTGTTACGACGG	387	Zhu et al., 2020
ISkpn6	F:GAAGATGCCAAGGTCAATGCCAGG R:TCACAGATACGCCATTCGCCTCAG	240	Zhu et al., 2020
ISkpn7	F:ATGTTGACCCAGGAGCAAACCGTG R:GAGGAAGGCGCGCAATAACGAGAG	300	Zhu et al., 2020
ISaba1	F:AATGATTGGTGACAATGAAG R:ATGCAGCGCTTCTTTGCAGG	372	Zhu et al., 2020
IS903	F:GCAATACGCACGCTTTCAGGC R:ACTGCACGGTTACGGTCTGCA	240	Zhu et al., 2020
intI1	F:TCTCGGGTAACATCAAGG R:GTTCTTCTACGGCAAGGT	243	Zou et al., 2018
intI2	F:CACGGATATGCGACAAAAAGGT R:GTAGCAAACGAGTGACGAAATG	233	Zou et al., 2018
intI3	F:AGTGGGTGGCGAATGAGTG R:TGTTCTTGTATCGGCAGGTG	600	Zou et al., 2018
In1	F:GGCATCCAAGCAGCAAG R:AAGCAGACTTGACCTGA	Variable	Zou et al., 2018
In2	F:CGGGATCCCGGACGGCATGCACG ATTTGTA P:GATGCCATCGCAAGTACGAG	Variable	Zou et al., 2018

DNA Extraction and High-Throughput Quantitative PCR for Antibiotic Resistance Genes Carried by the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

Total genomic DNA was extracted from the MDR *K. pneumonia* isolates based on the results of antimicrobial susceptibility test using the TIANamp Bacteria DNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. DNA samples were stored at −20°C.

To evaluate the abundance of ARGs in different MDR *K. pneumonia* isolates, high-throughput quantitative PCR (HT-qPCR) of ARGs was performed by Yearth Biotech (Changsha, China) using the SmartChip Real-time PCR (Warfergen Inc., United States) as described previously with a slight modification (Gao Q. et al., 2020). A total of 83 primer sets (including a 16S rRNA gene primer sets, not listed, see **Supplementary Table 1**) were used. HT-qPCR data was preprocessed as described previously, for each primer set, amplifications efficiency beyond the range (90∼110%) were discarded, and amplification was confirmed with more than two positive replicates. The relative copy number of ARGs was calculated and transformed to absolute copy numbers by normalizing to 16S rRNA gene copy numbers which were quantified separately from the Wafergen platform.

Detection of Mobile Genetic Elements Carried by the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

Twenty-one pairs of primers were designed to detect MGEs in the 30 MDR *K. pneumoniae* isolates by PCR (**Table 1**; Zhu et al., 2020). PCR amplification was performed in a total volume of 25 μL: 2 × Dream Taq Green PCR master mix (Thermo Fisher Scientific, United States) 12.5 μL, forward

and reverse primer each 1 μL, DNA template 2 μL, and ddH2O 8.5 μL. And ddH2O was set as a negative control. The PCR reaction conditions consisted of 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were subjected to electrophoresis in a 1% agarose gel stained with 4S Red Pus (Sangon Biotech, Shanghai, China), visualized under ultraviolet light and photographed using a gel documentation system (Bio-Rad, Hercules, United States).

Detection of the Integrons and Associated Cassette Arrays Carried by the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

All the MDR *K. pneumonia* isolates were also screened for the presence of int11, intI2 and intI3 genes. Moreover, strains that were positive for the intl1 or intl2 or intI3 genes were then evaluated for gene cassettes by PCR (**Table 1**; Zou et al., 2018). The PCR amplification, PCR reaction conditions and the analyses for PCR products were the same as described in section "Detection of Mobile Genetic Elements Carried by the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates."

Analysis of Clonal Relationship in the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates Based on the Multi-Locus Sequence Typing

MLST was performed to investigate the clonal relationship of the 30 MDR *K. pneumonia* isolates by amplifying the seven standard housekeeping loci *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* as described previously (Guo C. et al., 2015). The eBURST software was used to construct an evolutionary relationship map by analyzing the data of allelic sequence obtained in this study. That six of the seven sites were same was defined as a clone group. A type group containing 4 or more ST types was defined as a clonal complex (CC), of which one ST type was calculated to be the origin ST type, The other ST types was evolved based on the origin ST type. Compared with the original ST type, single-locus variant (SLV) has one different housekeeping gene locus. The single type does not belong to any clonal complex.

Sequence and Data Analysis

All positive PCR products (MGEs, integrons and MLST) were sequenced by Sangong Biotech (Shanghai, China) in both directions. Sequences of MGEs and gene cassettes were analyzed online using BLAST,[1] and sequences of MLST were analyzed online using MLST.[2]

Statistical Analysis

Data were analyzed by a χ^2-test using SPSS Statistics version 18.0. This test was used to compare the isolation rate of MDR *K. pneumonia* by sex in different age groups of the giant pandas by season. The difference of the number of antimicrobial resistances in MDR *K. pneumonia* strains from the individual giant pandas by sex, age and season groupings were tested with the "wilcox_test." function in R package "rstatix." The correlation between the antimicrobial resistant levels of MDR *K. pneumonia* and traits of the giant pandas by sex, age and season groupings using the Fisher's test with "fisher. test" in "stats" R package. Differences among groups were considered significant at $P < 0.05$.

[1]http://blast.ncbi.nlm.nih.gov

[2]http://bigsdb.pasteur.fr/klebsiella/klebsiella.html

RESULTS

The Isolation of the Multi-Drug-Resistant *Klebsiella pneumonia* Strains

A total of 182 *K. pneumonia* strains were isolated from the feces of captive giant pandas showing different degrees of resistance to 24 antimicrobials. Thirty strains which were identified as K1∼K30 were found to be multidrug resistant (MDR) (not listed, see **Supplementary Table 2**). The isolation rate of MDR *K. pneumonia* was 16.48% (30/182), of which the sub-adult group was 11.94% (8/67), adult group was 19.57% (18/92), and geriatric group was 17.39% (4/23), with the age of the giant panda having no significant effect on the isolation rate ($P > 0.05$). The isolation rate of MDR *K. pneumonia* was 16.82% (18/107) in female, and 16.00% (12/75) in male. There was no significant difference in the isolation rate based on the sex of the subject ($P > 0.05$); however, season had a significant effect on the isolation rate of MDR *K. pneumonia* ($P < 0.05$). The isolation rate was 14.52% (9/62) in spring, 11.25% (9/80) in summer, 24.14% (7/29) in autumn, 45.45% (5/11) in winter (**Figure 1**).

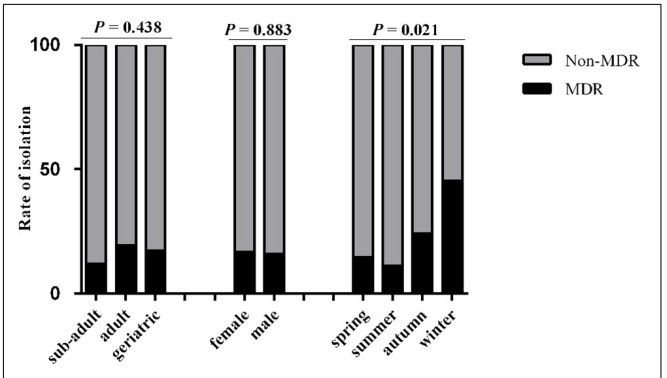


FIGURE 1 | The rate of isolation of non-MDR and MDR *K. pneumonia* from giant pandas of different ages and gender in four seasons. Sub-adult: giant pandas aged 1.5–5 years; Adult: giant pandas aged 5–19 years; Geriatric: giant pandas aged 20 years or older. Spring, March to May; Summer, June to August; Autumn, September to November; Winter, December to February. Gray, the non-MDR *K. pneumonia* from giant pandas; Black, the MDR *K. pneumonia* from giant pandas. Data were analyzed by a χ^2-test using SPSS Statistics version 18.0. $P < 0.05$: The age, sex and the season of sampling had a significant influence on the rate of isolation of non-MDR and MDR *K. pneumonia* from giant pandas.

The Comparison of Antimicrobial Resistances in Multi-Drug-Resistant Individual Giant Pandas

We further analyzed the effects of age, sex and season on the number of antimicrobial resistances in each MDR giant panda. It was found that the antimicrobial resistances in MDR male giant pandas was significantly higher than that of females ($P < 0.05$) (**Figure 2A**). Additionally, there was a significantly increasing trend of the number of antimicrobial resistances from spring to winter. Compared with other seasons, the antimicrobial resistance of giant pandas in winter was significantly higher ($P < 0.05$) (**Figure 2B**). However, age was not significantly correlated to the number of antimicrobial resistances ($P > 0.05$).

The Analysis of Antimicrobial Susceptibility in the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

Thirty MDR *K. pneumonia* strains showed resistance to AML (100.00%, 30/30), DOX (86.67%, 26/30), CHL (60.00%, 18/30), SXT (60.00%, 18/30), and TMP (56.67%, 17/30); resistance to the remaining 19 antimicrobials was observed in less than 50% of the samples (**Table 2**). Fisher's test showed that the antimicrobial resistance of MDR *K. pneumonia* strains to CRO, GM, and DOX was significantly related to age and season ($P < 0.05$) (**Figure 3**), while the remaining antimicrobials were not related to either factor ($P > 0.05$). Additionally, antimicrobial resistance of MDR *K. pneumonia* strains was not significantly correlated to sex of giant pandas ($P > 0.05$).

The Antibiotic Resistance Genes Prevalence of the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

We extracted DNA from the 30 MDR *K. pneumoniae* isolates and analyzed ARGs. Our results showed an abundance of various ARG types in the 30 MDR *K. pneumoniae* isolates, where a total of 50 different types of ARGs were detected, including 671 ARGs. The top ten resistance genes were: vanTC-02, aacC, blaCTX-M-04, blaSHV-01, blaSHV-02, ampC-04, blaOXY, tetD, blaTEM and tetA-02. 671 unique ARGs had potential to confer resistance against a range of antibiotics such as β-Lactamase resistance (41.58%), aminoglycosides resistance (22.21%), vancomycin resistance (11.18%), tetracycline resistance genes (18.93%), sulfonamide resistance (6.11%) (**Figure 4**).

The Mobile Genetic Elements Prevalence of the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

We extracted DNA from the 30 MDR *K. pneumoniae* isolates and analyzed MGEs. The results showed that 13 MGEs were detected among the 30 MDR isolates: IS26 (96.67%, $n = 29$), intI1 (96.67%, $n = 29$), tnpU (93.33%, $n = 28$), tnpA/Tn21 (93.33%, $n = 28$), IS903 (83.33%, $n = 25$), tbrC (36.67%, $n = 11$), ISEcp1 (30.00%, $n = 9$), merA (20.00%, $n = 6$), tnp513 (10.00%, $n = 3$), ISkpn7 (10.00%, $n = 3$), ISaba1 (10.00%, $n = 3$), ISCR3/14 (6.67%, $n = 2$), and ISCR1 (3.33%, $n = 1$). All isolates were negative for the traA, traF, tnsA, IS1133, ISpa7, ISkpn6, intI2, and intI3 genes (**Table 3**).

The Characterization of Integron Types and Gene Cassettes Carried by the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

We further studied integrons in the 30 MDR *K. pneumoniae* isolates: 96.67% (29 isolates) were positive for the intI1 genes, while intI2 and intI3 were not detected. We further analyzed antimicrobial resistance related to gene cassettes in the 29 intI1-positive isolates. The results showed that only in five strains were the drug resistance cassettes detected (dfrA12 + orfF + aadA2 in 2 isolates, and dfrA12 + orfF in 3 isolates) from the 29 intI1-positive isolates (**Table 3**).

The Multi-Locus Sequence Typing Characteristics of the Multi-Drug-Resistant *Klebsiella pneumonia* Isolates

We use MLST typing to understand the clonal relationship of the 30 MDR *K. pneumonia* strains isolated between different giant pandas. Twenty-two STs were identified, of which five strains belonged to ST37, three strains belonged to ST17, and three strains belonged to ST1558. Clonal Structure analysis through eBURST software showed that thirty MDR *K. pneumoniae* isolates were highly diverse, but ST37 had two SLVs (**Figure 5**).

DISCUSSION

MDR *K. pneumoniae* was first reported in the United States, followed by Europe, South America, and Asia (Winokur et al., 2001). The present study is the first to detect MDR *K. pneumonia* isolated from captive giant pandas, with an isolation rate of 16.48%. At the same time, our data illustrated that the isolation rate of MDR *K. pneumonia* was not significantly related to the age or sex of the giant panda. The isolation rate of MDR K. *pneumonia* was found to be 30–70% in a hospital located in Central China (Guo et al., 2017), which was higher than our study in giant pandas. The reason for the lower isolation rate of MDR *K. pneumonia* in giant pandas may be explained by the possibility that antibiotics were used less frequently to treat giant pandas, in addition, *K. pneumoniae* strains in the study were isolated from feces of healthy giant pandas, while *K. pneumoniae* strains in other researches were isolated from hospital materials.

In this study, MDR *K. pneumonia* strains showed resistance to AMX (100.00%), DOX (86.67%), CHL (60.00%), SXT (60.00%), and TMP (56.67%). Zhu et al. (2020) also found *E. coli* strains isolated from feces of giant pandas showed resistance to AMX, DOX, CHL, and SXT, but the resistance rates of these antibiotics were all lower than 50%. Our resistance rates to AMX, DOX, CHL, and SXT were higher than those of *E. coli* isolated from captive giant pandas. The antibiotics in our study were mainly distributed in β-lactams and tetracyclines, which was consistent with previous studies on *E. coli* (Zhu et al., 2020). We further

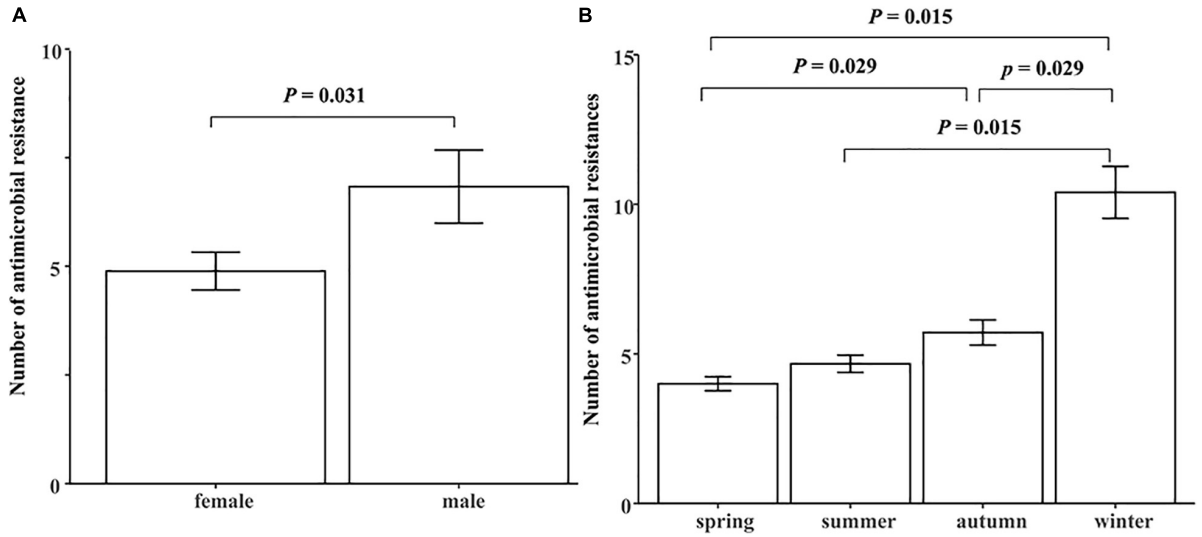


FIGURE 2 | The comparison of number of antimicrobial resistances in 30 MDR giant pandas in different sex and season groups. **(A)** The effect of sex on the number of antimicrobial resistances in each MDR giant panda. **(B)** The effect of season on the number of antimicrobial resistances in each MDR giant panda. Spring, March to May; Summer, June to August; Autumn, September to November; Winter, December to February. Data were analyzed with the "wilcox_test." function in R package "rstatix." $P < 0.05$: The sex or the season had a significant effect on the number of antimicrobial resistances in each MDR giant panda.

TABLE 2 | Antimicrobial susceptibility of 30 MDR *K. pneumoniae* isolates from the feces of captive giant pandas in four seasons in China.

Name of giant pandas	Time of sampling	Strains	Age group	Sex group	Resistance profile
P1	Winter	K1	Geriatric	M	PIC/AML/CTX/CEZ/CRO/CXM/CEC/GM/STR/AZM/SXT/TMP
P6	Summer	K2	Adult	M	AML/CHL/AZM/DOX/SXT/TMP
P14	Summer	K3	Adult	F	AML/CTX/CHL/DOX
P4	Spring	K4	Adult	F	AML/CHL/DOX/MH
P4	Summer	K5	Adult	F	PIC/AML/CHL/DOX
P8	Spring	K6	Sub-adult	M	AML/CHL/DOX/MH
P4	Winter	K7	Adult	F	AML/STR/OFX/NOR/LEV/CIP/CHL/AZM/DOX/SXT/TMP
P6	Spring	K8	Adult	F	AML/CHL/DOX/SXT/TMP
P8	Autum	K9	Sub-adult	M	AML/IPM/CHL/DOX/MH/SXT/TMP
P12	Spring	K10	Adult	F	AML/MEM/STR//DOX
P2	Winter	K11	Geriatric	M	PIC/AML/CTX/CEZ/CXM/CEC/GM/STR/AZM/SXT/TMP
P9	Summer	K12	Adult	F	AML/IPM/CHL/DOX
P8	Summer	K13	Sub-adult	M	AML/CHL/DOX/SXT/TMP
P14	Spring	K14	Adult	F	AML/STR/DOX
P7	Spring	K15	Geriatric	F	AML/KAN/DOX/MH
P5	Winter	K16	Adult	F	AML/STR/CHL/DOX/MH/SXT/TMP
P15	Spring	K17	Sub-adult	F	AML/GM/SXT/TMP
P16	Autum	K18	Sub-adult	M	AML/GM/SXT/TMP
P13	Summer	K19	Adult	F	AML/AZM/LEV/DOX
P9	Autum	K20	Adult	F	AML/STR/DOX/MH/SXT/TMP
P3	Winter	K21	Adult	M	PIC/AML/CEZ/CXM/CEC/SAM/CHL/DOX/MH/SXT/TMP
P12	Summer	K22	Adult	F	AML/STR/DOX/SXT/TMP
P13	Spring	K23	Adult	F	AML/CHL/DOX
P3	Spring	K24	Adult	M	AML/CHL/DOX/SXT/TMP
P7	Autum	K25	Geriatric	F	AML/CHL/DOX/SXT/TMP
P16	Summer	K26	Sub-adult	M	AML/CHL/DOX/SXT
P15	Summer	K27	Sub-adult	F	PIC/AML/DOX/MH/SXT/TMP
P12	Autum	K28	Adult	F	AML/CHL/DOX/SXT/TMP
P10	Autum	K29	Sub-adult	M	AML/SAM/DOX/MH/SXT/TMP
P6	Autum	K30	Adult	M	AML/KAN/LEV/CHL/DOX/SXT/TMP

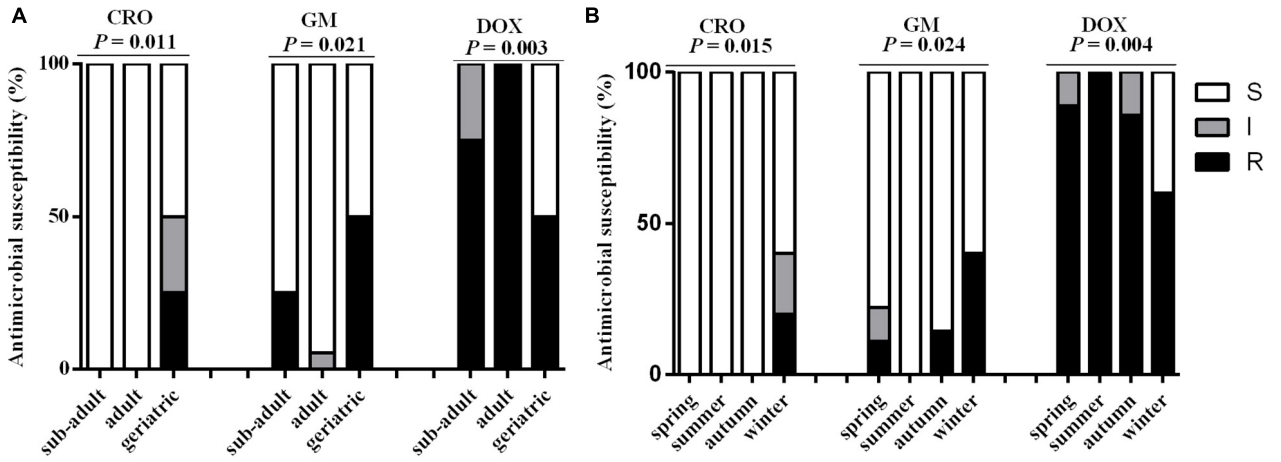


FIGURE 3 | The comparison of antimicrobial susceptibility in the 30 MDR *K. pneumonia* isolates in different age and season groups. **(A)** The effect of age on antimicrobial susceptibility in MDR *K. pneumonia* isolates. Sub-adult: 1.5–5 years; Adult: 5–19 years; Geriatric: 20 years or older. **(B)** The effect of season on antimicrobial susceptibility in MDR *K. pneumonia* isolates. Spring, March to May; Summer, June to August; Autumn, September to November; Winter, December to February. CRO, ceftriaxone, GM, gentamicin, DOX, doxycycline. White, MDR *K. Pneumonia* isolates which were sensitive to antibiotics; Gray, MDR *K. Pneumonia* isolates which were intermediate to antibiotics; Dark, MDR *K. Pneumonia* isolates which were resistant to antibiotics. Data were analyzed with the "fisher. test" in "stats" R package. $P < 0.05$: The age or the season had a significant infect on antimicrobial susceptibility in MDR *K. pneumonia* isolates.

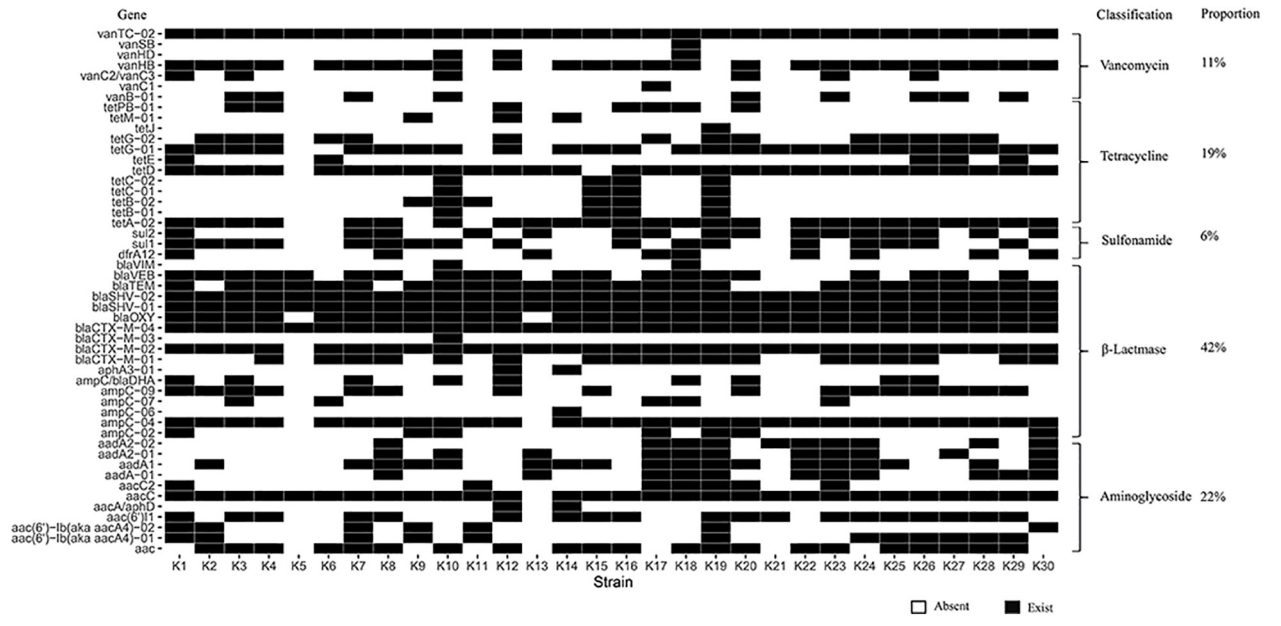


FIGURE 4 | Heat-map for the ARGs in the 30 MDR *K. pneumonia* isolated from the feces of giant pandas. Columns: K1∼K30 mean 30 MDR *K. pneumoniae* isolates. Rows: 50 different types of ARGs, the classification and proportion. White: The *K. pneumoniae* isolate which did not carry the ARG; Black: The *K. pneumoniae* isolate which carried the ARG.

found some strains were resistant to more than 10 antibiotics in this study, even though CHL was banned for animals, resistance to CHL was observed in 60% of the MDR strains. For these reasons, it is important to be prudent in the use of antimicrobials with giant pandas, and the supervision of antimicrobial resistance in isolates from giant pandas can be useful in choosing antibiotics for preventing *K. pneumonia* infections. In addition, our results also found that among the 24 antibiotics, the drug resistance rates of CRO, GM, and DOX were significantly related to the age of the giant panda. The possible reason was that the disease susceptibility of giant pandas differ by age, and the selections of antibiotics for clinical treatment were also different. At the same time, we found that the antimicrobial susceptibility of MDR *K. pneumonia* isolated from the geriatric groups of giant pandas to CRO and GM was significantly lower than that of the adult groups and sub-adult groups, which indicated that more

TABLE 3 | MGEs and integron gene cassettes in 30 MDR *K. pneumoniae* isolates from the feces of captive giant pandas in China.

Strains	Components of mobile genetic elements	Integron	Gene cassettes
K1	tnpU + tnpA/Tn21 + ISEcp1 + IS26 + ISkpn7 + IS903 + intI1	Class1	
K2	tnpU + tnpA/Tn21 + ISEcp1 + IS26 + IS903 + intI1	Class1	
K3	tnpU + tnpA/Tn21 + merA + IS26 + IS903 + intI1	Class1	
K4	tnpU + tnpA/Tn21 + merA + IS26 + intI1	Class1	
K5	tnpU + tnpA/Tn21 + IS903 + intI1	Class1	
K6	tnpU + tnpA/Tn21 + IS26 + IS903 + intI1	Class1	
K7	tnpU + tnpA/Tn21 + tnp513 + IS26 + IS903 + intI1	Class1	
K8	tbrC + tnpU + ISCR3/14 + IS26 + IS903 + intI1	Class1	dfrA12 + orfF + aadA2
K9	tnpU + tnpA/Tn21 + merA + IS26 + IS903 + intI1	Class1	
K10	tnp513 + IS1133 + ISEcp1 + IS26 + IS903		
K11	tnpU + tnpA/Tn21 + ISCR3/14 + IS1133 + ISEcp1 + IS26 + IS903 + intI1	Class1	
K12	tbrC + tnpU + tnpA/Tn21 + IS1133 + IS26 + IS903 + intI1	Class1	
K13	tnpU + tnpA/Tn21 + IS26 + IS903 + intI1	Class1	dfrA12 + orfF
K14	tnpU + tnpA/Tn21 + merA + IS26 + intI1	Class1	
K15	tbrC + tnpU + tnpA/Tn21 + IS26 + IS903 + intI1	Class1	
K16	tnpU + tnpA/Tn21 + IS26 + intI1	Class1	
K17	tbrC + tnpU + tnpA/Tn21 + merA + IS26 + ISaba1 + intI1	Class1	
K18	tbrC + tnpA/Tn21 + tnp513 + ISCR1 + ISEcp1 + IS26 + ISkpn7 + intI1	Class1	dfrA12 + orfF
K19	tbrC + tnpU + tnpA/Tn21 + IS1133 + ISEcp1 + IS26 + ISkpn7 + ISaba1 + IS903 + intI1	Class1	
K20	tnpU + tnpA/Tn21 + IS1133 + IS26 + IS903 + intI1	Class1	
K21	tbrC + tnpU + tnpA/Tn21 + IS1133 + IS26 + IS903 + intI1	Class1	
K22	tbrC + tnpU + tnpA/Tn21 + IS1133 + IS26 + IS903 + intI1	Class1	dfrA12 + orfF + aadA2
K23	tnpU + tnpA/Tn21 + IS26 + IS903 + intI1	Class1	
K24	tnpU + tnpA/Tn21 + ISEcp1 + IS26 + IS903 + intI1	Class1	dfrA12 + orfF
K25	tbrC + tnpU + tnpA/Tn21 + ISEcp1 + IS26 + ISaba1 + IS903 + intI1	Class1	
K26	tbrC + tnpU + tnpA/Tn21 + ISEcp1 + IS26 + IS903 + intI1	Class1	
K27	tnpU + tnpA/Tn21 + IS26 + IS903 + intI1	Class1	
K28	tbrC + tnpU + tnpA/Tn21 + IS26 + IS903 + intI1	Class1	
K29	tnpU + tnpA/Tn21 + IS26 + IS903 + intI1	Class1	
K30	tnpU + tnpA/Tn21 + merA + IS26 + IS903 + intI1	class1	

attention should be paid to the rational use of antibiotics for sick elderly pandas.

HT-qPCR enables a rapid and sensitive quantification of a number of ARGs, conferring resistance to almost all major classes of antibiotics and providing comprehensive profiles of ARGs (Su et al., 2015). In this study, fifty different types of ARGs were found, which included a total of 671 ARGs, which mainly belonged to β-Lactamase (41.58%), which was consistent with the findings of Hu et al. (2021). At the same time, the results of antimicrobial susceptibility test showed that the MDR isolates also had high resistance to β-lactam antibiotics, especially the resistance rate to AML, which was 100%. The resistance mechanism of *K. pneumonia* to β-lactam antibiotics is to mainly produce extended-spectrum β-lactamases encoded by extended-spectrum β-lactamase genes that destroy the β-lactam ring and lead to antibiotic inactivation (Fluit et al., 2001). Although β-lactam antibiotics and genes were diverse, the resistance phenotype and genotype of isolates roughly corresponded through this study. Given that rapid distribution and occurrence of novel ARGs among bacteria are intensifying problems throughout the world, it is necessary to monitor the resistance genes carried by the bacteria of giant pandas.

MGEs play an important role in the dissemination of ARGs among *K. pneumonia* strains. We had detected 13 MGEs among the 30 MDR isolates. The number of MGEs detected in this study was higher than the previous research on *E. coli*. strains isolated from giant panda, while traA, traF, tnsA, ISpa7, ISkpn6, and intI3 genes were all negative, which was consistent with previous findings (Zhu et al., 2020). The occurrence of MDR among microbes is considered to be associated with MGEs due to their ability to transfer several ARGs instantaneously (Martinez, 2009). Horizontal transfer of ARGs mediated by MGEs is one of the main mechanisms for the dissemination of ARGs (Chen et al., 2018). MGE as a carrier of ARGs plays an important role in capturing, accumulating and spreading, this can occur between cells of the same strain or between different *K. pneumonia* strains, enabling the rapid and widespread growth of antibiotic-resistant bacteria. In addition, the horizontal transfer of ARGs accelerates the emergence of drug-resistant and multi-drug-resistant bacteria in clinical practice (Chamosa et al., 2017). In this study, MDR *K. pneumonia* strains isolated from different giant panda carried some same ARGs, and a large number of MGEs were detected. Therefore, it can be speculated that there was the possibility of sharing the ARGs between giant pandas in

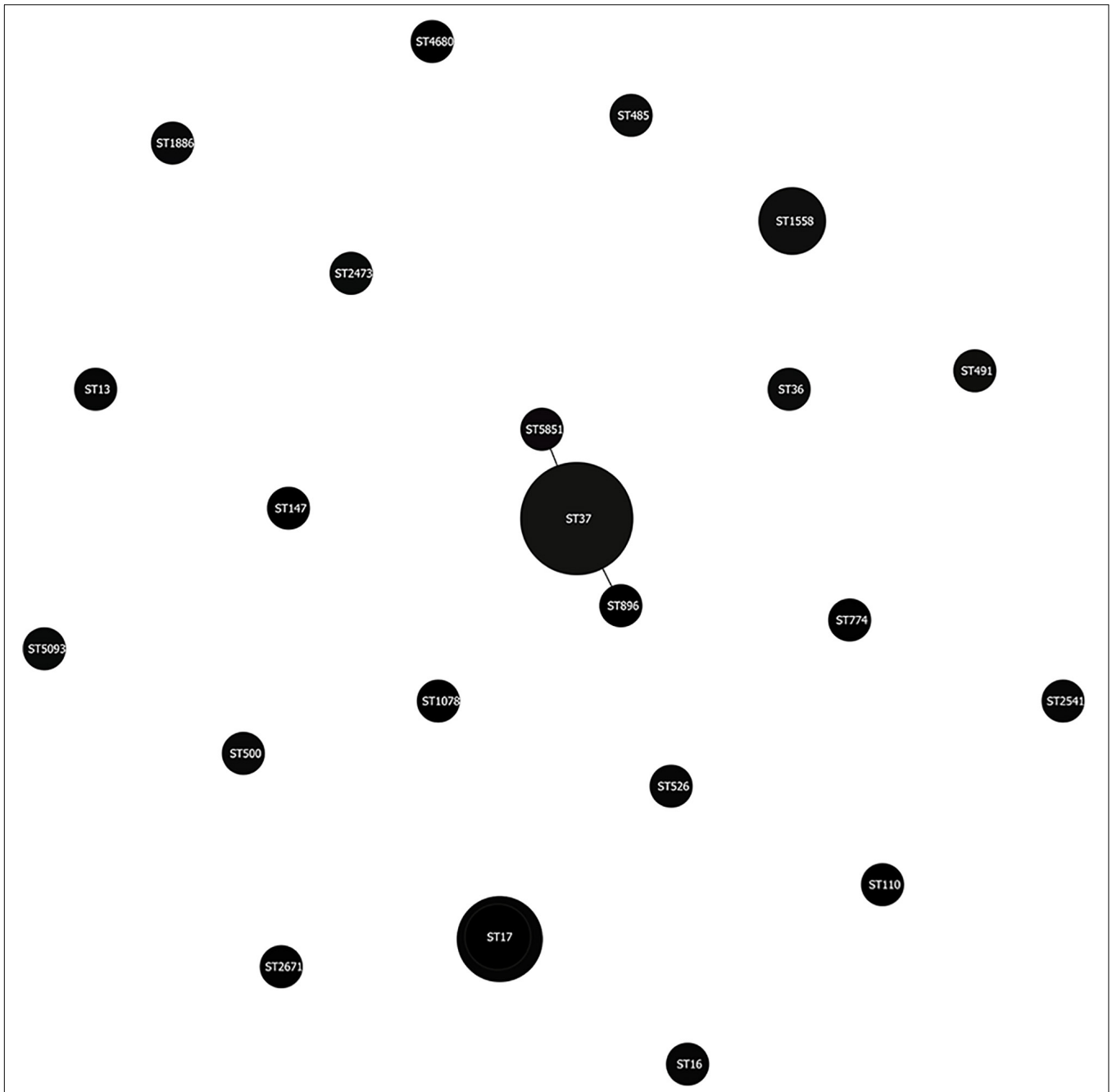


FIGURE 5 | MLST-based clonal Structure of the 30 MDR *K. pneumonia* isolates from the feces of giant pandas. The scheme was constructed using eBURST analysis. STs were symbolized by dots, the size of a dot corresponded to the number of isolates belonging to an ST. SLVs (single-locus variant: has one different housekeeping gene locus compared with the original ST type) were linked by solid lines.

this study. However, further investigation is needed to determine how the ARGs are shared.

We further analyzed antimicrobial resistance related to integrons in the 30 MDR *K. pneumonia* isolates. Integrons are genetic units consisting of determinants of a site-specific recombination event, which can mobilize and capture genes encoded on mobile elements known as gene cassettes (Gillings, 2014). In our study, the proportion of isolates containing the intI1 gene (96.67%, 29/30) was alarmingly higher than that previously reported in giant pandas (Zou et al., 2018; Zhu et al., 2020). We speculated the reason was that the intI1 gene was detected from MDR *K. pneumonia*. The diversity of gene cassettes are incorporated in the integrons and are able to encode antibiotics resistance to aminoglycoside, sulfonamides and trimethoprim. Accordingly, integrons were significantly connected with MDR (San et al., 2008; Tajbakhsh et al., 2015; Akrami et al., 2017).

The intI2 and intI3 genes were not detected in our study. Many studies revealed that the class 1 integron was more prevalent than the class 2 or 3 in gram-negative bacteria (Lavakhamseh et al., 2016; Chamosa et al., 2017; Rehman et al., 2017; Sidhu et al., 2017). Moreover, class 3 integrons are reported to be rare in animals and clinical samples. Therefore, the presence of only intl1 integrons and the absence of intl2 and intl3 integrons in the present study were consistent with other research (Chen et al., 2010, 2016; Rehman et al., 2017; Zou et al., 2018; Zhu et al., 2020). Sequencing analysis showed that there were only two types of gene cassettes (dfrA12 + orfF + aadA2 in 2 isolates, and dfrA12 + orfF in 3 isolates) from the 29 intI1-positive isolates in this study. The other 24 strains provided negative results for the gene cassettes likely due to retained empty or undetermined intl1 VRs, which was also reported in other studies (Zhang et al., 2019; Zhu et al., 2020). Previous studies had confirmed that the gene cassettes dfrA12-orfF-aadA2 and dfrA17-aadA5 were dominant in giant pandas. These gene cassettes have previously been reported in *E. coli* isolates (Koczura et al., 2015; Yu et al., 2016; Zou et al., 2018; Zhu et al., 2020), which were constituted by trimethoprim resistance genes (dfrA12, dfrA17), and also carried the aadA2 and aadA5 genes which led to resistance to aminoglycoside (example: streptomycin). In addition, these gene cassettes can code a reading frame (orfF) for a hypothetical protein with an unknown function (Zhang et al., 2019). As far as we know, the integron gene cassettes in our study exist widely in humans, animals, plants, and the environment, however, this is the first report of integron gene cassettes in *K. pneumonia* isolated from the feces of giant pandas. It is emphasized that we should focus more on intergrons which contribute to the effective spread of ARGs.

MLST is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of (usually) seven house-keeping genes (Jolley et al., 2018). In this study, thirty MDR *K. pneumoniae* isolates were divided into 22 STs, five of which belonged to ST37. Clonal Structure analysis through eBURST software showed that there was no clonal complex in thirty MDR *K. pneumoniae* isolates, but ST37 had two SLVs, which indicated that although MDR *Klebsiella pneumoniae* strains isolated from different giant pandas were highly diverse, some strains still had the same origin.

CONCLUSION

In our study, 30 MDR strains among 182 *K. pneumonia* strains isolated from the feces of captive giant pandas. All of the MDR *K. pneumonia* strains carried a large number of ARGs and MGEs, especially 29 of 30 MDR *K. pneumonia* strains carried integrons. In addition, two different types of gene cassettes were detected. What' more, MLST indicated that some MDR *K. pneumonia* strains had the same origin. To the best of our knowledge, MDR and a diversity of ARGs and MGEs existed in *K. pneumonia* isolates from captive giant pandas, and integrons were related to the emergence of MDR strains, and giant pandas may share the MDR *K. pneumonia* isolates. Therefore, it is essential that relevant precautionary measures be taken to prevent the spread of MDR bacteria, and monitor the emergence of MGEs integrons. Further studies are required to assess other factors involved in the transmission of linked resistance genes, and efforts should be made to eliminate or reduce drug resistance in captive giant pandas.

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 animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Chengdu Research Base of Giant Panda Breeding (No. 2018017).

AUTHOR CONTRIBUTIONS

XY, JD, ZR, XS, and RH contributed to conception and design of the study. SL and JD played a guiding role in carrying out the experiment. XY, YL, DZ, CY, and LL collected samples. XY and MY performed bacterial identification and isolation and related component testing. XY, XS, HD, YD, ZX, and XF performed the statistical analysis. XY wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This research was supported by the Chengdu Research Base of Giant Panda Breeding (project nos. 2021CPB-B15), the National Forestry and Grassland Administration and Sichuan Provincial Finance Department (project title: study on main epidemiological investigation and prevention of giant panda), and the Sichuan Agricultural University 2019 Disciplinary Construction Double Branch Plan (project nos. 035-1921993036).

ACKNOWLEDGMENTS

We would like to thank the veterinary staff and keepers of the Chengdu Panda Base for collecting samples, and James Edward Ayala for reviewing the manuscript, and Yulin Jiang and Huanxin Liu for their help in some experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.801292/full#supplementary-material

REFERENCES

Akrami, F., Shahandashti, E. F., Yahyapour, Y., Sadeghi, M., Khafri, S., Pournajaf, A., et al. (2017). Integron types, gene cassettes and antimicrobial resistance profile of *Acinetobacter baumannii* isolated from BAL samples in Babol, north of Iran. *Microb. Pathog.* 109, 35–38. doi: 10.1016/j.micpath.2017.05.005

Apondi, O. E., Oduor, O. C., Gye, B. K., and Kipkoech, M. K. (2016). High prevalence of multi-drug resistant *Klebsiella pneumoniae* in a tertiary teaching hospital in western kenya. *Afr. J. Infect. Dis.* 10, 89–95. doi: 10.21010/ajid.v10i2.3

Chamosa, L. S., Álvarez, V. E., Nardelli, M., Quiroga, M. P., Cassini, M. H., and Centrón, D. (2017). Lateral antimicrobial resistance genetic transfer is active in the open environment. *Sci. Rep.* 7:513. doi: 10.1038/s41598-017-00600-2

Chen, D., Zou, W., Xie, S., Kong, L., Chen, Y., Zhang, X., et al. (2018). Serotype and antimicrobial resistance of *Escherichia coli* isolated from feces of wild giant pandas (*ailuropoda melanoleuca*) in Sichuan Province, China. *J. Wildl. Dis.* 54, 691–699. doi: 10.7589/2017-07-165

Chen, H., Shu, W., Chang, X., Chen, J. A., Guo, Y., Tan, Y., et al. (2010). The profile of antibiotics resistance and integrons of extended-spectrum beta-lactamase producing thermotolerant coliforms isolated from the Yangtze River basin in Chongqing. *Environ. Pollut.* 158, 2459–2464. doi: 10.1016/j.envpol.2010.03.023

Chen, Q., An, X., Li, H., Su, J., Ma, Y., and Zhu, Y. G. (2016). Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. *Environ. Int.* 9, 1–10. doi: 10.1016/j.envint.2016.03.026

Clinical and Laboratory Standards Institute [CLSI] (2020). *Performance Standards For Antimicrobial Susceptibility Testing: Twenty - Fourth Informational Supplement CLSI document M100-S24*. Wayne, PA: CLSI.

Dolejska, M., Cizek, A., and Literak, I. (2007). High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from black-headed gulls in the Czech Republic. *J. Appl. Microbiol.* 103, 11–19. doi: 10.1111/j.1365-2672.2006.03241.x

Fluit, A. C., Visser, M. R., and Schmitz, F. J. (2001). Molecular detection of antimicrobial resistance. *Clin. Microbiol. Rev.* 14, 836–871. doi: 10.1128/CMR.14.4.836-871.2001

Gao, L., Lv, Y., and Li, Y. (2020). Analysis of the drug resistance of carbapenem-resistant *Klebsiella pneumoniae* in the China antimicrobial resistance surveillance trial program, 2007-2018. *Microb. Drug Resist.* 26, 944–950. doi: 10.1089/mdr.2019.0299

Gao, Q., Shen, Z., Qin, J., Liu, Y., and Li, M. (2020). Antimicrobial resistance and pathogenicity determination of community-acquired hypervirulent *Klebsiella pneumoniae*. *Microb. Drug Resist.* 26, 1195–1200. doi: 10.1089/mdr.2019.0439

Gillings, M. R. (2014). Integrons: past, present, and future. *Microbiol. Mol. Biol. Rev.* 78, 257–277. doi: 10.1128/MMBR.00056-13

Guo, C., Yang, X., Wu, Y., Yang, H., Han, Y., Yang, R., et al. (2015). MLST-based inference of genetic diversity and population structure of clinical *klebsiella pneumoniae*, China. *Sci. Rep.* 5:7612. doi: 10.1038/srep07612

Guo, L., Long, M., Huang, Y., Wu, G., Deng, W., Yang, X., et al. (2015). Antimicrobial and disinfectant resistance of *Escherichia coli* isolated from giant pandas. *J. Appl. Microbiol.* 119, 55–64. doi: 10.1111/jam.12820

Guo, X., Cao, Z., Dai, Z., Li, Y., He, X., Hu, X., et al. (2017). Antimicrobial susceptibility and molecular epidemiology of multidrug-resistant *Klebsiella pneumoniae* in central China. *Jpn. J. Infect. Dis.* 70, 229–234. doi: 10.7883/yoken.JJID.2016.049

Hu, T., Dai, Q., Chen, H., Zhang, Z., Dai, Q., Gu, X., et al. (2021). Geographic pattern of antibiotic resistance genes in the metagenomes of the giant panda. *Microb. Biotechnol.* 14, 186–197. doi: 10.1111/1751-7915.13655

Jeena, P., Thompson, E., Nchabeleng, M., and Sturm, A. (2001). Emergence of multi-drug-resistant *Acinetobacter anitratus* species in neonatal and paediatric intensive care units in a developing country: concern about antimicrobial policies. *Ann. Trop. Paediatr.* 21, 245–251. doi: 10.1080/02724930120077835

Jolley, K. A., Bray, J. E., and Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res.* 24:124. doi: 10.12688/wellcomeopenres.14826.1

Kaushik, M., Kumar, S., Kapoor, R. K., Virdi, J. S., and Gulati, P. (2018). Integrons in *Enterobacteriaceae*: diversity, distribution and epidemiology. *Int. J. Antimicrob. Agents* 51, 167–176. doi: 10.1016/j.ijantimicag.2017.10.004

Koczura, R., Krysiak, N., Taraszewska, A., and Mokracka, J. (2015). Coliform bacteria isolated from recreational lakes carry class 1 and class 2 integrons and virulence-associated genes. *J. Appl. Microbiol.* 119, 594–603. doi: 10.1111/jam.12838

Lavakhamseh, H., Mohajeri, P., Rouhi, S., Shakib, P., Ramazanzadeh, R., Rasani, A., et al. (2016). Multidrug-Resistant *Escherichia coli* strains isolated from patients are associated with class 1 and 2 integrons. *Chemotherapy* 61, 72–76. doi: 10.1159/000438666

Lu, M. G., Jiang, J., Liu, L., Ma, A. P., and Leung, F. C. (2015). Complete genome sequence of *Klebsiella pneumoniae* strain HKUOPLC, a cellulose-degrading bacterium isolated from giant panda feces. *Genome Announc.* 3, e01318–e01415. doi: 10.1128/genomeA.01318-15

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut.* 157, 2893–2902. doi: 10.1016/j.envpol.2009.05.051

Partridge, S. R., Tsafnat, G., Coiera, E., and Iredell, J. R. (2009). Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol. Rev.* 33, 757–784. doi: 10.1111/j.1574-6976.2009.00175.x

Peng, G. N., and Xiong, Y. (2000). The studying status quo on the panda *Klebsiella pneumoniae*. *Chin. J. Sichuan Anim. Vet. Sci.* 27, 86–87. doi: 10.3969/j.issn.1001-8964.2000.z1.044

Rehman, M. U., Zhang, H., Huang, S., Iqbal, M. K., Mehmood, K., Luo, H., et al. (2017). Characteristics of integrons and associated gene cassettes in antibiotic-resistant *Escherichia coli* isolated from free-ranging food animals in China. *J. Food Sci.* 82, 1902–1907. doi: 10.1111/1750-3841.13795

San, M. B., Lapierre, L., Cornejo, J., and Bucarey, S. (2008). Characterization of antibiotic resistance genes linked to class 1 and 2 integrons in strains of *Salmonella* spp. isolated from swine. *Can. J. Microbiol.* 54, 569–576. doi: 10.1139/w08-045

Sidhu, J., Jagals, P., Smith, A., and Toze, S. (2017). Comparative prevalence of *Escherichia coli* carrying virulence genes and class 1 and 2 integrons in sub-tropical and cool temperate freshwater. *Environ. Sci. Pollut. Res. Int.* 24, 18263–18272. doi: 10.1007/s11356-017-9497-0

Su, J. Q., Wei, B., Ou-Yang, W. Y., Huang, F. Y., Zhao, Y., Xu, H. J., et al. (2015). Antibiotic resistome and its association with bacterial communities during sewage sludge composting. *Environ. Sci. Technol.* 49, 7356–7363. doi: 10.1021/acs.est.5b01012

Su, X. Y., Li, Y. L., Yan, X., Zhang, D. S., Li, L., Hou, R., et al. (2021). Isolation and identification of extended-spectrum β-lactamases-producing *Klebsiella pneumoniae* in captive giant panda. *Chin. J Vet. Med.* 7, 1276–1281. doi: 10.16303/j.cnki.1005-4545.2021.07.08

Sun, F., Yang, S., and Chen, X. (2013). Detection of intestinal pathogenic *Klebsiella pneumoniae* from fecal samples of giant panda by polymerase chain reaction. *J. Biotech. Res.* 5, 10–15.

Tajbakhsh, E., Khamesipour, F., Ranjbar, R., and Ugwu, I. C. (2015). Prevalence of class 1 and 2 integrons in multi-drug resistant *Escherichia coli* isolated from aquaculture water in Chaharmahal Va Bakhtiari province, Iran. *Ann. Clin. Microbiol. Antimicrob.* 14:37. doi: 10.1186/s12941-015-0096-y

Wang, C. D., Lan, J. C., Luo, L., Yang, Z., and Zhang, R. R. (2006). *Klebsiella pneumonia*-pathogen of urogenital hematuria of giant panda. *Sichuan J. Zool.* 25, 83–85. doi: 10.3969/j.issn.1000-7083.2006.01.018

Wang, T., Xie, Y., Zheng, Y., Wang, C., Li, D., Koehler, A. V., et al. (2018). Parasites of the giant panda: a risk factor in the conservation of a species. *Adv. Parasitol.* 99, 1–33. doi: 10.1016/bs.apar.2017.12.003

Winokur, P. L., Canton, R., Casellas, J. M., and Legakis, N. (2001). Variations in the prevalence of strains expressing an extended-spectrum beta-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. *Clin. Infect. Dis.* 32(Suppl. 2), S94–S103. doi: 10.1086/320182

Yang, X., Cheng, G., Li, C., Yang, J., Li, J., Chen, D., et al. (2017). The normal vaginal and uterine bacterial microbiome in giant pandas (*Ailuropoda melanoleuca*). *Microbiol. Res.* 199, 1–9. doi: 10.1016/j.micres.2017.01.003

Yang, Y., Peng, Y., Jiang, J., Gong, Z., Zhu, H., Wang, K., et al. (2021). Isolation and characterization of multidrug-resistant *Klebsiella pneumoniae* from raw cow milk in Jiangsu and Shandong provinces, China. *Transbound. Emerg. Dis.* 68, 1033–1039. doi: 10.1111/tbed.13787

Yu, T., Zhang, J., Jiang, X., Wu, J., Dai, Z., Wu, Z., et al. (2016). Characterization and horizontal transfer of class 1 integrons in *Escherichia coli* isolates from cooked meat products. *J. Infect. Dev. Ctries.* 10, 68–73. doi: 10.3855/jidc.6858

Zhang, A. Y., Wang, H. N., Tian, G. B., Zhang, Y., Yang, X., Xia, Q. Q., et al. (2009). Phenotypic and genotypic characterisation of antimicrobial resistance in faecal bacteria from 30 Giant pandas. *Int. J. Antimicrob. Agents* 33, 456–460. doi: 10.1016/j.ijantimicag.2008.10.030

Zhang, S., Yang, H., Rehman, M. U., Yang, K., Dong, M., Yang, J., et al. (2019). Class 1 integrons as predominant carriers in *Escherichia coli* isolates from waterfowls in Hainan, China. *Ecotoxicol. Environ. Saf.* 183:109514. doi: 10.1016/j.ecoenv.2019.109514

Zhu, Z., Pan, S., Wei, B., Liu, H., Zhou, Z., Huang, X., et al. (2020). High prevalence of multi-drug resistances and diversity of mobile genetic elements in *Escherichia coli* isolates from captive giant pandas. *Ecotoxicol. Environ. Saf.* 198:110681. doi: 10.1016/j.ecoenv.2020.110681

Zou, W., Li, C., Yang, X., Wang, Y., Cheng, G., Zeng, J., et al. (2018). Frequency of antimicrobial resistance and integron gene cassettes in *Escherichia coli* isolated from giant pandas (*Ailuropoda melanoleuca*) in China. *Microb. Pathog.* 116, 173–179. doi: 10.1016/j.micpath.2018.01.034

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, Su, Ren, Fan, Li, Yue, Yang, Deng, Deng, Xu, Zhang, Li, Hou, Liu and Deng. 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 Microbiology

ORIGINAL RESEARCH
published: 18 March 2022
doi: 10.3389/fmicb.2022.838392

Population Diversity of Antibiotic Resistant *Enterobacterales* in Samples From Wildlife Origin in Senegal: Identification of a Multidrug Resistance Transposon Carrying $bla_{CTX-M-15}$ in *Escherichia coli*

*Rim Abdallah[1,2], Edmond Kuete Yimagou[1,2], Linda Hadjadj[1,2], Oleg Mediannikov[1,2], Ahmad Ibrahim[1,2], Bernard Davoust[1,2], Amanda Barciela[3], R. Adriana Hernandez-Aguilar[3,4], Georges Diatta[5], Cheikh Sokhna[2,5], Didier Raoult[1,2], Jean-Marc Rolain[1,2] and Sophie Alexandra Baron[1,2]**

[1] IRD, APHM, MEPHI, Faculté de Médecine et de Pharmacie, Aix Marseille University, Marseille, France, [2] IHU Méditerranée Infection, Marseille, France, [3] Dindefelo Biological Station, Jane Goodall Institute Spain and Senegal, Kedougou, Senegal, [4] Department of Social Psychology and Quantitative Psychology, Faculty of Psychology, Serra Hunter Programme, University of Barcelona, Barcelona, Spain, [5] VITROME IRD 257, Campus International de Recherche IRD-UCAD de Hann, Dakar, Senegal*

OPEN ACCESS

Edited by:
Teresa M. Coque,
Ramón y Cajal Institute for Health Research, Spain

Reviewed by:
Angela Novais,
University of Porto, Portugal
Justin Joseph Donato,
University of St. Thomas, United States

***Correspondence:**
Sophie Alexandra Baron
sophie.baron@ap-hm.fr

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 17 December 2021
Accepted: 18 February 2022
Published: 18 March 2022

Citation:
Abdallah R, Kuete Yimagou E, Hadjadj L, Mediannikov O, Ibrahim A, Davoust B, Barciela A, Hernandez-Aguilar RA, Diatta G, Sokhna C, Raoult D, Rolain JM and Baron SA (2022) Population Diversity of Antibiotic Resistant Enterobacterales in Samples From Wildlife Origin in Senegal: Identification of a Multidrug Resistance Transposon Carrying $bla_{CTX-M-15}$ in Escherichia coli. Front. Microbiol. 13:838392. doi: 10.3389/fmicb.2022.838392

Introduction: The role of wildlife in the transmission of antimicrobial resistant (AMR) is suspected but scarcely reported in current studies. Therefore, we studied the dynamics and prevalence of antibiotic-resistant *Enterobacterales* in antibiotic-limited areas of Senegal.

Materials and Methods: We collected fecal samples from monkeys and apes (N = 226) and non-fecal environmental samples (N = 113) from Senegal in 2015 and 2019. We grew the samples on selective media, subsequently isolated AMR *Enterobacterales*, and then sequenced their genomes.

Results: We isolated 72 different *Enterobacterales* among which we obtained a resistance rate of 65% for colistin (N = 47/72) and 29% for third generation-cephalosporin (C3G) (29%, N = 21/72). Interestingly, almost 46% of our isolates, among *Enterobacter* sp., *Citrobacter cronae* and *Klebsiella aerogenes*, belong to 34 new STs. Moreover, the genes $bla_{CTX-M-15}$, bla_{TEM1B}, *sul2*, *dfrA14*, *qnrs*, *aph*(3′′), *aph*(6), *tetA*, and *tetR* harbored within a transposon on the *IncY* plasmid of ST224 *Escherichia coli* were transferred and inserted into a ST10 *E. coli* phage coding region.

Conclusion: Wildlife constitutes a rich, unexplored reservoir of natural microbial diversity, AMR genes and international resistant clones pathogenic in humans. The presence of a transposon that carries AMR genes is intriguing since no antibiotics are used in the non-human primates we studied.

Keywords: antibiotic resistance, *Enterobacterales*, primates, environment, Senegal

INTRODUCTION

Antimicrobial resistant (AMR) bacteria are widely found in humans, animals, and the environment, which could be all part of the same ecosystem in certain areas (McEwen and Collignon, 2018). AMR genes can be transferred within this ecosystem, and homologous resistance genes could be found in pathogens, normal flora and soil bacteria (Woolhouse et al., 2015). AMR, even in a "One Health" perspective, is considered to be linked to human activity, in hospitals and livestock farms or the artificial environment created by people such as houses and cities. However, this often overlooks the role played by the wild environment, where human activity is lower than in artificial environments created by humans (Chokshi et al., 2019).

According to the World Health Organization (WHO), Africa has one of the largest gap in data on the incidence of AMR (World Health Organization, 2014) and genomic analysis of this AMR bacteria in African wildlife has rarely been explored, to our knowledge (Ramey and Ahlstrom, 2019; Miller et al., 2020). However, it is essential information for understanding the sources of antibiotic resistance, its spread and the factors involved in this spread. In Africa, and more precisely in Senegal, AMR has mainly been studied in relation to human medicine (Dromigny et al., 2005), as well as animal farming (Vounba et al., 2019). High levels of resistance in this country indicate that human hospital outbreaks are reservoirs of extended spectrum β-lactamases (ESBL)-*Enterobacterales* and are potential sources of colonization and infection (Chereau et al., 2015). The poor hygiene conditions that usually exist in hospitals in low-income countries, limited access to diagnostic tools, and reduced availability of second line antibiotics may promote the horizontal transmission of multidrug-resistant bacteria (Breurec et al., 2016; Bernabé et al., 2017). Other studies have shown this resistance is widespread on farms where animals are raised with the use of antibiotics as growth promoters (Vounba et al., 2019).

In a previous work, we collected samples from wildlife chimpanzees (*Pan troglodytes verus*) and termites in Senegal (Baron et al., 2021). We isolated enterobacteria carrying multiple antibiotic resistance genes from which we identified international high-risk clones (ST307 and ST147) of carbapenemase-producing *Klebsiella pneumoniae*. The high similarities between plasmids found in isolates from chimpanzees and termites suggest lateral gene exchanges between these two species. In this work, we pursued by exploring antibiotic resistance in other *Enterobacterales* isolated from the same specimens. We completed our study by analyzing another collection of samples isolated from Senegal wildlife [green monkeys (*Chlrocebus sabaeus*), baboons (*Papio papio*), chimpanzees (*Pan troglodytes verus*), and non-fecal environmental samples].

The objective of this work was to determine the level of resistance to antibiotics used in humans in *Enterobacterales* isolated from samples from wildlife in Senegal and to determine the genetic support of this resistance. Interestingly, we identified a transposon in *Escherichia coli* strains, located on a plasmid and in the chromosome. This observation supports the role of wildlife as a reservoir of resistance genes and as a place of exchange.

MATERIALS AND METHODS

Sample Collection

Between 26th and 30th of August 2019, 215 non-human primates (NHPs) fecal samples (110 baboons, 95 green monkeys, and 10 chimpanzees) and 113 non-fecal environmental samples (47 termites, 42 soil samples from termite mounds, and 24 samples including fruits, other plant parts, a centipede and a maggot) were collected in four sites in Senegal. The NHPs fecal samples were collected in three sites located in southern Senegal in the Niokolo-Koba National Park: Simenti, Dar Salam, and Niokolo Poste sites. The sample collectors observed the monkeys all day to collect their fresh stool in real time so that they could be sure that it belonged to that NHPs and not to others. The non-fecal environmental samples, with the exception of the centipede and the maggot, were NHP foods, were collected in one site in south-eastern Senegal, the Dindefelo Community Nature Reserve (12°22′01.4″N, 12°18′00.0″W), which is located on the border with Guinea Conakry about 35 km from the town of Kedougou. The permission to collect these samples was approved by the Direction des Parcs Nationaux and Direction des Eaux, Forêts, Chasse et de la Conservation des Sols of the Senegal Ministry for the Environment and Sustainable Development (002737/DEFCCS/DGF de la Direction des Eaux, Forêts, Chasses et de la Conservation des Sols du 27/06/2019). The monkey cohort consisted of 95 green monkey samples (*Chlrocebus sabaeus*) (Linnaeus, 1766) and 110 Guinea baboon samples (*Papio papio*) (Desmarest, 1820), while the ape cohort consists of 10 chimpanzee samples (*Pan troglodytes verus*)(Schwarz, 1934) (Primates: Hominidae). It should be noted that the morphological and molecular identifications of the non-fecal environmental samples have previously been reported (Baron et al., 2021).

In addition, fecal samples collected in 2015 from Niokolo Koba National Park, Senegal, were added to this study. This cohort included seven Guinea baboon samples and four green monkey samples. Permission to collect samples was granted by the National Parks Direction and Direction des Eaux, Forêts, Chasse et de la Conservation des Sols of the Senegal Ministry for the Environment and Sustainable Development (001914/DEF/DGF de la Direction des Eaux, Forêts, Chasses et de la Conservation des Sols du 05/06/2016). No other permissions were required, as this research was non-invasive and the collection of the samples did not disrupt the wild fauna. Samples were directly stored at +4°C and cultured 2 days after collection.

Sample Culture

Non-human primates samples were aliquoted and enriched in Tryptone Soy Broth (TSB, BioMérieux, Marcy l'Etoile, France) at 37°C for 72 h. Then, 20 μl was then inoculated on antibiotic-containing media: an LBJMR (Lucie Bardet Jean-Marc Rolain) plate (containing 4 μg/ml colistin and 100 μg/ml vancomycin) (Bardet et al., 2017), MacConkey (BioMérieux) + ertapenem (0.5 μg/ml) and MacConkey + cefotaxime (1 μg/ml). The critical concentrations selected were based on the European Committee

on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Version 11.0). Inoculation was followed by incubation at 37°C for 24 h. Colonies that grew on the different media were replicated on Columbia agar + 5% sheep blood (BioMérieux) for further analyses. Bacterial identification was performed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany), as described previously (Seng et al., 2009). Concerning non-fecal environmental samples, cultures were initially inoculated on MacConkey + cefotaxime and MacConkey + ertapenem and described in a previous study (Baron et al., 2021). Briefly, samples were crushed and put in TSB at 37°C for 72 h, then 20 µl were inoculated on selective media. In this study, we also cultured the non-fecal environmental samples on LBJMR media. Unique and common *Enterobacterales* strains of NHPs and the non-fecal environmental samples were visualized by Cytoscape program to show interaction between the two ecosystems (Shannon et al., 2003).

Antibiotic Susceptibility Test

Antibiotic Susceptibility Test (AST) were performed using the disk diffusion method following EUCAST recommendations. In case of resistance observed in disk diffusion method, minimum inhibitory concentrations (MIC) of imipenem and ertapenem were determined using the E-test method (BioMérieux), while colistin MIC was determined using the UMIC microdilution method (Biocentric, Bandol, France). The ESBL profile was detected by the observation of a champagne-cork or a keyhole between a third or fourth generation cephalosporin and clavulanic acid (Drieux et al., 2008). The ß-CARBA test (Biorad, Hercules, CA, United States) was performed to identify the strain with carbapenemase activity.

Genomic and Bioinformatic Analysis of Genomes of Interest

The DNA of enterobacterial isolates was extracted with the BioRobot EZ1 (Qiagen, Courtaboeuf, France) using a commercial EZ1 DNA extraction kit (Qiagen) and quantified by a Qubit assay (Life Technologies, Carlsbad, CA, United States). *De novo* sequencing was performed using MiSeq technology (Illumina Inc., San Diego, CA, United States) in a paired-end strategy. Libraries were prepared using the Nextera XR DNA sample prep kit (Illumina). Briefly, DNAs were fragmented and tagged with adapters and dual-index barcodes. Then, DNAs were purified using the AMPure XP beads (Beckman Coulter, Inc., Fullerton, CA, United States) and pooled in equimolar concentrations. An Illumina-generated PhiX control libraries was added to the libraries that was then 2 × 250 bp paired-end sequenced on an Illumina Miseq. Genomes have been deposited under the Bioproject number PRJNA738374 on the NCBI Database. The three *K. pneumoniae* strains (Q1947, Q1948, and Q1945) described previously (Baron et al., 2021) were deposited under genome accession numbers GCA_903166445.1, GCA_903166485.1, and GCA_903166415.1, respectively. They were then assembled using Spades (Galaxy Version 3.12.0 + galaxy1) (Bankevich et al., 2012), and annotated with Prokka (Galaxy Version 1.14.6 + galaxy1) (Seemann, 2014) using the Galaxy platform.[1] The detection of AMR genes, plasmids and virulence genes was performed with Abricate (Galaxy Version 1.0.1) using Resfinder, Plasmidfinder and VFDB databases, respectively. The sequence type (ST) of the isolates was determined using the pubMLST[2] and Pasteur Institute databases.[3] Pangenome analysis was performed using the Roary software (version 3.13.0) while preserving the default settings (95%: the minimum percentage of identity and 99%: a gene needs to be in to be core). The analysis included 51 genomes of sub-Saharan African strains, isolated from human, animal and the environment, that were available in PATRIC database (version 3.6.12) (accession numbers and genome details are available in **Supplementary Table 1**). We then used Fast tree software (version 2.1.10) to build a maximum likelihood phylogenetic tree with predefined parameters. It was computed using iTOL.[4] A core single nucleotide polymorphism (SNP) was performed using snippy (Galaxy Version 4.6.0 + galaxy0) for the mostly related strains. We sequenced the genomes of two strains, Q2160 (ST224) and Q2170 (ST10), using a MinION sequencer (Oxford Nanopore Technologies Inc., Oxford, United Kingdom) and assembled genomes using both Illumina and MinION reads to reconstitute the IncY plasmid and compare it to the transposon of the Q2170 isolate. Bacteriophages were detected using PHASTER (Arndt et al., 2016). The reconstitution of the genetic environment was performed using the Easyfig program (Sullivan et al., 2011).

Conjugation Experiment

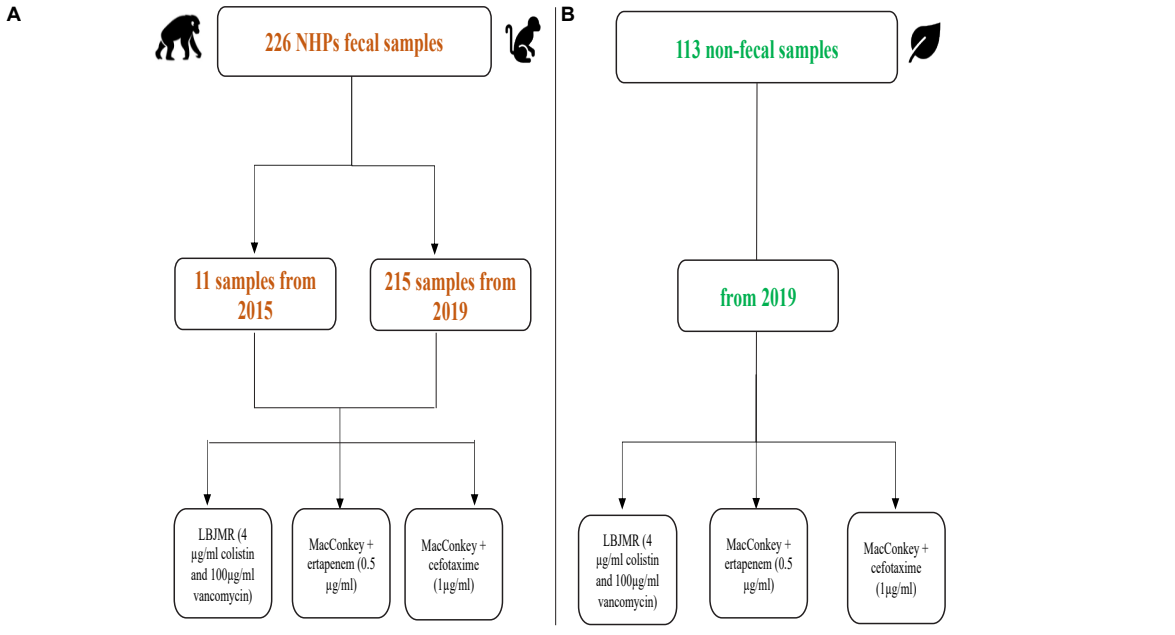
This experiment was done using the Q2160 *E. coli* strain containing the IncY plasmid as a donor strain and an acid-resistant *E. coli* J53 (F- pro Azi r) as the receiver strain. The strains were enriched in TSB for 24 h then mixed in the same tube (1 ml of the donor strain in 9 ml of the receiver strain) for another 24 h. Transconjugants were selected on LB agar, supplemented by 120 µg/mL of sodium acid and 1 µg/mL of cefotaxime (Sennati et al., 2016).

RESULTS

Sample Culture

From the 226 NHP samples, we isolated 617 bacteria including 590 Gram negative bacteria (GNB) and 27 Gram positive bacteria (GPB). The detail of the number of bacterial isolates on the different media is shown in **Supplementary Table 2** and **Figure 1**. Among these GNB, 234 were *Enterobacterales*, of which 29 that are not naturally resistant to the antibiotics in consideration, were studied in this work. Briefly, two of these 29 *Enterobacterales* grew on MacConkey + ertapenem medium: one *E. coli*, and one *Proteus* sp. On MacConkey + cefotaxime media, we isolated 16 *Enterobacterales* (12 *E. coli* and four *Morganella morganii*).

[1]https://usegalaxy.org.au/
[2]https://pubmlst.org/
[3]https://bigsdb.web.pasteur.fr/
[4]https://itol.embl.de/itol.cgi



Positive samples	218/227	215/227	177/227	78/113	63/113	70/113	**TOTAL**
Different colonies	233	276	108	82	60	64	**823**
Total colonies		**617**			**206**		
Isolates of interest	3 *E. coli* 8 *Enterobacter sp.*	1 *E. coli* 1 *P. mirabilis*	12 *E. coli* 4 *M. morganii*	1 *K. pneumoniae* 1 *K. aerogenes* 24 *Enterobacter sp.*	1 *K. aerogenes* 3 *Enterobacter sp.* 1 *C. freundii* 4 *M. morganii* 1 *S. marcescens*	3 *K. pneumoniae* 1 *Enterobacter sp.* 3 *C. freundii*	**17 *E.coli*, 34** *Enterobacter sp.* **1 *P. mirabilis*** **8 *M. morganii*** **4 *K. pneumoniae*** **2 *K. aerogenes*, 4 *C. freundii*** **1 *S. marcescens***
Total isolates	11	2	16	26	10	7	**72**
		29			43		

FIGURE 1 | Summary of positive non-human primates (NHPs) fecal samples **(A)** and non-fecal environmental samples **(B)** cultures on different selective media. Isolates of interest are those that were sequenced.

Finally, on LBJMR (Lucie Bardet Jean-Marc Rolain) medium, we isolated three *E. coli* and eight *Enterobacter* sp. (Three *E. cloacae*, three *E. quasiroggenkampii*, one *E. asburiae*, and one *E. bugandensis*) (**Supplementary Tables 3**, **4**).

From the 113 non-fecal environmental samples, we isolated 206 bacteria including 201 GNB and 5 GPB. The detail of the number of bacterial isolates on the different media is shown in **Supplementary Table 5** and **Figure 1**. Among the GNB, 84 were *Enterobacterales*, of which 43 non-naturally resistant were studied in this work. Briefly, ten *Enterobacterales* grew on MacConkey + ertapenem, including four *M. morganii*, three *Enterobacter sp.* (one *E. quasiroggenkampii*, one *E. cloacae*, and one *E. hormaechei*), one *Citrobacter cronae*, one *Klebsiella aerogenes*, and one *Serratia marcescens*. On MacConkey + cefotaxime, we isolated three *K. pneumoniae*, two *C. cronae*, and two *Enterobacter* sp. (one *E. quasiroggenkampii* and one *E. hormaechei*). Finally, on LBJMR, 25 *Enterobacter sp.* (nine *E. cloacae*, eight *E. quasiroggenkampii*, four *E. bugandensis*, two *E. roggenkampii*, and two *E. sichuanensis*), one *K. aerogenes* and one *K. pneumoniae* were isolated. Identical strains from the same origin are reported once. It should be noted that culture results from non-fecal environmental samples on MacConkey + ertapenem, MacConkey + cefotaxime, and MacConkey media alone have previously been described (Baron et al., 2021), but the strains of interest were sequenced for this study. Specific and common species between samples were represented in **Figure 2**.

Antibiotic Susceptibility Test

A total of 72 *Enterobacterales* were isolated from NHPs and non-fecal environmental samples. We confirmed the phenotypic resistance of these isolates by antibiotic susceptibility tests (AST) (**Supplementary Table 4**). Sixteen isolates were confirmed to be resistant to cefotaxime (N = 16/339 samples, 5%), two resistant to ertapenem (N = 2/340 samples, 0.6%), and 37 resistant to colistin (N = 37/339 samples, 11%). The ß-carba-test was negative for the two ertapenem-resistant isolates. These antibiotic-resistant isolates were isolated from different NHP

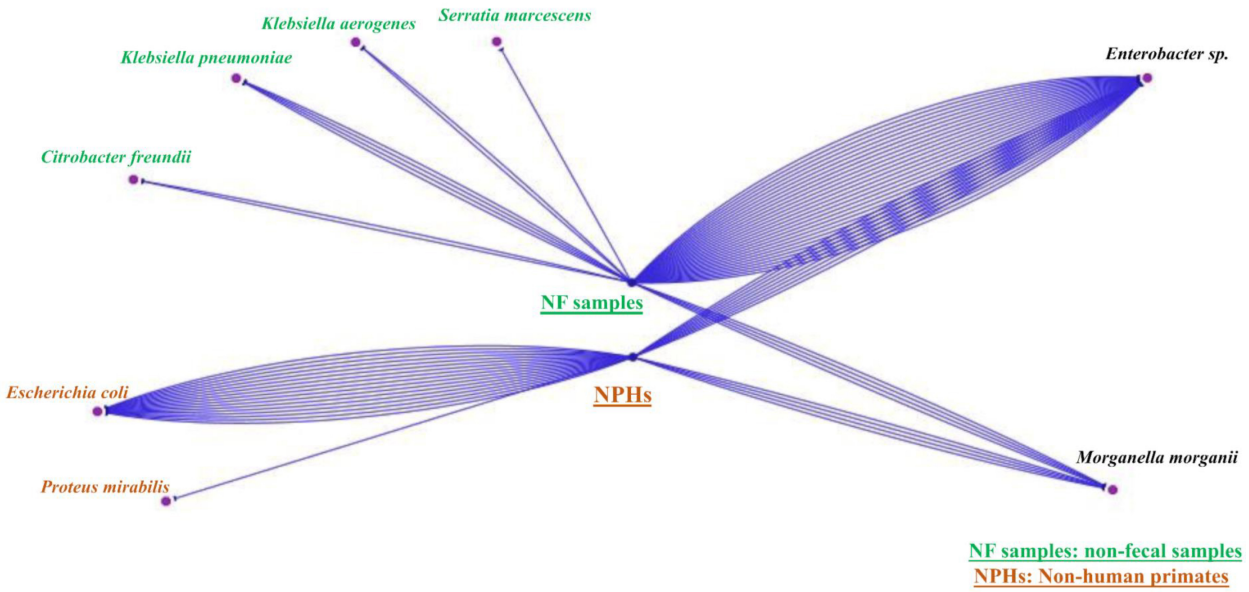


FIGURE 2 | Simple view of the Cytoscape visualizing unique and common species of the two samples' types (NHPs and non-feacal samples). The blue lines represent the number of strains found each type. There are strains that belong just to non-fecal environmental samples, others that belong just to NHPs and strains that can be found in both of them.

species and the environment. In chimpanzees, we isolated one ESBL-producing *E. coli* and six colistin-resistant *Enterobacter* sp. In green monkeys (*Chlorocebus sabaeus*), we identified seven *E. coli* and one *Morganella morganii* which produced ESBL, and one colistin-resistant *Enterobacter cloacae*. Four ESBL-producing and two colistin-resistant *E. coli* as well as one *Enterobacter asburiae* were found in Guinea baboons (*Papio papio*). From the non-fecal environmental samples, we isolated two ertapenem-resistant *M. morganii*, *27* colistin-resistant *Enterobacterales* including 25 *Enterobacter* sp., one *K. pneumoniae* and one *K. aerogenes* and three ESBL-producing *K. pneumoniae*.

Of these 72 *Enterobacterales*, 23 had a multidrug resistant profile (MDR) (Magiorakos et al., 2012) and were resistant to four or more antibiotic families, 46 to fewer than four antibiotic families, and three had no resistance. Resistance to third generation cephalosporins (3GC) was 29% (N = 21/72) whereas colistin resistance represented 65% (47/72) of the total strains. 3GC-resistant isolates, had co-resistance with tetracycline family (52%, N = 11/21) (doxycycline), fluoroquinolone (43%, N = 9) (ciprofloxacin) and trimethoprim-sulfamethoxazole (62%, N = 13). Resistance to amoxicillin was found in 75% of the *E. coli* strains (N = 12/16).

Population Analysis of Bacterial Species

We sequenced the genome of the 72 *Enterobacterales* and studied clonal population and AMR genes. Details of the assembly results are provided in **Supplementary Table 4**. The population distribution of *Enterobacterales* was mainly represented by new sequence types (STs), indicating the existence of a population specific to the ecosystem analyzed but we found clonal complexes that are associated with human infections for *E. coli* and *K. pneumoniae*. Of the 16 *E. coli*, six belonged to ST10, two to ST224, and nine to other unique STs (ST212, ST202, ST196, ST469, ST2803, ST642, ST10648, ST3580, and ST6611). For *K. pneumoniae*, three belonged to ST307 and had previously been described (Baron et al., 2021), but one had a new ST5460. Of the 37 *Enterobacter sp*. sequenced, only three strains belonged to a known ST (ST1084- Q3805, ST113- Q2141, and ST565-Q2153), while the remaining 34 isolates belonged to 29 new different STs. Interestingly, one group was containing strains belonged to the chimpanzee and non-fecal environmental samples. It consisted of 11 STs (ST1545, ST1547, ST1548, ST1549, ST1552, ST1554, ST1563, ST1565, ST1566, ST1567, and ST1570) combining strains of the environment origin (N = 7) and strains from chimpanzees (N = 4). Finally, two *K. aerogenes* and two *C. cronae* from the non-fecal environmental samples belonged to new STs ST225 and ST226 for *K. aerogenes* and ST574 and ST575 for *C. cronae* (**Supplementary Table 4**).

Antimicrobial Resistant Gene Circulation in Antibiotic-Resistant Isolates

We noticed that 53 of the 72 *Enterobacterales* had four or more resistance genes, some had the same association of genes, such as *E. coli* and *K. pneumoniae* (**Supplementary Table 4** and **Table 1**). We found a CTX-M-15 type β-lactamases in 12 *E. coli* from NHPs, three ST307 *K. pneumoniae* isolated from the environment (**Figure 3**). Four of the 12 *E. coli* (2 ST224, 1 ST202, and ST469) harbored an *IncY* plasmid of 91,959 bp length (GC% = 52.06%) carrying resistance genes to beta-lactam ($bla_{CTX-M-15}$, bla_{TEM1B}), sulfonamide (*sul2*, *dfrA14*), fluroquinolones (*qnrs*), tetracycline (*tetA*, *tetR*) and aminosides [*aph*(3″), *aph*(6)]. The plasmid IncY was non

TABLE 1 | Summary of antibiotic resistance genes in isolates of interest.

Species	Strain number	Samples	Sample origin	MLST	Antibiotic resistance genes
Escherichia coli	Q2154	SV 002	Green monkeys	ST469	*mdf(A)*, *sul2*, *aph (3'')-Ib*, *aph(6)-Id*, *blaTEM-1B*, *tet(A)*, *dfrA14*, *blaCTX-M-15*, and *qnrS1*
Escherichia coli	Q2155	SV 003	Green monkeys	ST3580	*aac(6')-Ib-cr*, *blaCTX-M-15*, *qnrS1*, *mdf(A)*, *dfrA17*, *aadA5*, and *mph(A)*
Escherichia coli	Q2156	SV 005	Green monkeys	ST10	*qnrS1*, *blaCTX-M-15*, *blaTEM-1B*, *mdf(A)*, *sul2*, *aph(3'')-Ib*, *aph(6)-Id*, *tet(A)*, and *dfrA14*
Escherichia coli	Q2157	SV 006	Green monkeys	ST10	*tet(A)*, *dfrA14*, *mdf(A)*, *blaTEM-1B*, *blaCTX-M-15*, *qnrS1*, *aph(6)-Id*, *aph(3'')-Ib*, and *sul2*
Escherichia coli	Q2160	SV 054	Green monkeys	ST224	*mdf(A)*, *tet(A)*, *sul2*, *aph(3'')-Ib*, *aph(6)-Id*, *blaTEM-1B*, *blaCTX-M-15*, *qnrS1*, and *dfrA14*
Escherichia coli	Q2170	SV 066	Green monkeys	ST10	*mdf(A)*, *sul2*, *aph(3'')-Ib*, *aph(6)-Id*, *tet(A)*, *blaTEM-1B*, *blaCTX-M-15*, *qnrS1*, and *dfrA14*
Escherichia coli	Q2161	SV 069	Green monkeys	ST224	*mdf(A)*, *sul2*, *aph(3'')-Ib*, *aph(6)-Id*, *blaTEM-1B*, *blaCTX-M-15*, *qnrS1*, *tet(A)*, and *dfrA14*
Escherichia coli	Q0179	MFB 3	Baboons	ST2803	*mdf(A)*
Escherichia coli	Q2163	BG 007	Baboons	ST202	*mdf(A)*, *qnrS1*, *blaCTX-M-15*, *blaTEM-1B*, *aph(6)-Id*, *aph(3'')-Ib*, *sul2*, *tet(A)*, and *dfrA14*
Escherichia coli	Q3820	BG 034	Baboons	ST6611	*ant(3'')-Ia*, *tet(A)*, and *mdf(A)*
Escherichia coli	Q3819	BG 046	Baboons	ST196	*mdf(A)*
Escherichia coli	Q2164	BG 049	Baboons	ST10	*mdf(A)*, *tet(A)*, *qnrS1*, and *blaCTX-M-15*
Escherichia coli	Q3830	BG 082	Baboons	ST10648	*mdf(A)*
Escherichia coli	Q2165	BG 085-A	Baboons	ST10	*mdf(A)*, *sul2*, *aph(3'')-Ib*, *aph(6)-Id*, *tet(A)*, *blaTEM-1B*, *blaCTX-M-15*, *qnrS1*, and *dfrA14*
Escherichia coli	Q3824	BG 104	Baboons	ST642	*mdf(A)*
Escherichia coli	Q3821	CH 002	Chimpanzees	ST202	*qnrS1*, *blaCTX-M-15*, and *mdf(A)*
Morganella morganii	Q2158	SV 017	Green monkeys	–	*blaMOR-2*, *catA2*, and *tet(D)*
Morganella morganii	Q2159	SV 028	Green monkeys	–	*blaMOR-2*
Morganella morganii	Q2169	SV 054	Green monkeys	–	*catA2* and *blaDHA-12*
Morganella morganii	Q2162	SV 094	Green monkeys	–	*blaDHA-12*, *tet(D)*, and *catA2*
Morganella morganii	Q2142	NCS 002B	Plant	–	*blaDHA-12* and *catA2*
Morganella morganii	Q2143	NCS 002C	Plant	–	*catA2*, *blaDHA-12*
Morganella morganii	Q2144	NCS 003A	Plant	–	*blaDHA-12*, *catA2*
Morganella morganii	Q2147	NCS 010	Plant	–	*blaDHA-12* and *catA2*
Proteus mirabilis	Q3812	SV 035	Green monkeys	–	*tet(J)* and *cat*
Serratia ureilytica	Q2145	NCS 006A	Termites	–	*aac(6')-Ic*, *blaSST-1*, *oqxB*, and *tet(41)*
Citrobacter cronae	Q2146	NCS 009	Fruit	ST574	*qnrB12* and *blaCMY-98*
Citrobacter cronae	Q2150	NCS 033	Termites	ST575	*qnrB12* and *blaCMY-98*
Citrobacter cronae	Q2168	NCS 037 (1)	Termites	ST575	*qnrB34* and *blaCMY-98*
Enterobacter asburiae	Q0180	MFB 6	Baboons	ST1543	*fosA*, *oqxB*, *oqxA*, and *blaACT-6*
Enterobacter cloacae	Q0181	AGM 2	Green monkeys	ST1544	*fosA*, *oqxB*, *oqxA*, and *blaCMH-3*
Enterobacter quasiroggenkampii	Q3813	CH 001	Chimpanzees	ST1545	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter cloacae	Q3814	CH 002	Chimpanzees	ST1546	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*
Enterobacter quasiroggenkampii	Q3810	CH 003	Chimpanzees	ST1547	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter cloacae	Q3804	CH 005	Chimpanzees	ST1548	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter bugandensis	Q3805	CH 009	Chimpanzees	ST1084	*oqxB*, *oqxA*, *fosA*, and *blaACT-6*
Enterobacter quasiroggenkampii	Q3803	CH 010	Chimpanzees	ST1549	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter hormaechei	Q2141	NCS 001	Fruit	ST113	*blaTEM-1B*, *aph(6)-Id*, *aph(3'')-Ib*, *oqxB*, *oqxA*, *fosA*, *blaACT-15*, *sul2*, *dfrA14*, and *tet(D)*
Enterobacter bugandensis	Q3822	NCS 005	Fruit	ST1550	*blaACT-6*, *oqxA*, *oqxB*, and *fosA*
Enterobacter cloacae	Q3807	NCS 006B	Termite mounds	ST1551	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*
Enterobacter quasiroggenkampii	Q2148	NCS 013B	Termite mounds	ST1552	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter cloacae	Q3806	NCS 018B	Termites	ST1553	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*
Enterobacter quasiroggenkampii	Q3808	NCS 019B	Termite mounds	ST1554	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter cloacae	Q3794	NCS 022A	Fruit	ST1570	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter cloacae	Q3796	NCS 023	Fruit	ST1555	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*

(Continued)

TABLE 1 | (Continued)

Species	Strain number	Samples	Sample origin	MLST	Antibiotic resistance genes
Enterobacter cloacae	Q3818	NCS 24A	Fruit	ST1556	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*
Enterobacter cloacae	Q3831	NCS 027C	Termite mounds	ST1557	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*
Enterobacter sichuanensis	Q3826	NCS 028B	Termites	ST1558	*oqxA*, *oqxB*, *fosA*, and *blaACT-6*
Enterobacter roggenkampii	Q3823	NCS 030C	Termite mounds	ST1559	*oqxA*, *oqxB*, *fosA*, and *blaMIR-1*
Enterobacter cloacae	Q3809	NCS 031A	Termites	ST1565	*fosA*, *blaCMG*[1], *oqxA*, and *oqxB*
Enterobacter quasiroggenkampii	Q3802	NCS 034A	Termites	ST1554	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter quasiroggenkampii	Q3827	NCS 034B	Termite mounds	ST1554	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter bugandensis	Q3815	NCS 035A	Termites	ST1561	*oqxA*, *oqxB*, *fosA*, and *blaACT-6*
Enterobacter sichuanensis	Q3801	NCS 036A	Termite mounds	ST1562	*oqxA*, *oqxB*, *fosA*, and *blaACT-6*
Enterobacter quasiroggenkampii	Q2151	NCS 037A	Termites	ST1563	*oqxA*, *oqxB*, *fosA*, and *blaACT-6*
Enterobacter cloacae	Q2152	NCS 039B	Termite mounds	ST1564	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*
Enterobacter quasiroggenkampii	Q3799	NCS 040 B	Termite mounds	ST1554	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter quasiroggenkampii	Q3798	NCS 045C	Termite mounds	ST1566	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter quasiroggenkampii	Q3817	NCS 046B	Termite mounds	ST1567	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter bugandensis	Q3825	NCS 049A	Termites	ST1568	*oqxA*, *oqxB*, *fosA*, and *blaACT-6*
Enterobacter cloacae	Q3795	NCS 050D	Termite mounds	ST1569	*oqxA*, *oqxB*, *fosA*, and *blaCMH-3*
Enterobacter hormaechei	Q2153	NCS 050 (19)	Termites	ST565	*oqxA*, *oqxB*, *fosA*, and *blaACT-16*
Enterobacter quasiroggenkampii	Q3828	NCS 051A	Termites	ST1570	*oqxA*, *oqxB*, *fosA*, and *blaCMG*[1]
Enterobacter roggenkampii	Q3829	NCS 053A	Termites	ST1571	*oqxA*, *oqxB*, *fosA*, and *blaMIR-2*
Enterobacter bugandensis	Q3800	NCS 053B	Termites	ST1572	*oqxA*, *oqxB*, *fosA*, and *blaACT-6*
Klebsiella aerogenes	Q1946	NCS 028A	Termites	ST225	*oqxA*, *oqxB*, and *fosA*
Klebsiella aerogenes	Q2149	NCS 033A	Termites	ST226	*fosA*, *oqxA*, and *oqxB*
Klebsiella quasivariicola	Q2548	NCS 42B	Termite mounds	ST5460	*oqxA*, *oqxB*, *fosA*, and *blaLEN26*
Klebsiella pneumoniae	Q1947	NCS 043A	Termite mounds	ST307	*blaTEM-1B*, *blaSHV-106*, *blaOXA-1*, *blaCTX-M-15*, *aac(3)-IIa*, *aac(6')Ib-cr*, *aac(6')Ib-cr*, *qnrB1*, *dfrA14*, *sul2*, and *tet(A)*
Klebsiella pneumoniae	Q1948	NCS 043B	Termite mounds	ST307	*blaTEM-1B*, *blaSHV-106*, *blaOXA-1*, *blaCTX-M-15*, *aac(3)-Iia*, *aac(6')Ib-cr*, *aac(6')Ib-cr*, *qnrB1*, *dfrA14*, *sul2*, and *tet(A)*
Klebsiella pneumoniae	Q1945	NCS 045A	Termites	ST307	*blaTEM-1B*, *blaSHV-106*, *blaOXA-1*, *blaCTX-M-15*, *aac(3)-Iia*, *aac(6')Ib-cr*, *aac(6')Ib-cr*, *qnrB1*, *dfrA14*,*sul2*, and *tet(A)*

[1] *These sequences have 99.62% identity with bla_{CMG} (accession number AY266892) and 98.34% with bla_{ACT-62} (MH469270.1).*

conjugative, did not have a complete conjugative apparatus, and conjugation to an *E. coli* J53 failed on three attempts. It was found in three green monkey and one baboon samples (**Figure 3**). A Tn2 transposon of 30,340 bp (GC% = 52.05%) containing the same AMR genes found on the IncY plasmid [$bla_{CTX-M-15}$, bla_{TEM1B}, *sul2*, *dfrA14*, *qnrs1*, *aph*(3″), *aph*(6), *tetA*, and *tetR*] was observed in the chromosome of four of the six ST10 isolates (Q2156, Q2170, Q2165, and Q2157). These four strains differed from 1 to 14 SNPs in their core genome suggesting they had the same origin (**Figure 3**). Interestingly, this transposon was similar to that present in the plasmid IncY (**Figure 4**) but was inserted in a SfII-like prophage (KC736978) of 35.5 bp (GC% = 50.23%) in the chromosome of the ST10 isolate (Q2170). On the 5′–3′ locus, an insertion sequence (IS)26 inserted in an integrase (INT) of the phage, causing a deletion of 8 bp in the integrase sequence, shifting the reading frame and leading to the synthesis of 187 amino acid residues of the integrase protein against 216 amino acid residues in the reference phage. On the 3′–5′ locus, the IS26 inserted before the tail fiber assembly protein (TfaE), leading to the truncation of a hypothetical protein located between the integrase (INT) and the TfaE protein. Interestingly, the intact SFII-like prophage was found in a β-lactamases susceptible *E. coli* isolate (Q3820) that was only resistant to colistin (**Figure 4**). Finally, the four other *E. coli* isolates carrying CTX-M-15 enzymes belonged to different STs [two ST10 (Q2157, Q2164), one ST3580 (Q2155), and one ST212 (Q3821)], had no IncY plasmid and did not have the same transposon. Three of them carried one to five plasmids (IncF, Col, and IncB plasmids, details in **Supplementary Table 4**), but the location of $bla_{CTX-M-15}$ on the plasmids could not be identified in these genomes. The conjugation experiment showed negative results as no growth has been detected for a J53 *E. coli* receiving the cefotaxime resistant gene.

Regarding other *Enterobacterales*, three *C. cronae* genomes harbored a fluroquinolone-resistance gene (*qnrB12*) as well as the chromosomal beta-lactamase $bla_{CMY\ -98_1.}$ In the 37 *Enterobacter* spp., we identified four different chromosomal *ampC* genes, namely $bla_{CMH-3-1}$ (N = 10), $bla_{ACT-6-1}$ (N = 10), bla_{CMG-1} (N = 14), and bla_{MIR-1} (N = 2). None of these

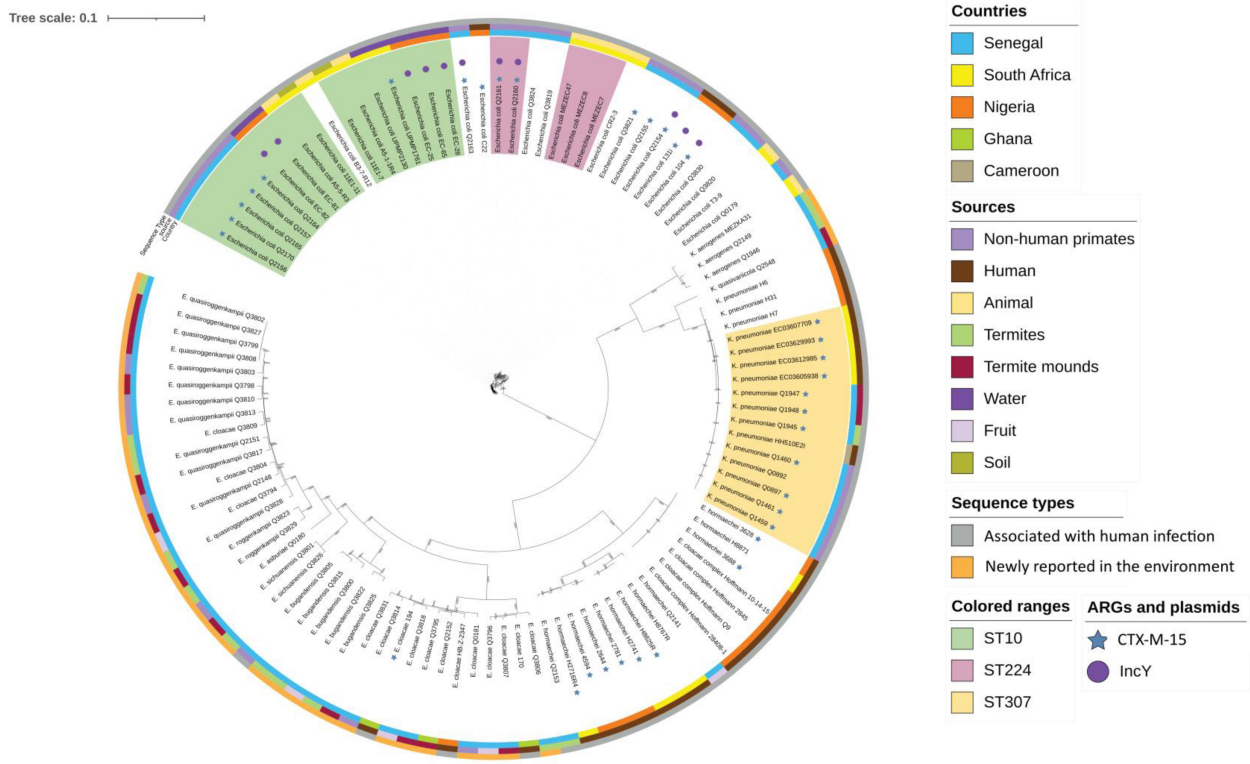


FIGURE 3 | Maximum likelihood phylogenetic tree representation of the 9 *Klebsiella* spp., 16 *E. coli*, and 37 *Enterobacter* spp. strains compared with strains from different origin of the Sub-Saharan region. The tree was generated and annotated with the iTOL tool (https://itol.embl.de/tree/).

strains had an inducible expression of their AmpC β-lactamase with a phenotypic resistance to 3 GC. In the eight *M. morganii* genomes isolated from green monkeys and non-fecal environmental samples, we found the *ampC* β-lactamase (bla_{MOR-2}, bla_{DHA12}), conferring resistance to 3 GC. The summary of antibiotic resistance genes in these strains is presented in **Table 1**. The bla_{CMG} gene shows 98.34% similarity with bla_{ACT-62} suggesting that they could be allelic variants of the same AmpC gene.

Genome Comparison With Published Bacterial Genomes From Sub-Saharan Africa From Human, Animal, and Environmental Origins

We compared the genomes of *E. coli*, *K. pneumonia*, and *Enterobacter* spp. isolated in our work with 51 genomes from different origin isolated in sub-Saharan Africa (**Figure 3**). *Enterobacter* isolates clustered together, far from the human isolates which were consistent with the ST results. *E. coli* and *K. pneumoniae* isolates were more related with human isolates but their core genome differed by more than 20,000 SNPs suggesting there were not directly related. We have also included in this analysis two strains of *E. coli* (131i and 104) that were isolated from animals use for human consumption in Nigeria (Sharma et al., 2020) that were interesting because they carried an IncY plasmid, the antibiotic resistance genes included in the transposon [$bla_{CTX-M-15}$, bla_{TEM1B}, *sul2*, *dfrA14*, *qnrs1*, *aph*(3″), *aph*(6), *tetA* and *tetR*] and a SfII-like prophage. However, these isolates did not cluster with isolates that contained the transposon (Q2154, Q2156, Q2160, Q2161, Q2163, Q2165, Q2157, and Q2170). Unfortunately, we were not able to find the antibiotic resistance genes location in these two isolates.

DISCUSSION

Microbial diversity in natural environments is considered to be the largest unexplored reservoir of biodiversity on earth. Many studies have described bacterial diversity in primates, but few of them have focused on their genomic characteristics. This explains the large number of new STs of *Enterobacter* sp., *C. cronae*, and *K. aerogenes* found in our study. *E. coli* [ST10 (Mohsin et al., 2017) and ST224 (Cao et al., 2014)] and *K. pneumoniae* [ST307 and ST147 (Peirano et al., 2020)] found in our previous study (Baron et al., 2021) are reported to cause human infections. Although our newly described STs have not yet been found in humans and transmission cannot be excluded.

Interestingly, in our study, 11 green monkeys, three baboons and one chimpanzee carried an *Enterobacterales* with a MDR profile, often an ESBL profile. This MDR profile was also found in the *Enterobacterales* of nine non-fecal environmental samples

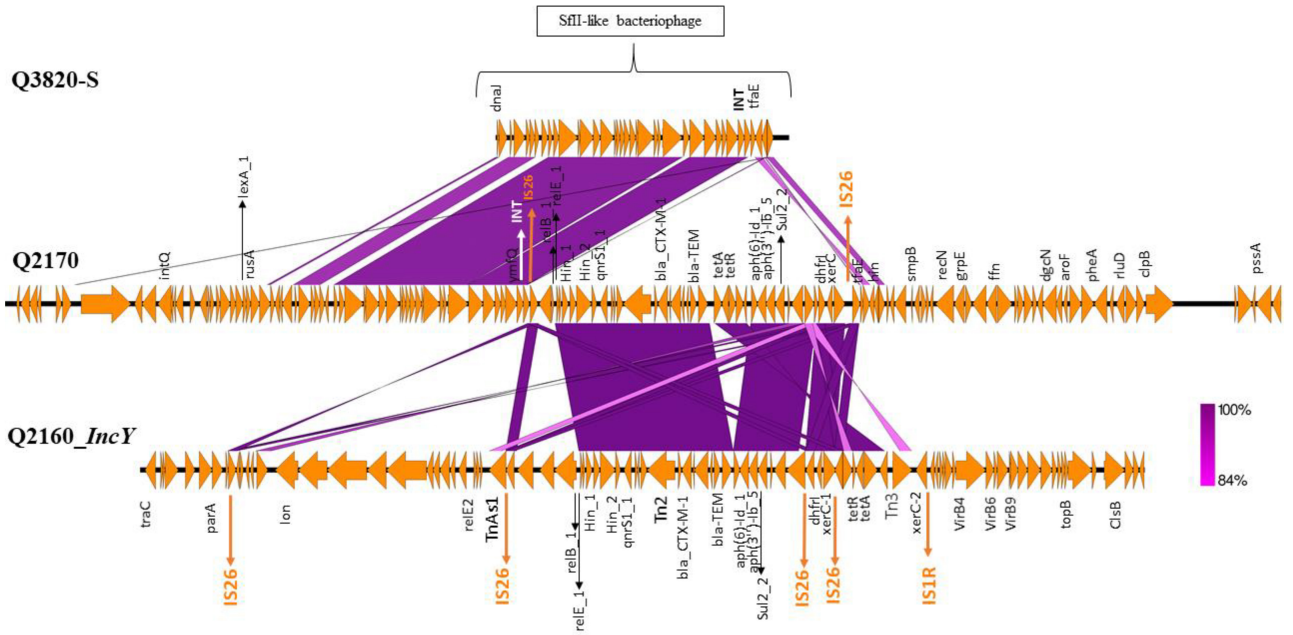


FIGURE 4 | Genomic comparison of the SFII-like bacteriophage region of the 3GC-susceptible *Escherichia coli* strain (Q3820), the transposon integrated in the SFII-like prophage region of the ST10 3GC-resistant *E. coli*(Q2170) and the IncY plasmid sequence harboring the same transposon of the ST224 *E. coli* (Q2160). This comparison shows the putative transfer of resistance genes between these two strains. The figure was made using the Easyfig program.

showing resistance to 3GC, tetracycline and fluoroquinolones. Moreover, the rate of resistance was relatively high for a cohort far from massive antibiotic exposure, especially for colistin but also for 3GC (29%, N = 21/72). Moreover, 18% (N = 13/72) of the isolated strains were resistant to tetracycline and 18% (N = 13/72) were resistant to sulphonamides. According to the WHO report published in 2014 highlighting resistance levels to the most widely-used antibiotics, fluoroquinolones and 3GC antibiotics are known to be less used in Africa, where the resistance rate is lower than in Europe (Bassoum et al., 2019). Our results show that 13% (N = 9/72) of the isolates were fluoroquinolone-resistant and 29% (21/72) were C3G-resistant. These phenotypic results were confirmed genotypically, where AMR genes were found to be consistent with isolate antibiotic resistance. 3GC-resistance is often co-presented in this study with tetracycline, fluoroquinolone and sulphonamide resistance, conferring a MDR profile for the strain, which was demonstrated in the IncY plasmid that carries all the AMR genes conferring resistance to these antibiotics at the same time. In contrast, mobile colistin resistance (*mcr*) genes were not found and further analysis will be needed in order to decipher the resistance mechanisms of *Enterobacter* sp.

The IncY plasmid has been found in the environment (Ma et al., 2021), including in water samples (Moremi et al., 2016) and in animals such as camels in Tunisia (Saidani et al., 2019). Although it could be mobilizable *in silico* because of the current flanking sequence surrounding this DNA element, the conjugation experimental attempt to mobilize it has failed. This plasmid is often associated with ESBL-resistance genes, especially $bla_{CTX-M-15}$ (Rasheed et al., 2020). Here, AMR genes clustered in this plasmid within a transposon [$bla_{CTX-M-15}$, bla_{TEM1B}, *sul2*, *dfrA14*, *qnrs1_1*, *aph*(3′′), *aph*(6), *tetA*, and *tetR*] in four *E. coli* strains, and were surrounded by IS26 from each side. Interestingly, we showed that this transposon was inserted in a SfII-like phage on the chromosome of a ST10 isolate (**Figure 4**) leading to nucleotides deletion that modified the prophage integrase (INT) amino-acid sequence from a side and truncated a hypothetical protein from the other side. Phages are believed to play a critical role in the horizontal transfer of bacterial genes, including antibiotic resistance genes (Kondo et al., 2021). The *SfII* phage is known to play a role in virulence spread (Mavris et al., 1997). This finding demonstrates that intermediate steps between transposons and the genomes of the phage can also be found and the recombination site of a prophage is vulnerable and can receive mobile elements (Brown-Jaque et al., 2015). Some studies have shown the integration of the transposon next to the recombination site of a phage (Huang et al., 2017) but none has shown the insertion of the transposon inside the phage to our knowledge. We were interested in the two *E. coli* strains that were isolated from poultry in Nigeria (Sharma et al., 2020) and contained the same phage, plasmid and antibiotic resistance genes that our *E. coli* isolates but we were unable to verify if they have the same transposon and its location. These findings, however, suggest a circulation of these mobile elements in bacteria from animals in Africa.

Antimicrobial resistant in humans is a current issue linked to the overuse of antibiotics in clinical, veterinary and agricultural practices, which has been considered to be the main selective pressure for AMR strains and AMR gene dissemination since the 1950s (Thaller et al., 2010). However, the literature is rich in cases

reporting the emergence of AMR in the absence of antibiotic use, as is the case for colistin resistance (Olaitan et al., 2016). Other factors, such as anthropological and socioeconomic factors (Collignon et al., 2018) and cross-resistance with other antibiotics may be involved (Napier et al., 2013). The "One Health" approach has emerged several years ago (Thamlikitkul et al., 2015), and puts the issue of AMR in a more global context. This approach has already been well studied for farm animals but remains poorly studied for wild animals (Diallo et al., 2020). The study of NHPs is even more interesting since they are phylogenetically close to humans and may be colonized or infected by species that are close or identical to those that are pathogenic to humans (Wolfe et al., 1998). Since NHPs live in areas with different protection status and human impact, they are a good model for studying the phenomenon of AMR, especially since they also live in groups and display behaviors similar to our own.

CONCLUSION

Our study shows that AMR can be found in wildlife and that this emergence, in places where human selection and pressure is reduced, is a public health concern. The wild environment is not well studied and hosts a large, undescribed microbial diversity. Transfer of several AMR genes via transposon in plasmids and via integrated prophages should be further explored to understand this dynamic in natural environments.

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 animal study was reviewed and approved by the Direction des Parcs Nationaux and Direction des Eaux, Forêts, Chasse et de la Conservation des Sols of the Senegal Ministry for the Environment and Sustainable Development.

AUTHOR CONTRIBUTIONS

SB, J-MR, and DR designed the study, drafted, and revised the manuscript. OM, BD, AB, GD, and CS collected the samples. RA drafted the manuscript. RA, EK, LH, and AI performed microbiology analyses. RH-A facilitated sample collection and revised the manuscript. All authors have read and approved the final manuscript.

FUNDING

This work was supported by the French Government under the "Investissements d'Avenir" (Investments for the Future) programme managed by the Agence Nationale de la Recherche (ANR, fr: National Agency for Research), (reference: Méditerranée Infection 10-IAHU-03) and by the IHU Mediterranean Foundation.

ACKNOWLEDGMENTS

We would like to thank Mouna Hamel for her committed assistance in the laboratory. We are grateful to the Direction des Parcs Nationaux and Direction des Eaux, Forêts, Chasse et de la Conservation des Sols of the Senegal Ministry for the Environment and Sustainable Development for their support. We also thank the MLST and Pasteur Institute databases for helping us submit the new ST profiles. We thank Mamadou Samba Sylla from the Jane Goodall Institute Spain and Senegal for help with sample collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.838392/full#supplementary-material

Supplementary Tables 1 | List of reference bacterial genomes used for phylogenetic comparison (**Figure 3**).

Supplementary Table 2 | List of bacterial isolates in NHPs fecal samples on different media.

Supplementary Table 3 | Summary of the *Enterobacterales* isolated on selective media (Source, date and localisation were presented).

Supplementary Table 4 | Characteristics summary of *Enterobacterales* isolated on selective media. Antibiotic susceptibility tests to provide resistance profile and genomic analysis results (antibiotic resistance genes, plasmid and virulence genes as well as allelic profile) were presented.

Supplementary Table 5 | List of bacterial isolates contained in non-fecal environmental samples on different media.

REFERENCES

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bardet, L., Le Page, S., Leangapichart, T., and Rolain, J.-M. (2017). LBJMR medium: a new polyvalent culture medium for isolating and selecting vancomycin and colistin-resistant bacteria. *BMC Microbiol.* 17:220. doi: 10.1186/s12866-017-1128-x

Baron, S. A., Mediannikov, O., Abdallah, R., Kuete Yimagou, E., Medkour, H., Dubourg, G., et al. (2021). Multidrug-resistant *Klebsiella pneumoniae* clones from wild chimpanzees and termites in senegal. *Antimicrob. Agents Chemother.* 65:e0255720. doi: 10.1128/AAC.02557-20

Bassoum, O., Lèye, M. M. M., Sougou, N. M., Diongue, M., Niang, K., Tine, J. A. D., et al. (2019). Practices about antibiotic use among urban residents: a cross-sectional survey in rufisque, Senegal. *Central Afr. J. Public Health* 5:1. doi: 10.11648/j.cajph.20190501.11

Bernabé, K. J., Langendorf, C., Ford, N., Ronat, J.-B., and Murphy, R. A. (2017). Antimicrobial resistance in West Africa: a systematic review and meta-analysis. *Int. J. Antimicrob. Agents* 50, 629–639. doi: 10.1016/j.ijantimicag.2017.07.002

Breurec, S., Bouchiat, C., Sire, J.-M., Moquet, O., Bercion, R., Cisse, M. F., et al. (2016). High third-generation cephalosporin resistant *Enterobacteriaceae* prevalence rate among neonatal infections in Dakar, Senegal. *BMC Infect. Dis.* 16:587. doi: 10.1186/s12879-016-1935-y

Brown-Jaque, M., Calero-Cáceres, W., and Muniesa, M. (2015). Transfer of antibiotic-resistance genes via phage-related mobile elements. *Plasmid* 79, 1–7. doi: 10.1016/j.plasmid.2015.01.001

Cao, X., Zhang, Z., Shen, H., Ning, M., Chen, J., Wei, H., et al. (2014). Genotypic characteristics of multidrug-resistant *Escherichia coli* isolates associated with urinary tract infections. *APMIS* 122, 1088–1095. doi: 10.1111/apm.12260

Chereau, F., Herindrainy, P., Garin, B., Huynh, B.-T., Randrianirina, F., Padget, M., et al. (2015). Colonization of extended-spectrum-β-lactamase- and NDM-1-Producing *Enterobacteriaceae* among Pregnant Women in the Community in a Low-Income Country: a potential reservoir for transmission of multiresistant *Enterobacteriaceae* to Neonates. *Antimicrob. Agents Chemother.* 59, 3652–3655. doi: 10.1128/AAC.00029-15

Chokshi, A., Sifri, Z., Cennimo, D., and Horng, H. (2019). Global contributors to antibiotic resistance. *J. Glob. Infect. Dis.* 11, 36–42. doi: 10.4103/jgid.jgid_110_18

Collignon, P., Beggs, J. J., Walsh, T. R., Gandra, S., and Laxminarayan, R. (2018). Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis. *Lancet Planet. Health* 2:e398–e405. doi: 10.1016/S2542-5196(18)30186-4

Diallo, O. O., Baron, S. A., Abat, C., Colson, P., Chaudet, H., and Rolain, J.-M. (2020). Antibiotic resistance surveillance systems: a review. *J. Glob. Antimicrob. Resist.* 23, 430–438. doi: 10.1016/j.jgar.2020.10.009

Drieux, L., Brossier, F., Sougakoff, W., and Jarlier, V. (2008). Phenotypic detection of extended-spectrum β-lactamase production in *Enterobacteriaceae*: review and bench guide. *Clin. Microbiol. Infect.* 14, 90–103. doi: 10.1111/j.1469-0691.2007.01846.x

Dromigny, J. A., Nabeth, P., Juergens-Behr, A., and Perrier-Gros-Claude, J. D. (2005). Risk factors for antibiotic-resistant *Escherichia coli* isolated from community-acquired urinary tract infections in Dakar, Senegal. *J. Antimicrob. Chemother.* 56, 236–239. doi: 10.1093/jac/dki158

Huang, W., Wang, G., Sebra, R., Zhuge, J., Yin, C., Aguero-Rosenfeld, M. E., et al. (2017). Emergence and Evolution of multidrug-resistant *Klebsiella pneumoniae* with both blaKPC and blaCTX-M Integrated in the Chromosome. *J. Antimicrob. Chemother.* 61:e00076-17. doi: 10.1128/AAC.00076-17

Kondo, K., Kawano, M., and Sugai, M. (2021). Distribution of antimicrobial resistance and virulence genes within the prophage-associated regions in nosocomial pathogens. *mSphere* 6:e00452-21. doi: 10.1128/mSphere.00452-21

Ma, Y., Chen, J., Fong, K., Nadya, S., Allen, K., Laing, C., et al. (2021). Antibiotic Resistance in Shiga Toxigenic *Escherichia coli* Isolates from Surface Waters and Sediments in a Mixed Use Urban Agricultural Landscape. *Antibiotics (Basel)* 10:237. doi: 10.3390/antibiotics10030237

Magiorakos, A.-P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Mavris, M., Manning, P. A., and Morona, R. (1997). Mechanism of bacteriophage SfII-mediated serotype conversion in *Shigella flexneri*. *Mol. Microbiol.* 26, 939–950. doi: 10.1046/j.1365-2958.1997.6301997.x

McEwen, S. A., and Collignon, P. J. (2018). Antimicrobial resistance: a one health perspective. *Microbiol. Spectr.* 6:2017. doi: 10.1128/microbiolspec.ARBA-0009-2017

Miller, E. A., Ponder, J. B., Willette, M., Johnson, T. J., and VanderWaal, K. L. (2020). Merging metagenomics and spatial epidemiology to understand the distribution of antimicrobial resistance genes from *Enterobacteriaceae* in wild owls. *Appl. Environ. Microbiol.* 86:e00571-20. doi: 10.1128/AEM.00571-20

Mohsin, J., Pál, T., Petersen, J. E., Darwish, D., Ghazawi, A., Ashraf, T., et al. (2017). Plasmid-mediated colistin resistance gene mcr-1 in an *Escherichia coli* ST10 bloodstream isolate in the sultanate of Oman. *Microbial. Drug Resist.* 24, 278–282. doi: 10.1089/mdr.2017.0131

Moremi, N., Manda, E. V., Falgenhauer, L., Ghosh, H., Imirzalioglu, C., Matee, M., et al. (2016). Predominance of CTX-M-15 among ESBL producers from environment and fish gut from the shores of Lake Victoria in Mwanza, Tanzania. *Front. Microbiol.* 7:1862. doi: 10.3389/fmicb.2016.01862

Napier, B. A., Burd, E. M., Satola, S. W., Cagle, S. M., Ray, S. M., McGann, P., et al. (2013). Clinical use of colistin induces cross-resistance to host antimicrobials in *Acinetobacter baumannii*. *mBio* 4:e00021-13. doi: 10.1128/mBio.00021-13

Olaitan, A. O., Morand, S., and Rolain, J.-M. (2016). Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. *Int. J. Antimicrob. Agents* 47, 1–3. doi: 10.1016/j.ijantimicag.2015.11.009

Peirano, G., Chen, L., Kreiswirth, B. N., and Pitout, J. D. D. (2020). Emerging antimicrobial-resistant high-risk *Klebsiella pneumoniae* clones ST307 and ST147. *Antimicrob. Agents Chemother.* 64:e01148-20. doi: 10.1128/AAC.01148-20

Ramey, A. M., and Ahlstrom, C. A. (2019). Antibiotic resistant bacteria in wildlife: perspectives on trends, acquisition and dissemination, data gaps, and future directions. *J. Wildlife Dis.* 56, 1–15. doi: 10.7589/2019-04-099

Rasheed, F., Saeed, M., Alikhan, N.-F., Baker, D., Khurshid, M., Ainsworth, E. V., et al. (2020). Emergence of Resistance to fluoroquinolones and third-generation cephalosporins in *Salmonella* Typhi in Lahore, Pakistan. *Microorganisms* 8:1336. doi: 10.3390/microorganisms8091336

Saidani, M., Messadi, L., Mefteh, J., Chaouechi, A., Soudani, A., Selmi, R., et al. (2019). Various Inc-type plasmids and lineages of *Escherichia coli* and *Klebsiella pneumoniae* spreading blaCTX-M-15, blaCTX-M-1 and mcr-1 genes in camels in Tunisia. *J. Glob. Antimicrob. Resist.* 19, 280–283. doi: 10.1016/j.jgar.2019.05.007

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.-E., Rolain, J. M., et al. (2009). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis.* 49, 543–551. doi: 10.1086/600885

Sennati, S., Riccobono, E., Di Pilato, V., Villagran, A. L., Pallecchi, L., Bartoloni, A., et al. (2016). pHN7A8-related multiresistance plasmids (blaCTX-M-65, fosA3 and rmtB) detected in clinical isolates of *Klebsiella pneumoniae* from Bolivia: intercontinental plasmid dissemination? *J. Antimicrob. Chemother.* 71, 1732–1734. doi: 10.1093/jac/dkv506

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Sharma, P., Gupta, S. K., Adenipekun, E. O., Barrett, J. B., Hiott, L. M., Woodley, T. A., et al. (2020). Genome analysis of multidrug-resistant *Escherichia coli* isolated from poultry in Nigeria. *Foodborne Pathog. Dis.* 17, 1–7. doi: 10.1089/fpd.2019.2659

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039

Thaller, M. C., Migliore, L., Marquez, C., Tapia, W., Cedeño, V., Rossolini, G. M., et al. (2010). Tracking acquired antibiotic resistance in commensal bacteria of galápagos land iguanas: no man, no resistance. *PLoS One* 5:e8989. doi: 10.1371/journal.pone.0008989

Thamlikitkul, V., Rattanaumpawan, P., Boonyasiri, A., Pumsuwan, V., Judaeng, T., Tiengrim, S., et al. (2015). Thailand antimicrobial resistance containment and prevention program. *J. Glob. Antimicrob. Resist.* 3, 290–294. doi: 10.1016/j.jgar.2015.09.003

Vounba, P., Arsenault, J., Bada-Alambédji, R., and Fairbrother, J. M. (2019). Prevalence of antimicrobial resistance and potential pathogenicity, and possible spread of third generation cephalosporin resistance, in *Escherichia coli* isolated from healthy chicken farms in the region of Dakar, Senegal. *PLoS One* 14:e0214304. doi: 10.1371/journal.pone.0214304

Wolfe, N. D., Escalante, A. A., Karesh, W. B., Kilbourn, A., Spielman, A., and Lal, A. A. (1998). Wild primate populations in emerging infectious disease research: the missing link? *Emerg. Infect. Dis. J.* 4, 149–158. doi: 10.3201/eid0402.980202

Woolhouse, M., Ward, M., van Bunnik, B., and Farrar, J. (2015). Antimicrobial resistance in humans, livestock and the wider environment. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370:20140083. doi: 10.1098/rstb.2014.0083

World Health Organization (2014). *Antimicrobial Resistance: Global Report on Surveillance*. Geneva: World Health Organization.

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 Abdallah, Kuete Yimagou, Hadjadj, Mediannikov, Ibrahim, Davoust, Barciela, Hernandez-Aguilar, Diatta, Sokhna, Raoult, Rolain and Baron. 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 Microbiology

ORIGINAL RESEARCH
published: 29 March 2022
doi: 10.3389/fmicb.2022.818548

Molecular Epidemiology and Colistin-Resistant Mechanism of *mcr*-Positive and *mcr*-Negative *Escherichia coli* Isolated From Animal in Sichuan Province, China

*Fulei Li[1,2], Ping Cheng[1,2], Xiaoting Li[1,2], Ruimeng Liu[1,2], Haibin Liu[1,2] and Xiuying Zhang[1,2]**

[1] Heilongjiang Key Laboratory for Animal Disease Control and Pharmaceutical Development, Northeast Agricultural University, Harbin, China, [2] Department of Basic Veterinary Science, College of Veterinary Medicine, Northeast Agricultural University, Harbin, China

OPEN ACCESS

Edited by:
Ziad Daoud, Central Michigan University, United States

Reviewed by:
Thongpan Leangapichart, Norwegian Veterinary Institute (NVI), Norway
Christopher Morton Thomas, University of Birmingham, United Kingdom

***Correspondence:**
Xiuying Zhang
zhangxiuying@neau.edu.cn

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *19 November 2021*
Accepted: *02 February 2022*
Published: *29 March 2022*

Citation:
Li F, Cheng P, Li X, Liu R, Liu H and Zhang X (2022) Molecular Epidemiology and Colistin-Resistant Mechanism of mcr-Positive and mcr-Negative Escherichia coli Isolated From Animal in Sichuan Province, China. Front. Microbiol. 13:818548. doi: 10.3389/fmicb.2022.818548

Colistin is the last line of defense for the treatment of multidrug-resistant gram-negative bacterial infections. However, colistin resistance is gradually increasing worldwide, with resistance commonly regulated by two-component system and *mcr* gene. Thus, this study aimed to investigate molecular epidemiology and colistin-resistant mechanism of *mcr*-positive and *mcr*-negative *Escherichia coli* isolates from animal in Sichuan Province, China. In this study, a total of 101 colistin-resistant *E. coli* strains were isolated from 300 fecal samples in six farms in Sichuan Province. PCR was used to detect *mcr* gene (*mcr-1* to *mcr-9*). The prevalence of *mcr-1* in colistin-resistant *E. coli* was 53.47% (54/101), and the prevalence of *mcr-3* in colistin-resistant *E. coli* was 10.89% (11/101). The colistin-resistant *E. coli* and *mcr-1*–positive *E. coli* showed extensive antimicrobial resistance profiles. For follow-up experiments, we used 30 *mcr*-negative and 30 *mcr-1*–positive colistin-resistant *E. coli* isolates and *E. coli* K-12 MG1655 model strain. Multi-locus sequence typing (MLST) of 30 strains carrying *mcr-1* as detected by PCR identified revealed six strains (20%) of ST10 and three strains (10%) of each ST206, ST48, and ST155 and either two (for ST542 and 2539) or just one for all other types. The conjugation experiment and plasmid replicon type analysis suggest that *mcr-1* was more likely to be horizontally transferred and primarily localized on IncX4-type and IncI2-type plasmid. The ST diversity of the *mcr-1* indicated a scattered and non-clonal spreading in *mcr-1*–positive *E. coli.* Twenty-eight *mcr*-negative colistin-resistant *E. coli* isolates carried diverse amino acid alterations in PmrA, PmrB, PhoP, PhoQ, and MgrB, whereas no mutation was found in the remaining isolates. The finding showed the high prevalence of colistin resistance in livestock farm environments in Sichuan Province, China. Our study demonstrates that colistin resistance is related to chromosomal point mutations including the two-component systems PhoP/PhoQ, PmrA/PmrB, and their regulators MgrB. These point mutations may confer colistin resistance in *mcr*-negative *E. coli*. These findings help in gaining insight of chromosomal-encoded colistin resistance in *E. coli*.

Keywords: colistin resistant mechanism, *mcr*, *Escherichia coli*, multidrug resistant, two-component systems

INTRODUCTION

Colistin is the last line of defense for the treatment of multidrug-resistant (MDR) gram-negative bacteria (Paterson et al., 2020). The rate of colistin resistance has risen to 30% of Carbapenem-resistant Enterobacteriaceae (CRE) isolates in Italy, Spain, and Greece over the past decade (Stefaniuk and Tyski, 2019). Colistin resistance is a growing public health concern worldwide. Colistin is a cyclic polycationic peptide that interacts with anionic lipopolysaccharide (LPS) molecules (Olaitan et al., 2014). However, overuse and misuse of colistin accelerate propagation of antibiotic resistance genes. The exact mechanism of colistin resistance is not well understood. It has been shown that an altered outer membrane, a loss of lipid A, and increase in drug efflux pumps are associated with colistin resistance (Raetz et al., 2007; Moffatt et al., 2010; Padilla et al., 2010).

The plasmid-mediated colistin resistance was not demonstrated until 2015. Liu et al. (2016) first systematically reported *mcr-1* gene. Over the past few years, *mcr-1* became common all over the world. The *mcr-1* has the potential to spread rapidly by horizontal transfer and may pose a significant public health risk (Rapoport et al., 2016; Rolain et al., 2016; Sonnevend et al., 2016; Borowiak et al., 2017). In the past few years, novel plasmid-encoded colistin resistance genes have also been identified. So far, novel colistin resistance genes (*mcr-2* to *mcr-10*) have been reported (Xavier et al., 2016; Carattoli et al., 2017; Yin et al., 2017; AbuOun et al., 2018; Yang et al., 2018; Wang et al., 2019, 2020). The *mcr-2* to *mcr-9* genes encode the proteins MCR-2 to MCR-9, which share 81, 32.5, 34, 36, 83, 35, 31, and 36% amino acid sequence identity with MCR-1, respectively (Carroll et al., 2019; Nang et al., 2019). MCR-10 is encoded by *mcr-10*, which contains 82.93% amino acids identical to that in MCR-9 (Wang et al., 2020). The *mcr-1* has spread to more than 60 countries (Cheng et al., 2021). It has been reported that prevalence of *mcr-1*–positive bacteria ranged from 0.35 to 36.00% in pigs and 2.40–30.00% in poultry (Xiaomin et al., 2020). To date, *mcr-1* was identified in many different plasmid types, including IncI2, IncHI2, IncX4, IncP, IncY, and IncF (Shen et al., 2018).

Before the discovery of *mcr* gene, clinical colistin resistance was associated with mutation of chromosomal gene (Kim et al., 2019; Mendes Oliveira et al., 2019). Chromosome-mediated colistin resistance had been linked to LPS modifications, which was associated with PmrAB or PhoPQ two-component systems (Poirel et al., 2017). MgrB is a negative regulator of the PhoPQ system. The inactivation of MgrB leads to over expression of the phoPQ operon (Cheng et al., 2010; Cannatelli et al., 2013). Moreover, amino acid substitutions of PmrA and/or PmrB have been reported in clinical isolates of *Acinetobacter baumanni* and *Klebsiella pneumoniae* (Poirel et al., 2017). First, amino acid substitutions may affect protein function. In addition, amino acid substitutions in MgrB, PmrA/B, and PhoP/Q are a common mechanism of colistin resistance among *K. pneumoniae* in clinical settings (Luo et al., 2017). However, amino acid substitutions in MgrB, PmrA/B, and PhoP/Q are rarely reported among *mcr*-negative colistin-resistant *Escherichia coli* isolates. A recent study showed that missense mutations may be responsible for colistin resistance (Olaitan et al., 2014).

This study aimed to investigate molecular epidemiology and colistin-resistant mechanism of *mcr*-positive and *mcr*-negative *E. coli* isolated from animals in Sichuan Province, China.

MATERIALS AND METHODS

Sample Collection

From January 2016 to March 2018, a total of 300 fecal swabs were collected from six different farms in Sichuan Province. The samples were kept on ice and were immediately brought to laboratory. The samples were cultured on MacConkey agar at 37°C for 18–24 h, and then, five colonies with typical *E. coli* morphology were selected. The *E. coli* were identified using biochemical methods and confirmed by PCR amplification of *16S rRNA* and sequencing. The primer of *16S rRNA* is listed in **Supplementary Table 1**. The protocols used during this study were approved by the Northeast Agricultural University Institutional Animal Care and Use Committee, and all the animal care and treatment methods complied with the standards described in the Laboratory Animal Management Regulations (revised 2016) of Heilongjiang Province, China.

Detection of Colistin-Resistant *Escherichia coli* Isolates and *mcr* Gene

To screen colistin-resistant *E. coli*, *E. coli* were cultured on MacConkey agar at 37°C for 18–24 h. To determine the colistin minimum inhibitory concentration (MIC), *E. coli* isolates (>4μg/ml) were served as colistin-resistant *E. coli*. A DNA extraction kit (TIANGEN, Beijing, China) was used to extract genomic DNA of colistin-resistant *E. coli* isolates. The *mcr*-harboring isolates were screened by PCR amplification and were validated by sequencing. The primers of *mcr* gene are listed in **Supplementary Table 1**.

Antimicrobial Susceptibility Testing

The susceptibility of colistin-resistance *E. coli* isolates to 23 antibiotics, namely, Nitrofurantoin, Ciprofloxacin, Levofloxacin, Kanamycin, Amikacin, Amoxicillin, Tigecycline, Cefepime, Chloramphenicol, Fosfomycin, Aztreonam, Ampicillin, Ampicillin/sulbactam, Cefoxitin, Doxycycline, Streptomycin, Ceftriaxone, Florfenicol, Cefuroxime, Sulfamethoxazole, Gentamicin, and Tetracycline, was determined by the standard disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI).[1] *Escherichia coli* ATCC 25922 was served as a quality control strain for susceptibility testing.

Detection of Antimicrobial Resistance Genes

The presence of the β-lactamase genes (bla_{CTX-M}, bla_{TEM}, and bla_{SHV}) (Dallenne et al., 2010), aminoglycoside resistance

[1] https://clsi.org/

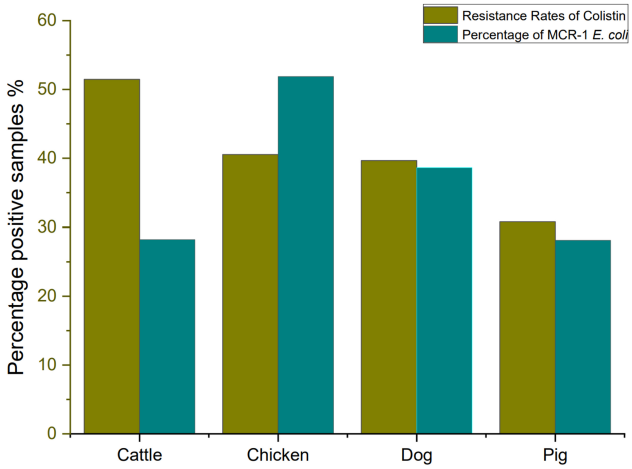


FIGURE 1 | The resistance rate to colistin and percentage of *mcr-1*–positive *Escherichia coli*.

genes (*strA*, *strB*, *aacC2*, and *aacC4*) (Kozak et al., 2009), tetracycline resistance genes (*tetA*, *tetB*, and *tetC*) (Ji et al., 2020), fluoroquinolone resistance genes [*qnrS*, *oqxA*, *oqxB*, *qepA*, and *aac(6′)-Ib-cr*] (Ciesielczuk et al., 2013), florfenicol resistance gene (*floR*) (Lu et al., 2018), and sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) (Hammerum et al., 2006) were examined by PCR amplification and were validated by sequencing. The primers of resistance genes are listed in **Supplementary Table 2**. The obtained DNA sequences were analyzed using ChromasPro software and were compared with published sequences by BLAST.

Detection of *pmrA/B*, *mgrB*, and *PhoP/Q* Amino Acid Variants

The entire *pmrAB*, *mgrB*, and *phoPQ* genes were amplified of colistin-resistant isolates using the primers listed in **Supplementary Table 3**. The amplification products were validated with Sanger sequencing. Amino acid sequences of *mcr*-negative colistin-resistant *E. coli* isolates were compared with the reference strain *E. coli* K-12 MG1655. Missense mutations of PmrA/B, PhoP/Q, and MgrB identified in *mcr*-negative colistin-resistant *E. coli* were analyzed using the PROVEAN (Choi and Chan, 2015) prediction software. In addition, amino acid substitutions were considered "deleterious" if the PROVEAN score was $\leq$-2.5 and "neutral replacements" if the PROVEAN score was $>$-2.5 (Choo et al., 2016; Higuchi et al., 2016). We used PROVEAN bioinformatic tool[2] to predict whether amino acid substitutions in MgrB, PmrA/B, and PhoP/Q affect protein function (Choi et al., 2012). SMART (Simple Modular Architecture Research Tool) is a web resource,[3] providing simple identification and extensive annotation of protein domains and the exploration of protein domain architectures (Schultz et al., 1998; Letunic et al., 2015). SMART analysis was performed to determine the domain architectures of PmrA, PmrB, PhoP, PhoQ, and MgrB protein.

Conjugation Experiment and Plasmid Replicon Type Analysis

The transferability of *mcr-1* was tested by conjugation experiment with 30 *mcr-1*–positive *E. coli* (MCRPEC) as donors and *E. coli* J53 as recipient strains. MacConkey agar plates containing rifampicin (256 μg/ml) and colistin (4 μg/ml) were used to select *mcr-1*–positive transconjugants. The *mcr-1* gene of transconjugants was examined by PCR amplification and was validated by sequencing. The replicon types of the transconjugants were determined according to previous studies (Carattoli et al., 2005).

Multi-Locus Sequence Typing Analysis

Sequence type of 30 MCRPEC was determined according to the primers and protocol specified in *E. coli* multi-locus sequence typing (MLST) database website, which is based on the housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*. The primers of housekeeping gene are listed in **Supplementary Table 4**. The obtained DNA sequence alignments were performed

TABLE 1 | Number of *mcr*-positive and *mcr*-negative colistin-resistant *Escherichia coli* in this study.

Origin	Period of isolation	No. *E. coli*	No. of *mcr*-positive *E. coli*			No. *mcr*-negative colistin-resistant *E. coli*
			mcr-1	*mcr-3*	*mcr-1/3*	
Chicken	2016–2018	37	12	–	–	4
Pig	2016–2018	91	16	–	–	12
Cattle	2016–2018	68	12	4	4	23
Dog	2016–2018	58	14	7	7	9

mcr-1/3 strains have both mcr-1 and mcr-3 gene.

[2]http://sift.jcvi.org

[3]http://smart.embl.de/

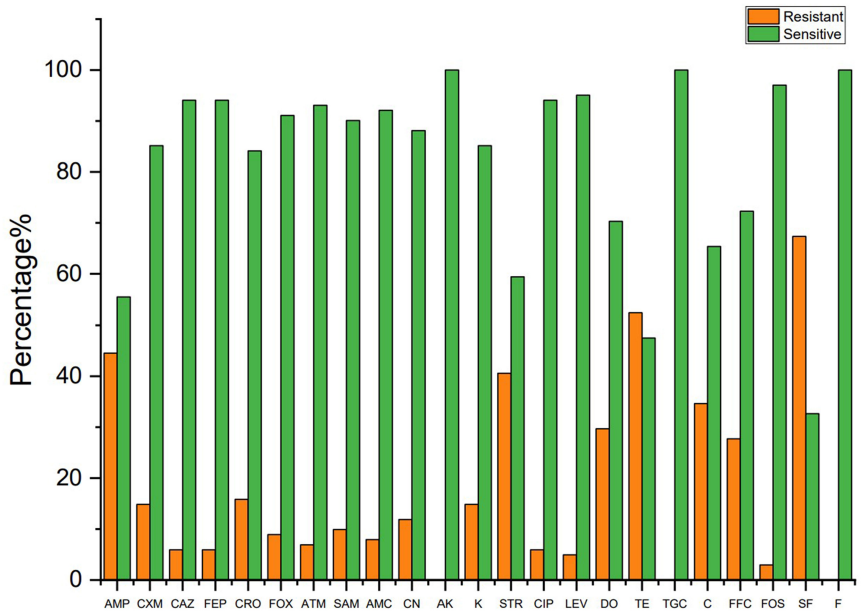


FIGURE 2 | The resistant rate of colistin resistance *E. coli* to other antibiotics. AMP, ampicillin; CXM, cefuroxime; CAZ, ceftazidime; FEP, cefepime; CRO, ceftriaxone; FOX, cefoxitin; ATM, aztreonam; SAM, ampicillin-sulbactam; AMC, amoxicillin–clavulanic acid; CN, gentamicin; AK, amikacin; K, kanamycin; STR, streptomycin; CIP, ciprofloxacin; LEV, levofloxacin; TE, tetracycline; DO, doxycycline; TGC, tigecycline; C, chloramphenicol; FFC, florfenicol; FOS, fosfomycin; SF, sulfisoxazole; F, nitrofurantoin.

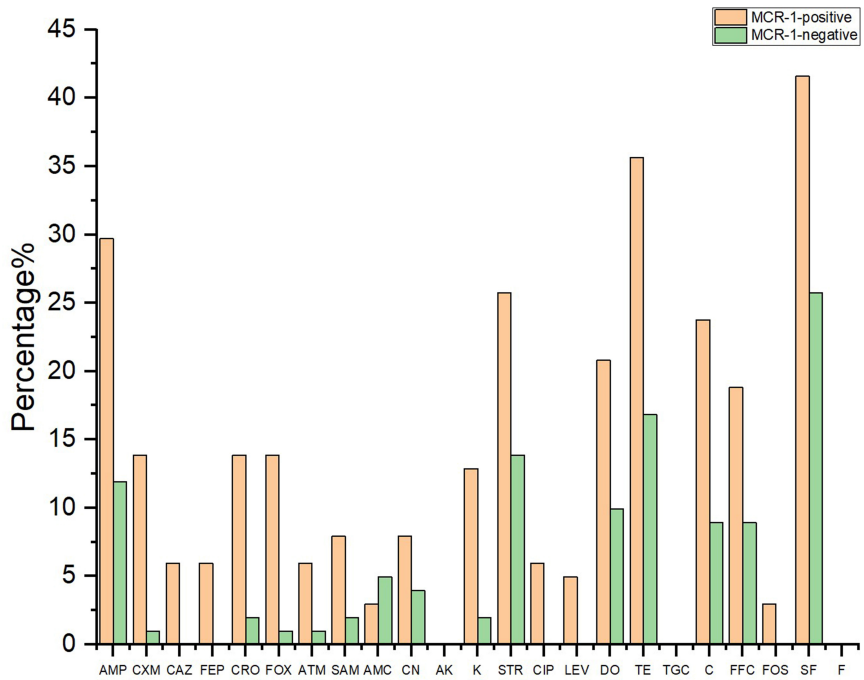


FIGURE 3 | The resistant rate of *mcr-1*–positive and *mcr-1*–negative *E. coli* to 23 antibiotics. AMP, ampicillin; CXM, cefuroxime; CAZ, ceftazidime; FEP, cefepime; CRO, ceftriaxone; FOX, cefoxitin; ATM, aztreonam; SAM, ampicillin–sulbactam; AMC, amoxicillin–clavulanic acid; CN, gentamicin; AK, amikacin; K, kanamycin; STR, streptomycin; CIP, ciprofloxacin; LEV, levofloxacin; TE, tetracycline; DO, doxycycline; TGC, tigecycline; C, chloramphenicol; FFC, florfenicol; FOS, fosfomycin; SF, sulfisoxazole; F, nitrofurantoin.

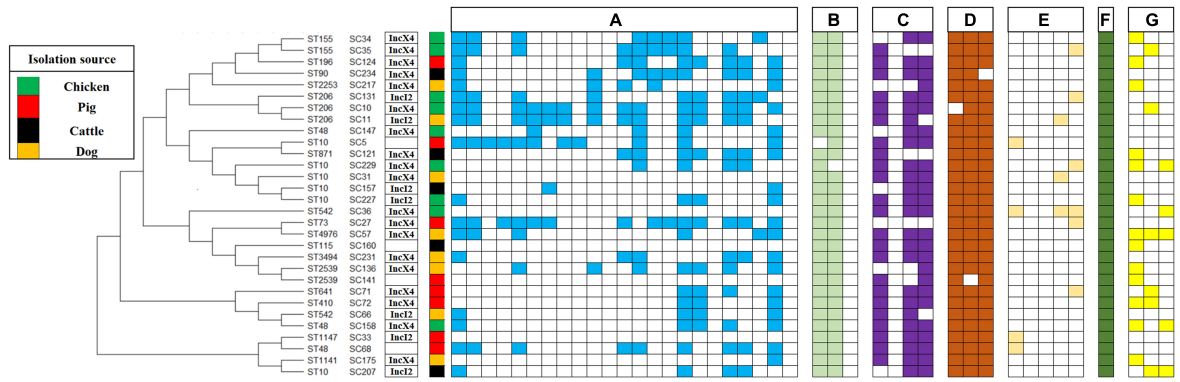


FIGURE 4 | Phylogeny of *mcr-1*–positive *E. coli* (sequence types, strain number, plasmid type, source of isolation, and map of phenotypic resistance and resistance genes). Full and empty square mean presence and absence of antimicrobial resistance (AMR) gene, respectively, whereas empty space means non-defined. **(A)** Resistance profiles: ampicillin, cefuroxime, ceftazidime, cefepime, ceftriaxone, cefoxitin, aztreonam, ampicillin-sulbactam, amoxicillin-clavulanic acid, gentamicin, amikacin, kanamycin, streptomycin, ciprofloxacin, levofloxacin, tetracycline, doxycycline, tigecycline, chloramphenicol, florfenicol, fosfomycin, sulfisoxazole, and nitrofurantoin. **(B)** Beta-lactam resistance genes: bla_{CTX-M}, bla_{TEM} and bla_{SHV}. **(C)** Aminoglycoside resistance genes: *strA*, *strB*, *aacC2*, and *aacC4*. **(D)** Tetracycline resistance genes: *tetA*, *tetB*, and *tetC*. **(E)** Fluoroquinolone resistance genes: *qnrS*, *oqxA*, *oqxB*, *qepA*, and *aac(6′)-Ib-cr*. **(F)** Florfenicol resistance gene: *floR*. **(G)** Sulfonamide resistance genes: *sul1*, *sul2*, and *sul3*.

by using ChromasPro software. The phylogenetic tree was constructed by the neighbor-joining method.

RESULTS AND DISCUSSION

Prevalence of *mcr*-Positive *Escherichia coli* Isolated From Animal

In this study, we investigate colistin resistance rate in *E. coli* isolated from six different farms in Sichuan Province during 2016–2018. A total of 254 *E. coli* strains were isolated from 300 fecal samples, including 37 chickens, 91 pigs, 68 cattle, and 58 dogs. The *E. coli* isolates from cattle showed a high resistance rate to colistin (51.47%, 35/68), followed by *E. coli* isolates from chicken (40.54%, 15/37), *E. coli* isolates from dog (39.66%, 23/58), and *E. coli* isolates from pig (30.77%, 28/91). However, it has been reported that the prevalence of colistin resistance in *E. coli* isolated from farms in different areas of China during 2013–2014, which revealed that colistin resistance rates in *E. coli* from pigs, chickens, and cattle were 26.5, 14.0, and 0.9%, respectively (Zhuge et al., 2019). Our data also showed that colistin resistance rate has risen significantly high. Colistin-resistant *E. coli* isolates (MIC of colistin $\geq$ 4 μg/ml) are listed in **Supplementary Table 5**. The resistance rate to colistin and percentage of MCRPEC are shown in **Figure 1**.

However, discoveries of plasmid-mediated colistin resistance gene in many countries have heralded a significant threat to public health worldwide (Kai and Wang, 2020). As shown in **Table 1**, *mcr-1* gene was detected in 54 (53.47%) out of the selected 101 isolates, and *mcr-3* gene was detected in 11 (10.89%) out of the selected 101 isolates. Eleven *E. coli* isolates have both *mcr-1* and *mcr-3* gene. No other *mcr* genes were found in this survey. Similar to our result, a surveillance of colistin resistance performed in Jiangsu Province revealed that the *mcr-1* prevalence was 68.86% in pigs (Zhang et al., 2019). More detailed studies of *mcr-1* have been performed in colistin-resistant isolates (Tong et al., 2018). The previous study showed that *mcr-1* had been identified in approximately 60 countries across five different continents (Cao et al., 2018; Sun et al., 2018; Wang et al., 2018). The finding of mobilizable *mcr*-like genes became a global concern due to the possibility of horizontal transfer of the plasmid that often carry resistance determinants to beta-lactams and/or quinolones (Mendes Oliveira et al., 2019).

Antimicrobial Susceptibility of Colistin-Resistance and *mcr-1*–Positive *Escherichia coli*

The susceptibility of 101 colistin-resistant *E. coli* isolates to 23 antimicrobials was determined by the standard disk diffusion method. **Figure 2** shows a significant difference in resistance rate of the *E. coli* isolates to 23 antibiotics. Among the colistin-resistant *E. coli* isolates, all isolates were susceptible to amikacin, tigecycline, and nitrofurantoin. Antibiotic sensitivity tests revealed that colistin-resistant *E. coli* isolates have a highly resistance to sulfisoxazole; a moderate rate of resistance to ampicillin, streptomycin, and tetracycline; and a low rate of resistance to cefuroxime, ceftazidime, cefepime, ceftriaxone, cefoxitin, aztreonam, ampicillin-sulbactam, amoxicillin-clavulanic acid, gentamicin, kanamycin, ciprofloxacin, and levofloxacin. The colistin-resistant *E. coli* isolates displayed high resistance rates to antibiotics that are commonly used in veterinary medicine in Sichuan Province (Ma et al., 2017; Zhang et al., 2021), including sulfisoxazole, ampicillin, streptomycin, tetracycline, and chloramphenicol. The antimicrobial resistance rates have reached to 30%, which may be due to the breadth of our sample or because of the generally high use of antibiotics in Sichuan Province. Three studies reported the rate of resistance to streptomycin was within the range 0–7% (Routman et al., 1985; Sayah et al., 2005; Tong et al., 2018). However, the rate

TABLE 2 | Mutations of PmrA in *mcr*-negative colistin-resistant *E. coli* isolates.

Isolates	PmrA										
	S29	T31	T79	T85	L116	R118	E126	I128	R139	G144	T151
Escherichia coli K12	S	T	T	T	L	R	E	I	R	G	T
Escherichia coli SC8	G										
Escherichia coli SC9	G			A				N	P	S	A
Escherichia coli SC10											
Escherichia coli SC11										S	
Escherichia coli SC12	G										
Escherichia coli SC13	G										
Escherichia coli SC14	G										
Escherichia coli SC15	G										
Escherichia coli SC16	G										
Escherichia coli SC17	G										
Escherichia coli SC18	G						K				
Escherichia coli SC19	G					F					
Escherichia coli SC20	G		A		V						
Escherichia coli SC21	G										
Escherichia coli SC22	G										
Escherichia coli SC23	G										
Escherichia coli SC24	G										
Escherichia coli SC25	G										
Escherichia coli SC26	G										
Escherichia coli SC27											
Escherichia coli SC28	G										
Escherichia coli SC29	G										
Escherichia coli SC30	G										
Escherichia coli SC31											
Escherichia coli SC32											
Escherichia coli SC33	G	S									
Escherichia coli SC34											
Escherichia coli SC35	G										
Escherichia coli SC36	G										
Escherichia coli SC37	G										

of resistance to streptomycin has reached 30% in this study. This difference may reflect difference in antimicrobial use in different livestock.

Figure 3 shows the resistance rate of the MCRPEC isolates to 23 antibiotics. MCRPEC isolate have a moderate rate of resistance (20–60%) to ampicillin, streptomycin, doxycycline, tetracycline, and sulfisoxazole; and a low rate of resistance (<20%) to cefuroxime, ceftazidime, cefepime, ceftriaxone, cefoxitin, aztreonam, ampicillin-sulbactam, amoxicillin-clavulanic acid, gentamicin, kanamycin, ciprofloxacin, levofloxacin, and fosfomycin. All the MCRPEC isolate were susceptible to amikacin, tigecycline, and nitrofurantoin. The resistance phenotype of *mcr-1* positive *E. coli* is shown in **Supplementary Table 6**.

Detection of Antimicrobial Resistance Genes of *mcr-1*–Positive *Escherichia coli*

The prevalence of additional antimicrobial resistance genes in 30 MCRPEC is shown in **Figure 4**. Overall, bla_{TEM} (n = 29, 96.67%) and bla_{CTX-M} (n = 29, 96.67%) were the most common ESBL genes, and bla_{SHV} was not detected in this study. The aminoglycoside resistance genes with the highest detection rate were *aacC2* (n = 24, 80.00%) and *aacC4* (n = 25, 83.33%), followed by *strA* (n = 23, 76.67%), and *strB* were not detected. Among tetracycline resistance genes, *tetA* (n = 29, 96.67%), *tetB* (n = 29, 96.67%), and *tetC* (n = 29, 96.67%) were the most common tetracycline resistance genes. The fluoroquinolone resistance genes with the highest detection rate were *aac(6′)-Ib-cr* (n = 6, 20.00%), followed by *qepA* (n = 4, 13.33%) and *qnrS* (n = 4, 13.33%), and *oqxA* and *oqxB* were not detected in our study. The florfenicol resistance gene *floR* detection rate was (n = 30, 100.00%). The sulfonamide resistance genes with the highest detection rate were *sul1* (n = 13, 43.33%), followed by *sul2* (n = 7, 23.33%) and *sul3* (n = 6, 20.00%). In our study, a high prevalence of ESBL genes (bla_{TEM} and bla_{CTX-M}), aminoglycoside resistance genes (*aacC2*, *aacC4*, and *strA*), tetracycline resistance genes (*tetA*, *tetB*, and *tetC*), and florfenicol resistance gene *floR* were found in 30 MCRPEC. This

TABLE 3 | Mutations of *PmrB*, *PhoP*, and *PhoQ* in *mcr*-negative colistin-resistant *E. coli* isolates.

Isolates	PmrB				MgrB		PhoP	PhoQ
	T235	D283	V351	Y358	M1	V8	A416	K46
Escherichia coli K12	T	D	V	Y	M	V	A	K
Escherichia coli SC8								
Escherichia coli SC9		G	I		V			
Escherichia coli SC10					V	A		
Escherichia coli SC11								
Escherichia coli SC12								
Escherichia coli SC13					V			
Escherichia coli SC14					V			
Escherichia coli SC15					V			
Escherichia coli SC16					V			
Escherichia coli SC17					V			
Escherichia coli SC18		G		N	V			
Escherichia coli SC19					V			
Escherichia coli SC20					V			
Escherichia coli SC21		G		N	V			
Escherichia coli SC22					V			
Escherichia coli SC23					V		T	
Escherichia coli SC24	N	G		N	V			
Escherichia coli SC25		G		N	V			
Escherichia coli SC26		G		N	V			
Escherichia coli SC27		G		N				
Escherichia coli SC28		G		N	V			T
Escherichia coli SC29		G		N	V			
Escherichia coli SC30		G		N	V			
Escherichia coli SC31		G		N				
Escherichia coli SC32					V			
Escherichia coli SC33								
Escherichia coli SC34								
Escherichia coli SC35					V			
Escherichia coli SC36					V			
Escherichia coli SC37					V			

result may reflect that plasmid harboring *mcr-1* usually carry other resistance genes (Rozwandowicz et al., 2018; Cheng et al., 2021).

Transferability and Plasmid Replicon Types of *mcr-1*–Positive *Escherichia coli*

As shown in **Figure 4**, the conjugation result showed that 27 isolates successfully conjugated among 30 representative MCRPEC. The success rate of conjugation was as high as 90%. *mcr-1* has been observed in two types plasmids, including IncX4 (n = 19) and IncI2 (n = 7). In this study, the transferability of MCRPEC was assessed among 30 representative strains. The result was in line with previous study, which showed that majority of *mcr-1*–carrying plasmids were transferable (Cheng et al., 2021). Previous research has shown *mcr-1*–carrying plasmids belong to different replicon types, including IncI2 (Matamoros et al., 2017), IncHI1 (Zurfluh et al., 2016), IncHI2 (Matamoros et al., 2017), IncFIB (Wang et al., 2017; Khezri et al., 2020), IncFII (Xavier et al., 2016), IncP (Zhao et al., 2017), IncX4 (Gao et al., 2016), and IncY (Shen et al., 2018).

Molecular Genotyping of *mcr-1*–Positive *Escherichia coli*

As shown in **Figure 4**, 30 MCRPEC isolates were assigned to 17 STs. ST10 (n = 6) was the most numerous ST in this study, followed by ST206 (n = 3), ST48 (n = 3), ST155 (n = 3), ST542 (n = 2), and ST2539 (n = 2), and then by single ST type isolates, including ST196, ST90, ST2253, ST871, ST73, ST4976, ST3494, ST641, ST410, ST1147, and ST1141. Hence, colistin-resistant *E. coli* isolates comprised a variety of STs and were therefore genetically different, with the nosocomial transmission excluded. The ST diversity of the *mcr-1*–harboring *E. coli* isolates indicated a scattered and non-clonal prevalence. The discovery of the superbug MCRPEC has triggered a huge amount of innovative scientific inquiry (Yuan et al., 2021). Recently, a study analyzed 616 whole genomes of MCRPEC isolates from NCBI online database. Similar to our result, ST10 was the most common ST among the *mcr-1*–positive isolates (Zhuge et al., 2019).

Amino Acid Variations of *PmrA/B*, *PhoP/Q*, and *MgrB* in *mcr*-Negative Isolates

As shown before, colistin resistance was reported to be associated with chromosomal mutations. It is well-known that two-component PhoPQ and PmrAB were associated with LPS modification (Luo et al., 2017). LPS may play an important regulatory role in colistin-resistance isolates (Schurek et al., 2009; Kandehkar Ghahraman et al., 2021). To determine chromosome-mediated colistin-resistant mechanism in *mcr*-negative colistin-resistant isolates, we explored whether alterations in amino acid of PmrAB, PhoPQ, and MgrB affect protein function. We selected K-12 MG1655 *E. coli* as negative controls. The mutations are shown in **Tables 2**, **3**, and we found that many variations were synonymous and non-synonymous mutations. The *mgrB* gene encodes a short 47–amino acid transmembrane protein. MgrB is a small transmembrane protein of 47 amino acids, which acts as negative feedback regulator of the PhoPQ two-component regulatory system (Lippa and Goulian, 2009). The multiple sequence alignment tool MEGA-X was used for multiple protein sequence alignment. Two mutations M1V (23 *mcr*-negative isolates) and V8A (one *mcr*-negative isolate) were detected. The mutation M1V [PROVEAN score = -4.670 (cutoff = -2.5)] and V8A [PROVEAN score = -2.808 (cutoff = -2.5)] were deleterious affecting protein function. We found similar mutations in MgrB (V8A) with previous studies (Delannoy et al., 2017; Luo et al., 2017). The mutation V8A may confer colistin resistance in *mcr*-negative *E. coli*.

The *pmrA* gene encodes a short 222–amino acid transmembrane protein. The multiple sequence alignment tool MEGA-X was used for multiple protein sequence alignment. As shown in **Table 2**, 11 mutations S29G (24 *mcr*-negative isolates), T31S (one *mcr*-negative isolate), T79A (one *mcr*-negative isolate), T85A (one *mcr*-negative isolate), L116V (one *mcr*-negative isolate), R118F (one *mcr*-negative isolate), E126K

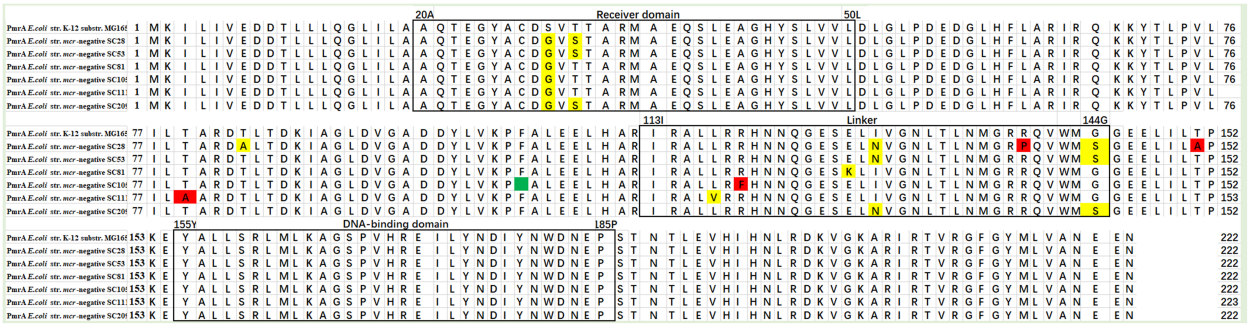


FIGURE 5 | Multiple sequence alignment of PmrA across seven *mcr*-negative colistin-resistant *E. coli*. Mutations are shown with yellow boxes. The red boxes show that mutations were deleterious, affecting protein function. Amino acid deletion is shown with green box.

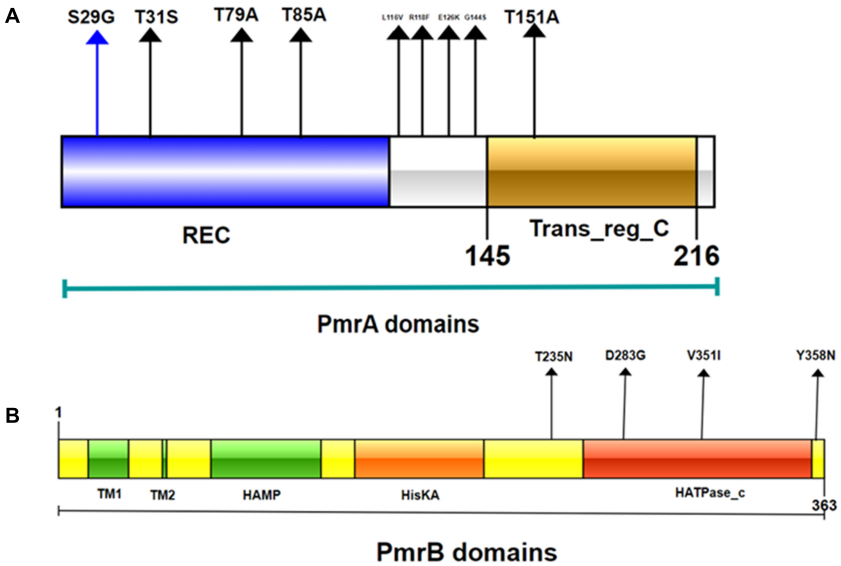


FIGURE 6 | Domains of the PmrA/PmrB two-component system and positions of all mutations conferring colistin resistance to *E. coli*. **(A)** PmrA domains, cheY-homologous receiver domain [REC]; aa 1-112. Transcriptional regulatory protein, C-terminal domain [Trans_reg_C]; aa 145–216. S29G, T31S, T79A, T85A, L116V, R118F, E126K, I128N, G144S, and T151A represent mutation site. **(B)** PmrB domains, first transmembrane domain [TM1]; aa 15–34; second transmembrane domain [TM2]; aa 66–68. histidine kinases, adenylyl cyclases, methyl binding proteins, phosphatases [HAMP domain]; aa 89-141. Histidine kinase A (phosphoacceptor) domain [HisKA]; aa 142–202. Histidine kinase-like ATPases [HATPase_c]; aa 249-357. D283G, V351I, and Y358N represent smutation site.

(one *mcr*-negative isolate), I128N (one *mcr*-negative isolate), R139P (one *mcr*-negative isolate), G144S (two *mcr*-negative isolates), and T151A (one *mcr*-negative isolate) were detected. The mutations T79A [PROVEAN score = -4.586 (cutoff = -2.5)], R118F [PROVEAN score = -7.740 (cutoff = -2.5)], R139P [PROVEAN score = -3.456 (cutoff = -2.5)], and T151A [PROVEAN score = -2.790 (cutoff = -2.5)] were deleterious, affecting protein function. **Figure 5** shows multiple sequence alignment of PmrA across seven *mcr-1*–negative colistin-resistant isolates. Mutations are shown with yellow boxes. The red boxes show that mutations were deleterious, affecting protein function. Amino acid deletion is shown with green box. In this study, 11 different mutations in PmrA (S29G, T31S, T79A, T85A, L116V, R118F, E126K, I128N, R139P, G144S, and T151A) were observed in 24 *mcr*-negative colistin-resistant isolates. Of them, four mutations, namely, T79A, R118F, R139P, and T151A, were deleterious, affecting protein function. In addition, we found similar mutations with previous study in PmrA (S29G, T31S, I128N, and G144S) (Luo et al., 2017). However, we also found many novel mutations in PmrA (T79A, T85A, L116V, R118F, E126K, R139P, and T151A). The seven mutations have not been reported in *mcr*-negative colistin-resistant *E. coli*. These mutations may confer colistin resistance in *mcr*-negative *E. coli*.

The *pmrB* gene encodes a short 363–amino acid transmembrane protein. Four mutations T235N (one *mcr*-negative isolate), D283G (11 *mcr*-negative isolates), V351I (one *mcr*-negative isolate), and Y358N (10 *mcr*-negative isolates) were detected. We found similar mutations in PmrB (D283G, V351I, and Y358N) with previous studies (Delannoy et al., 2017; Luo et al., 2017).

The *phoP* gene encodes a short 456–amino acid transmembrane protein. One mutation A416T (one *mcr-1*–negative isolate) was detected. The *phoQ* gene encodes a short 486–amino acid transmembrane protein. One mutation K46T (one *mcr*-negative isolate) was detected. The mutation K46T [PROVEAN score = -3.746 (cutoff = -2.5)] was deleterious, affecting protein function.

Domain Architectures of *PmrAB*, *PhoPQ*, and *MgrB* in *mcr*-Negative Isolates

SMART analysis was performed to determine the domain architectures of PmrA, PmrB, PhoP, PhoQ, and MgrB. As shown in **Figure 6**, we found that mutations have occurred in different domains of both PmrA and PmrB. In addition, *pmrA* appears to be the most commonly mutated gene in *E. coli*. The mutations T79A, R118F, R139P, and T151A were unique in PmrA. T91A is located in the cheY-homologous receiver domain. This domain contains a phosphoacceptor site that is phosphorylated by histidine kinase homologs. R118F, R139P, and T151A were located in transcriptional regulatory protein, C terminal. This domain is almost always found associated with the response regulator receiver domain. It may play a role in DNA binding. SMART analysis suggests that mutations T235N, V351I, and Y358N occurred in the HATPase_c domain in PmrB. The mutation V8A located in transmembrane region in MgrB, which starts at position 7 and ends at position 24. In summary, this study revealed diverse genetic mutations in two-component systems PmrAB and PhoPQ and their regulators MgrB in *mcr*-negative colistin-resistant *E. coli* isolates from Sichuan, China. However, mutated PmrB proteins do not contribute to colistin resistance (Wang et al., 2021). Although PmrAB, MgrB, and PhoPQ may be responsible for *mcr*-negative colistin-resistant *E. coli*, the mechanisms of colistin resistance appear to be highly diverse. These results suggest that the mechanisms underlying colistin resistance remain to be discovered in *E. coli*.

CONCLUSION

The finding of this study showed the high prevalence of colistin in farms in Sichuan, China. The conjugation experiment and plasmid replicon type analysis suggest that the *mcr-1* gene is more likely to be horizontally transferred. The ST diversity of the *mcr-1* indicated a scattered and non-clonal. In addition, this study demonstrates diverse genetic mutations in two-component systems PmrAB and PhoPQ and their regulators MgrB of *mcr*-negative colistin-resistant *E. coli* isolates. In this study, we found several novel mutations, which have not been reported in *mcr*-negative colistin-resistant *E. coli*. These substitutions may confer colistin resistance in *mcr*-negative *E. coli*. To confirm our findings, further studies elucidating the resistance mechanism of *mcr*-negative *E. coli* to colistin are under way.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

FL conceived and designed the experiments and wrote the manuscript. PC, XL, RL, and HL provided assistance of the experiments and collected the samples. XZ supervised the experiments and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National 13th Five-Year Key R&D Program Special, Project under grant: 2018YFD0500306.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.818548/full#supplementary-material

REFERENCES

AbuOun, M., Stubberfield, E. J., Duggett, N. A., Kirchner, M., Dormer, L., Nunez-Garcia, J., et al. (2018). mcr-1 and mcr-2 (mcr-6.1) variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015. *J. Antimicrob. Chemother.* 73:2904. doi: 10.1093/jac/dky272

Borowiak, M., Fischer, J., Hammerl, J. A., Hendriksen, R. S., Szabo, I., and Malorny, B. (2017). Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. enterica serovar Paratyphi B. *J. Antimicrob. Chemother.* 72, 3317–3324. doi: 10.1093/jac/dkx327

Cannatelli, A., D'Andrea, M. M., Giani, T., Di Pilato, V., Arena, F., Ambretti, S., et al. (2013). In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. *Antimicrob. Agents Chemother.* 57, 5521–5526. doi: 10.1128/aac.01480-13

Cao, L., Li, X., Xu, Y., and Shen, J. (2018). Prevalence and molecular characteristics of mcr-1 colistin resistance in *Escherichia coli*: isolates of clinical infection from a Chinese University Hospital. *Infect. Drug Resist.* 11, 1597–1603. doi: 10.2147/idr.S166726

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods* 63, 219–228. doi: 10.1016/j.mimet.2005.03.018

Carattoli, A., Villa, L., Feudi, C., Curcio, L., Orsini, S., Luppi, A., et al. (2017). Novel plasmid-mediated colistin resistance mcr-4 gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill.* 22:30589. doi: 10.2807/1560-7917.Es.2017.22.31.30589

Carroll, L. M., Gaballa, A., Guldimann, C., Sullivan, G., Henderson, L. O., and Wiedmann, M. (2019). Identification of Novel Mobilized Colistin Resistance Gene mcr-9 in a Multidrug-Resistant, Colistin-Susceptible *Salmonella enterica* Serotype Typhimurium Isolate. *mBio* 10:19. doi: 10.1128/mBio.00853-19

Cheng, H. Y., Chen, Y. F., and Peng, H. L. (2010). Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in

Klebsiella pneumoniae CG43. *J. Biomed. Sci.* 17:60. doi: 10.1186/1423-0127-17-60

Cheng, P., Yang, Y., Cao, S., Liu, H., Li, X., Sun, J., et al. (2021). Prevalence and Characteristic of Swine-Origin mcr-1-Positive *Escherichia coli* in Northeastern China. *Front. Microbiol.* 12:712707. doi: 10.3389/fmicb.2021.712707

Choi, Y., and Chan, A. P. (2015). PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* 31, 2745–2747. doi: 10.1093/bioinformatics/btv195

Choi, Y., Sims, G. E., Murphy, S., Miller, J. R., and Chan, A. P. (2012). Predicting the functional effect of amino acid substitutions and indels. *PLoS One* 7:e46688. doi: 10.1371/journal.pone.0046688

Choo, S. W., Rayko, M., Tan, T. K., Hari, R., Komissarov, A., Wee, W. Y., et al. (2016). Pangolin genomes and the evolution of mammalian scales and immunity. *Genome Res.* 26, 1312–1322. doi: 10.1101/gr.203521.115

Ciesielczuk, H., Hornsey, M., Choi, V., Woodford, N., and Wareham, D. W. (2013). Development and evaluation of a multiplex PCR for eight plasmid-mediated quinolone-resistance determinants. *J. Med. Microbiol.* 62(Pt 12), 1823–1827. doi: 10.1099/jmm.0.064428-0

Dallenne, C., Da Costa, A., Decré, D., Favier, C., and Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 65, 490–495. doi: 10.1093/jac/dkp498

Delannoy, S., Le Devendec, L., Jouy, E., Fach, P., Drider, D., and Kempf, I. (2017). Characterization of Colistin-Resistant *Escherichia coli* Isolated from Diseased Pigs in France. *Front. Microbiol.* 8:2278. doi: 10.3389/fmicb.2017.02278

Gao, R., Hu, Y., Li, Z., Sun, J., Wang, Q., Lin, J., et al. (2016). Dissemination and Mechanism for the MCR-1 Colistin Resistance. *PLoS Pathog.* 12:e1005957. doi: 10.1371/journal.ppat.1005957

Hammerum, A. M., Sandvang, D., Andersen, S. R., Seyfarth, A. M., Porsbo, L. J., Frimodt-Møller, N., et al. (2006). Detection of sul1, sul2 and sul3 in sulphonamide resistant *Escherichia coli* isolates obtained from healthy humans, pork and pigs in Denmark. *Int. J. Food Microbiol.* 106, 235–237. doi: 10.1016/j.ijfoodmicro.2005.06.023

Higuchi, Y., Hashiguchi, A., Yuan, J., Yoshimura, A., Mitsui, J., Ishiura, H., et al. (2016). Mutations in MME cause an autosomal-recessive Charcot-Marie-Tooth disease type 2. *Ann. Neurol.* 79, 659–672. doi: 10.1002/ana.24612

Ji, K., Xu, Y., Sun, J., Huang, M., Jia, X., Jiang, C., et al. (2020). Harnessing efficient multiplex PCR methods to detect the expanding Tet(X) family of tigecycline resistance genes. *Virulence* 11, 49–56. doi: 10.1080/21505594.2019.1706913

Kai, J., and Wang, S. (2020). Recent progress on elucidating the molecular mechanism of plasmid-mediated colistin resistance and drug design. *Int. Microbiol.* 23, 355–366. doi: 10.1007/s10123-019-00112-1

Kandehkar Ghahraman, M. R., Hosseini-Nave, H., Azizi, O., Shakibaie, M. R., Mollaie, H. R., and Shakibaie, S. (2021). Stereochemical Trajectories of a Two-Component Regulatory System PmrA/B in a Colistin-Resistant *Acinetobacter baumannii* Clinical Isolate. *Iran Biomed J.* 25, 193–201. doi: 10.29252/ibj.25.3.157

Khezri, A., Avershina, E., and Ahmad, R. (2020). Plasmid Identification and Plasmid-Mediated Antimicrobial Gene Detection in Norwegian Isolates. *Microorganisms* 9:microorganisms9010052. doi: 10.3390/microorganisms9010052

Kim, S., Woo, J. H., Kim, N., Kim, M. H., Kim, S. Y., Son, J. H., et al. (2019). Characterization Of Chromosome-Mediated Colistin Resistance In *Escherichia coli* Isolates From Livestock In Korea. *Infect. Drug Resist.* 12, 3291–3299. doi: 10.2147/idr.S225383

Kozak, G. K., Boerlin, P., Janecko, N., Reid-Smith, R. J., and Jardine, C. (2009). Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. *Appl. Environ. Microbiol.* 75, 559–566. doi: 10.1128/aem.01821-08

Letunic, I., Doerks, T., and Bork, P. (2015). SMART: recent updates, new developments and status in 2015. *Nucleic Acids Res.* 43, D257–D260. doi: 10.1093/nar/gku949

Lippa, A. M., and Goulian, M. (2009). Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet.* 5:e1000788. doi: 10.1371/journal.pgen.1000788

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/s1473-3099(15)00424-7

Lu, J., Zhang, J., Xu, L., Liu, Y., Li, P., Zhu, T., et al. (2018). Spread of the florfenicol resistance floR gene among clinical *Klebsiella pneumoniae* isolates in China. *Antimicrob. Resist. Infect. Control* 7:127. doi: 10.1186/s13756-018-0415-0

Luo, Q., Yu, W., Zhou, K., Guo, L., Shen, P., Lu, H., et al. (2017). Molecular Epidemiology and Colistin Resistant Mechanism of mcr-Positive and mcr-Negative Clinical Isolated *Escherichia coli*. *Front. Microbiol.* 8:2262. doi: 10.3389/fmicb.2017.02262

Ma, S., Lei, C., Kong, L., Jiang, W., Liu, B., Men, S., et al. (2017). Prevalence, Antimicrobial Resistance, and Relatedness of *Salmonella* Isolated from Chickens and Pigs on Farms, Abattoirs, and Markets in Sichuan Province, China. *Foodborne Pathog. Dis.* 14, 667–677. doi: 10.1089/fpd.2016.2264

Matamoros, S., van Hattem, J. M., Arcilla, M. S., Willemse, N., Melles, D. C., Penders, J., et al. (2017). Global phylogenetic analysis of *Escherichia coli* and plasmids carrying the mcr-1 gene indicates bacterial diversity but plasmid restriction. *Sci. Rep.* 7:15364. doi: 10.1038/s41598-017-15539-7

Mendes Oliveira, V. R., Paiva, M. C., and Lima, W. G. (2019). Plasmid-mediated colistin resistance in Latin America and Caribbean: A systematic review. *Travel Med. Infect. Dis.* 31:101459. doi: 10.1016/j.tmaid.2019.07.015

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D., Vinogradov, E., Seemann, T., et al. (2010). Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob. Agents Chemother.* 54, 4971–4977. doi: 10.1128/aac.00834-10

Nang, S. C., Li, J., and Velkov, T. (2019). The rise and spread of mcr plasmid-mediated polymyxin resistance. *Crit. Rev. Microbiol.* 45, 131–161. doi: 10.1080/1040841x.2018.1492902

Olaitan, A. O., Morand, S., and Rolain, J. M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front. Microbiol.* 5:643. doi: 10.3389/fmicb.2014.00643

Padilla, E., Llobet, E., Doménech-Sánchez, A., Martínez-Martínez, L., Bengoechea, J. A., and Albertí, S. (2010). *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob. Agents Chemother.* 54, 177–183. doi: 10.1128/aac.00715-09

Paterson, D. L., Isler, B., and Stewart, A. (2020). New treatment options for multiresistant gram negatives. *Curr. Opin. Infect. Dis.* 33, 214–223. doi: 10.1097/qco.0000000000000627

Poirel, L., Jayol, A., and Nordmann, P. (2017). Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. *Clin. Microbiol. Rev.* 30, 557–596. doi: 10.1128/cmr.00064-16

Raetz, C. R., Reynolds, C. M., Trent, M. S., and Bishop, R. E. (2007). Lipid A modification systems in gram-negative bacteria. *Annu. Rev. Biochem.* 76, 295–329. doi: 10.1146/annurev.biochem.76.010307.145803

Rapoport, M., Faccone, D., Pasteran, F., Ceriana, P., Albornoz, E., Petroni, A., et al. (2016). First Description of mcr-1-Mediated Colistin Resistance in Human Infections Caused by *Escherichia coli* in Latin America. *Antimicrob. Agents Chemother.* 60, 4412–4413. doi: 10.1128/aac.00573-16

Rolain, J. M., Kempf, M., Leangapichart, T., Chabou, S., Olaitan, A. O., Le Page, S., et al. (2016). Plasmid-Mediated mcr-1 Gene in Colistin-Resistant Clinical Isolates of *Klebsiella pneumoniae* in France and Laos. *Antimicrob. Agents Chemother.* 60, 6994–6995. doi: 10.1128/aac.00960-16

Routman, E., Miller, R. D., Phillips-Conroy, J., and Hartl, D. L. (1985). Antibiotic resistance and population structure in *Escherichia coli* from free-ranging African yellow baboons. *Appl. Environ. Microbiol.* 50, 749–754. doi: 10.1128/aem.50.4.749-754.1985

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., et al. (2018). Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 73, 1121–1137. doi: 10.1093/jac/dkx488

Sayah, R. S., Kaneene, J. B., Johnson, Y., and Miller, R. (2005). Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. *Appl. Environ. Microbiol.* 71, 1394–1404. doi: 10.1128/aem.71.3.1394-1404.2005

Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U S A.* 95, 5857–5864. doi: 10.1073/pnas.95.11.5857

Schurek, K. N., Sampaio, J. L., Kiffer, C. R., Sinto, S., Mendes, C. M., and Hancock, R. E. (2009). Involvement of pmrAB and phoPQ in polymyxin B adaptation

and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 4345–4351. doi: 10.1128/aac.01267-08

Shen, Y., Zhou, H., Xu, J., Wang, Y., Zhang, Q., Walsh, T. R., et al. (2018). Anthropogenic and environmental factors associated with high incidence of mcr-1 carriage in humans across China. *Nat. Microbiol.* 3, 1054–1062. doi: 10.1038/s41564-018-0205-8

Sonnevend, Á, Ghazawi, A., Alqahtani, M., Shibl, A., Jamal, W., Hashmey, R., et al. (2016). Plasmid-mediated colistin resistance in *Escherichia coli* from the Arabian Peninsula. *Int. J. Infect. Dis.* 50, 85–90. doi: 10.1016/j.ijid.2016.07.007

Stefaniuk, E. M., and Tyski, S. (2019). Colistin Resistance in Enterobacterales Strains - A Current View. *Pol. J. Microbiol.* 68, 417–427. doi: 10.33073/pjm-2019-055

Sun, J., Zhang, H., Liu, Y. H., and Feng, Y. (2018). Towards Understanding MCR-like Colistin Resistance. *Trends Microbiol.* 26, 794–808. doi: 10.1016/j.tim.2018.02.006

Tong, H., Liu, J., Yao, X., Jia, H., Wei, J., Shao, D., et al. (2018). High carriage rate of mcr-1 and antimicrobial resistance profiles of mcr-1-positive *Escherichia coli* isolates in swine faecal samples collected from eighteen provinces in China. *Vet. Microbiol.* 225, 53–57. doi: 10.1016/j.vetmic.2018.09.018

Wang, C. H., Siu, L. K., Chang, F. Y., Chiu, S. K., and Lin, J. C. (2021). A Resistance Mechanism in Non-mcr Colistin-Resistant *Escherichia coli* in Taiwan: R81H Substitution in PmrA Is an Independent Factor Contributing to Colistin Resistance. *Microbiol. Spectr.* 9:e0002221. doi: 10.1128/Spectrum.00022-21

Wang, C., Feng, Y., Liu, L., Wei, L., Kang, M., and Zong, Z. (2020). Identification of novel mobile colistin resistance gene mcr-10. *Emerg. Microbes Infect.* 9, 508–516. doi: 10.1080/22221751.2020.1732231

Wang, Q., Sun, J., Li, J., Ding, Y., Li, X. P., Lin, J., et al. (2017). Expanding landscapes of the diversified mcr-1-bearing plasmid reservoirs. *Microbiome* 5:70. doi: 10.1186/s40168-017-0288-0

Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., et al. (2018). Emergence of a novel mobile colistin resistance gene, mcr-8, in NDM-producing *Klebsiella pneumoniae*. *Emerg. Microbes Infect.* 7:122. doi: 10.1038/s41426-018-0124-z

Wang, X., Wang, Y., Zhou, Y., Wang, Z., Wang, Y., Zhang, S., et al. (2019). Emergence of Colistin Resistance Gene mcr-8 and Its Variant in Raoultella ornithinolytica. *Front. Microbiol.* 10:228. doi: 10.3389/fmicb.2019.00228

Xavier, B. B., Lammens, C., Butaye, P., Goossens, H., and Malhotra-Kumar, S. (2016). Complete sequence of an IncFII plasmid harbouring the colistin resistance gene mcr-1 isolated from Belgian pig farms. *J. Antimicrob. Chemother.* 71, 2342–2344. doi: 10.1093/jac/dkw191

Xiaomin, S., Yiming, L., Yuying, Y., Zhangqi, S., Yongning, W., and Shaolin, W. (2020). Global impact of mcr-1-positive *Enterobacteriaceae* bacteria on "one health". *Crit. Rev. Microbiol.* 46, 565–577. doi: 10.1080/1040841x.2020.1812510

Yang, Y. Q., Li, Y. X., Lei, C. W., Zhang, A. Y., and Wang, H. N. (2018). Novel plasmid-mediated colistin resistance gene mcr-7.1 in *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* 73, 1791–1795. doi: 10.1093/jac/dky111

Yin, W., Li, H., Shen, Y., Liu, Z., Wang, S., Shen, Z., et al. (2017). Novel Plasmid-Mediated Colistin Resistance Gene mcr-3 in *Escherichia coli*. *mBio* 8:17. doi: 10.1128/mBio.00543-17

Yuan, J., Wang, X., Shi, D., Ge, Q., Song, X., Hu, W., et al. (2021). Extensive antimicrobial resistance and plasmid-carrying resistance genes in mcr-1-positive E. coli sampled in swine, in Guangxi, South China. *BMC Vet. Res.* 17:86. doi: 10.1186/s12917-021-02758-4

Zhang, S., Chen, S., Abbas, M., Wang, M., Jia, R., Chen, S., et al. (2021). High incidence of multi-drug resistance and heterogeneity of mobile genetic elements in *Escherichia coli* isolates from diseased ducks in Sichuan province of China. *Ecotoxicol. Environ. Saf.* 222:112475. doi: 10.1016/j.ecoenv.2021.112475

Zhang, X., Zhang, B., Guo, Y., Wang, J., Zhao, P., Liu, J., et al. (2019). Colistin resistance prevalence in *Escherichia coli* from domestic animals in intensive breeding farms of Jiangsu Province. *Int. J. Food Microbiol.* 291, 87–90. doi: 10.1016/j.ijfoodmicro.2018.11.013

Zhao, F., Feng, Y., Lü, X., McNally, A., and Zong, Z. (2017). IncP Plasmid Carrying Colistin Resistance Gene mcr-1 in *Klebsiella pneumoniae* from Hospital Sewage. *Antimicrob. Agents Chemother.* 61:16. doi: 10.1128/aac.02229-16

Zhuge, X., Ji, Y., Tang, F., Sun, Y., Jiang, M., Hu, W., et al. (2019). Population structure and antimicrobial resistance traits of avian-origin mcr-1-positive *Escherichia coli* in Eastern China, 2015 to 2017. *Transbound Emerg. Dis.* 66, 1920–1929. doi: 10.1111/tbed.13222

Zurfluh, K., Klumpp, J., Nüesch-Inderbinen, M., and Stephan, R. (2016). Full-Length Nucleotide Sequences of mcr-1-Harboring Plasmids Isolated from Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* Isolates of Different Origins. *Antimicrob. Agents Chemother.* 60, 5589–5591. doi: 10.1128/aac.00935-16

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 Li, Cheng, Li, Liu, Liu 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 Microbiology

REVIEW
published: 31 March 2022
doi: 10.3389/fmicb.2022.859854

The Dynamics of the Antimicrobial Resistance Mobilome of *Salmonella enterica* and Related Enteric Bacteria

*Suad Algarni[1,2], Steven C. Ricke[3], Steven L. Foley[1,2] and Jing Han[1]**

[1]Division of Microbiology, FDA National Center for Toxicological Research, Jefferson, AR, United States, [2]Cellular and Molecular Biology Graduate Program, University of Arkansas, Fayetteville, AR, United States, [3]Meat Science and Animal Biologics Discovery Program, Department of Animal and Dairy Sciences, University of Wisconsin, Madison, WI, United States

The foodborne pathogen *Salmonella enterica* is considered a global public health risk. *Salmonella enterica* isolates can develop resistance to several antimicrobial drugs due to the rapid spread of antimicrobial resistance (AMR) genes, thus increasing the impact on hospitalization and treatment costs, as well as the healthcare system. Mobile genetic elements (MGEs) play key roles in the dissemination of AMR genes in *S. enterica* isolates. Multiple phenotypic and molecular techniques have been utilized to better understand the biology and epidemiology of plasmids including DNA sequence analyses, whole genome sequencing (WGS), incompatibility typing, and conjugation studies of plasmids from *S. enterica* and related species. Focusing on the dynamics of AMR genes is critical for identification and verification of emerging multidrug resistance. The aim of this review is to highlight the updated knowledge of AMR genes in the mobilome of *Salmonella* and related enteric bacteria. The mobilome is a term defined as all MGEs, including plasmids, transposons, insertion sequences (ISs), gene cassettes, integrons, and resistance islands, that contribute to the potential spread of genes in an organism, including *S. enterica* isolates and related species, which are the focus of this review.

Keywords: mobilome, conjugation, mobile genetic elements, *Salmonella enterica*, horizontal gene transfer

OPEN ACCESS

Edited by:
Ziad Daoud,
Central Michigan University,
United States

Reviewed by:
Valentine Usongo,
Health Canada, Canada
Ben Pascoe,
University of Bath, United Kingdom

***Correspondence:**
Jing Han
jing.han1@fda.hhs.gov

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *21 January 2022*
Accepted: *07 March 2022*
Published: *31 March 2022*

Citation:
Algarni S, Ricke SC, Foley SL and Han J (2022) The Dynamics of the Antimicrobial Resistance Mobilome of Salmonella enterica and Related Enteric Bacteria. Front. Microbiol. 13:859854. doi: 10.3389/fmicb.2022.859854

INTRODUCTION TO *Salmonella enterica*

Salmonella enterica is a facultative intracellular microorganism that is one of the more important etiological agents of foodborne illnesses and has a significant impact on global human health. *Salmonella* can be classified, based on the acquisition of *Salmonella* pathogenicity islands (SPIs), into two species: *S. enterica* and *Salmonella bongori* (Majowicz et al., 2010; Moreno Switt et al., 2012). Annually, more than 90 million human cases of *Salmonella* gastroenteritis are estimated globally (Mather et al., 2013). *Salmonella* infections cost an estimated $3 billion per year in both the European Union and in the United States (U.S.). There are around 1.03 million infections and 400 deaths annually in the United States due to mortality, disability, medical and productivity costs, resulting in the loss of about 16,782 quality-adjusted life years (Hoffmann et al., 2012; Foley et al., 2013; Mather et al., 2013).

Salmonella isolates can be differentiated into more than 2,600 serotypes based on their surface antigen profiles. The most common serotypes associated with human infections in the

United States include Typhimurium, Enteritidis, Newport, Javiana, and Heidelberg (Foley and Lynne, 2008; Foley et al., 2013). These serotypes have shown the ability to infect multiple host species while other serotypes have a narrow host range; for example, *Salmonella* Typhi and *Salmonella* Paratyphi are human-associated serotypes that typically are not associated with foodborne transmission (Lynne et al., 2009; Foley et al., 2013). According to data from the United States Centers for Disease Control and Prevention (CDC) Food Net Program, greater than half of the human infections involving narrow host range serotypes caused invasive infection, rather than gastroenteritis, which leads to more severe disease outcomes (Vugia et al., 2004; Foley et al., 2013).

Over the past several decades, the incidence of multidrug-resistant (MDR) *Salmonella* infections has exhibited a steady rise in many regions including Europe and North America (Helms et al., 2005) and they have been associated with a number of outbreaks in the United States (Nair et al., 2018). Several studies have indicated that *S. enterica* resistance to antimicrobial drugs are correlated with an increased need for hospitalization, higher risk of invasive illness and deaths, as well as increased treatment costs (Helms et al., 2005; Mather et al., 2013). Based on epidemiological data, the CDC estimates the incidence of *Salmonella* infections per 100,000 population ranged from 11 to 15 during the years of 1996 to 2011 (Boore et al., 2015). There were 91,408 confirmed clinical cases of *Salmonella* foodborne illnesses in the European Union in 2014 (Pornsukarom et al., 2018).

THE RISE OF AMR

Over the past several decades, antimicrobial agents have been a cornerstone of modern medicine and used extensively in animal production, veterinary medicine and industrial production (van Schaik, 2015). Antimicrobial therapies target specific aspects of bacterial physiology which selectively impacts the pathogens, while having less deleterious effects on the vertebrate host, but potentially can impact the commensal microbiome of the host (Jernberg et al., 2010). Antimicrobial agents are categorized based on multiple features involving their structure, ranges of target organisms (spectrum of activity), and mode of action. Most act by specifically binding to their targets, which plays a vital role in bacterial growth and survival, thereby preventing the physiological function of these targets and becoming lethal to the bacterial cells or inhibitory to cell growth (Lambert, 2005). The efficacy of an antimicrobial is related largely to its mechanisms of action, which include: (i) the inhibition of cell wall biosynthesis (penicillins and other β-lactams); (ii) protein synthesis inhibition by the targeting of the 16S rRNA (A-site) of the 30S ribosomal subunits (tetracyclines and aminoglycosides) or prevention of the 50S ribosomal subunit function (macrolides and chloramphenicol); (iii) inhibition of nucleic acid biosynthesis including inhibition of RNA transcription (rifampicin) or inhibition of DNA synthesis (quinolones and fluoroquinolones); (iv) inhibition of the metabolic pathways (including folic acid analogs such as sulfonamides and trimethoprim); and (v) by damaging the bacterial cell membrane structure (polymyxins; Bockstael and Van Aerschot, 2009; Sultan et al., 2018; **Figure 1**). As a result of their therapeutic use, antimicrobial agents have led to increased human life expectancy and decreased human morbidity and mortality (van Hoek et al., 2011).

However, due to overuse or misuse of the antimicrobials, antimicrobial resistance (AMR; a situation in which the drugs are unable to inhibit bacterial growth and lose the ability to be used for treating a bacterial infection) has emerged worldwide, and has become a major public health concern (Blair et al., 2015; Ventola, 2015; Zaman et al., 2017). In the United States, MDR bacteria infections have led to increased mortality and a strained health care system, with an estimated economic impact of over $20 billion associated with more than two-million infections and approximately 23,000 deaths each year (Blair et al., 2015).

The mechanisms of acquired resistance to antimicrobial agents are broadly classified into three categories: (i) use of energy-dependent efflux pumps to extrude the antimicrobials; (ii) production of hydrolytic or modifying enzymes to inactivate antimicrobials; and (iii) modifying antimicrobial targets (Blanco et al., 2016; Reygaert, 2018; **Figure 2**). In addition, many Gram-negative bacteria are naturally resistant to some larger antimicrobials, such as vancomycin, due to cell membrane characteristics that prevent entry into the cell and subsequent effectiveness.

Efflux pumps are bacterial transport proteins that are encoded by genes located on the bacterial chromosome and/or plasmids. The efflux pumps function primarily to extrude substrates (antimicrobials) from the cellular interior to the external environment, therefore imparting the efflux pump-expressing bacteria with an AMR phenotype. While some efflux pumps are expressed constitutively, others are induced under certain environmental stimuli or when a suitable substrate is present (Blanco et al., 2016). Many of these efflux pumps can transport a large variety of compounds (MDR efflux pumps), resulting in the acquisition of MDR by bacteria. In order to transport substrates against a concentration gradient, these efflux pumps are energy-dependent. Based on the type of the energy used, the efflux pumps are broadly classified into two categories, the primary efflux pumps which utilize energy from active hydrolysis of ATP and the secondary efflux pumps which derive energy from chemical gradients formed by either protons or ions such as sodium (Sharma et al., 2019).

In prokaryotes, five major families of efflux pumps have been described: (i) ATP binding cassette (ABC), which are primary active transporters; (ii) small MDR family; (iii) multidrug and toxin extrusion (MATE) family; (iv) major facilitator superfamily (MFS); and (v) resistance nodulation cell division (RND) family, which are all secondary active transporters (Sharma et al., 2019). Based on sequence similarity, the nine functional drug efflux pumps identified in *S. enterica* (AcrAB, AcrD, AcrEF, MdtABC, MdsAB, EmrAB, MdfA, MdtK, and MacAB) either belong to the MFS family (EmrAB and MdfA), the RND family (AcrAB, AcrD, AcrEF, MdtABC, and MdsAB), the MATE family (MdtK); or the ABC family (MacAB), respectively (Horiyama et al., 2010). Among these efflux pumps,

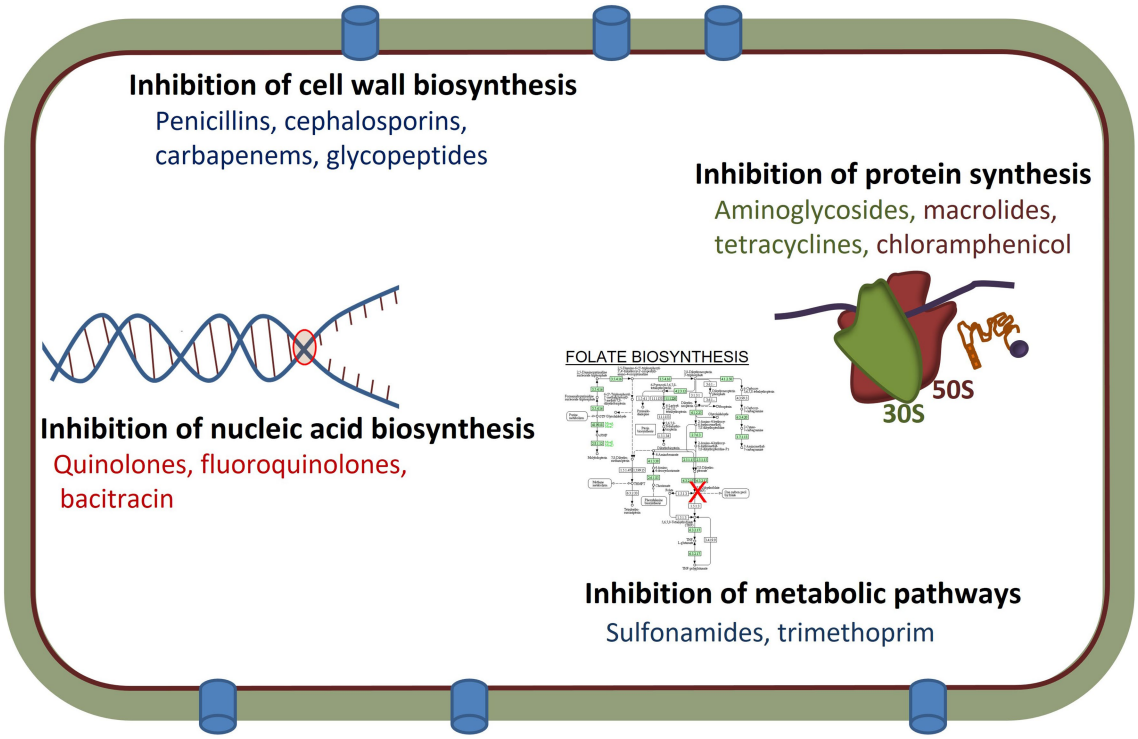


FIGURE 1 | Mechanisms of actions and targets of different antimicrobial agents used in human and/or veterinary medicine.

the AcrAB-TolC efflux pump (a tripartite complex consisting of a periplasmic membrane fusion protein AcrA, a cytoplasmic membrane transporter protein AcrB, and an outer membrane channel TolC) is the most effective in causing MDR *Salmonella* and has been shown to directly contribute to resistance to fluoroquinolones, chloramphenicol, and tetracyclines in *Salmonella* (Piddock, 2006; Horiyama et al., 2010; Shen et al., 2017). The OqxAB efflux pump also mediates MDR in various bacteria, including *Salmonella* (Aljahdali et al., 2019). It belongs to the RND family and significantly contributes to reduced susceptibility to olaquindox, nalidixic acid, tigecycline, nitrofurantoin, chloramphenicol, and facilitates the development of high-level fluoroquinolone resistance (Li et al., 2013; Aljahdali et al., 2019). The *oqxAB* gene is located either on the chromosomal DNA of *Salmonella* (Wong and Chen, 2013) or on plasmids with other AMR genes (Li et al., 2013; Wong et al., 2016). The carriage of *oqxAB* on transferable plasmids would facilitate its transmission *via* horizontal gene transfer and the emergence of MDR strains (Wong et al., 2016; Aljahdali et al., 2019). Plasmids from the incompatibility group (Inc) HI2 have been shown to play a pivotal role in dissemination of *oqxAB* in *Salmonella* spp. (Wong et al., 2016).

The resistance to antimicrobials that target the ribosomal subunits interferes with the ability to bind to the ribosome. This binding interference is due to ribosomal mutation (aminoglycosides and oxazolidinones), ribosomal subunit methylation (aminoglycosides, macrolides, oxazolidinones, and streptogramins) most commonly involving *erm* genes, or ribosomal protection (tetracyclines; Reygaert, 2018). The tetracycline family of antimicrobials inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) and is widely used because it offers a broad spectrum of activity against Gram-positive and Gram-negative bacteria. However, the increasing incidence of resistance to tetracyclines in *Salmonella* spp. of human and animal origins has been reported worldwide. In addition to efflux pump-mediated resistance mechanisms, resistance to tetracyclines can involve ribosomal protection proteins (RPPs), which are a group of cytoplasmic proteins that can bind to the ribosome, resulting in the release of tetracycline from the ribosome, enabling protein synthesis to proceed (Warburton et al., 2016). To date, 12 tetracycline resistance genes encoding RPPs have been reported, including *tet*M, O, Q, S, T, W, 32, 36, 44, *B*(P), *otr*(A), and *tet* (Warburton et al., 2016). Many of the RPP determinants are located on mobile genetic elements (MGEs) within *Salmonella*, including transposons or plasmids, which may have facilitated their spread throughout the eubacteria *via* lateral gene transfer events (Gargano et al., 2021). Another example of modification of antimicrobial targets is the wide dissemination of the plasmid-encoded chloramphenicol-florfenicol resistance (cfr) methyltransferase, which specifically methylates the adenine at position 2,503 in the 23S rRNA, thereby conferring resistance to a wide range of ribosome-targeted antimicrobials, including the phenicols, streptogramins, macrolides, and oxazolidinones (such as linezolid; Kaminska et al., 2010).

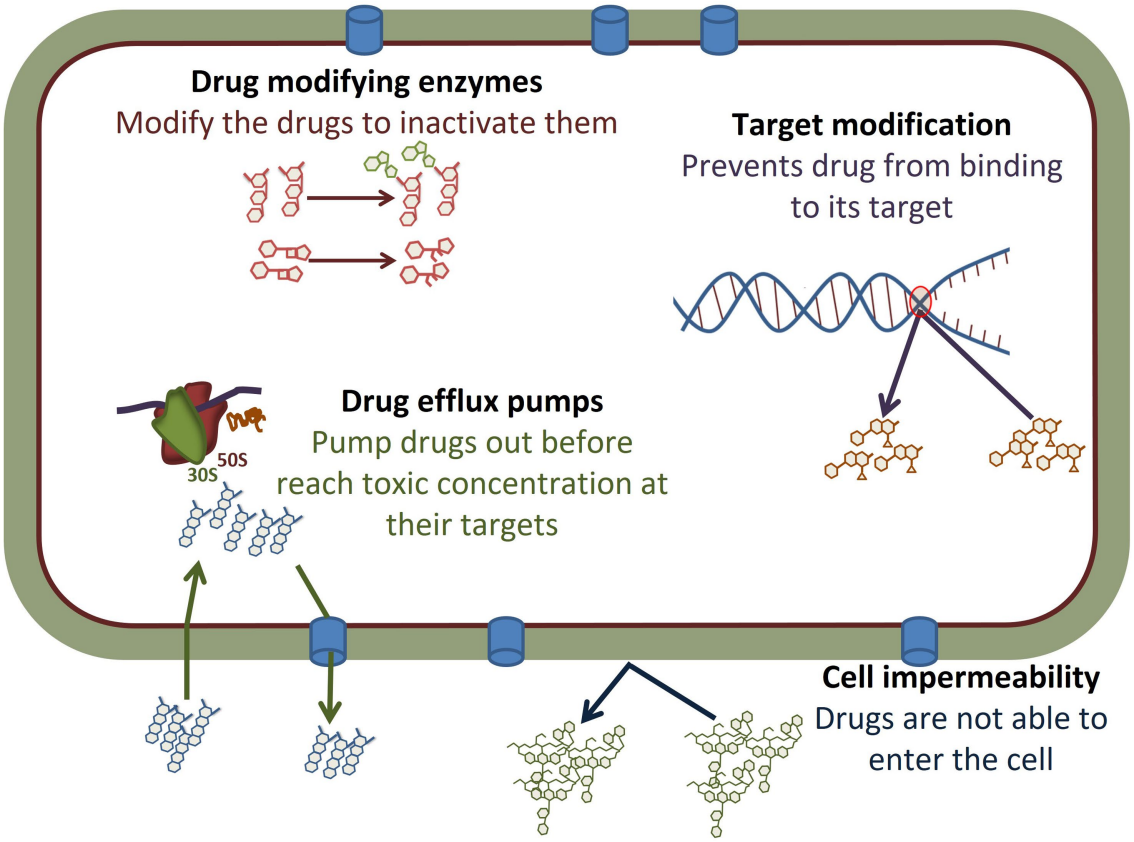


FIGURE 2 | Antimicrobial resistance mechanisms observed in *Salmonella*. The different mechanisms are described in detail in the body of the manuscript. Briefly, drugs such as many aminoglycosides can be modified by the addition of functional groups, while others such as β-lactam antibiotics can be cleaved by enzymes, thus modifying their structures and inhibiting function. Target modification, such as can occur in metabolic pathways or in the nucleic acid replication machinery, can limit the ability of antimicrobials to bind to their targets to prevent efficacy. A number of other drugs are impacted by drug efflux pumps that prevent drugs such as tetracyclines and chloramphenicol from reaching their targets, such as the ribosomes, in a high enough concentration which limits their effectiveness. Other drugs, such as vancomycin, are too large to enter through the Gram-negative cell wall and *Salmonella* are intrinsically resistant due to inability to enter the cell.

For antimicrobials that target nucleic acid synthesis (for example, fluoroquinolones), resistance is primarily associated with chromosomal mutations in the bacterial genes encoding targeted enzymes, DNA gyrase (such as *gyrA* in Gram-negative bacteria, including *Salmonella*) or topoisomerase IV (such as *grlA* in Gram-positive bacteria or *parC* in Gram-negative bacteria). Many of these mutations occur in the quinolone resistance determining region (QRDR) of the gyrase and topoisomerase genes and cause changes in the structure of the respective proteins that decreases or eliminates the ability of the antimicrobials to bind to the enzymes (Lee et al., 2021). The emergence of fluoroquinolone-resistant *Salmonella* has resulted in treatment failure and high mortality rates (Lee et al., 2021).

Antimicrobials such as the sulfonamides and trimethoprim act as competitive inhibitors of essential steps in the folate biosynthetic pathway in bacteria. These drugs are structural analogs of the natural substrates (sulfonamides for p-amino-benzoic acid and trimethoprim for dihydrofolate) and bind to their respective enzymes through competitive inhibition by binding to the active site of the enzymes dihydropteroate synthase and dihydrofolate reductase, respectively. Resistance to these antimicrobial that inhibit metabolic pathways can be due to mutations in enzymes, such as dihydropteroate synthase or dihydrofolate reductase, that prevent binding of the competitive inhibitor (Reygaert, 2018). Mutations in these enzymes at or near the active site result in structural changes in the enzyme that interfere with antimicrobial binding, while still allowing the natural substrate to bind (Reygaert, 2018). Additionally, overproduction of dihydrofolate reductase or dihydropteroate synthase can limit the competitive impact of sulfonamides and trimethoprim on the pathway function (Reygaert, 2018).

Antimicrobial inactivation by enzymes is another critical resistance mechanism. There are two main ways in which bacteria inactivate drugs, these include actual degradation of the drug or by the transfer of a chemical group (most commonly acetyl, phosphoryl, and adenyl groups) to the antimicrobials altering their function (Reygaert, 2018). The β-lactamases are a very large group of drug hydrolyzing enzymes that inactivate β-lactam drugs (including penicillin, ampicillin, cephalosporins, carbapenems, and ceftazidime) by hydrolyzing a specific site

in the β-lactam ring structure, causing the ring to open, resulting in the inability of the antimicrobials to bind to their target penicillin-binding protein (PBP). To date, more than 1,300 distinct β-lactamases have been identified in clinical isolates, of which the most deleterious are the extended-spectrum β-lactamases (ESBLs) that hydrolyze most penicillins, cephalosporins, and the carbapenemases that can inactivate all β-lactam classes of drugs (Bush, 2013). The production of β-lactamases, which can be encoded by genes located on the chromosome (such as $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-9a}}$, and bla_{MIR}) or on plasmids (for example, bla_{TEM} or $bla_{\text{CTX-M-15}}$), is the most common resistance mechanism used by Gram-negative bacteria against β-lactam drugs (Bush and Bradford, 2016; Reygaert, 2018). Combined with decreased uptake or increased efflux of the drugs, resistance to the β-lactams in Gram-negative bacteria continues to be on the rise with the high-level resistance being a major clinical problem (Bush and Bradford, 2016).

Inactivation by enzymatic modification is also the most prevalent mechanism of resistance to aminoglycoside antimicrobials, which is currently considered to be one of the more formidable broad-spectrum antimicrobials used in the treatment of life-threatening infections in the clinical setting (Ramirez and Tolmasky, 2010; Aishwarya et al., 2020). Aminoglycoside-modifying enzymes (AMEs) are the most common cause of resistance to aminoglycosides (Ramirez and Tolmasky, 2010). AMEs catalyze the covalent modification of aminoglycosides as they transport across the cytoplasmic membrane by modifying the amino or hydroxyl groups. There are three kinds of AMEs: (1) N-acetyltransferases (AAC), which acetylate the –NH2 (amino) group by N-acetylation; (2) O-nucleotidyltranferases (ANT), which adenylate the hydroxyl groups by O-nucleotidylation; and (3) O-phosphotranferases (APH), which phosphorylate the hydroxyl groups by O-phosphorylation (Ramirez and Tolmasky, 2010). The modifications of the aminoglycoside reduces drug binding to the ribosome, which results in high levels of resistance. The genes coding for AMEs are highly mobile with the ability to transfer as part of integrons, gene cassettes, transposons, integrative conjugative elements, or through conjugation as part of mobilizable or conjugative plasmids (Ramirez and Tolmasky, 2010). To date, more than 85 AMEs have been reported in both Gram-positive and Gram-negative bacteria (Aishwarya et al., 2020). The combination of large numbers, the ability of the genes coding for these enzymes to evolve, as well as the numerous mobile elements where they are located, result in a high adaptability AMEs to efficiently disseminate among bacteria, which has greatly reduced the efficacy of several aminoglycosides (Ramirez and Tolmasky, 2010). Tetracyclines can also be inactivated by hydrolyzation *via* the TetX enzyme, whose gene is located on plasmid that catalyzes tetracycline degradation (Moore et al., 2005).

Antimicrobial inactivation by the transfer of a chemical group is another key resistance challenge, as a large number of transferases that have been identified contribute to resistance. Among them, acetylation is the most diversely used mechanism and has led to resistance against aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones (Reygaert, 2018). The primary resistance mechanism for chloramphenicol is the enzymatic inactivation by acetylation of the antimicrobial drug molecule *via* different types of chloramphenicol acetyltransferases (CATs; Schwarz et al., 2004). There are two different types of CAT enzymes which are genetically unrelated and encoded by *catA* and *catB* groups, respectively (Roberts and Schwarz, 2017). Both *catA* and *catB* genes are often associated with mobile elements such as plasmids, transposons, or gene cassettes and are able to be transferred between bacteria of different species and genera (Roberts and Schwarz, 2017). In *Salmonella*, besides mutations in the QRDR and plasmid-mediated quinolone resistance (PMQR) determinants *qnr* genes; *aac(6′)-Ib-cr* encodes acetyltransferase which can acetylate the quinolone antimicrobials that reduces their activities and contribute to quinolone resistance (Robicsek et al., 2006).

The detection of AMR genes in bacteria has mainly relied on molecular methods. Due to their rapidity and sensitivity, nucleic acid-based detection methods have played an important role in the elucidation of resistance mechanisms (Fluit et al., 2001). Based on the amplification of target genes, traditional PCR, multiplex PCR, qPCR (real-time PCR), Reverse Transcriptase PCR (RT-PCR), approaches have been developed and widely used in the laboratory to rapidly identify AMR genes (Galhano et al., 2021). DNA microarray and hybridization approaches have also been used to simultaneously detect a large number different AMR genes in a short time. Microarrays for resistance detection in both gram-negative and gram-positive bacteria have been developed (Perreten et al., 2005; Card et al., 2013; Galhano et al., 2021). Microarray detection methods have been largely replaced by whole-genome sequencing (WGS) approaches that have become widely used and effective tools in detection of AMR genes (Galhano et al., 2021). To detect AMR genes in WGS data, several AMR gene databases, such as ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation), RGI/CARD (Comprehensive Antibiotic Resistance Database), NCBI-AMRFinder, and PointFinder have been developed to identify AMR genes in the WGS contigs (Gupta et al., 2014; Zankari et al., 2017; Alcock et al., 2020; Feldgarden et al., 2021). WGS has been proven to be an effective technique to detect AMR genes within bacteria such as *Salmonella* (Galhano et al., 2021).

Mobilome Among *Salmonella enterica* Isolates

The term "mobilome" refers to all MGEs and genes including plasmids, integrons, transposons, insertion sequences (ISs), integrative and conjugative elements (ICEs), and resistance islands and/or genomic islands (GIs) in bacteria which can translocate within and transfer between genomes (Frost et al., 2005; Cabezón et al., 2017). MGEs play a key role in both the development and spread of AMR genes among the bacterial populations as well as in the evolution of bacterial genomes which rapidly respond to a selective pressure. These responses include alteration of antimicrobial exposure in different genera, species, and strains, including those of *Salmonella* isolates (Davies and Davies, 2010).

TABLE 1 | Approaches to detect plasmid-based antimicrobial resistance (AMR) genes.

Approach	Sample types	Advantage	Limitation	References
Illumina-based WGS	Food/clinical samples	Fast, applicable for studying the genetic diversity evolutionary history of plasmid type and epidemiological tracking.	Challenge for plasmid detection in short read length.	Almeida et al., 2018
Oxford Nanopore Technology (ONT)	Food/clinical samples	Rapid tool Low instrument Cost and small footprint	Incomplete fragmentation of plasmid. High error rate. Performance often needs to be optimized with MiSeq reads.	Lemon et al., 2017
Pacific bioscience single-molecule real-time sequencing (SMRT)	Food/clinical samples	Generate long-read sequencing data that can lead to closed plasmid sequences.	The reads length of data relies on the individual polymerase.	Chen et al., 2019

TABLE 2 | *In silico* analysis of resistance plasmid gene content and epidemiology.

Web server	Advantages	Links	References
PLSDB	Used for short sequences to identify the specific AB genes in the plasmids.	https://ccb-microbe.cs.uni-saarland.de/plsdb/	Galata et al., 2019
ATLAS	Detect the plasmids carrying the antimicrobials resistance genes, and plasmid network.	http://www.patlas.site	Jesus et al., 2019
PlasmidSeeker	Able to identify the novel plasmids and detect plasmids from bacterial WGS data without read assembly.	https://github.com/bioinfo-ut/PlasmidSeeker	Roosaare et al., 2018
FeatureExtract	Able to identify the antimicrobial resistance genes and conjugal transfer system (IV).	https://services.healthtech.dtu.dk/service.php?FeatureExtract-1.2	Wernersson, 2005

THE MOBILITY OF THE AMR GENES THROUGH MGEs

Plasmids

Understanding the transfer dynamics of AMR plasmids requires detailed monitoring of different pathogenic bacteria in both clinical and non-clinical environments. Plasmid-mediated transfer plays a critical role in the spread of AMR in *S. enterica* and related species (Han et al., 2012; San Millan et al., 2015; San Millan, 2018). Several studies and review articles have described the importance of plasmid dynamics in Gram-negative bacteria, in particular *S. enterica*, and many have utilized DNA sequencing to help examine resistance transfer (Zhao et al., 2003; Han et al., 2012; Lerminiaux and Cameron, 2019; McMillan et al., 2019). Baker et al. (2018) highlights the importance of WGS as a key technology for understanding the spatial dynamics of AMR evolution and for genomic epidemiology (Baker et al., 2018). **Tables 1**, **2** present multiple sequencing platforms and computational-based approaches that can be used for the detection of plasmid-based AMR genes.

In studies of MDR plasmid transfer in different *Salmonella* strains, isolates have been sequenced and diverse AMR genes associated with chloramphenicol, tetracyclines, ampicillin, streptomycin, kanamycin, and β-lactam antimicrobials have been identified (Han et al., 2012). When the rate of horizontal transfer outweighs the costs they impose on their bacterial hosts, plasmids are favored in the new hosts (Svara and Rankin, 2011). Stalder et al. (2017) reported that common mechanisms of plasmid stabilization in bacterial pathogens could influence plasmid-host coevolutionary dynamics (Stalder et al., 2017). Various concentrations of antimicrobials are encountered by bacteria in different environments and may affect the dynamics of the microbial population. For example, plasmid transfer between bacteria may lead to acquired resistance to different antimicrobials through the transfer of AMR determinants in response to selective pressure (Martinez, 2008). Svara and Rankin (2011) designed a model to examine the effect of antimicrobial treatment, specifically the dosage of antimicrobials and the interval between treatments, on the evolution of plasmid-borne resistance (Svara and Rankin, 2011). Their results showed that different treatment regimens (different interval between antimicrobial treatments and the dosage of antimicrobials) can select for either plasmid-carried or chromosome-carried resistance. In the absence of competing non-resistant plasmids, high transmission favors plasmid-borne resistance. While in the presence of other plasmids, plasmid-borne resistance was favored over chromosomal resistance in the low frequency antimicrobial treatments, but a high intensity of antimicrobial treatments is required for resistance plasmids to outcompete non-resistance plasmids (Svara and Rankin, 2011). Therefore, changes in natural ecosystems, including the release of large amounts of antimicrobials, might alter the population dynamics of antimicrobial resistant microorganisms, which will affect the evolution and dissemination of AMR in nature (Martinez, 2008). Taken together, these findings emphasize the necessity of new therapeutics to reduce the spread of AMR.

According to their transmissibility by conjugation, there are two classes of transmissible plasmids: (1) conjugative plasmids, which contain a full set of conjugation genes; and (2) mobilizable plasmids, which contain only a minimal set of genes that allow them to be mobilized by conjugation when they coexist in the same donor cell with a conjugative plasmid (Garcillan-Barcia et al., 2011). Conjugative plasmids possess a generally

low copy number, while mobilizable plasmids tend to be a high copy number (Watve et al., 2010; van Hoek et al., 2011). Both conjugative and mobilizable plasmids can encode AMR genes and transfer them to a new host (van Hoek et al., 2011). Over the last decade, more than 1,000 unique plasmids have been identified in the family of *Enterobacteriaceae*, some of the most common ones include IncA/C, HI2, HI1, I1-γ, X, L/M, N, FIA, FIB, FIC, W, Y, P, T, K, B/O, FI1, U, R, ColE, and Q (Garcia-Fernandez and Carattoli, 2010). Among these, some Inc. types can be clustered into groups based on the genetic and pilus structure: (1) IncF group (consisting of IncF, D, J, and S); (2) IncI group (includes IncB, I, and K); and (3) IncP group (containing IncM, P, U, and W); and Ti (tumor inducing) that may have implications on plasmid transfer dynamics (van Hoek et al., 2011). Specifically, many of the plasmids that have been identified in *Salmonella* strains include representatives of IncA/C, F, H, I, L/M, N, R, and X groups (Fricke et al., 2009; Foley et al., 2013).

In the 1970s, IncA/C plasmids were initially described as large-molecular-weight (140–200 kb) and low copy number plasmids present in fish pathogens such as *Aeromonas hydrophila* and *Vibrio* spp. (Carraro et al., 2015; Ruggiero et al., 2018). Subsequently, IncA/C plasmids were found to encode resistance genes to antimicrobial agents in food animals and agricultural settings (Johnson and Lang, 2012). To date, research on conjugative IncA/C plasmids has determined that these plasmids play an essential role in the spread of MDR among the species of *Enterobacteriaceae*, including *S. enterica* and *Vibrio cholerae*, which is an important concern in the public health community (Carraro et al., 2015; Han et al., 2018). The core genetic backbones of IncA/C plasmids are generally highly conserved, with greater than 98% nucleotide identity across the plasmid backbone. Most examples of IncA/C plasmids among non-typhoidal *S. enterica* isolates in the United States carry transfer-associated genes which accelerate conjugal transfer (Welch et al., 2007; Fricke et al., 2009; Wiesner et al., 2011; Han et al., 2018; Ruggiero et al., 2018). Wiesner et al. (2011) described a MDR *Salmonella* Typhimurium ST213 strain in Mexico, which was associated with the carriage of IncA/C plasmids that carried a plasmid-borne bla_{CMY-2} gene encoding resistance to extended-spectrum cephalosporins (Wiesner et al., 2011). As predicted by Han et al. (2018), many IncA/C plasmids encode their own conjugal machinery and are able to be transferred within bacterial communities. However, others without the full cohort of transfer genes required other conjugative plasmids in the bacterial cell for transfer of the plasmids (Han et al., 2018). Rankin et al. (2002) indicated that IncA/C plasmids were responsible for MDR phenotypes in *Salmonella* Newport isolates and their resistance genes could be transferred both to other *Salmonella* as well as other susceptible organisms under antimicrobial selection pressure in the same environments (Rankin et al., 2002). In *S. enterica* serovar Newport isolates, IncA/C plasmids emerged as the source of MDR phenotypes after 1980 (Johnson and Lang, 2012). Moreover, these plasmids are considered as broad host range plasmids due to their ability to spread by conjugative transfer among the bacterial communities, meanwhile under *in vitro* conditions some IncA/C plasmids exhibit variation in their ability to transfer between *Escherichia coli* and *S. enterica* (Johnson and Lang, 2012). In addition to their presence in food animal environments, cases have also been documented of plasmids spreading in hospital environments (Johnson and Lang, 2012). In some of these studies it was demonstrated that IncA/C plasmids have mobilizable genes conferring resistance to a number of different antimicrobial agents including β-lactams, aminoglycosides, chloramphenicol, sulfonamide, trimethoprim, quinolones, and tetracyclines (Carraro et al., 2015). These studies have been somewhat hampered by the presence of a large number of AMR elements carried on the plasmids. These AMR elements have been a challenge for studying their basic molecular biology and regulatory mechanisms because most of the molecular biology tools that are available rely on the antimicrobial selection for mutational studies (Johnson and Lang, 2012; Carraro et al., 2015).

Conjugative Plasmids

One of the main mechanisms of the horizontal gene transfer is bacterial conjugation (Frost et al., 2005; Cabezón et al., 2017). Bacterial conjugation is a highly specific process that occurs by transferring plasmid DNA from one bacterial cell (donor) to another bacterium (recipient) through a direct cell-to-cell contact (Willetts and Wilkins, 1984; Partridge et al., 2018). Conjugative plasmids are considered to be self-transmissible and carry the genes, known as mobilization (MOB) or DNA-transfer replication (Dtr) genes, that are required for DNA transfer and mating-pair apparatus formation to initiate the transfer process at OriT (Jain and Srivastava, 2013). The MOB functionality has also been used for the identification/classification/typing of plasmids and epidemiological tracking based on the conjugative and mobilization relaxase genes (Partridge et al., 2018). The fundamental principle of the bacterial conjugation system involves the merging of the two archaic bacterial systems: rolling-circle replication (RCR) and the type IV secretion system (T4SS; Llosa et al., 2002). The T4SS forms a complex protein encoded by numerous genes called mating pore formation genes (MPF; Cabezón et al., 2017). In the conjugation machinery, RCR has a similar function to a relaxosome, which initiates transfer through the T4SS (Llosa et al., 2002). Howard et al. (1995) demonstrated that the two proteins (TraYp and TraLp) assemble the relaxosome site and bind at the origin of the transfer which causes a strand-specific nick found within conjugation machinery in the *E. coli* F plasmids (Howard et al., 1995). There are two basic regions that contribute to conjugation machinery: TraI, also known as TrhX, which carries OriT, relaxosome genes and some MPF components; and Tra2, or TrhZ, which consists of genes encoding MPF proteins (Partridge et al., 2018). It has been reported that the evolution of conjugation mobility systems, with their relaxases and type IV coupling protein (T4CPs) diverged from the prototypical T4SS (Smillie et al., 2010). Study in our lab demonstrated that some *Salmonella* strains with plasmids carrying a VirB/D4 T4SS survived better in epithelial cells and macrophages than those without the plasmid (Gokulan et al., 2013).

TABLE 3 | Examples of mobilizable IncQ plasmids identified in *Salmonella enterica* strains.

Plasmid names	Antimicrobial resistance genes	Size (bp)	*Salmonella enterica* serotype	References
IncQ1	*sul2*, *strAB*, and *tetAR*	10,867 bp	Reading	Miller et al., 2020
C0144011- and C01RNA1-like	bla_{TEM-1C}	10,384 bp	Reading	Miller et al., 2020
P28321a-1	*aph(3′)-Ia*, *aph(6)-Id*, bla_{TEM-1}, *aph(3″)-Ib*, *sul2*, *tetB*, and *aac(3)-IId*	219,745 bp	Typhimurium	Zhao et al., 2020
PSTU288-2	*sulII*, *strA*, *strB*, *tetA*, and *cat*	11,067 bp	Typhimurium	Hooton et al., 2014

Mobilizable Plasmids

Unlike conjugative plasmids which are self-transmissible, mobilizable plasmids lack the necessary genes for complete conjugation and are therefore not self-transmissible (Francia et al., 2004). Mobilizable plasmids have an origin of transfer site (*oriT*), a region essential for replication, and a mobilization gene (*mob*). They have an ability to exploit conjugative plasmids for horizontal dissemination, but are non-mobile in cells that lack mobile elements carrying compatible mating-pore genes (Ramsay et al., 2016). Like conjugative plasmids, mobilizable plasmids in *Salmonella* also can carry AMR genes.

The IncQ1 plasmids are examples of mobilizable plasmids. They are small in size (10–12 kb) and have broad host range (McMillan et al., 2020). Generally, they are associated with AMR genes including *strAB*, *tetAR*, and *sul2*, and can be mobilized by large plasmids including the IncF, I1, N, P, W, or X plasmids (Francia et al., 2004; McMillan et al., 2020). Baker et al. (2008) demonstrated that a small region in *Salmonella* pathogenicity island 7 (SPI-7) in *Salmonella* Typhi was able to mobilize IncQ plasmid R300B (Baker et al., 2008). Several examples of the the IncQ plasmid (MoB_Q) in different *Salmonella* strains are presented in **Table 3**. Other examples of mobilizable plasmids are the IncR plasmids (40–160 kb), which were first identified in 2009 from *Klebsiella* and *Salmonella* Montevideo (Garcia-Fernandez et al., 2009). IncR plasmids have been reported to carry genes conferring resistance to antimicrobials belonging to numerous classes including: β-lactams, sulphonamides, quinolones, aminoglycosides, tetracyclines, chloramphenicol, and trimethoprim (Rozwandowicz et al., 2018; Plumb et al., 2019).

Integrons

Integrons are mobile genetic elements containing a site-recombination system that is able to integrate, express, and exchange specific DNA known as gene cassettes (Silva et al., 2012; Ravi et al., 2014). Gene cassettes are defined as the smallest mobile elements with an AMR gene; they are located between two recombination sites (*attI* and *attC* 59-base elements; Rodriguez et al., 2006; Gillings, 2014).

Generally, integrons contain three important major elements: (1) the integrase, which is a gene encoding integration into the host genetic backbone, (2) the recombination site, which serves as the primary site to capture the resistance gene cassettes, and (3) the promoter. Each of these elements is required for transcription and drives expression of gene cassettes (Rodriguez et al., 2006; Silva et al., 2012; Gillings, 2014). Integrons are divided into two different types: (1) mobile integrons (MIS), which carry a limited number of AMR gene cassettes (these include the class I integron) and are involved in the dissemination of AMR; and (2) chromosomal integrons (CIS), which have a different number of gene cassettes and are not involved in the dissemination of AMR (Domingues et al., 2012). Moreover, integrons are found in a wide diversity of clinical bacterial strains and environmental isolates in both chromosomes and within MGE (Gillings, 2013). The first discovered chromosomally-located superintegrons were identified in *V. cholerae* isolates (Ravi et al., 2014). These superintegrons are located on the chromosome and typically carry more than 20 gene cassettes with unknown function (Ravi et al., 2014). Integrons are immobile on their own and yet have been observed to transfer across numerous bacterial genomes (Gillings, 2014). Integrons are divided into five classes based on the type of gene capture mechanism, gene cassettes, and the similarity of sequence which encodes integrases to facilitate insertion of the target DNA (Gillings, 2013, 2014; Ravi et al., 2014). Each of the five different classes of integrons (class I–V) encodes a distinct integrase gene. The class I integrons play an important role in the spread of AMR genes and are the most-studied types from clinical samples (Gillings, 2013). They are referred to as broad host range as they have been found both in commensal and pathogenic bacteria (Ravi et al., 2014) and are widely found across different *S. enterica* isolates (Rodriguez et al., 2006).

Recent comparative studies described how integrons contribute to MDR phenotypes (Gillings, 2014; Ravi et al., 2014; Deng et al., 2015). The integron location within MGEs and the chromosome has a unique advantage for generating genomic and phenotypic diversity (Gillings, 2014). Three different classes of mobile integrons (class I, II, and III) can contribute to the MDR phenotypes (Mazel, 2006). For example, class I and class II integrons are associated with the bacteria from animals and have an impact on the human gastrointestinal microbiota by transmission of AMR through the food supply. The horizontal transfer of class I integrons to commensal organisms *via* conjugation facilitates the development and spread of resistant bacteria (de Paula et al., 2018; Miller and Harbottle, 2018). Class I integrons can also play a key role in the transmission of AMR in the aquatic environment and in fish pathogens, as well as in terrestrial pathogens such as *S. enterica*, *E. coli*, and other bacteria species (Miller and Harbottle, 2018). Evershed et al. (2009) discovered IncA/C plasmids, which play an important role in the dissemination of AMR genes, have an unusual cassette in their class I integrons, with most carrying the same set of resistance genes including: *aacU*, *aphA*, *hph*, *sul2*, and *tetA*(D) (Evershed et al., 2009). Similar integrons

have also been identified in *S. enterica* serovars Senftenberg and Ohio. IncA/C plasmids have a broad host range, having been recovered from numerous species, their conjugal transfer capacities are variable (Evershed et al., 2009; Wiesner et al., 2013).

Moreover, Villa and Carattoli (2005) reported that some plasmids in MDR *S.* Typhimurium strains carry *sprC*, *rck*, and *pefA* virulence genes, in addition to two class I integrons carrying AMR genes within the Tn22 and Tn1696 transposons (Villa and Carattoli, 2005). Thus, it was shown that the association between MDR and virulence determinants may contribute to virulence plasmid evolution (Villa and Carattoli, 2005). Likewise, Rodriguez et al. (2006) reported that the class II integrons are present on self-transferable plasmids of *Salmonella* (Rodriguez et al., 2006). It is well-known, that the diversity of integrons plays a critical role in the spread of AMR genes and can drive evolution in *S. enterica*. Several integrons (both class I and class II) have been detected in clinical or environment isolates of *Salmonella* (Yeh et al., 2018) and other *Enterobacteriaceae* from different geographical locations (Adelowo et al., 2018; Gomi et al., 2018; Leungtongkam et al., 2018; Phoon et al., 2018).

Transposons

Transposons, commonly referred to as "jumping gene systems," provide functionality for the transfer of a segment of bacterial DNA to and from chromosomal or plasmid DNA and often carry AMR genes (Bennett, 2008; Giedraitiene et al., 2011; Sultan et al., 2018). Transposons are made up of a transposase and inverted repeat sequences on their ends. The inverted repeat sequences can accelerate target recognition and recombination; while the transposase catalyzes the movement of DNA segments to another part of the genome by a cut-and-paste mechanism, or by a replicative transposition mechanism, and functional genes (such as AMR genes; Giedraitiene et al., 2011). Furthermore, transposable elements (TEs) consist of a set of mobile elements that include small cryptic elements, IS elements, transposons, and transposing bacteriophages. The bacteriophage is a bacterial virus, which is also known as a transposing phage, that infects and replicates its own sequences within bacteria through either a lysogenic or lytic cycle (Balcazar, 2014). In the lysogenic cycle, the phage is referred to as temperate or non-virulent, and occurs following the injection of bacteriophage DNA into the bacteria cell. During this phase, the bacteriophage integrates its genome into the host genome *via* the phage-encoded integrases to form prophage, where it replicates passively as part of the host genome. As the phage excises from the host chromosome, it may transport bacterial genes from one bacteria strain to another *via* a process called transduction (Bennett, 2008; Giedraitiene et al., 2011). In the lytic cycle, also referred to as virulent infection, immediately after injecting into the host cell, the bacteriophage genome synthesizes what are termed early proteins instead of integrating its DNA into the host genome. These early proteins function to break down the host DNA allowing the bacteriophage to control the host's cellular machinery to synthesize the proteins required to build new phage particles. Eventually, the destruction of the infected bacteria cell results in the release of the new phage progeny. Transduction mediated by temperate bacteriophages can serve as a horizontal gene transfer mechanisms and has played an important role in the dissemination of AMR genes among bacterial populations (Bennett, 2008; Giedraitiene et al., 2011; Colavecchio et al., 2017). Multiple bacteriophage and prophages have been reported in *Salmonella* strains, such as Fels-1, Gifsy-2, P22, and FelixO1 (Switt et al., 2013; Colavecchio et al., 2017). Among them, *Salmonella* phage P22 has been widely used in molecular biology for its ability to introduce foreign genes, including AMR genes into recipient cells by transduction (Switt et al., 2013). Since its emergence in the 1990s, the MDR ACSSuT-type (resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline) *S.* Typhimurium phage type DT104, has become widely distributed worldwide. Studies showed that the ACSSuT-type region of DT104 was derived from two separate evolutionary events. One was the integration of a 43-kb *Salmonella* genomic island (SGI) 1 that carries multiple AMR genes and the other is the integration of a P22-like phage into the chromosome to form prophage PDT17 or ST104 (Chiu et al., 2006). More information about the contribution of bacteriophages to the spread of AMR genes among foodborne pathogens can be found in a review article wrote by Colavecchio et al. (2017).

The sizes of transposon sequences range from 3 to 40 kb depending on the variability of the genes present in the transposons (Pagano et al., 2016). **Figure 3** shows a representative transposon (Tn21) that is located within an IncA/C plasmid and carries a class 1 integron as well as multiple antimicrobial and disinfectant resistance genes (Han et al., 2012). There are two major types of TEs: (1) composite transposons (class I) and (2) complex transposons (class II). Class I transposons contain a variety of resistance genes and have little DNA homology in their central regions, which are flanked by IS elements (Pagano et al., 2016; Sultan et al., 2018). In *S. enterica* serovars, such as Typhimurium and Choleraesuis, class I transposons often reside in conjugative plasmids, such as IncHI2, and are responsible for AMR gene dissemination (Sultan et al., 2018). In contrast, class II transposons, constituting of three dissimilar, but interrelated families (Tn3, Tn21, and Tn2501), have more diverse genetic structures than class I transposons (Pagano et al., 2016; Sultan et al., 2018). A wide diversity of class I transposons (for example, Tn1696, Tn10, Tn6088, Tn3, Tn9, and Tn 1721-like transposon) carrying a variety of different AMR genes have been identified in *Salmonella* serovar (Frech and Schwarz, 1999; Orman et al., 2002; Cain et al., 2010; Cain and Hall, 2012; Chen et al., 2020). The TEs that are transferred include: (i) the integrative and conjugative elements (ICEs), (ii) the integrative and mobilizable elements (IMEs), and (iii) decayed elements derived from ICEs or IMEs, such as cis-mobilizable elements (CIMEs; Lao et al., 2020). CIMEs are decayed elements originating from ICEs or IMEs that have lost their integration and transfer genes, but retained *attL* and *attR* sites (Guedon et al., 2017). The CIMEs are known to be major vehicles for acquisition of a broad spectrum of AMR genes among bacteria and many other genes that can be advantageous for their hosts (Guedon et al., 2017). The recombination between related or unrelated ICEs, IMEs, and CIMEs, likely integrated in tandem,

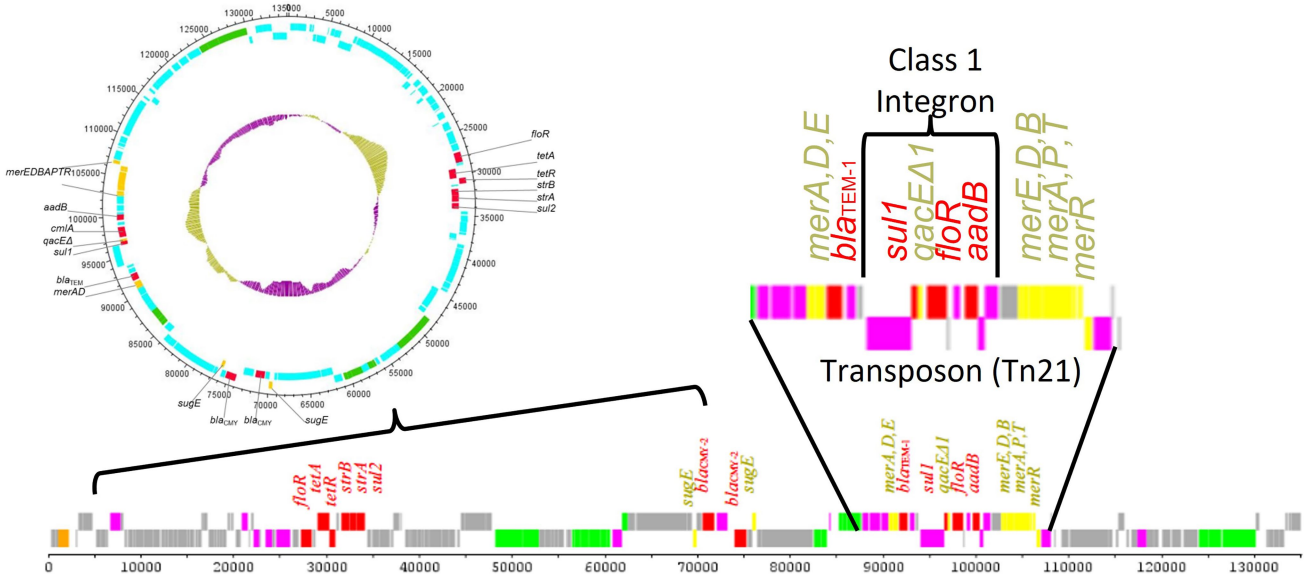


FIGURE 3 | Results of the sequencing of a transmissible antimicrobial resistance plasmid from a *Salmonella enterica* strain (Han et al., 2012). The plasmid contains multiple antimicrobial resistance (denoted in red) and heavy metal/disinfectant (denoted in yellow) resistance genes, including those carried in a Tn21 transposon. Within the Tn21 transposon, there was a class 1 integron carrying multiple antimicrobial resistance genes. This nesting of MGEs within other MGEs provides significant opportunities for the generation and dissemination of diverse antimicrobial resistance elements. AMR regions and mobile genetic elements also often have a higher G+C content compared to the plasmid backbone as is highlighted in the inner ring of the circular plasmid diagram (gold vs. purple bars).

plays a major role in the evolution and plasticity of ICEs and IMEs (Bellanger et al., 2014; Lao et al., 2020).

Integrative and Conjugative Elements

Integrative and conjugative elements, also known as conjugative transposons, are genetic elements that are self-transmissible between chromosomes of different cells and are widely distributed among both Gram-negative and Gram-positive bacteria (van Hoek et al., 2011; Seth-Smith et al., 2012; Johnson and Grossman, 2015). Contrary to conjugative plasmids which are extrachromosomal DNA elements within a cell that are physically separated from the host chromosome and replicate independently, ICEs are typically found integrated in the host bacterial chromosome (Johnson and Grossman, 2015). They contain genes required for their integration and excision, through which they can excise from the host chromosome to form a circular product prior to conjugal transfer (Johnson and Grossman, 2015). Normally, the repression of transcription prevents the expression of the excision and conjugation genes and the ICEs are maintained as inert elements in the host chromosome (Johnson and Grossman, 2015). The de-repression/activation of expression of ICE genes under certain conditions lead to the induction of ICE genes, which in turn leads to their excision from the donor chromosome, formation of a circular intermediate, transfer by conjugation, and reintegrated into the recipient chromosome (Johnson and Grossman, 2015). In addition to an integrase and excisionase genes, ICEs also encode a functional type IV secretion system, which facilitates the conjugative transfer (Johnson and Grossman, 2015). While integrated in the chromosome, ICEs are passively propagated during chromosomal replication, segregation, and cell division (Johnson and Grossman, 2015). Each ICE also contains a variety of cargo genes that confer different phenotypes to host cells, for example, virulence determinants, antimicrobial-resistant factors and/or genes coding for other beneficial traits, indicating they play important roles for bacterial evolution (Arai et al., 2019). A well-known example is the *Salmonella* pathogenicity island (SPI)-7, which possesses features indicative of an ICE and carries genes implicated in virulence and has been found within some pathogenic strains of *S. enterica* (Seth-Smith et al., 2012). Seth-Smith et al. (2012) identified and analyzed ICEs related to SPI-7 within the genus *Salmonella* and other *Enterobacteriaceae* and discovered two new ICEs with high levels of identity to SPI-7, which indicates that these elements may be more common than previously thought in the *Salmonella* (Seth-Smith et al., 2012). They also identified more distantly related ICEs, with distinct cargo regions in other strains of *Salmonella* and members of the *Enterobacteriaceae* (Seth-Smith et al., 2012).

Salmonella genomic island 3 has been demonstrated to be an ICE and increases copper and arsenic tolerance in *Salmonella* strains (Arai et al., 2019). ICEkp, an ICE in *Klebsiella pneumoniae*, mobilizes a *ybt* locus which encodes biosynthesis of the siderophore yersiniabactin and its receptor. Yersiniabactin and other siderophore systems are key bacterial virulence factors as they provide mechanisms for scavenging iron (an essential nutrient) from host transport proteins, thereby enhancing the ability of bacteria to survive and replicate within the host (Lam et al., 2018). SXT/R391 family of ICEs, that is mainly associated with *Vibrio* spp., is one of the largest of the ICE families with more than 100 elements (Marrero and Waldor, 2007; Ryan et al., 2016). These elements integrate into an *attB* site in the 5′ end of the chromosomal *prfC* gene by site-specific

recombination with their *attP* site, catalyzed by the IntSXT tyrosine recombinase (Partridge et al., 2018). ICEs of SXT/R391 family are able to mobilize adjacent sequences and have been recognized as major drivers of the dissemination of AMR genes among several species of *Enterobacteriaceae* and *Vibrionaceae* (Carraro et al., 2016; Partridge et al., 2018).

Integrative Mobilizable Elements/Mobilizable Transposons

Similar to ICEs, the IMEs, also known as mobilizable transposons, are mobile elements that lack the recombination module that allows for their autonomous integration and excision into host sequence (Guedon et al., 2017; Lao et al., 2020). Unlike ICEs, which are self-transferable, IMEs cannot self-transfer. Instead they can be mobilized *in trans* by subverting the conjugation machinery of related or unrelated co-resident conjugative element to promote their own transfer (Guedon et al., 2017; Lao et al., 2020). Beside AMR genes, IMEs can carry genes conferring various known or unknown functions that may enhance the fitness of their hosts and contribute to their maintenance in bacterial populations (Doublet et al., 2005; Guedon et al., 2017). An example is SGI1, which was classified within the group of IMEs since it is not self-transmissible, but mobilizable (Doublet et al., 2005; de Curraize et al., 2021). SGI1 could be conjugally transferred from an *S. enterica* donor to *E. coli* recipient strains (Doublet et al., 2005). SGI1 has been shown to exploit the conjugal apparatus of the IncA/C plasmids to facilitate SGI1 being conjugally mobilized (Douard et al., 2010; Harmer et al., 2016; Siebor et al., 2016). SGI1 is extremely stable in the host chromosome; however, the presence of an IncA/C plasmid enables excision of SGI1 *via* the action of the IncC transcriptional activator complex AcaCD. This complex is required for expression of *tra* genes and hence for conjugative activity of IncC plasmids (Harmer et al., 2016; Huguet et al., 2016). AcaCD binds to SGI1 upstream of *xis*, which encodes the recombination directionality factor Xis, resulting in the activation of *xis* expression and hence SGI1 excision which enables subsequent mobilization of SGI1 (Kiss et al., 2015). The extra-chromosomal circle of SGI1 is integrated into the recipient chromosome in a site-specific manner (Doublet et al., 2005). Paradoxically, although SGI1 needs a co-resident IncA/C plasmid for its excision from the host chromosome and transfer to a new host, it is incompatible with A/C plasmids (Harmer et al., 2016; Huguet et al., 2016, 2020). SGI1 promotes the loss of IncA/C plasmid, which is associated with SGI1 replication. The high-copy replication of SGI1 prevents the co-transfer of IncA/C plasmid into a recipient cell (Harmer et al., 2016; Huguet et al., 2016, 2020). The mobilization of SGI1 from *Salmonella* to the other bacterial pathogens probably contributes to the spread of AMR genes among them.

Insertion Sequences

In general, IS elements are small mobile elements (at least 0.5 kb) that perform as a complex type of transposable element and typically carry one or two transposase (Tnp) genes (Pagano et al., 2016; Partridge et al., 2018). These elements are defined as the simplest autonomous mobile elements in bacterial genomes (He et al., 2015). They are classified into different groups based on active site motifs in Tnp, designated by the key amino acids which are present in the active site including DDE (Asp and Glu), DEDO and H4H (a His split by a large collection of hydrophobic amino acids); and/or on whether transposition is a conservative, cut-and-paste mechanism, where the IS is simply excised from the donor and inserted into the recipient, or is replicative (Seth-Smith et al., 2012). The transposition can be replicated by a copy-and-paste mechanism or copy-out-paste-in mechanism. In the copy-and-paste mechanism, the IS is replicated to join the donor and recipient in a co-integrate, which is subsequently resolved to provide the original donor plus the recipient with the IS; while in the copy-out-paste-in mechanism, the IS is replicated into a double-stranded circular intermediate that then integrates into the recipient (Partridge et al., 2018). IS elements are widespread and can occur in very high numbers in bacterial genomes and have had an important impact on genome structure and function (Siguier et al., 2014). IS-mediated gene inactivation can affect bacterial virulence, AMR and metabolism (Vandecraen et al., 2017). For example, IS1 or IS10 insertion can up-regulate the AcrAB-TolC efflux pump in *Salmonella*, resulting in increased AMR (Ramirez-Santos et al., 1992; Siguier et al., 2014). IS1, which is approximately 768 bp, can be detected in the majority of the family *Enterobacteriaceae* including *E. coli*, *Salmonella*, *Shigella*, and *Klebsiella* (Ramirez-Santos et al., 1992). IS1 can be found in the chromosome and plasmids, respectively, and some strains carried IS1 in both the chromosome and plasmids (Ramirez-Santos et al., 1992). Ramirez-Santos et al. (1992) reported that the frequency of IS1 was higher in *Salmonella* than in *E. coli* and *Shigella*.

The relatively high prevalence of IS1 in plasmids of MDR clinical isolates suggests its role in the dissemination of AMR genes (Ramirez-Santos et al., 1992). ISPa12, a member of IS4 family, can induce expression of ESBL PER-1 in a series of Gram-negative isolates, including *Salmonella*, resulting in resistance to penicillins, cefotaxime, ceftibuten, ceftazidime, and aztreonam (Poirel et al., 2005). IS200 is a *Salmonella*-specific IS that is found in almost all *Salmonella* serotypes examined, but is absent from most other enteric bacteria, thus it has been used as fingerprinting tool that is applied for strain discrimination (Beuzon et al., 2004). IS elements have been reviewed thoroughly in previous publications (Siguier et al., 2014; Vandecraen et al., 2017).

Resistance Islands/*Salmonella* Genomic Island

Resistance islands are genomic islands that contain multiple resistance genes. They have been described mainly in the Proteobacteria, including *Salmonella*, *Shigella flexneri*, *V. cholerae*, *Staphylococcus aureus*, and *Acinetobacter baumannii* (Pagano et al., 2016). Several AMR islands, also called SGIs, have been identified in *Salmonella* strains (Miriagou et al., 2006; Silva et al., 2012). The best known is SGI1, which was initially described in the epidemic MDR *Salmonella* Typhimurium

phage-type DT104 (Boyd et al., 2001; Doublet et al., 2005). Since then, several variants of SGI1 have been described in a wide variety of *Salmonella* serovars, including Typhimurium, Agona, Paratyphi B, Albany, Meleagridis, and Newport, which indicates its horizontal transfer potential (Levings et al., 2005). SGI1 is 43 kb and contains 44 open reading frames (ORFs), many of which show no homology to known gene sequences. As mentioned above, SGI1 is an integrative mobilizable element that contains a complex class 1 integron named In104, located within the AMR gene cluster which is 13 kb and is located at the 3′ end of SGI1 (Doublet et al., 2005; Levings et al., 2005). The SGI1 antimicrobial resistance gene cluster contains a complex class 1 integron that harbors five resistance genes [*aadA2*, *sul1*, *floR*, *tetA(G)*, *blaPSE-1*] encoding ampicillin, chloramphenicol, florfenicol, streptomycin/spectinomycin, sulphonamides, and tetracycline (ACSSuT) resistance, the so-called ACSSuT resistance type (Doublet et al., 2005; Levings et al., 2005; de Curraize et al., 2021).

FACTORS THAT IMPACT RESISTANCE TRANSFER

Environmental and genetic factors that regulate horizontal transfer of AMR genes in bacterial populations remain mostly unknown. MDR *Salmonella* often have multiple resistance-encoding plasmids. The exposure to some antimicrobial agents appears to influence plasmid transfer in enteric bacteria under certain circumstances (Schuurmans et al., 2014; Moller et al., 2017; Lu et al., 2018; Shun-Mei et al., 2018; Liu et al., 2019). Several factors have been shown to contribute to the dissemination of resistance plasmids and other MGEs. Quorum-sensing (QS) and SOS responses have been known to promote horizontal gene transfer, which plays an essential role for the AMR development and dissemination among bacterial populations (Lu et al., 2017; Shun-Mei et al., 2018; Liu et al., 2019).

Quorum-Sensing System

Quorum-sensing is an intercellular cell–cell communication mechanism that allows bacteria to control the expression of genes involved in a variety of cellular processes and plays a critical role in the adaption and survival of bacteria in their environment (Kendall and Sperandio, 2007). QS is mediated by secreted chemical signals called autoinducers (AIs), which are small diffusible molecules that are synthesized and released from bacterial cells in accordance with cell number and accumulate in the external environment (Taga et al., 2003). When bacterial cell density reaches to a certain level that AIs concentration is over a minimal threshold, AIs bind to cognate receptors to alter and regulate bacterial gene expression accordingly in response to their population density (Taga et al., 2003; Kendall and Sperandio, 2007). A large number of physiological processes in bacteria, including biofilm formation, virulence factor production, bioluminescence, sporulation, motility, and antibiotic production, are regulated by QS systems (Rutherford and Bassler, 2012; Wang et al., 2019a).

While most QS signals are species-specific, AI-2 has been observed throughout the bacterial kingdom and is considered as a universal QS signal (Wang et al., 2019a). It is involved in interspecies cell-to-cell communication, as AI-2 produced by one species can influence gene expression in another. AI-2 is a metabolic byproduct of a *luxS* gene-encoded synthase, which is an enzyme that has been found in more than 55 bacterial species and is involved primarily in the conversion of ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor of AI-2 (Vendeville et al., 2005). The LuxS/AI-2 QS system is biologically important as it is involved in numerous physiological processes such as altering bacterial growth characteristics, biofilm formation, conjugation, virulence regulation, AMR, and metabolism in *Salmonella* (Taga et al., 2001; Choi et al., 2007; Widmer et al., 2007; Chen et al., 2008; Karavolos et al., 2008; Jesudhasan et al., 2010; Ju et al., 2018; Shi et al., 2018). It contributes to AMR through different mechanisms: MGE, efflux pumps, the VraSR two-component system, inhibition of the folate synthesis pathway, and biofilm formation. Detailed information on the relationships between the LuxS/AI-2 system and drug resistance has been reviewed by Wang et al. (2019a). In brief, the LuxS/AI-2 system has been shown to impact the transfer of resistance plasmids and the LuxS/AI-2-based QS enhances the expression of genes involved in conjugation-related activities, leading to increased conjugation frequency (Lu, 2004).

In the presence of AI-2, the conjugation frequency of the plasmid RP4 carrying the *tet(A)* gene in bacteria exposed to tetracycline increased significantly compared to a non-exposed control (Lu, 2004). Increased AI-2 concentrations led to increased expression of plasmid-transfer associated gene *trbC*. Similar studies conducted by another group also showed that exposure to sub-MIC tetracyclines could facilitate the conjugative transfer of plasmid RP4 in *E. coli* and this process could be enhanced by AIs, but inhibited by quorum sensing inhibitors (QSIs; Zhang et al., 2018). Therefore, the LuxS/AI-2 could be a potential target for preventing the spread of bacterial resistance.

SOS Response

The SOS response is a global stress response to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis is induced (Beaber et al., 2004; Baharoglu et al., 2010; Zgur-Bertok, 2013; Qin et al., 2015). It is an inducible DNA repair pathway controlled by two key regulators, LexA, a repressor, and RecA, an inducer (Podlesek and Žgur Bertok, 2020; **Figure 4**). In bacteria, upon DNA damage, SOS is induced when an increase in intracellular single-stranded DNA (ssDNA) is generated when DNA polymerase III stalls at a lesion while helicase continues unwinding DNA (Podlesek and Žgur Bertok, 2020). Stimulated by ssDNA, RecA is activated by binding to ssDNA (Maslowska et al., 2019). The formation of a ssDNA/RecA nucleofilament stimulates auto-proteolysis of the LexA repressor, leading to de-repression of genes comprising the SOS regulon (Baharoglu et al., 2010; Podlesek and Žgur Bertok, 2020). In addition to several endogenous triggers (for example, UV irradiation, oxidants, and chemical mutagens) that cause the accumulation of ssDNA, thus inducing the SOS response,

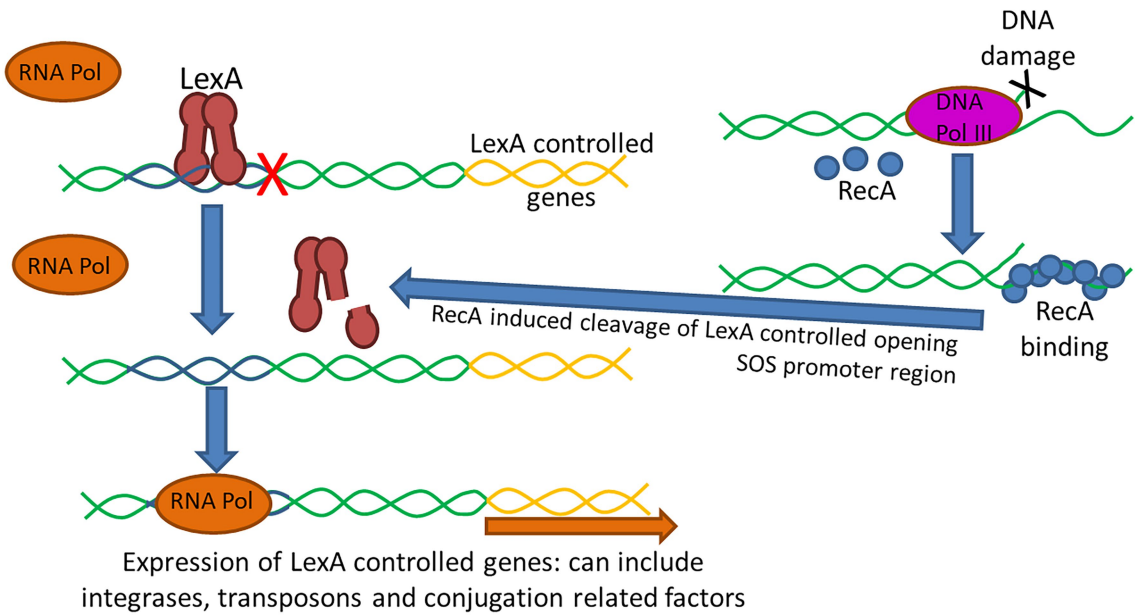


FIGURE 4 | The SOS response is an inducible DNA repair pathway controlled by two key regulators, the LexA repressor of gene expression and the RecA inducer. Under normal conditions, LexA is bound to the promoter of SOS-associated genes preventing binding of RNA polymerase (RNA Pol) and expression of the regulated genes (Maslowska et al., 2019). Following DNA damage, such as following certain antimicrobial exposures, the DNA polymerase III (DNA Pol III) enzyme senses the damage and the increase in single-stranded DNA (ssDNA) causes RecA to become activated and bind to the ssDNA. The ssDNA/RecA complex leads to proteolysis of the LexA repressor allowing RNA polymerase to bind facilitating expression of genes under LexA control. LexA has been shown to impact the expression of integrases and transposases, as such the activation of the SOS response has been shown to promote horizontal dissemination of resistance genes. Additionally, conjugation can also trigger the SOS response as ssDNA is generated and transferred during the conjugation process which can upregulate integrase and transposase expression and triggering recombination of plasmids in the recipient cells.

ssDNA is also produced by several mechanisms of exogenous DNA uptake involved in lateral gene transfer, including conjugation, transformation, and occasionally transduction (Baharoglu et al., 2010). The SOS response can also be induced by numerous antimicrobials, presumably because these antimicrobials cause the production of ssDNA. At least two classes of antimicrobials, fluoroquinolones (such as ciprofloxacin) and dihydrofolate reductase inhibitors (such as trimethoprim), can activate the SOS response (Beaber et al., 2004; Qin et al., 2015). Besides being initially recognized as a regulator of DNA damage repair, it has been noted that the SOS response plays a much broader role in bacteria. As an error-prone repair system, the SOS response contributes significantly to DNA changes observed in a wide range of species by promoting an elevated mutation rate which generates genetic diversity and adaptation, including the development of resistance to antimicrobials (Podlesek and Žgur Bertok, 2020). The SOS response also plays a key role in AMR by stimulating gene transfer, inducing mutation and genomic rearrangements, and the formation of biofilms which are highly recalcitrant to antimicrobials (Podlesek and Žgur Bertok, 2020).

The SOS response has been shown to promote horizontal dissemination of resistance genes in the presence of antimicrobial agents (Beaber et al., 2004; Hastings et al., 2004). Guerin et al. (2009) observed that the antimicrobial compounds found in manure can activate the SOS response in bacteria, which then increases the activity of integrases and transposases, and thereby transposition rates of gene cassettes and other translocative elements (Guerin et al., 2009). They concluded that LexA controlled expression of most integron integrases and consequently regulated cassette recombination. Baharoglu et al. (2010) found that conjugative transfer of plasmids R388, R6Kdrd, and RP4 in *E. coli* and *V. cholerae* induces the SOS response and up-regulates integrase expression, triggering cassette recombination in recipient cells (Baharoglu et al., 2010). Additional studies have shown that the SOS response can lead to an increase in cell membrane permeability and downregulation of the *korA* repressor of various transfer associated genes in the RP4 plasmid (Wang et al., 2019b).

The SOS induction leads to genetic diversification of these mobile elements and their transfer to surrounding bacteria, resulting in the dissemination of AMR genes (Baharoglu et al., 2010). It was also observed that ICEs could utilize the SOS response to mobilize themselves from the bacterial chromosome and infect other cells (Hastings et al., 2004). The transfer of SXT, an approximately 100 kb ICE derived from *V. cholerae* that encodes a variety of antimicrobial resistance genes, is markedly enhanced when the SOS response is induced by two DNA-damaging antimicrobials (mitomycin C and ciprofloxacin), resulting in the transfer of MDR genes (Hastings et al., 2004).

Other studies have outlined the connections between horizontal gene transfer, bacterial stress response (SOS), and recombination of gene cassettes in integrons, which provides new insights into the development of the AMR within a population (Guerin et al., 2009; Baharoglu et al., 2010). Since several commonly used

TABLE 4 | Summary of terms and definitions of mobile genetics elements (MGEs) in *Salmonella enterica*.

Mobile genetic elements	Definition
Plasmids	Plasmids are genetic elements that are typically circular DNA in a cell that can replicate independently of the chromosomes. They have variable host-ranges and can be conjugative and replicate in diverse genera.
Integrons	Integrons are genetic elements with a site-specific recombination system that can integrate, express and exchange specific DNA elements (gene cassettes), including resistance genes.
Transposable elements/ Transposons (Tn)	Transposable elements/ Transposons (Tn) are known as "jumping genes" that can move themselves (and associated resistance genes) almost randomly to new locations in the same or different DNA molecules within a single cell.
Resistance islands	Resistance islands are genomic islands (GIs) containing MDR genes.
Bacteriophages	Bacteriophages are bacterial viruses that infect and replicate only in bacterial cells.
Insertion sequences	Insertion sequences are small mobile elements (generally 700 ~ 2,500 bp) and only code for proteins implicated in the transposition activity. They can move themselves almost randomly to new locations in the same or different DNA molecules within a single cell.

antimicrobials can induce the SOS response, which in turn can enhance the conjugative transfer of the plasmids, the use of certain antimicrobial agents, either clinically or in agricultural settings, might potentiate the horizontal dissemination of AMR genes to a broad range of bacterial species and hasten genetic change and the evolution to resistance in pathogenic populations.

CONCLUSION

Antimicrobial resistance acquisition can occur *via* either vertical or horizontal transfer. In vertical transmission, the accumulated genetic errors in existing genes (either in the chromosome or plasmid) is passed on from parent cells to progeny cells, leading to the observed resistance. While in the horizotal transfer, also called as acquired resistance, the AMR genes are exchanged within and among different bacteria species (Founou et al., 2016). The resistant genes harbored on MGEs can be acquired by the recipient strains. Both vertical transmission and horizontal transfer of AMR genes play important roles in AMR acquisition. The MGE-mediated horizontal transfer of foreign genes among different bacteria plays an important role in bacterial evolution and contributes signaficatnly in both the AMR and virulence gene acquisition (Gyles and Boerlin, 2014). This review article examined MGEs that can be found in *Salmonella* as well as discussed their roles in the development and dissemination of antimicrobial resistance. Key examples of MGEs that are important in antimicrobial resistance dissemination include SG1, which has been associated with the stable acquisition of multiple resistance genes at a site in the chromosome, yet requires an IncC resistance plasmid to facilitate its transfer. Plasmids are likely the most important vehicle of resistance transfer between different *Salmonella* strains and/or from related species. Plasmids can harbor other MGEs including transposons and integrons that can lead to the transfer of genes between the chromosome and other co-resident plasmids, thus providing more genetic diversity among the bacterial populations. The dynamic resistance of *Salmonella* isolates to antimicrobial agents is considered a serious problem and developing methods and strategies to reduce and control the spread of resistance genes and their evolution are vital. Another trend observed in *S. enterica* is IS element activation which plays a critical role in efflux mechanisms. A recent study suggested that a combination of conjugation inhibition and plasmid elimination would be considered an effective method to reduce the conjugation-assisted persistence of antimicrobial resistance (Lopatkin et al., 2017; San Millan, 2018). Since the induction of SOS response promotes horizontal dissemination of AMR genes, inhibiting the bacterial SOS response would be a suitable target to prevent the acquisition of AMR genes, and could be used in combination with antibiotics for the treatment of infections (Baharoglu et al., 2010). The different types MGEs are summarized in **Table 4**, which describes the antimicrobial resistance mobilome of zoonotic pathogens, particularly in *S. enterica*, that is critical to understand the epidemiology, dynamics and evolution of antimicrobial resistance.

AUTHOR CONTRIBUTIONS

SA and JH contributed equally to this work and manuscript writing. SR and SF conceived the idea and assisted with the manuscript writing. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors thank the graduate committee of Suad Algarni for their input and guidance on this review article. We also thank Ashraf Khan and Kidon Sung for their critical review of the manuscript.

REFERENCES

Adelowo, O. O., Helbig, T., Knecht, C., Reincke, F., Mäusezahl, I., and Müller, J. A. (2018). High abundances of class 1 integrase and sulfonamide resistance genes, and characterisation of class 1 integron gene cassettes in four urban wetlands in Nigeria. *PLoS One* 13:e0208269. doi: 10.1371/journal.pone.0208269

Aishwarya, K. V. L., Geetha, P. V., Eswaran, S., Mariappan, S., and Sekar, U. (2020). Spectrum of aminoglycoside modifying enzymes in gram-negative bacteria causing human infections. *J. Lab. Physicians* 12, 27–31. doi: 10.1055/s-0040-1713687

Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., Tsang, K. K., Bouchard, M., Edalatmand, A., et al. (2020). CARD 2020: antibiotic resistome surveillance

with the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 48, D517–D525. doi: 10.1093/nar/gkz935

Aljahdali, N. H., Kaldhone, P. R., Foley, S. L., and Khajanchi, B. K. (2019). Whole-genome sequences of 35 incompatibility group I1 plasmid-carrying *Salmonella enterica* isolates from food animal and clinical sources. *Microbiol. Resour. Announc.* 8, e00831–e00919. doi: 10.1128/MRA.00831-19

Almeida, F., Seribelli, A. A., Medeiros, M. I. C., Rodrigues, D. D. P., de MelloVarani, A., Luo, Y., et al. (2018). Phylogenetic and antimicrobial resistance gene analysis of *Salmonella* Typhimurium strains isolated in Brazil by whole genome sequencing. *PLoS One* 13:e0201882. doi: 10.1371/journal.pone.0201882

Arai, N., Sekizuka, T., Tamamura, Y., Kusumoto, M., Hinenoya, A., Yamasaki, S., et al. (2019). *Salmonella* genomic Island 3 is an integrative and conjugative element and contributes to copper and arsenic tolerance of *Salmonella enterica*. *Antimicrob. Agents Chemother.* 63, e00429–e00519. doi: 10.1128/AAC.00429-19

Baharoglu, Z., Bikard, D., and Mazel, D. (2010). Conjugative DNA transfer induces the bacterial SOS response and promotes antibiotic resistance development through integron activation. *PLoS Genet.* 6:e1001165. doi: 10.1371/journal.pgen.1001165

Baker, S. A.-O., Thomson, N., Weill, F. X., and Holt, K. E. (2018). Genomic insights into the emergence and spread of antimicrobial-resistant bacterial pathogens. *Science* 360, 733–738. doi: 10.1126/science.aar3777

Baker, S., et al. (2008). Mobilization of the incQ plasmid R300B with a chromosomal conjugation system in *Salmonella enterica* serovar typhi. *J. Bacteriol.* 190, 4084–4087. doi: 10.1128/JB.00065-08

Balcazar, J. L. (2014). Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS Pathog.* 10:e1004219. doi: 10.1371/journal.ppat.1004219

Beaber, J. W., Hochhut, B., and Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* 427, 72–74. doi: 10.1038/nature02241

Bellanger, X., Payot, S., Leblond-Bourget, N., and Guédon, G. (2014). Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. *FEMS Microbiol. Rev.* 38, 720–760. doi: 10.1111/1574-6976.12058

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* 153, S347–S357. doi: 10.1038/sj.bjp.0707607

Beuzon, C. R., Chessa, D., and Casadesus, J. (2004). IS200: an old and still bacterial transposon. *Int. Microbiol.* 7, 3–12.

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. J. V. (2015). Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* 13, 42–51. doi: 10.1038/nrmicro3380

Blanco, P., Hernando-Amado, S., Reales-Calderon, J., Corona, F., Lira, F., Alcalde-Rico, M., et al. (2016). Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. *Microorganisms* 4:14. doi: 10.3390/microorganisms4010014

Bockstael, K., and Van Aerschot, A. (2009). Antimicrobial resistance in bacteria. *Cent. Eur. J. Med.* 4, 141–155. doi: 10.2478/s11536-008-0088-9

Boore, A. L., Hoekstra, R. M., Iwamoto, M., Fields, P. I., Bishop, R. D., and Swerdlow, D. L. (2015). *Salmonella enterica* infections in the United States and assessment of coefficients of variation: a novel approach to identify epidemiologic characteristics of individual serotypes, 1996-2011. *PLoS One* 10:e0145416. doi: 10.1371/journal.pone.0145416

Boyd, D., Peters, G. A., Cloeckaert, A., Boumedine, K. S., Chaslus-Dancla, E., Imberechts, H., et al. (2001). Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J. Bacteriol.* 183, 5725–5732. doi: 10.1128/JB.183.19.5725-5732.2001

Bush, K. (2013). Proliferation and significance of clinically relevant beta-lactamases. *Ann. N. Y. Acad. Sci.* 1277, 84–90. doi: 10.1111/nyas.12023

Bush, K., and Bradford, P. A. (2016). Beta-lactams and beta-lactamase inhibitors: an overview. *Cold Spring Harb. Perspect. Med.* 6:a025247. doi: 10.1101/cshperspect.a025247

Cabezón, E., Cruz, F., and Arechaga, I. (2017). Conjugation inhibitors and their potential use to prevent dissemination of antibiotic resistance genes in bacteria. *Front. Microbiol.* 8:2329. doi: 10.3389/fmicb.2017.02329

Cain, A. K., and Hall, R. M. (2012). Evolution of IncHI2 plasmids via acquisition of transposons carrying antibiotic resistance determinants. *J. Antimicrob. Chemother.* 67, 1121–1127. doi: 10.1093/jac/dks004

Cain, A. K., Liu, X., Djordjevic, S. P., and Hall, R. M. (2010). Transposons related to Tn1696 in IncHI2 plasmids in multiply antibiotic resistant *Salmonella enterica* serovar Typhimurium from Australian animals. *Microb. Drug Resist.* 16, 197–202. doi: 10.1089/mdr.2010.0042

Card, R., Zhang, J., Das, P., Cook, C., Woodford, N., and Anjum, M. F. (2013). Evaluation of an expanded microarray for detecting antibiotic resistance genes in a broad range of gram-negative bacterial pathogens. *Antimicrob. Agents Chemother.* 57, 458–465. doi: 10.1128/AAC.01223-12

Carraro, N., Matteau, D., Burrus, V., and Rodrigue, S. (2015). Unraveling the regulatory network of IncA/C plasmid mobilization: when genomic islands hijack conjugative elements. *Mob. Genet. Elem.* 5, 1–5. doi: 10.1080/2159256X.2015.1045116

Carraro, N., Rivard, N., Ceccarelli, D., Colwell, R. R., and Burrus, V. (2016). IncA/C conjugative plasmids mobilize a new family of multidrug resistance Islands in clinical vibrio cholerae non-O1/non-O139 isolates from Haiti. *MBio* 7, e00509–e00516. doi: 10.1128/mBio.00509-16

Chen, K., Dong, N., Chan, E. W.-C., and Chen, S. (2019). Transmission of ciprofloxacin resistance in *Salmonella* mediated by a novel type of conjugative helper plasmids. *Emerg. Microbes Infect.* 8, 857–865. doi: 10.1080/22221751.2019.1626197

Chen, M. H., Lin, Z. H., Peng, X. W., and Pan, X. Z. (2008). Role of Luxs gene on regulation of virulence factors in *Salmonella* typhimurium. *Zhonghua Yi Xue Za Zhi* 88, 2581–2583.

Chen, C. L., Su, L. H., Janapatla, R. P., Lin, C. Y., and Chiu, C. H. (2020). Genetic analysis of virulence and antimicrobial-resistant plasmid pOU7519 in *Salmonella enterica* serovar Choleraesuis. *J. Microbiol. Immunol. Infect.* 53, 49–59. doi: 10.1016/j.jmii.2017.11.004

Chiu, C. H., Su, L.-H., Chu, C.-H., Wang, M.-H., Yeh, C.-M., Weill, F.-X., et al. (2006). Detection of multidrug-resistant *Salmonella enterica* serovar typhimurium phage types DT102, DT104, and U302 by multiplex PCR. *J. Clin. Microbiol.* 44, 2354–2358. doi: 10.1128/JCM.00171-06

Choi, J., Shin, D., and Ryu, S. (2007). Implication of quorum sensing in *Salmonella enterica* serovar typhimurium virulence: the luxS gene is necessary for expression of genes in pathogenicity island 1. *Infect. Immun.* 75, 4885–4890. doi: 10.1128/IAI.01942-06

Colavecchio, A., Cadieux, B., Lo, A., and Goodridge, L. D. (2017). Bacteriophages contribute to the spread of antibiotic resistance genes among foodborne pathogens of the enterobacteriaceae family—a review. *Front. Microbiol.* 8:1108. doi: 10.3389/fmicb.2017.01108

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–433. doi: 10.1128/MMBR.00016-10

de Curraize, C., Siebor, E., and Neuwirth, C. (2021). Genomic islands related to *Salmonella* genomic island 1; integrative mobilisable elements in trmE mobilised in trans by A/C plasmids. *Plasmid* 114:102565. doi: 10.1016/j.plasmid.2021.102565

de Paula, A. C. L., Medeiros, J. D., de Azevedo, A. C., de Assis Chagas, J. M., da Silva, V. L., and Diniz, C. G. (2018). Antibiotic resistance genetic markers and integrons in white soft cheese: aspects of clinical resistome and potentiality of horizontal gene transfer. *Gene* 9:106. doi: 10.3390/genes9020106

Deng, Y., Bao, X., Ji, L., Chen, L., Liu, J., Miao, J., et al. (2015). Resistance integrons: class 1, 2 and 3 integrons. *Ann. Clin. Microbiol. Antimicrob.* 14:45. doi: 10.1186/s12941-015-0100-6

Domingues, S., da Silva, G. J., and Nielsen, K. M. (2012). Integrons: vehicles and pathways for horizontal dissemination in bacteria. *Mob. Genet. Elem.* 2, 211–223. doi: 10.4161/mge.22967

Douard, G., Praud, K., Cloeckaert, A., and Doublet, B. (2010). The *Salmonella* genomic island 1 is specifically mobilized in trans by the IncA/C multidrug resistance plasmid family. *PLoS One* 5:e15302. doi: 10.1371/journal.pone.0015302

Doublet, B., Boyd, D., Mulvey, M. R., and Cloeckaert, A. (2005). The *Salmonella* genomic island 1 is an integrative mobilizable element. *Mol. Microbiol.* 55, 1911–1924. doi: 10.1111/j.1365-2958.2005.04520.x

Evershed, N. J., Levings, R. S., Wilson, N. L., Djordjevic, S. P., and Hall, R. M. (2009). Unusual class 1 integron-associated gene cassette configuration found in IncA/C plasmids from *Salmonella enterica*. *Antimicrob. Agents Chemother.* 53, 2640–2642. doi: 10.1128/AAC.00233-09

Feldgarden, M., Brover, V., Gonzalez-Escalona, N., Frye, J. G., Haendiges, J., Haft, D. H., et al. (2021). AMRFinderPlus and the reference gene catalog facilitate examination of the genomic links among antimicrobial resistance, stress response, and virulence. *Sci. Rep.* 11:12728. doi: 10.1038/s41598-021-91456-0

Fluit, A. C., Visser, M. R., and Schmitz, F. J. (2001). Molecular detection of antimicrobial resistance. *Clin. Microbiol. Rev.* 14, 836–871. doi: 10.1128/CMR.14.4.836-871.2001

Foley, S. L., Johnson, T. J., Ricke, S. C., Nayak, R., and Danzeisen, J. (2013). *Salmonella* pathogenicity and host adaptation in chicken-associated serovars. *Microbiol. Mol. Biol. Rev.* 77, 582–607. doi: 10.1128/MMBR.00015-13

Foley, S. L., and Lynne, A. M. (2008). Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *J. Anim. Sci.* 86, E173–E187. doi: 10.2527/jas.2007-0447

Founou, L. L., Founou, R. C., and Essack, S. Y. (2016). Antibiotic resistance in the food chain: a developing country-perspective. *Front. Microbiol.* 7:1881. doi: 10.3389/fmicb.2016.01881

Francia, M. V., Varsaki, A., Garcillán-Barcia, M. P., Latorre, A., Drainas, C., and de la Cruz, F. (2004). A classification scheme for mobilization regions of bacterial plasmids. *FEMS Microbiol. Rev.* 28, 79–100. doi: 10.1016/j.femsre.2003.09.001

Frech, G., and Schwarz, S. (1999). Plasmid-encoded tetracycline resistance in *Salmonella enterica* subsp. enterica serovars choleraesuis and typhimurium: identification of complete and truncated Tn1721 elements. *FEMS Microbiol. Lett.* 176, 97–103. doi: 10.1111/j.1574-6968.1999.tb13648.x

Fricke, W. F., Welch, T. J., McDermott, P. F., Mammel, M. K., LeClerc, J. E., White, D. G., et al. (2009). Comparative genomics of the IncA/C multidrug resistance plasmid family. *J. Bacteriol.* 191, 4750–4757. doi: 10.1128/JB.00189-09

Frost, L. S., Leplae, R., Summers, A. O., and Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* 3, 722–732. doi: 10.1038/nrmicro1235

Galata, V., Fehlmann, T., Backes, C., and Keller, A. (2019). PLSDB: a resource of complete bacterial plasmids. *Nucleic Acids Res.* 47, D195–D202. doi: 10.1093/nar/gky1050

Galhano, B. S. P., Ferrari, R. G., Panzenhagen, P., de Jesus, A. C. S., and Conte-Junior, C. A. (2021). Antimicrobial resistance gene detection methods for bacteria in animal-based foods: a brief review of highlights and advantages. *Microorganisms* 9:923. doi: 10.3390/microorganisms9050923

Garcia-Fernandez, A., and Carattoli, A. (2010). Plasmid double locus sequence typing for IncHI2 plasmids, a subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum beta-lactamase and quinolone resistance genes. *J. Antimicrob. Chemother.* 65, 1155–1161. doi: 10.1093/jac/dkq101

Garcia-Fernandez, A., Fortini, D., Veldman, K., Mevius, D., and Carattoli, A. (2009). Characterization of plasmids harbouring qnrS1, qnrB2 and qnrB19 genes in *Salmonella*. *J. Antimicrob. Chemother.* 63, 274–281. doi: 10.1093/jac/dkn470

Garcillan-Barcia, M. P., Alvarado, A., and de la Cruz, F. (2011). Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiol. Rev.* 35, 936–956. doi: 10.1111/j.1574-6976.2011.00291.x

Gargano, V., Sciortino, S., Gambino, D., Costa, A., Agozzino, V., Reale, S., et al. (2021). Antibiotic susceptibility profile and tetracycline resistance genes detection in *Salmonella* spp. strains isolated from animals and food. *Antibiotics* 10:809. doi: 10.3390/antibiotics10070809

Giedraitiene, A., Vitkauskienė, A., Naginienė, R., and Pavilonis, A. (2011). Antibiotic resistance mechanisms of clinically important bacteria. *Medicina* 47, 137–146. doi: 10.3390/medicina47030019

Gillings, M. R. (2013). Evolutionary consequences of antibiotic use for the resistome, mobilome and microbial pangenome. *Front. Microbiol.* 4:4. doi: 10.3389/fmicb.2013.00004

Gillings, M. R. (2014). Integrons: past, present, and future. *Microbiol. Mol. Biol. Rev.* 78, 257–277. doi: 10.1128/MMBR.00056-13

Gokulan, K., et al. (2013). Impact of plasmids, including those encodingVirB4/D4 type IV secretion systems, on *Salmonella enterica* serovar heidelberg virulence in macrophages and epithelial cells. *PLoS One* 8:e77866. doi: 10.1371/journal.pone.0077866

Gomi, R., Matsuda, T., Yamamoto, M., Chou, P. H., Tanaka, M., Ichiyama, S., et al. (2018). Characteristics of carbapenemase-producing Enterobacteriaceae in wastewater revealed by genomic analysis. *Antimicrob. Agents Chemother.* 62, e02501–e02517. doi: 10.1128/AAC.02501-17

Guedon, G., Libante, V., Coluzzi, C., Payot, S., and Leblond-Bourget, N. (2017). The obscure world of integrative and mobilizable elements, highly widespread elements that pirate bacterial conjugative systems. *Gene* 8:337. doi: 10.3390/genes8110337

Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., da Re, S., et al. (2009). The SOS response controls integron recombination. *Science* 324:1034. doi: 10.1126/science.1172914

Gupta, S. K., Padmanabhan, B. R., Diene, S. M., Lopez-Rojas, R., Kempf, M., Landraud, L., et al. (2014). ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob. Agents Chemother.* 58, 212–220. doi: 10.1128/AAC.01310-13

Gyles, C., and Boerlin, P. (2014). Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. *Vet. Pathol.* 51, 328–340. doi: 10.1177/0300985813511131

Han, J., Lynne, A. M., David, D. E., Tang, H., Xu, J., Nayak, R., et al. (2012). DNA sequence analysis of plasmids from multidrug resistant *Salmonella enterica* serotype heidelberg isolates. *PLoS One* 7:e51160. doi: 10.1371/journal.pone.0051160

Han, J., Pendleton, S. J., Deck, J., Singh, R., Gilbert, J., Johnson, T. J., et al. (2018). Impact of co-carriage of IncA/C plasmids with additional plasmids on the transfer of antimicrobial resistance in *Salmonella enterica* isolates. *Int. J. Food Microbiol.* 271, 77–84. doi: 10.1016/j.ijfoodmicro.2018.01.018

Harmer, C. J., Hamidian, M., Ambrose, S. J., and Hall, R. M. (2016). Destabilization of IncA and IncC plasmids by SGI1 and SGI2 type *Salmonella* genomic islands. *Plasmid* 87–88, 51–57. doi: 10.1016/j.plasmid.2016.09.003

Hastings, P. J., Rosenberg, S. M., and Slack, A. (2004). Antibiotic-induced lateral transfer of antibiotic resistance. *Trends Microbiol.* 12:15337159, 401–404. doi: 10.1016/j.tim.2004.07.003

He, S., Hickman, A. B., Varani, A. M., Siguier, P., Chandler, M., Dekker, J. P., et al. (2015). Insertion sequence IS26 reorganizes plasmids in clinically isolated multidrug-resistant bacteria by replicative transposition. *mBio* 6:e00762. doi: 10.1128/mBio.00762-15

Helms, M., Ethelberg, S., and Mølbak, K.DT104 Study Group (2005). International *Salmonella* Typhimurium DT104 infections, 1992-2001. *Emerg. Infect. Dis.* 11, 859–867. doi: 10.3201/eid1106.041017

Hoffmann, S., Batz, M. B., and Morris, J. G. (2012). Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *J. Food Prot.* 75, 1292–1302. doi: 10.4315/0362-028X.JFP-11-417

Hooton, S. P., Timms, A. R., Cummings, N. J., Moreton, J., Wilson, R., and Connerton, I. F. (2014). The complete plasmid sequences of *Salmonella enterica* serovar Typhimurium U288. *Plasmid* 76, 32–39. doi: 10.1016/j.plasmid.2014.08.002

Horiyama, T., Yamaguchi, A., and Nishino, K. (2010). TolC dependency of multidrug efflux systems in *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother.* 65, 1372–1376. doi: 10.1093/jac/dkq160

Howard, M. T., Nelson, W. C., and Matson, S. W. (1995). Stepwise assembly of a relaxosome at the F plasmid origin of transfer (*). *J. Biol. Chem.* 270, 28381–28386. doi: 10.1074/jbc.270.47.28381

Huguet, K. T., Gonnet, M., Doublet, B., and Cloeckaert, A. (2016). A toxin antitoxin system promotes the maintenance of the IncA/C-mobilizable *Salmonella* genomic island 1. *Sci. Rep.* 6:32285. doi: 10.1038/srep32285

Huguet, K. T., Rivard, N., Garneau, D., Palanee, J., and Burrus, V. (2020). Replication of the *Salmonella* genomic island 1 (SGI1) triggered by helper IncC conjugative plasmids promotes incompatibility and plasmid loss. *PLoS Genet.* 16:e1008965. doi: 10.1371/journal.pgen.1008965

Jain, A., and Srivastava, P. (2013). Broad host range plasmids. *FEMS Microbiol. Lett.* 348, 87–96. doi: 10.1111/1574-6968.12241

Jernberg, C., Löfmark, S., Edlund, C., and Jansson, J. K. (2010). Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* 156, 3216–3223. doi: 10.1099/mic.0.040618-0

Jesudhasan, P. R., Cepeda, M. L., Widmer, K., Dowd, S. E., Soni, K. A., Hume, M. E., et al. (2010). Transcriptome analysis of genes controlled by luxS/autoinducer-2 in *Salmonella enterica* serovar Typhimurium. *Foodborne Pathog. Dis.* 7, 399–410. doi: 10.1089/fpd.2009.0372

Jesus, T. F., Ribeiro-Gonçalves, B., Silva, D. N., Bortolaia, V., Ramirez, M., and Carriço, J. A. (2019). Plasmid ATLAS: plasmid visual analytics and identification in high-throughput sequencing data. *Nucleic Acids Res.* 47, D188–D194. doi: 10.1093/nar/gky1073

Johnson, C. M., and Grossman, A. D. (2015). Integrative and conjugative elements (ICEs): what they do and how they work. *Annu. Rev. Genet.* 49, 577–601. doi: 10.1146/annurev-genet-112414-055018

Johnson, T. J., and Lang, K. L. (2012). IncA/C plasmids: an emerging threat to human and animal health? *Mob. Genet. Elem.* 2, 55–58. doi: 10.4161/mge.19626

Ju, X., Li, J., Zhu, M., Lu, Z., Lv, F., Zhu, X., et al. (2018). Effect of the luxS gene on biofilm formation and antibiotic resistance by *Salmonella* serovar Dublin. *Food Res. Int.* 107, 385–393. doi: 10.1016/j.foodres.2018.02.039

Kaminska, K. H., Purta, E., Hansen, L. H., Bujnicki, J. M., Vester, B., and Long, K. S. (2010). Insights into the structure, function and evolution of the radical-SAM 23S rRNA methyltransferase Cfr that confers antibiotic resistance in bacteria. *Nucleic Acids Res.* 38, 1652–1663. doi: 10.1093/nar/gkp1142

Karavolos, M. H., Bulmer, D. M., Winzer, K., Wilson, M., Mastroeni, P., Williams, P., et al. (2008). LuxS affects flagellar phase variation independently of quorum sensing in *Salmonella enterica* serovar typhimurium. *J. Bacteriol.* 190, 769–771. doi: 10.1128/JB.01253-07

Kendall, M. M., and Sperandio, V. (2007). Quorum sensing by enteric pathogens. *Curr. Opin. Gastroenterol.* 23, 10–15. doi: 10.1097/MOG.0b013e3280118289

Kiss, J., Papp, P. P., Szabó, M., Farkas, T., Murányi, G., Szakállas, E., et al. (2015). The master regulator of IncA/C plasmids is recognized by the *Salmonella* genomic island SGI1 as a signal for excision and conjugal transfer. *Nucleic Acids Res.* 43, 8735–8745. doi: 10.1093/nar/gkv758

Lam, M. M. C., Wick, R. R., Wyres, K. L., Gorrie, C. L., Judd, L. M., Jenney, A. W. J., et al. (2018). Genetic diversity, mobilisation and spread of the yersiniabactin-encoding mobile element ICEKp in *Klebsiella pneumoniae* populations. *Microb. Genom.* 4:e000196. doi: 10.1099/mgen.0.000196

Lambert, P. A. (2005). Bacterial resistance to antibiotics: modified target sites. *Adv. Drug Deliv. Rev.* 57, 1471–1485. doi: 10.1016/j.addr.2005.04.003

Lao, J., Guédon, G., Lacroix, T., Charron-Bourgoin, F., Libante, V., Loux, V., et al. (2020). Abundance, diversity and role of ICEs and IMEs in the adaptation of *Streptococcus salivarius* to the environment. *Gene* 11:999. doi: 10.3390/genes11090999

Lee, S., Park, N., Yun, S., Hur, E., Song, J., Lee, H., et al. (2021). Presence of plasmid-mediated quinolone resistance (PMQR) genes in non-typhoidal *Salmonella* strains with reduced susceptibility to fluoroquinolones isolated from human salmonellosis in Gyeonggi-do, South Korea from 2016 to 2019. *Gut Pathog.* 13:35. doi: 10.1186/s13099-021-00431-7

Lemon, J. K., Khil, P. P., Frank, K. M., and Dekker, J. P. (2017). Rapid nanopore sequencing of plasmids and resistance gene detection in clinical isolates. *J. Clin. Microbiol.* 55, 3530–3543. doi: 10.1128/JCM.01069-17

Lerminiaux, N. A., and Cameron, A. D. S. (2019). Horizontal transfer of antibiotic resistance genes in clinical environments. *Can. J. Microbiol.* 65, 34–44. doi: 10.1139/cjm-2018-0275

Leungtongkam, U., Thummeepak, R., Tasanapak, K., and Sitthisak, S. (2018). Acquisition and transfer of antibiotic resistance genes in association with conjugative plasmid or class 1 integrons of *Acinetobacter baumannii*. *PLoS One* 13:e0208468. doi: 10.1371/journal.pone.0208468

Levings, R. S., Lightfoot, D., Partridge, S. R., Hall, R. M., and Djordjevic, S. P. (2005). The genomic island SGI1, containing the multiple antibiotic resistance region of *Salmonella enterica* serovar Typhimurium DT104 or variants of it, is widely distributed in other *S. enterica* serovars. *J. Bacteriol.* 187, 4401–4409. doi: 10.1128/JB.187.13.4401-4409.2005

Li, L., Liao, X., Yang, Y., Sun, J., Li, L., Liu, B., et al. (2013). Spread of oqxAB in *Salmonella enterica* serotype Typhimurium predominantly by IncHI2 plasmids. *J. Antimicrob. Chemother.* 68, 2263–2268. doi: 10.1093/jac/dkt209

Liu, G., Bogaj, K., Bortolaia, V., Olsen, J. E., and Thomsen, L. E. (2019). Antibiotic-induced, increased conjugative transfer is common to diverse naturally occurring ESBL plasmids in *Escherichia coli*. *Front. Microbiol.* 10:2119. doi: 10.3389/fmicb.2019.02119

Llosa, M., Gomis-Rüth, F. X., Coll, M., and de la Cruz Fd, F. (2002). Bacterial conjugation: a two-step mechanism for DNA transport. *Mol. Microbiol.* 45, 1–8. doi: 10.1046/j.1365-2958.2002.03014.x

Lopatkin, A. J., Meredith, H. R., Srimani, J. K., Pfeiffer, C., Durrett, R., and You, L. (2017). Persistence and reversal of plasmid-mediated antibiotic resistance. *Nat. Commun.* 8:1689. doi: 10.1038/s41467-017-01532-1

Lu, L. (2004). *Autoinducer 2-based quorum sensing respose of Escherichia coli to sub-therapeutic tetracycling exposure*. Texas A&M University.

Lu, J., Wang, Y., Li, J., Mao, L., Nguyen, S. H., Duarte, T., et al. (2018). Triclosan at environmentally relevant concentrations promotes horizontal transfer of multidrug resistance genes within and across bacterial genera. *Environ. Int.* 121, 1217–1226. doi: 10.1016/j.envint.2018.10.040

Lu, Y., Zeng, J., Wang, L., Lan, K., Shunmei, E., Wang, L., et al. (2017). Antibiotics promote *Escherichia coli-Pseudomonas aeruginosa* conjugation through inhibiting quorum sensing. *Antimicrob. Agents Chemother.* 61, e01284–e01317. doi: 10.1128/AAC.01284-17

Lynne, A. M., Kaldhone, P., David, D., White, D. G., and Foley, S. L. (2009). Characterization of antimicrobial resistance in *Salmonella enterica* serotype heidelberg isolated from food animals. *Foodborne Pathog. Dis.* 6, 207–215. doi: 10.1089/fpd.2008.0172

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., et al. (2010). The global burden of nontyphoidal *Salmonella gastroenteritis*. *Clin. Infect. Dis.* 50, 882–889. doi: 10.1086/650733

Marrero, J., and Waldor, M. K. (2007). The SXT/R391 family of integrative conjugative elements is composed of two exclusion groups. *J. Bacteriol.* 189, 3302–3305. doi: 10.1128/JB.01902-06

Martinez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321, 365–367. doi: 10.1126/science.1159483

Maslowska, K. H., Makiela-Dzbenska, K., and Fijalkowska, I. J. (2019). The SOS system: a complex and tightly regulated response to DNA damage. *Environ. Mol. Mutagen.* 60, 368–384. doi: 10.1002/em.22267

Mather, A. E., Reid, S. W. J., Maskell, D. J., Parkhill, J., Fookes, M. C., Harris, S. R., et al. (2013). Distinguishable epidemics of multidrug-resistant *Salmonella* Typhimurium DT104 in different hosts. *Science* 341, 1514–1517. doi: 10.1126/science.1240578

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.* 4, 608–620. doi: 10.1038/nrmicro1462

McMillan, E. A., Gupta, S. K., Williams, L. E., Jové, T., Hiott, L. M., Woodley, T. A., et al. (2019). Antimicrobial resistance genes, cassettes, and plasmids present in *Salmonella enterica* associated with United States food animals. *Front. Microbiol.* 10:832. doi: 10.3389/fmicb.2019.00832

McMillan, E. A., Jackson, C. R., and Frye, J. G. (2020). Transferable plasmids of *Salmonella enterica* associated with antibiotic resistance genes. *Front. Microbiol.* 11:562181. doi: 10.3389/fmicb.2020.562181

Miller, E. A., Elnekave, E., Flores-Figueroa, C., Johnson, A., Kearney, A., Munoz-Aguayo, J., et al. (2020). Emergence of a novel salmonella enterica serotype reading clonal group is linked to its expansion in commercial Turkey production, resulting in unanticipated human illness in North America. *mSphere* 5, e00056–e00120. doi: 10.1128/mSphere.00056-20

Miller, R. A., and Harbottle, H. (2018). Antimicrobial drug resistance in fish pathogens. *Microbiol. Spectr.* 6:2017. doi: 10.1128/microbiolspec.ARBA-0017-2017

Miriagou, V., Carattoli, A., and Fanning, S. (2006). Antimicrobial resistance islands: resistance gene clusters in *Salmonella* chromosome and plasmids. *Microbes Infect.* 8, 1923–1930. doi: 10.1016/j.micinf.2005.12.027

Moller, T. S. B., Liu, G., Boysen, A., Thomsen, L. E., Lüthje, F. L., Mortensen, S., et al. (2017). Treatment with Cefotaxime affects expression of conjugation associated proteins and conjugation transfer frequency of an IncI1 plasmid in *Escherichia coli*. *Front. Microbiol.* 8:2365. doi: 10.3389/fmicb.2017.02365

Moore, I. F., Hughes, D. W., and Wright, G. D. (2005). Tigecycline is modified by the flavin-dependent monooxygenase TetX. *Biochemistry* 44, 11829–11835. doi: 10.1021/bi0506066

Moreno Switt, A. I., den Bakker, H. C., Cummings, C. A., Rodriguez-Rivera, L. D., Govoni, G., Raneiri, M. L., et al. (2012). Identification and characterization of novel Salmonella mobile elements involved in the dissemination of genes linked to virulence and transmission. *PLoS One* 7:e41247. doi: 10.1371/journal.pone.0041247

Nair, D. V. T., Venkitanarayanan, K., and Johny, A. K. (2018). Antibiotic-resistant *Salmonella* in the food supply and the potential role of antibiotic alternatives for control. *Foods* 7:167. doi: 10.3390/foods7100167

Orman, B. E., Piñeiro, S. A., Arduino, S., Galas, M., Melano, R., Caffer, M. I., et al. (2002). Evolution of multiresistance in nontyphoid *Salmonella* serovars from 1984 to 1998 in Argentina. *Antimicrob. Agents Chemother.* 46, 3963–3970. doi: 10.1128/AAC.46.12.3963-3970.2002

Pagano, M., Martins, A. F., and Barth, A. L. (2016). Mobile genetic elements related to carbapenem resistance in *Acinetobacter baumannii*. *Braz. J. Microbiol.* 47, 785–792. doi: 10.1016/j.bjm.2016.06.005

Partridge, S. R., Kwong, S. M., Firth, N., and Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* 31, e00088–e00117. doi: 10.1128/CMR.00088-17

Perreten, V., Vorlet-Fawer, L., Slickers, P., Ehricht, R., Kuhnert, P., and Frey, J. (2005). Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. *J. Clin. Microbiol.* 43, 2291–2302. doi: 10.1128/JCM.43.5.2291-2302.2005

Phoon, H. Y. P., Hussin, H., Hussain, B. M., and Thong, K. L. (2018). Molecular characterization of extended-spectrum beta lactamase- and carbapenemase-producing *Pseudomonas aeruginosa* strains from a Malaysian tertiary hospital. *Microb. Drug Resist.* 24, 1108–1116. doi: 10.1089/mdr.2017.0258

Piddock, L. J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.* 19, 382–402. doi: 10.1128/CMR.19.2.382-402.2006

Plumb, I. D., Schwensohn, C. A., Gieraltowski, L., Tecle, S., Schneider, Z. D., Freiman, J., et al. (2019). Outbreak of *Salmonella* newport infections with decreased susceptibility to azithromycin linked to beef obtained in the United States and soft cheese obtained in Mexico—United States, 2018–2019. *MMWR Morb. Mortal. Wkly Rep.* 68, 713–717. doi: 10.15585/mmwr.mm6833a1

Podlesek, Z., and Žgur Bertok, D. (2020). The DNA damage inducible SOS response is a key player in the generation of bacterial persister cells and population wide tolerance. *Front. Microbiol.* 11:1785. doi: 10.3389/fmicb.2020.01785

Poirel, L., Cabanne, L., Vahaboglu, H., and Nordmann, P. (2005). Genetic environment and expression of the extended-spectrum beta-lactamase blaPER-1 gene in gram-negative bacteria. *Antimicrob. Agents Chemother.* 49, 1708–1713. doi: 10.1128/AAC.49.5.1708-1713.2005

Pornsukarom, S., van Vliet, A. A.-O., and Thakur, S. A.-O. (2018). Whole genome sequencing analysis of multiple *Salmonella serovars* provides insights into phylogenetic relatedness, antimicrobial resistance, and virulence markers across humans, food animals and agriculture environmental sources. *BMC Genomics* 19:801. doi: 10.1186/s12864-018-5137-4

Qin, T. T., Kang, H. Q., Ma, P., Li, P. P., Huang, L. Y., and Gu, B. (2015). SOS response and its regulation on the fluoroquinolone resistance. *Ann. Transl. Med.* 3:358. doi: 10.3978/j.issn.2305-5839.2015.12.09

Ramirez, M. S., and Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug Resist. Updat.* 13, 151–171. doi: 10.1016/j.drup.2010.08.003

Ramirez-Santos, J., Alvarez, G., Cisneros, E., and Gomez-Eichelmann, M. C. (1992). Distribution of insertion sequence IS1 in multiple-antibiotic resistant clinical Enterobacteriaceae strains. *FEMS Microbiol. Lett.* 72, 189–193. doi: 10.1016/0378-1097(92)90527-u

Ramsay, J. P., Kwong, S. M., Murphy, R. J. T., Eto, K. Y., Price, K. J., Nguyen, Q. T., et al. (2016). An updated view of plasmid conjugation and mobilization in *Staphylococcus*. *Mob. Genet. Elem.* 6:e1208317. doi: 10.1080/2159256X.2016.1208317

Rankin, S. C., Aceto, H., Cassidy, J., Holt, J., Young, S., Love, B., et al. (2002). Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype newport isolates from animals in Pennsylvania. *J. Clin. Microbiol.* 40, 4679–4684. doi: 10.1128/JCM.40.12.4679-4684.2002

Ravi, A., Avershina, E., Ludvigsen, J., L'Abée-Lund, T., and Rudi, K. (2014). Integrons in the intestinal microbiota as reservoirs for transmission of antibiotic resistance genes. *Pathogens* 3, 238–248. doi: 10.3390/pathogens3020238

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol.* 4, 482–501. doi: 10.3934/microbiol.2018.3.482

Roberts, M. C., and Schwarz, S. (2017). "Tetracycline and chloramphenicol resistance mechanisms," in *Antimicrobial Drug Resistance: Mechanisms of Drug Resistance*, *Vol. 1*. eds. D. L. Mayers, D. Marchaim, J. D. Sobel, M. Ouellette and S. Kaye (Cham: Springer International Publishing), 231–243.

Robicsek, A., Strahilevitz, J., Jacoby, G. A., Macielag, M., Abbanat, D., Hye Park, C., et al. (2006). Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat. Med.* 12, 83–88. doi: 10.1038/nm1347

Rodriguez, I., Martín, M. C., Mendoza, M. C., and Rodicio, M. R. (2006). Class 1 and class 2 integrons in non-prevalent serovars of *Salmonella enterica*: structure and association with transposons and plasmids. *J. Antimicrob. Chemother.* 58, 1124–1132. doi: 10.1093/jac/dkl400

Roosaare, M., Puustusmaa, M., Möls, M., Vaher, M., and Remm, M. (2018). PlasmidSeeker: identification of known plasmids from bacterial whole genome sequencing reads. *PeerJ* 6:e4588. doi: 10.7717/peerj.4588

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., et al. (2018). Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *J. Antimicrob. Chemother.* 73, 1121–1137. doi: 10.1093/jac/dkx488

Ruggiero, M., Girlich, D., Dabos, L., Power, P., Naas, T., and Gutkind, G. (2018). Complete sequence of the IncA/C1 plasmid pCf587 carrying blaPER-2 from *Citrobacter freundii*. *Antimicrob. Agents Chemother.* 62, e00006–e00018. doi: 10.1128/AAC.00006-18

Rutherford, S. T., and Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* 2:a012427. doi: 10.1101/cshperspect.a012427

Ryan, M. P., Armshaw, P., and Pembroke, J. T. (2016). SXT/R391 integrative and conjugative elements (ICEs) encode a novel 'trap-door' strategy for mobile element escape. *Front. Microbiol.* 7:829. doi: 10.3389/fmicb.2016.00829

San Millan, A. (2018). Evolution of plasmid-mediated antibiotic resistance in the clinical context. *Trends Microbiol.* 26, 978–985. doi: 10.1016/j.tim.2018.06.007

San Millan, A., Toll-Riera, M., Qi, Q., and MacLean, R. C. (2015). Interactions between horizontally acquired genes create a fitness cost in *Pseudomonas aeruginosa*. *Nat. Commun.* 6, 6845. doi: 10.1038/ncomms7845

Schuurmans, J. M., van Hijum, S. A. F. T., Piet, J. R., Händel, N., Smelt, J., Brul, S., et al. (2014). Effect of growth rate and selection pressure on rates of transfer of an antibiotic resistance plasmid between *E. coli* strains. *Plasmid* 72, 1–8. doi: 10.1016/j.plasmid.2014.01.002

Schwarz, S., Kehrenberg, C., Doublet, B., and Cloeckaert, A. (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol. Rev.* 28, 519–542. doi: 10.1016/j.femsre.2004.04.001

Seth-Smith, H. M., Fookes, M. C., Okoro, C. K., Baker, S., Harris, S. R., Scott, P., et al. (2012). Structure, diversity, and mobility of the *Salmonella* pathogenicity island 7 family of integrative and conjugative elements within Enterobacteriaceae. *J. Bacteriol.* 194, 1494–1504. doi: 10.1128/JB.06403-11

Sharma, A., Gupta, V. K., and Pathania, R. (2019). Efflux pump inhibitors for bacterial pathogens: from bench to bedside. *Indian J. Med. Res.* 149, 129–145. doi: 10.4103/ijmr.IJMR_2079_17

Shen, J., Yang, B., Gu, Q., Zhang, G., Yang, J., Xue, F., et al. (2017). The role of AcrAB-TolC efflux pump in mediating fluoroquinolone resistance in naturally occurring salmonella isolates from China. *Foodborne Pathog. Dis.* 14, 728–734. doi: 10.1089/fpd.2017.2291

Shi, C., Li, M., Muhammad, I., Ma, X., Chang, Y., Li, R., et al. (2018). Combination of berberine and ciprofloxacin reduces multi-resistant *Salmonella* strain biofilm formation by depressing mRNA expressions of luxS, rpoE, and ompR. *J. Vet. Sci.* 19, 808–816. doi: 10.4142/jvs.2018.19.6.808

Shun-Mei, E., Zeng, J. M., Yuan, H., Lu, Y., Cai, R. X., and Chen, C. (2018). Sub-inhibitory concentrations of fluoroquinolones increase conjugation frequency. *Microb. Pathog.* 114, 57–62. doi: 10.1016/j.micpath.2017.11.036

Siebor, E., de Curraize, C., Amoureux, L., and Neuwirth, C. (2016). Mobilization of the *Salmonella* genomic island SGI1 and the proteus genomic island PGI1 by the A/C2 plasmid carrying blaTEM-24 harboured by various clinical species of Enterobacteriaceae. *J. Antimicrob. Chemother.* 71, 2167–2170. doi: 10.1093/jac/dkw151

Siguier, P., Gourbeyre, E., and Chandler, M. (2014). Bacterial insertion sequences: their genomic impact and diversity. *FEMS Microbiol. Rev.* 38, 865–891. doi: 10.1111/1574-6976.12067

Silva, C., Wiesner, M., and Calva, E. (2012). "The importance of mobile genetic elements in the evolution of *Salmonella*: pathogenesis, antibiotic resistance and host adaptation," in *Salmonella*. ed. Y. Kumar.

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. C., and de la Cruz, F. (2010). Mobility of plasmids. *Microbiol. Mol. Biol. Rev.* 74, 434–452. doi: 10.1128/MMBR.00020-10

Stalder, T., Rogers, L. M., Renfrow, C., Yano, H., Smith, Z., and Top, E. M. (2017). Emerging patterns of plasmid-host coevolution that stabilize antibiotic resistance. *Sci. Rep.* 7:4853. doi: 10.1038/s41598-017-04662-0

Sultan, I., Rahman, S., Jan, A. T., Siddiqui, M. T., Mondal, A. H., and Haq, Q. M. R. (2018). Antibiotics, resistome and resistance mechanisms: a bacterial perspective. *Front. Microbiol.* 9:2066. doi: 10.3389/fmicb.2018.02066

Svara, F., and Rankin, D. J. (2011). The evolution of plasmid-carried antibiotic resistance. *BMC Evol. Biol.* 11:130. doi: 10.1186/1471-2148-11-130

Switt, A. I. M., Orsi, R. H., den Bakker, H. C., Vongkamjan, K., Altier, C., and Wiedmann, M. (2013). Genomic characterization provides new insight into *Salmonella* phage diversity. *BMC Genomics* 14:481. doi: 10.1186/1471-2164-14-481

Taga, M. E., Miller, S. T., and Bassler, B. L. (2003). Lsr-mediated transport and processing of AI-2 in *Salmonella* typhimurium. *Mol. Microbiol.* 50, 1411–1427. doi: 10.1046/j.1365-2958.2003.03781.x

Taga, M. E., Semmelhack, J. L., and Bassler, B. L. (2001). The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella* typhimurium. *Mol. Microbiol.* 42, 777–793. doi: 10.1046/j.1365-2958.2001.02669.x

van Hoek, A. H., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., and Aarts, H. J. M. (2011). Acquired antibiotic resistance genes: an overview. *Front. Microbiol.* 2:203. doi: 10.3389/fmicb.2011.00203

van Schaik, W. (2015). The human gut resistome. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 370:20140087. doi: 10.1098/rstb.2014.0087

Vandecraen, J., Chandler, M., Aertsen, A., and van Houdt, R. (2017). The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit. Rev. Microbiol.* 43, 709–730. doi: 10.1080/1040841X.2017.1303661

Vendeville, A., Winzer, K., Heurlier, K., Tang, C. M., and Hardie, K. R. (2005). Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. *Nat. Rev. Microbiol.* 3, 383–396. doi: 10.1038/nrmicro1146

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P T* 40, 277–283.

Villa, L., and Carattoli, A. (2005). Integrons and transposons on the *Salmonella enterica* serovar typhimurium virulence plasmid. *Antimicrob. Agents Chemother.* 49, 1194–1197. doi: 10.1128/AAC.49.3.1194-1197.2005

Vugia, D. J., Samuel, M., Farley, M. M., Marcus, R., Shiferaw, B., Shallow, S., et al. (2004). Invasive *Salmonella* infections in the United States, FoodNet, 1996-1999: incidence, serotype distribution, and outcome. *Clin. Infect. Dis.* 38, S149–S156. doi: 10.1086/381581

Wang, Y., Liu, B., Grenier, D., and Yi, L. (2019a). Regulatory mechanisms of the LuxS/AI-2 system and bacterial resistance. *Antimicrob. Agents Chemother.* 63, e01186–e01219. doi: 10.1128/AAC.01186-19

Wang, Y., Lu, J., Mao, L., Li, J., Yuan, Z., Bond, P. L., et al. (2019b). Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. *ISME J.* 13, 509–522. doi: 10.1038/s41396-018-0275-x

Warburton, P. J., Amodeo, N., and Roberts, A. P. (2016). Mosaic tetracycline resistance genes encoding ribosomal protection proteins. *J. Antimicrob. Chemother.* 71, 3333–3339. doi: 10.1093/jac/dkw304

Watve, M. M., Dahanukar, N., and Watve, M. G. (2010). Sociobiological control of plasmid copy number in bacteria. *PLoS One* 5:e9328. doi: 10.1371/journal.pone.0009328

Welch, T. J., Fricke, W. F., McDermott, P. F., White, D. G., Rosso, M. L., Rasko, D. A., et al. (2007). Multiple antimicrobial resistance in plague: an emerging public health risk. *PLoS One* 2:e309. doi: 10.1371/journal.pone.0000309

Wernersson, R. (2005). FeatureExtract--extraction of sequence annotation made easy. *Nucleic Acids Res.* 33, W567–W569. doi: 10.1093/nar/gki388

Widmer, K. W., Jesudhasan, P. R., Dowd, S. E., and Pillai, S. D. (2007). Differential expression of virulence-related genes in a *Salmonella enterica* serotype typhimurium luxS mutant in response to autoinducer AI-2 and poultry meat-derived AI-2 inhibitor. *Foodborne Pathog. Dis.* 4, 5–15. doi: 10.1089/fpd.2006.40

Wiesner, M., Calva, E., Fernández-Mora, M., Cevallos, M. A., Campos, F., Zaidi, M. B., et al. (2011). *Salmonella* Typhimurium ST213 is associated with two types of IncA/C plasmids carrying multiple resistance determinants. *BMC Microbiol.* 11:9. doi: 10.1186/1471-2180-11-9

Wiesner, M., Fernández-Mora, M., Cevallos, M. A., Zavala-Alvarado, C., Zaidi, M. B., Calva, E., et al. (2013). Conjugative transfer of an IncA/C plasmid-borne blaCMY-2 gene through genetic re-arrangements with an IncX1 plasmid. *BMC Microbiol.* 13:264. doi: 10.1186/1471-2180-13-264

Willetts, N., and Wilkins, B. (1984). Processing of plasmid DNA during bacterial conjugation. *Microbiol. Rev.* 48, 24–41. doi: 10.1128/mr.48.1.24-41.1984

Wong, M. H., Chan, E. W. C., Xie, L., Li, R., and Chen, S. (2016). IncHI2 plasmids are the key vectors responsible for oqxAB transmission among *Salmonella* species. *Antimicrob. Agents Chemother.* 60, 6911–6915. doi: 10.1128/AAC.01555-16

Wong, M. H., and Chen, S. (2013). First detection of oqxAB in *Salmonella* spp. isolated from food. *Antimicrob. Agents Chemother.* 57, 658–660. doi: 10.1128/AAC.01144-12

Yeh, J. C., Chen, C.-L., Chiou, C.-S., Lo, D.-Y., Cheng, J.-C., and Kuo, H.-C. (2018). Comparison of prevalence, phenotype, and antimicrobial resistance of *Salmonella* serovars isolated from turkeys in Taiwan. *Poult. Sci.* 97, 279–288. doi: 10.3382/ps/pex293

Zaman, S. B., Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T., and Hossain, N. (2017). A review on antibiotic resistance: alarm bells are ringing. *Cureus* 9:e1403. doi: 10.7759/cureus.1403

Zankari, E., Allesøe, R., Joensen, K. G., Cavaco, L. M., Lund, O., and Aarestrup, F. M. (2017). PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. *J. Antimicrob. Chemother.* 72, 2764–2768. doi: 10.1093/jac/dkx217

Zgur-Bertok, D. (2013). DNA damage repair and bacterial pathogens. *PLoS Pathog.* 9:e1003711. doi: 10.1371/journal.ppat.1003711

Zhang, Y., Ma, Q., Su, B., Chen, R., Lin, J., Lin, Z., et al. (2018). A study on the role that quorum sensing play in antibiotic-resistant plasmid conjugative transfer in *Escherichia coli*. *Ecotoxicology* 27, 209–216. doi: 10.1007/s10646-017-1886-0

Zhao, S., Datta, A. R., Ayers, S., Friedman, S., Walker, R. D., and White, D. G. (2003). Antimicrobial-resistant *Salmonella* serovars isolated from imported foods. *Int. J. Food Microbiol.* 84, 87–92. doi: 10.1016/s0168-1605(02)00402-6

Zhao, S., Li, C., Hsu, C. H., Tyson, G. H., Strain, E., Tate, H., et al. (2020). Comparative genomic analysis of 450 strains of *Salmonella enterica* isolated from diseased animals. *Gene* 11:1025. doi: 10.3390/genes11091025

Author Disclaimer: This manuscript reflects the views of the authors and does not necessarily reflect those of the United States Food and Drug Administration. Any mention of commercial products is for clarification only and is not intended as approval, endorsement, or recommendation.

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 Algarni, Ricke, Foley and Han. 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 Microbiology

ORIGINAL RESEARCH
published: 31 March 2022
doi: 10.3389/fmicb.2022.803043

Identifying the Sources of Intestinal Colonization With Extended-Spectrum β-Lactamase-Producing *Escherichia coli* in Healthy Infants in the Community

Mohammed Badrul Amin[1], Kazi Injamamul Hoque[1], Subarna Roy[1], Sumita Rani Saha[1], Md. Rayhanul Islam[1], Timothy R. Julian[2,3,4] and Mohammad Aminul Islam[1,5]**

[1] *Laboratory of Food Safety and One Health, Laboratory Sciences and Services Division, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b), Dhaka, Bangladesh,* [2] *Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland,* [3] *Swiss Tropical and Public Health Institute, Basel, Switzerland,* [4] *University of Basel, Basel, Switzerland,* [5] *Paul G. Allen School for Global Health, Washington State University, Pullman, DC, United States*

OPEN ACCESS

Edited by:
Ziad Daoud, Central Michigan University, United States

Reviewed by:
Christopher Morton Thomas, University of Birmingham, United Kingdom
Tarek Moussa, Cairo University, Egypt

***Correspondence:**
Mohammed Badrul Amin
badrul.amin@icddrb.org
Mohammad Aminul Islam
amin.islam@wsu.edu

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *27 October 2021*
Accepted: *31 January 2022*
Published: *31 March 2022*

Citation:
Amin MB, Hoque KI, Roy S, Saha SR, Islam MR, Julian TR and Islam MA (2022) Identifying the Sources of Intestinal Colonization With Extended-Spectrum β-Lactamase-Producing Escherichia coli in Healthy Infants in the Community. Front. Microbiol. 13:803043. doi: 10.3389/fmicb.2022.803043

The prevalence of fecal colonization with extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-Ec) among children in low- and middle-income countries is alarmingly high. This study aimed to identify the sources of ESBL-Ec colonization in children < 1 year old through comparative analysis of *E. coli* isolates from child stool, child's mother stool, and point-of-use drinking water from 46 rural households in Bangladesh. The pairwise similarity in antibiotic susceptibility of *E. coli* from all three sources was evaluated, followed by phylogenetic clustering using enterobacterial repetitive intergenic consensus polymerase chain reaction and whole-genome sequence analysis of the isolates. Matching antibiotic susceptibility and enterobacterial repetitive intergenic consensus polymerase chain reaction patterns were found among ESBL-Ec isolates from child–mother dyads of 24 and 11 households, respectively, from child–water dyads of 5 and 4 households, respectively, and from child–mother–water triads of 3 and 4 households, respectively. Whole-genome sequence analysis of 30 isolates from 10 households revealed that ESBL-Ec from children in five households (50%) was clonally related to ESBL-Ec either from their mothers (2 households), drinking water sources (2 households), or both mother and drinking-water sources (1 household) based on serotype, phylogroup, sequence type, antibiotic resistance genes, mobile genetic elements, core single-nucleotide polymorphisms, and whole-genome multilocus sequence typing. Overall, this study provides empirical evidence that ESBL-Ec colonization in children is linked to the colonization status of mothers and exposure to the household environments contaminated with ESBL-Ec. Interventions such as improved hygiene practices and a safe drinking water supply may help reduce the transmission of ESBL-Ec at the household level.

Keywords: antibiotic resistance, transmission, colonization, whole genome sequencing, *E. coli*, ESBL, children, drinking water

INTRODUCTION

Antimicrobial resistance is a growing public health challenge expected to disproportionately affect low- and middle-income countries in the coming decades (Nadimpalli et al., 2021). The rate of intestinal colonization with antibiotic-resistant organisms has been increasing globally, especially in low- and middle-income countries where both exposure to antibiotic-resistant organisms and use of antibiotics are high (Woerther et al., 2013). Children younger than 5 years, including infants, are frequent carriers of antibiotic-resistant bacteria and antimicrobial resistance genes (ARGs) in their gut (Kaarme et al., 2013; Fernandez-Reyes et al., 2014; Farra et al., 2016; Islam et al., 2018). In rural Bangladesh, we previously found that around 78% of healthy infants (asymptomatic) younger than 1 year were colonized by extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-Ec) with a mean of 6.21 $\log_{10}$ CFU/g wet weight of stool (Islam et al., 2019). Moreover, ESBL-Ec encompassed an average of one-third of the total culturable *E. coli* found in stool samples. Intestinal colonization with ESBL-Ec is associated with an increased risk of drug-resistant infections, treatment complications, and mortality (Ben-Ami et al., 2009; Freeman et al., 2012; Van Aken et al., 2014; Rottier et al., 2015; Isendahl et al., 2019), as it has been shown that multidrug-resistant (MDR) commensal *E. coli* causes extraintestinal infections such as urinary tract infection, bloodstream infections, and neonatal meningitis (Poolman and Wacker, 2016). Additionally, gut colonization with ESBL-Ec represents a gene pool for ARGs that can be exchanged with human pathogens in the gut (Gekenidis et al., 2020).

Escherichia coli is one of the most common commensal gut microflora that account for a significant proportion of antibiotic-resistant bacteria both in infants and adults (Moore et al., 2013; Backhed et al., 2015; Pärnänen et al., 2018). *E. coli* is also one of the first bacterial species to colonize an infant's gut through exposure to maternal fecal flora and/or environmental bacteria (Ducluzeau, 1993; Nowrouzian et al., 2003; Hetzer et al., 2019). *E. coli* also share antibiotic resistance determinants with other pathogenic and non-pathogenic organisms *via* horizontal gene transfer of mobile genetic elements (MGEs), including plasmids, integrons, or transposons (Wright, 2007). Therefore, reducing the level of gut colonization with antibiotic-resistant organisms is considered an effective strategy to control infections caused by MDR organisms in hospitals and in the community (Karanika et al., 2016). Identifying the sources and transmission routes of resistant organisms that colonize infants' gut will be helpful for designing effective intervention strategies.

The antibiotic resistome (collection of antibiotic resistance genes) and mobilome (collection of MGEs) present in a child's gut are influenced by diet and the external environment (Wu et al., 2016; Li et al., 2021). For example, maternal gut microbiome and breast milk microflora influence child microbiomes (Pärnänen et al., 2018). The gut microbiome of children in resource-poor settings is also impacted by environmental flora, as children are frequently exposed to contaminated environments such as courtyard soil, toys and other inanimate objects, contaminated complementary foods, and drinking water (Hartinger et al., 2021). In low-income settings, complementary foods are often contaminated with microorganisms that can cause diseases in infants, and unhygienic food preparation practices are potential sources of contamination (Islam et al., 2012).

In both urban and rural Bangladesh, point-of-use drinking water is found to be frequently contaminated with fecal indicator bacteria that are resistant to antibiotics (Hoque et al., 2006; Talukdar et al., 2013). Given the frequent contamination of point-of-use drinking water within households, as demonstrated by higher levels of contamination compared with water at the source, drinking water may be an important route of exposure to antibiotic-resistant bacteria (Ercumen et al., 2015). However, empirical evidence demonstrating household stored water as a reservoir of antibiotic-resistant bacteria is scarce.

In this study, we analyzed *E. coli* isolates from mother–child dyads along with stored drinking water in a number of rural households in Bangladesh. The findings of the study provide important insights into the possible intra-household transmission of ESBL-Ec from mother and/or household drinking water to children.

MATERIALS AND METHODS

Study Participants Enrollment and Sample Collection

A total of 100 households having an infant ($\leq$ 1 year) were randomly selected during the period from March to October 2017 in rural villages of Hajigonj (n = 50) and Matlab (n = 50), two subdistricts of Chandpur, Bangladesh. Infant's mothers were enrolled in this study upon written consent either by signature or by thumbprint if they were not literate and were approached for stool sample collection in a sterile container provided by our field staff. Infant demographic information, including age, mode of delivery, feeding practices, and antibiotic consumption within 3 months before sampling, was collected (**Supplementary Table 1**). Three types of samples, including mother stool (MS), child stool (CS), and point-of-use drinking water (WU), were collected on the same day from each household and marked as Hajigonj and Matlab sample ID as RH and RM, respectively. All samples were immediately kept in a cold box (4–8°C) and transported to the laboratory within 4–6 h.

Detection and Isolation of *Escherichia coli*

Escherichia coli was isolated from stool and water samples following a standard culture-based procedure described previously (Islam et al., 2019). Briefly, 50 μl of each of four 10-fold serial dilutions (10^{-1} to 10^{-4}) of each stool sample from mothers and children was inoculated using drop plate techniques onto MacConkey agar plates (Becton Dickson, MD, United States) and then incubated at 37°C for 18 h. For water samples, 100 ml was filtered through a 0.45-μm membrane filter, which was placed on modified membrane thermotolerant *E. coli* agar (m-TEC) (Becton Dickinson, MD, United States) according

to the procedure described by the US Environmental Protection Agency (Method 1603, EPA) (US Environmental Protection Agency, 2002). At least one well-isolated colony typical of *E. coli* from each MacConkey and m-TEC plate was tested by API-20E (bioMerieux, France) for confirmation and stored at −80°C.

Antibiotics Susceptibility Test

Standard disk diffusion technique following the Clinical and Laboratory Standards Institute guidelines was used to determine antibiotic susceptibility (AS) of *E. coli* (Patel, 2017). The list of antibiotics used in this study included ampicillin (10 μg), gentamycin (10 μg), tetracycline (30 μg), meropenem (10 μg), imipenem (10 μg), ceftriaxone (30 μg), cefotaxime (30 μg), cefepime (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), azithromycin (15 μg), trimethoprim/sulfamethoxazole (25 μg), and chloramphenicol (30 μg) (Oxoid, Hampshire, United Kingdom). The isolates were classified as resistant or sensitive by measuring the zone of inhibition according to the Clinical and Laboratory Standards Institute (Patel, 2017). If an isolate was resistant to at least one agent in three or more classes of antibiotics, it was categorized as MDR (Magiorakos et al., 2012). Third-generation cephalosporin-resistant *E. coli* isolates were further tested for ESBL using the combined disk test described previously (Patel, 2017).

Typing of *Escherichia coli* Isolates by Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction

All *E. coli* isolates were analyzed using enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) according to the procedure described earlier (Morales-Erasto et al., 2011). ERIC-PCR was carried out in a Thermal Cycler system (C1000 Touch, BioRad, Hercules, CA, United States), and amplified products were separated in 1.5% agarose gel, normalized using the 100-bp DNA ladder as an external reference standard, stained with Midori Green, and visualized by FastGene Blue/Green LED Gel Illuminator (Nippon Genetics, Tokyo, Japan). The TIF formatted image was analyzed with BioNumerics version 4.5 (Applied Maths, Kortrijk, Belgium) to determine the similarity among the isolates. A dendrogram showing the degree of similarity among isolates was generated by Dice, and clustering correlation coefficients were calculated by the unweighted pair group method with arithmetic averages. In ERIC-PCR typing, clusters were considered at approximately 85% similarity in banding patterns between *E. coli* isolates (Casarez et al., 2007).

DNA Extraction and Whole-Genome Sequencing of *Escherichia coli* Isolates

DNA was extracted from an overnight culture of *E. coli* isolates using the Maxwell culture DNA extraction kit and Maxwell automated nucleic acid extraction system (Promega, Madison, USA) following the manufacturer's instruction. The purity and concentration of the extracted DNA were evaluated using NanoDrop spectrophotometer (Thermo Scientific, United States) and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, United States), respectively. Libraries were prepared using the Nextera XT kit, and paired-end sequencing was performed using the Illumina NextSeq500 platform (Illumina, San Diego, CA, United States). Whole-genome sequencing (WGS) data of 30 *E. coli* isolates from 10 households were submitted to the National Center for Biotechnology Information under the accession numbers listed in **Supplementary Table 2**. The size of the assembled genome of *E. coli* isolates ranged from 4.39 to 5.43 Mb with a mean guanine–cytosine (GC) content of 49.6%.

Bioinformatics Analyses

FastQC version 0.11.4 was used for assessing the quality of the reads[1]. Reads were *de novo* assembled using ABySS assembler version 2.2.3 (Simpson et al., 2009). Contigs generated by ABySS were ordered and orientated using ABACAS (Assefa et al., 2009). Prokka 1.12 was used for genome annotation (Seemann, 2014). ParSNP was used for identification of core genome single-nucleotide polymorphisms (SNPs) by comparing to the reference genome of strain *E. coli* SEC470, which showed closed similarity with our study isolates, and construction of phylogenetic tree was done based on aligned core-SNPs (Treangen et al., 2014; Liu et al., 2016). Furthermore, SNP difference was assessed using snp-dists available at https://github.com/tseemann/snp-dists. The sequence types (STs) and serotypes of the isolates were determined using the Achtman scheme and SRST2 v0.2.0[2], respectively (Inouye et al., 2014). ARGs were identified using ARIBA available at https://github.com/sanger-pathogens/ariba considering identity and coverage threshold greater than 90% against the Comprehensive Antibiotic Resistance Database (Hunt et al., 2017). The presence of virulence genes associated with intestinal pathogenic *E. coli* and plasmid replicons was identified using VirulenceFinder and PlasmidFinder database (Carattoli et al., 2014; Joensen et al., 2014). ClermonTyping was used for *E. coli* phylogrouping (Beghain et al., 2018). For MGEs, annotation of transposons was done using ISsaga, and integrons were identified using the database reported earlier (Varani et al., 2011; Cury et al., 2016). Whole-genome multilocus sequence typing (wgMLST) of isolates was carried out using Enterobase schemes with 25,002 loci for *E. coli* and minimum spanning tree based on wgMLST generated using MSTree Algorithm (Zhou et al., 2020).

Statistical Analysis

Data were entered in SPSS 20.0 (IBM Inc., Chicago, IL, United Ststes). Data cleaning statistical analysis was done in R-3.4.2 (R Core Team, 2013). Data visualization was done using MS Office Excel 2010 (Microsoft, Washington, United States). Jaccard/Tanimoto similarity test was done for similarity index analysis among *E. coli* strains isolated from CS, MS, and WU sample types (Chung et al., 2019). AS patterns of isolates were analyzed for similarity using the Jaccard index (J-index), where $J > 0.6$ is considered a statistically significant association (Kain et al., 2016; Lavers and Bond, 2016).

[1]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2]https://github.com/katholt/srst2

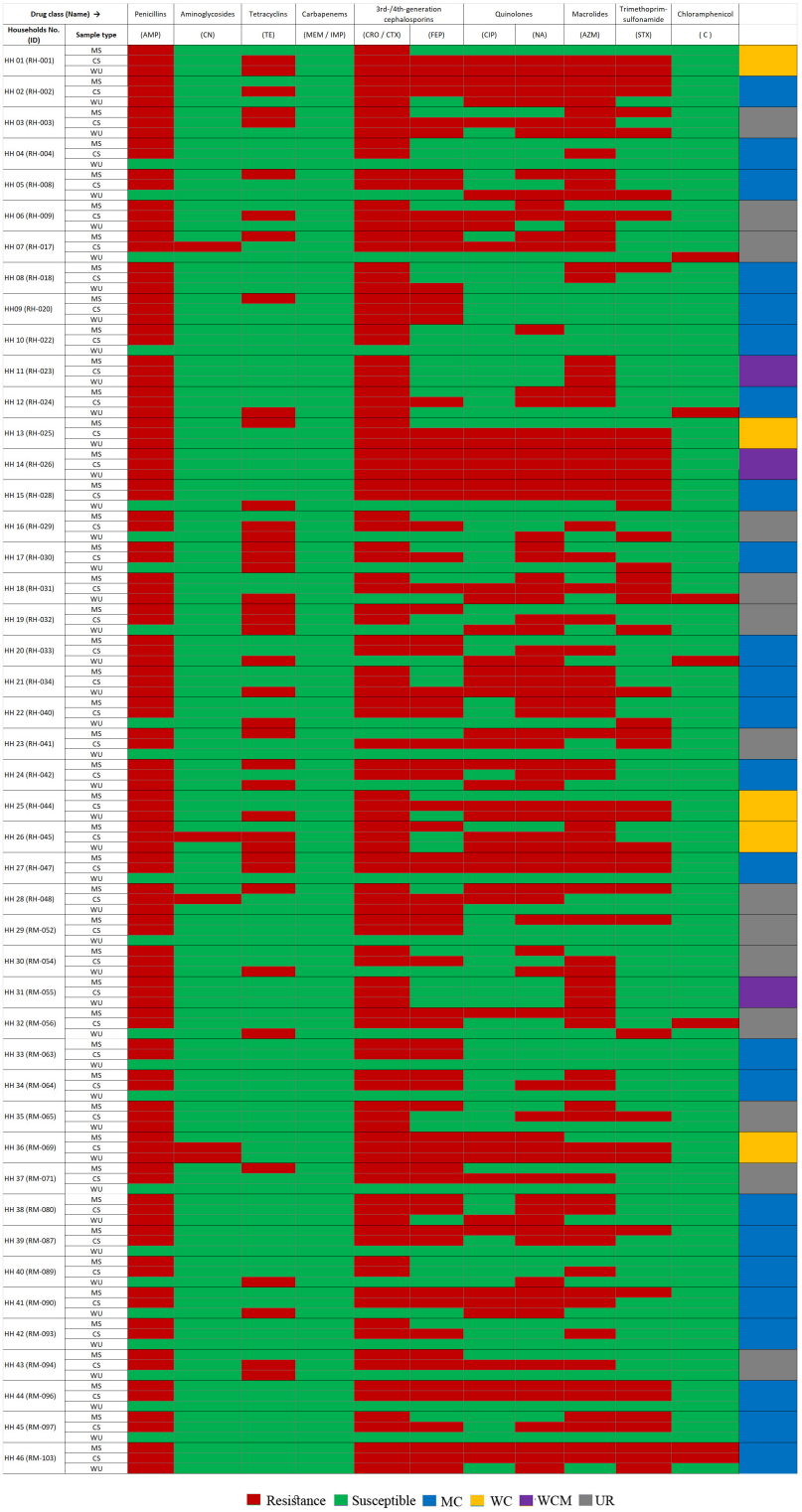


FIGURE 1 | Heatmap showing antibiotic susceptibility (AS) pattern of ESBL-Ec isolated from stool samples of mother–child dyads and point-of-use drinking water samples from 46 households. Amp, ampicillin; CN, gentamycin; TE, tetracycline; MEM, meropenem; IMP, imipenem; CRO, ceftriaxone; CTX, cefotaxime, FEP, cefepime; CIP, ciprofloxacin; NA, nalidixic acid; AZM, azithromycin; STX, trimethoprim/sulfamethoxazole; C, chloramphenicol; HH, households; MS, mother stool; CS, child stool; WCM, AS patterns matched among *E. coli* isolates from mother–child gut and point-of-use drinking water origin; MC, AS pattern matched between *E. coli* isolates of mother–child gut origin; WC; AS pattern matched between *E. coli* strains from child-gut and point-of-use drinking water origin; UR; AS pattern among *E. coli* strains from all sample sources were diverse.

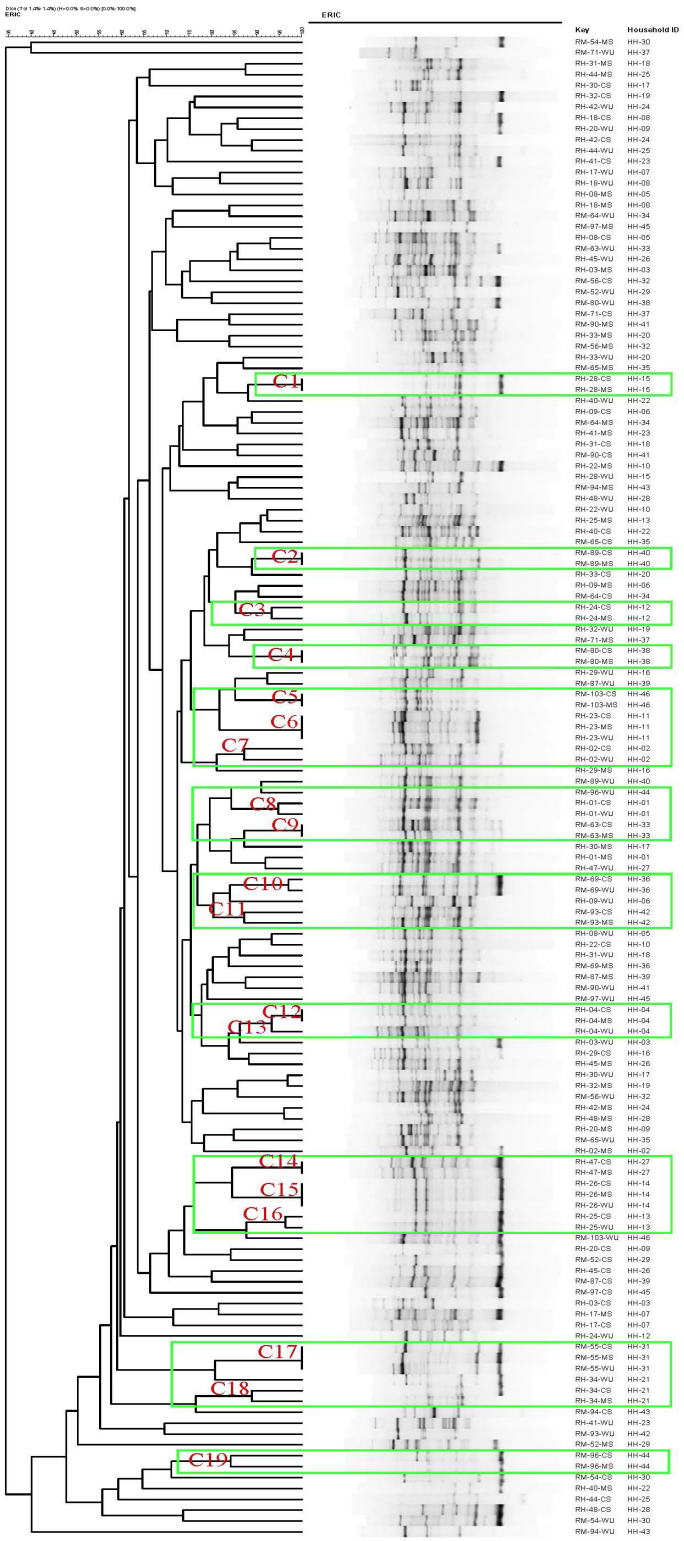


FIGURE 2 | Dendrogram generated by BioNumerics software, showing distances calculated by Dice similarity index of enterobacterial repetitive intergenic consensus polymerase chain reaction banding patterns of *E. coli* strains obtained from stool samples of mother and child and point-of-use drinking water samples from 46 households. Degree of similarity (%) is shown on scale.

TABLE 1 | Distribution of serotype (O and H types), sequence type (ST), phylogroup, and pathotype in 30 *E. coli* isolates obtained from 10 households.

HH[a] No.	HH ID	H type	O type	ST[b] type	Phylogroup[c]	Pathotype[d]
HH-01	RH-01-MS	H21	O28ac-O42	Unknown	B1	-
	RH-01-CS	H9	Onovel14	410	C	-
	RH-01-WU	H9	Onovel14	410	C	-
HH-04	RH-04-MS	H27	O51	226	A	-
	RH-04-CS	H10	Onovel17	226	A	-
	RH-04-WU	H42	O157	3,744	A	astA(STEC)
HH-12	RH-24-MS	H33	O99	10	A	aaiC (EAEC), astA(STEC)
	RH-24-CS	H33	O99	10	A	aaiC (EAEC), astA(STEC)
	RH-24-WU	H21	Onovel4	602	B1	
HH-26	RH-45-MS	H10	O45	226	A	astA(STEC)
	RH-45-CS	H30	O153	315	D	-
	RH-45-WU	H9	Onovel32	10	A	-
HH-28	RH-48-MS	H23	O8	224	B1	-
	RH-48-CS	H4	O25	131	B2	aatA(EAEC)
	RH-48-WU	H41	O137	3,018	E	
HH-31	RM-55-MS	H18	O17-O44-O77	394	D	aatA(EAEC), astA(STEC)
	RM-55-CS	H18	O17-O44-O77	394	D	aatA(EAEC), astA(STEC)
	RM-55-WU	H18	O17-O44-O77	394	D	aatA(EAEC), astA(STEC)
HH-35	RM-65-MS	H48	O164	2,705	A	-
	RM-65-CS	H15	O23	70	D	astA(STEC)
	RM-65-WU	H12	O8	3,580	B1	-
HH-36	RM-69-MS	H21	not found	443	B1	-
	RM-69-CS	H4	O25	131	B2	-
	RM-69-WU	H4	O25	131	B2	-
HH-44	RM-96-MS	H30	O153	38	D	aatA(EAEC)
	RM-96-CS	H30	O153	38	G	aatA(EAEC)
	RM-96-WU	H16	O185	2,280	B1	-
HH-46	RM-103-MS	H19	Onovel1	1,290	A	-
	RM-103-CS	H19	Onovel1	1,290	A	-
	RM-103-WU	H18	O17-O44-O77	8,131	G	aatA(EAEC), astA(STEC)

[a] *Household number.*
[b] *Sequence type (ST) based on multilocus sequence typing Achtman scheme (Achtman et al., 2012).*
[c] *Phylogenetic group based on Clermon Typing (Beghain et al., 2018).*
[d] *Pathotype: EPEC: eae, bfp, and perA; EAEC: aatA; EIEC: ipaH and ial; ETEC: eltA, eltB, and lt; EHEC: espK, espN, nleA, nlec, and nleG; STEC: astA, aaic, stx1a, stx1b, stx2a, and stx2db (Joensen et al., 2014).*

RESULTS

Antibiotic Susceptibility Profiles of *Escherichia coli* From Child Stool, Mother Stool, and Point-of-Use Drinking Water Samples

Of 100 households enrolled in this study, *E. coli* was detected in samples from all three sources (MS, CS, and WU samples) of 55 households. AS tests of *E. coli* from these 55 households showed that all *E. coli* isolates from MS samples were resistant to ampicillin, followed by 98% (n = 54) to third-generation cephalosporins (3GC), 55% (n = 30) to azithromycin, 53% (n = 29) to fourth-generation cephalosporins (4GC), 31% (n = 17) to sulfamethoxazole–trimethoprim, 29% (n = 16) to tetracycline, and 27% (n = 15) to fluoroquinolones. *E. coli* isolates from CS samples were all resistant to ampicillin, followed by 95% (n = 52) to 3GC, 85% (n = 47) to azithromycin, 71% (n = 39) to 4GC, 49% (n = 27) to fluoroquinolones, 40% (n = 22) to sulfamethoxazole–trimethoprim, and 27% (n = 15) to tetracycline. For *E. coli* from WU samples, 55% (n = 30) isolates were resistant to ampicillin, followed by 42% (n = 23) to 3GC, 35% (n = 19) to sulfamethoxazole–trimethoprim, 33% (n = 18) to fluoroquinolones, and 24% (n = 13) to 4GC. The prevalence of MDR *E. coli* was high (98%) among isolates from CS samples, followed by MS (87%) and WU (55%). Of 55 households, CS and MS samples from 46 households (designated as HH1 to HH46) were positive for ESBL-Ec, of which 23 households had ESBL-Ec in WU sample.

Among these 46 households, *E. coli* isolates from CS and MS samples in 24 households (52%) had matching AS profiles, whereas similar results were observed between CS and WU isolates only in five households (11%) based on the J-index similarity matrix (**Supplementary Table 3**). Furthermore, *E. coli* from all three sources (CS, MS, and WU) with matching AS profiles was found only in three households (6%) (**Figure 1**).

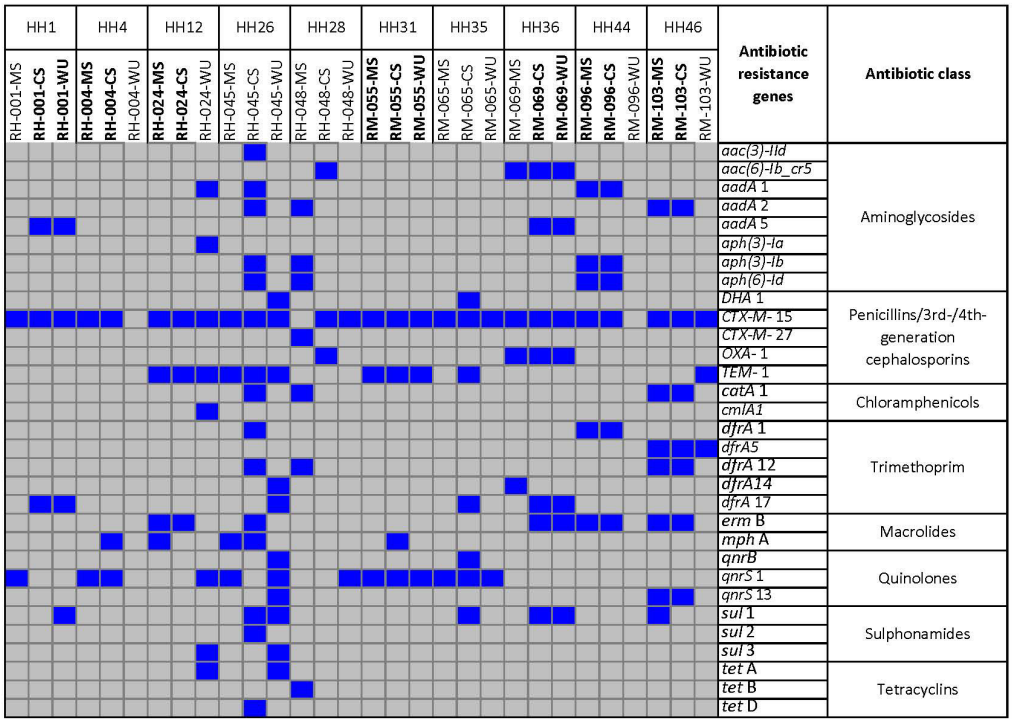


FIGURE 3 | Distribution of antibiotic resistance genes retrieved from whole-genome sequence of 30 *E. coli* strains isolated from CS, MS, and WU samples from 10 households. Blue and gray boxes indicate presence and absence of genes, respectively. HH, households; MS, mother stool; CS, child stool; WU, point-of-use drinking water.

Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction Patterns of *E. coli* Isolates From Child Stool, Mother Stool, and Point-of-Use Drinking Water Samples

Considering a similarity cutoff of 85% among ERIC-PCR banding patterns, a total of 138 isolates consisting of 92 from CS and MS (46 each) and 46 from WU (23 each of ESBL-Ec and non-ESBL-Ec) were grouped into 19 major clusters designated as C1–C19 (**Figure 2**). CS isolates from 11 (24%) and 4 (9%) households had matching ERIC-PCR patterns with their corresponding MS and WU isolates, respectively. Furthermore, isolates from all three sources (MS, CS, and WU) from four additional households had identical ERIC-PCR profiles. Overall, ESBL-Ec from children of 19 households (41%) were related to isolates either from their mothers or drinking water or both, whereas isolates from the remaining 27 households (59%) were unrelated to each other (**Figure 2**).

Genomic Analysis of *Escherichia coli* Isolates From Child Stool, Mother Stool, and Point-of-Use Drinking Water Samples

Based on ERIC-PCR clustering, we selected 30 isolates from 10 households for WGS analysis. Of these, four households had matching CS-MS isolates (HH4, HH12, HH44, and HH46), two had matching CS–WU isolates (HH1 and HH36), one had matching CS–MS–WU isolates (HH31), and three had unmatched isolates (HH26, HH28, and HH35). Of these 30 isolates, 28 were ESBL-Ec, and the remaining two were non-ESBL-Ec.

WGS analysis revealed that *E. coli* isolates from CS–MS dyads in four households (HH4, HH12, HH44, and HH46) had similar serotype, ST, phylogroup, and pathotype except for HH4 and HH44, where isolates from CS–MS dyads differ only in serotype and phylogroup, respectively. Similarly, *E. coli* from WU–CS dyads from two households (HH1 and HH36) and CS–MS–WU triad from one household (HH31) were identical based on serotype, ST, phylogroup, and pathotype (**Table 1**).

A total of 31 different antibiotic resistance genes were found among these *E. coli* isolates (**Figure 3**). Antibiotic resistance genes that were commonly found in both CS and MS isolates were as follows: $bla_{\mathrm{CTX-M-15}}$ (β-lactum) and *qnrS*1 (quinolones) in HH4; $bla_{\mathrm{CTX-M-15}}$, $bla_{\mathrm{TEM-1}}$ (β-lactum), and *ermB* (macrolide) in HH12; *aadA*1, *aph(3)-Ib*, *aph(6)-Id* (aminoglycosides), $bla_{\mathrm{CTX-M-15}}$, *dfrA*1 (trimethoprim), and *ermB* in HH44; and *aadA*2, $bla_{\mathrm{CTX-M-15}}$, *catA*1 (chloramphenicol), *dfrA*5, *dfrA*12, *ermB*, and *qnrS*13 for HH46. The common genes among CS–WU isolates from HH1 were *aadA*1, $bla_{\mathrm{CTX-M-15}}$, and *dfrA*17, whereas *aac(6)-Ib-cr*5 (aminoglycoside), $bla_{\mathrm{CTX-M-15}}$, $bla_{\mathrm{OXA-1}}$, *dfrA*17, *ermB*, and *sul*1 (sulphonamides) were common in HH36. Furthermore, $bla_{\mathrm{CTX-M-15}}$, $bla_{\mathrm{TEM-1}}$,

TABLE 2 | Distribution of mobile genetic elements in 30 *E. coli* isolates of 10 households.

HH ID	*E. coli* isolates ID	Plasmid Inc[a]	Integron	Transposon
HH-01	RH-001-MS	N/F	-	Tn3(R)
	RH-001-CS	IncFIA, IncFIB, IncFII	*Int* 1, *int* 2	Tn3(CR)
	RH-001-WU	IncFIA, IncFIB, IncFII	*Int* 1, *int* 2	Tn3 (CR)
HH-04	RH-004-MS	N/F	-	Tn3(R)
	RH-004-CS	N/F	-	Tn3(R)
	RH-004-WU	N/F	-	-
HH-12	RH-024-MS	IncFII, IncI1, IncX4	-	Tn3(R)
	RH-024-CS	IncFII, IncI1, IncX4	-	Tn3(R)
	RH-024-WU	IncFIB, IncFIC, IncX1	*Int* 1	Tn3(R)
HH-26	RH-045-MS	IncFIA, IncFII	-	Tn3(R)
	RH-045-CS	IncFIB, IncFII	*Int* 1, *int* 2	Tn3(R)
	RH-045-WU	IncFIB, IncFII, IncX1	Int 1	Tn3(R)
HH-28	RH-048-MS	IncFIA, IncFIB, IncFII	*Int* 1	-
	RH-048-CS	IncFIA, IncFIB, IncFII	*Int* 1	-
	RH-048-WU	IncX4	-	Tn3(R)
HH-31	RM-055-MS	IncFIB, IncFII	-	Tn3(R)
	RM-055-CS	IncFIB, IncFII	-	Tn3(R)
	RM-055-WU	IncFIB, IncFII	-	Tn3(R)
HH-35	RM-065-MS	N/F	-	Tn3(R)
	RM-065-CS	IncB/O/K/Z, IncFII	*Int* 1	Tn3(R)
	RM-065-WU	N/F	-	Tn3(R)
HH-36	RM-069-MS	IncFIA, IncFIB, IncFII	*Int* 1	Tn3(R)
	RM-069-CS	IncFIA, IncFII	*Int* 1, *int* 2, *Int*3	Tn3(CR)
	RM-069-WU	IncFIA, IncFII	*Int* 1, *int* 2, *Int*3	Tn3(R)
HH-44	RM-096-MS	IncFIB, IncFII	*Int* 1	Tn3(R)
	RM-096-CS	IncFIB, IncFII	*Int* 1	Tn3(R)
	RM-096-WU	N/F	-	Tn3(R)
HH-46	RM-103-MS	IncB/O/K/Z	*Int* 1	Tn3(R)
	RM-103-CS	IncB/O/K/Z	*Int* 1	Tn3(R)
	RM-103-WU	IncB/O/K/Z, IncFIB, IncFII	-	-

[a] *Plasmid Inc type based on Carottoli (Carattoli et al., 2014).*

and *qnrS*1 genes were commonly found in ESBL-Ec from CS–MS–WU isolates in HH31.

Same MGEs were found among isolates from multiple sources within the same households. Plasmids that belonged to the IncF family (FIA, FIB, FIC, and FII) along with the class I integron (*Int*1) and Tn3 transposon were most commonly found in isolates from different households (**Table 2**). In one household (HH46), IncB/O/K/Z plasmid was detected in both CS and MS isolates.

Genetic relatedness among *E. coli* isolates was determined using core genome SNPs and wgMLST (**Figure 4**). Core SNPs analysis of the isolates produced seven clusters (C1–C7), each representing one household (**Figure 4A**). CS isolates from 4 (HH04, HH12, HH44, and HH46), 2 (HH01 and HH36), and 1 (HH31) households were clustered together with isolates from the corresponding MS, WU, and both, respectively. Pairwise core SNP difference among isolates in clusters C1 and C3–7 was minimum (less than 21 SNPs) and monophyletic (share most common recent ancestor and include all its descendants) in phylogenetic analysis, suggesting clonality among the isolates. The SNP difference among isolates in cluster C2 was relatively higher (1,231 SNPs), and they had a paraphyletic (share a common ancestor, but some descendants are excluded) relationship, suggesting that these isolates are distantly related. The core SNP difference among isolates in the remaining three households (HH26, HH28, and HH35) was very high (> 18,000), suggesting that they are unrelated to each other (**Supplementary Table 4**). A minimum spanning tree was also generated based on wgMLST to further assess the genetic relatedness (**Figure 4B**). wgMLST result shows that allelic distances among CS isolates in five households were < 5 loci from their corresponding MS (HH12 and HH46) or WU (HH1 and HH36) or both (HH31) isolates, indicating their clonal relationship.

DISCUSSION

In this study, we investigated genomic characteristics of *E. coli* isolates from MS, CS, and WU samples from the same households in rural Bangladesh to identify the potential sources of ESBL-Ec colonized in the intestine of children (Islam et al., 2019). We found concordant results between ERIC-PCR typing and WGS based analysis of isolates.

WGS analysis has increasingly been used as an essential tool for microbial source tracking (Rantsiou et al., 2018). The previous report suggests that isolates are predicted to be clonal or originated from the same ancestral clone when pairwise SNP difference is < 21, the allelic difference in wgMLST is 0–17 loci, and monophyletic topology is present in the phylogenetic tree (Pightling et al., 2018; Blanc et al., 2020). The report also added that a more precise decision on the clonality of isolates could be made from WGS-based analysis when it is interpreted along with available epidemiological data. In this study, 15 (33%) of 46 households had identical ERIC-PCR banding patterns of ESBL-Ec isolates from mother–child dyads, indicating possible transmission of organisms from one source to the other within the same household. The result is also supported by WGS analysis, where three households (HH12, HH31, and HH46) had identical ESBL-Ec strains in mother and child pairs based on the core-SNPs and wgMLST analysis. Clonal relationship between isolates from CS–MS dyads in the same households supports vertical transmission, although the cross-sectional design of the study did not permit us to determine the directionality of transmission. This finding is concordant with the prior work, which showed that there is a significant impact of mother's gut microflora on the colonization with the antibiotic-resistant microbial community in infant's gut (Zhang et al., 2011; Denkel et al., 2014; Pärnänen et al., 2018).

In 8 (35%) of 23 households, CS *E. coli* isolates had ERIC-PCR patterns identical to isolates from WU samples, indicating that drinking water could be a potential source of gut colonization with these organisms (Günther and Schipper, 2013). The observation is also supported by WGS analysis where ESBL-Ec from WU samples were found similar with ESBL-Ec isolates from CS samples in three representative households (HH01, HH31, and HH36) based on core-SNP, wgMLST, serotype distribution, phylogroup, antibiotic resistance genes, and MGE characteristics.

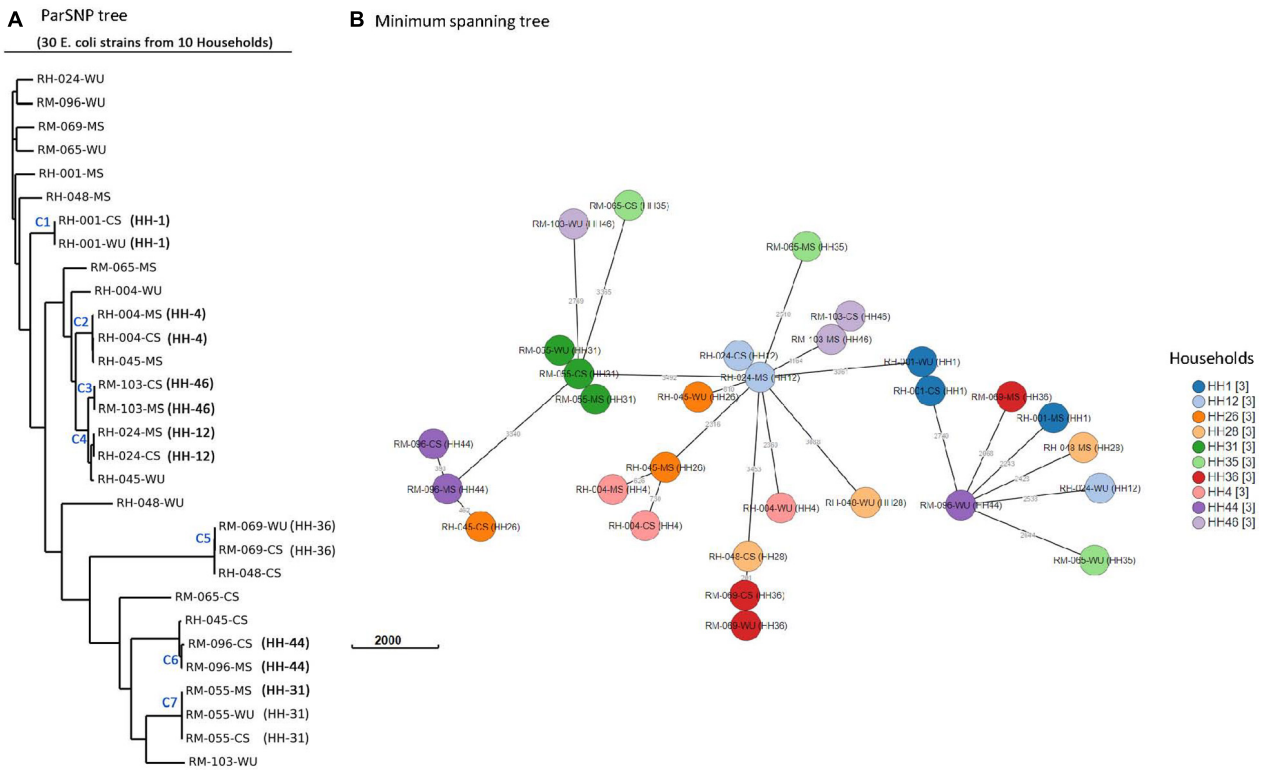


FIGURE 4 | Phylogenetic analysis of 30 *E. coli* isolates. **(A)** Core-genome SNP tree using ParSNP alignment. **(B)** Minimum spanning tree generated by wgMLST. Isolates from same household are shown by circles of identical color. Numbers next to branches indicate allelic differences according to wgMLST analysis.

MGEs such as plasmids, integrons, and transposons play an important role in the transmission of antibiotic resistance in the bacterial community. In this study, the majority of ESBL-Ec isolates possessed IncF type plasmids, which are commonly found in *E. coli* from different human specimens (e.g., rectal samples, gastric aspirate samples, and vaginal samples) and animal sources (e.g., poultry feces) (Marcadé et al., 2008). Furthermore, the IncF plasmid family is predominantly associated with the transfer of antibiotic resistance traits (Johnson et al., 2007; Lyimo et al., 2016) and can persist under selective environments because of the addiction systems harbored by these plasmids (Yang et al., 2015). Apart from plasmids, *E. coli* isolates possessed mostly *int*1, which is mainly involved in the spread of antibiotic resistance (Domingues et al., 2012). In the present study, all the isolates have *int*1 except one harbored *aadA* encoding aminoglycosides and *dfr*A encoding trimethoprim resistance, which is concordant with the previous studies (Lee et al., 2006; Vinué et al., 2008; Bailey et al., 2010). Transposon Tn3 family and Tn3-like subfamilies play an important role in the dissemination of antibiotic resistance genes, especially the β-lactam and aminoglycoside resistance genes (Tran van Nhieu and Collatz, 1987; Tolmasky, 2000; Partridge and Hall, 2005).

Analysis of WGS data also revealed that 8 of 10 households had at least one *E. coli* positive for pathogenic genes, either *aat*A or *ast*A or both. *aat*A encodes for an autotransporter predominantly found in avian pathogenic *E. coli* and enteroaggregative *E. coli* (EAEC), causing diarrhea in humans. *ast*A is found in multiple pathotypes, including EAEC, enterohemorrhagic *E. coli*, and enteropathogenic *E. coli*. EAEC has been shown to cause subclinical infection, is associated with growth decrements in children in Bangladesh, and plays a significant role as a co-pathogen (Lima et al., 2018). Moreover, *aat*A- and *ast*A-carrying *E. coli* are also prevalent in household environments, including courtyard soil that is commonly impacted by feces of domestic poultry carrying avian pathogenic *E. coli* isolates (Montealegre et al., 2020). Children have direct exposure to soil, as reported by a previous study that more than 80% of children < 30 months of age in rural Bangladesh were observed mouthing soil, objects with visible soil, or food with visible soil (Morita et al., 2017). It is also likely that children < 1 year of age can get the ESBL-Ec present in courtyard soil through their mothers, as women in rural areas are regularly exposed to soil (Montealegre et al., 2018).

Our findings should be interpreted in the context of this study's limitations. First, only one colony/isolate per sample was analyzed, which is not sufficient to describe the genetic heterogeneity among *E. coli* strains present in a sample. The selection of colonies with different morphological characteristics from each sample would have given us more confidence in drawing the conclusion on the intra-household transmission of ESBL-Ec, which has been underrepresented in this study.

Second, only MS and WU samples were included for comparative analysis, and sampling of other household environments such as soil and domestic animals was not done, which might be the potential sources of antibiotic resistance acquisition. Nevertheless, this study was the first attempt to identify potential sources of colonization with ESBL-Ec among children in Bangladesh. Intestinal colonization with ESBL-Ec among children is linked to the colonization status of mothers and exposure to the contaminated household environments, which are exacerbated by the lack of improved sanitation, safe drinking water supply, and hygienic practices. Further studies could be designed to longitudinally follow up the intestinal colonization with ESBL-Ec in children, including other members of the household, to understand the persistence and dynamics of colonization by these organisms in this setting.

DATA AVAILABILITY STATEMENT

All relevant data presented in this study are within the manuscript. In addition, the draft genome sequences of the 30 *E. coli* isolates are available in GenBank under accession numbers CP050193-CP050222. All the samples are part of BioProject PRJNA607650 and correspond to BioSample IDs SAMN14342432-SAMN14342461 (**Supplementary Table 2**).

ETHICS STATEMENT

The study (PR-16086) involving human participants was reviewed and approved by the research and the ethical review committees of icddr,b. The review committees also monitored the progress of this study. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MBA and MAI contributed to the conceptualization and initial study design. MBA wrote the first draft and was involved in methodology and data analysis. KIH performed data analysis and methodology. SR and SRS conducted lab experiment. KIH and MRI performed the statistical analysis. TRJ performed review and editing. MAI was involved in draft review and editing, methodology, project administration, and investigation. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the REACH catalyst grant, UK (GRANT no. GR-01507).

ACKNOWLEDGMENTS

icddr,b acknowledges with gratitude the funding support of the REACH program, United Kingdom. icddr,b is also thankful to the following donors: the governments of Bangladesh, Canada, Sweden, and the United Kingdom for providing core/unrestricted support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.803043/full#supplementary-material

REFERENCES

Achtman, M., Wain, J., Weill, F. X., Nair, S., Zhou, Z., Sangal, V., et al. (2012). Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. *PLoS Pathog* 8:e1002776. doi: 10.1371/journal.ppat.1002776

Assefa, S., Keane, T. M., Otto, T. D., Newbold, C., and Berriman, M. (2009). ABACAS: algorithm-based automatic contiguation of assembled sequences. *Bioinformatics* 25, 1968–1969. doi: 10.1093/bioinformatics/btp347

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17:852.

Bailey, J. K., Pinyon, J. L., Anantham, S., and Hall, R. M. (2010). Commensal *Escherichia coli* of healthy humans: a reservoir for antibiotic-resistance determinants. *J. Med. Microbiol.* 59, 1331–1339. doi: 10.1099/jmm.0.022475-0

Beghain, J., Bridier-Nahmias, A., Le Nagard, H., Denamur, E., and Clermont, O. (2018). ClermonTyping: an easy-to-use and accurate in silico method for *Escherichia* genus strain phylotyping. *Microbial Genomics* 4:e000192. doi: 10.1099/mgen.0.000192

Ben-Ami, R., Rodríguez-Baño, J., Arslan, H., Pitout, J. D., Quentin, C., Calbo, E. S., et al. (2009). A multinational survey of risk factors for infection with extended-spectrum beta-lactamase-producing *enterobacteriaceae* in nonhospitalized patients. *Clin. Infect. Dis.* 49, 682–690. doi: 10.1086/604713

Blanc, D. S., Magalhães, B., Koenig, I., Senn, L., and Grandbastien, B. (2020). Comparison of Whole Genome (wg-) and Core Genome (cg-) MLST (BioNumericsTM) versus snp variant calling for epidemiological investigation of *Pseudomonas aeruginosa*. *Front. Microbiol.* 11:1729. doi: 10.3389/fmicb.2020.01729

Carattoli, A., Zankari, E., García-Fernández, A., Larsen, M. V., Lund, O., Villa, L., et al. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrobial Agents Chemotherapy* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Casarez, E. A., Pillai, S. D., Mott, J. B., Vargas, M., Dean, K. E., and Di Giovanni, G. D. (2007). Direct comparison of four bacterial source tracking methods and use of composite data sets. *J. Appl. Microbiol.* 103, 350–364. doi: 10.1111/j.1365-2672.2006.03246.x

Chung, N. C., Miasojedow, B., Startek, M., and Gambin, A. (2019). Jaccard/Tanimoto similarity test and estimation methods for biological presence-absence data. *BMC Bioinformatics* 20:644. doi: 10.1186/s12859-019-3118-5

Cury, J., Jové, T., Touchon, M., Néron, B., and Rocha, E. P. (2016). Identification and analysis of integrons and cassette arrays in bacterial genomes. *Nucleic Acids Res.* 44, 4539–4550. doi: 10.1093/nar/gkw319

Denkel, L. A., Schwab, F., Kola, A., Leistner, R., Garten, L., Von Weizsäcker, K., et al. (2014). The mother as most important risk factor for colonization of very low birth weight (VLBW) infants with extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-E). *J. Antimicrob Chemother* 69, 2230–2237. doi: 10.1093/jac/dku097

Domingues, S., Da Silva, G. J., and Nielsen, K. M. (2012). Integrons: vehicles and pathways for horizontal dissemination in bacteria. *Mob Genet Elements* 2, 211–223. doi: 10.4161/mge.22967

Ducluzeau, R. (1993). [Development, equilibrium and role of microbial flora in the newborn]. *Annales Pediatrie* 40, 13–22.

Ercumen, A., Naser, A. M., Unicomb, L., Arnold, B. F., Colford, J. M. Jr., et al. (2015). Effects of source- versus household contamination of tubewell water on child diarrhea in rural Bangladesh: a randomized controlled trial. *PLoS One* 10:e0121907. doi: 10.1371/journal.pone.0121907

Farra, A., Frank, T., Tondeur, L., Bata, P., Gody, J. C., Onambele, M., et al. (2016). High rate of faecal carriage of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in healthy children in Bangui, Central African Republic. *Clin. Microbiol. Infect.* 22, 891.e1–891.e4. doi: 10.1016/j.cmi.2016.07.001

Fernandez-Reyes, M., Vicente, D., Gomariz, M., Esnal, O., Landa, J., Onate, E., et al. (2014). High rate of fecal carriage of extended-spectrum-beta-lactamase-producing *Escherichia coli* in healthy children in Gipuzkoa, northern Spain. *Antimicrob. Agents Chemother.* 58, 1822–1824. doi: 10.1128/AAC.01503-13

Freeman, J. T., Mcbride, S. J., Nisbet, M. S., Gamble, G. D., Williamson, D. A., Taylor, S. L., et al. (2012). Bloodstream infection with extended-spectrum beta-lactamase-producing *Enterobacteriaceae* at a tertiary care hospital in New Zealand: risk factors and outcomes. *Int. J. Infect. Dis.* 16, e371–e374.

Gekenidis, M. T., Kläui, A., Smalla, K., and Drissner, D. (2020). Transferable extended-spectrum β-Lactamase (ESBL) plasmids in *Enterobacteriaceae* from irrigation water. *Microorganisms* 8:978. doi: 10.3390/microorganisms8070978

Günther, I., and Schipper, Y. (2013). Pumps, germs and storage: the impact of improved water containers on water quality and health. *Health Econ.* 22, 757–774. doi: 10.1002/hec.2852

Hartinger, S. M., Medina-Pizzali, M. L., Salmon-Mulanovich, G., Larson, A. J., Pinedo-Bardales, M., Verastegui, H., et al. (2021). Antimicrobial resistance in humans, animals, water and household environs in rural andean peru: exploring dissemination pathways through the one health lens. *Int. J. Environ. Res. Public Health* 18:4604. doi: 10.3390/ijerph18094604

Hetzer, B., Orth-Höller, D., Würzner, R., Kreidl, P., Lackner, M., Müller, T., et al. (2019). Enhanced acquisition of antibiotic-resistant intestinal *E. coli* during the first year of life assessed in a prospective cohort study. *Antimicrobial Resistance Infection Control* 8:79. doi: 10.1186/s13756-019-0522-6

Hoque, B. A., Hallman, K., Levy, J., Bouis, H., Ali, N., Khan, F., et al. (2006). Rural drinking water at supply and household levels: quality and management. *Int. J. Hyg. Environ. Health* 209, 451–460. doi: 10.1016/j.ijheh.2006.04.008

Hunt, M., Mather, A. E., Sánchez-Busó, L., Page, A. J., Parkhill, J., Keane, J. A., et al. (2017). ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. *Microbial Genomics* 3:e000131. doi: 10.1099/mgen.0.000131

Inouye, M., Dashnow, H., Raven, L.-A., Schultz, M. B., Pope, B. J., Tomita, T., et al. (2014). SRST2: rapid genomic surveillance for public health and hospital microbiology labs. *Genome Med.* 6:90. doi: 10.1186/s13073-014-0090-6

Isendahl, J., Giske, C. G., Hammar, U., Sparen, P., Tegmark Wisell, K., Ternhag, A., et al. (2019). Temporal dynamics and risk factors for bloodstream infection with extended-spectrum β-Lactamase-producing bacteria in previously-colonized individuals: national population-based cohort study. *Clin. Infect. Dis.* 68, 641–649. doi: 10.1093/cid/ciy539

Islam, M. A., Amin, M. B., Roy, S., Asaduzzaman, M., Islam, M. R., Navab-Daneshmand, T., et al. (2019). Fecal colonization with multidrug-resistant *E. coli* among healthy infants in rural Bangladesh. *Front. Microbiol.* 10:640. doi: 10.3389/fmicb.2019.00640

Islam, M., Ahmed, T., Faruque, A., Rahman, S., Das, S., Ahmed, D., et al. (2012). Microbiological quality of complementary foods and its association with diarrhoeal morbidity and nutritional status of Bangladeshi children. *Eur. J. Clin. Nutr.* 66, 1242–1246. doi: 10.1038/ejcn.2012.94

Islam, S., Selvarangan, R., Kanwar, N., Mchenry, R., Chappell, J. D., Halasa, N., et al. (2018). Intestinal carriage of third-generation cephalosporin-resistant and extended-spectrum β-Lactamase-producing *Enterobacteriaceae* in healthy US children. *J. Pediatric Infectious Dis. Soc.* 7, 234–240. doi: 10.1093/jpids/pix045

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Nielsen, E. M., et al. (2014). Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 52, 1501–1510. doi: 10.1128/JCM.03617-13

Johnson, T. J., Wannemuehler, Y. M., Johnson, S. J., Logue, C. M., White, D. G., Doetkott, C., et al. (2007). Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. *Appl. Environ. Microbiol.* 73, 1976–1983. doi: 10.1128/AEM.02171-06

Kaarme, J., Molin, Y., Olsen, B., and Melhus, A. (2013). Prevalence of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in healthy Swedish preschool children. *Acta Paediatr.* 102, 655–660. doi: 10.1111/apa.12206

Kain, E. C., Lavers, J. L., Berg, C. J., Raine, A. F., Bond, A. L. J. E. S., and Research, P. (2016). Plastic ingestion by Newell's (*Puffinus newelli*) and wedge-tailed shearwaters (*Ardenna pacifica*) in Hawaii. *Environ. Sci. Pollut. Res. Int.* 23, 23951–23958. doi: 10.1007/s11356-016-7613-1

Karanika, S., Karantanos, T., Arvanitis, M., Grigoras, C., and Mylonakis, E. (2016). Fecal colonization with extended-spectrum beta-lactamase–producing *Enterobacteriaceae* and risk factors among healthy individuals: a systematic review and metaanalysis. *Clin. Infect. Dis.* 63, 310–318. doi: 10.1093/cid/ciw283

Lavers, J. L., and Bond, A. L. (2016). Selectivity of flesh-footed shearwaters for plastic colour: evidence for differential provisioning in adults and fledglings. *Mar. Environ. Res.* 113, 1–6. doi: 10.1016/j.marenvres.2015.10.011

Lee, J. C., Kang, H. Y., Oh, J. Y., Jeong, J. H., Kim, J., Seol, S. Y., et al. (2006). Antimicrobial resistance and integrons found in commensal *Escherichia coli* isolates from healthy humans. *J. Bacteriol. Virol.* 36, 133–139.

Li, X., Stokholm, J., Brejnrod, A., Vestergaard, G. A., Russel, J., Trivedi, U., et al. (2021). The infant gut resistome associates with E. coli, environmental exposures, gut microbiome maturity, and asthma-associated bacterial composition. *Cell Host Microbe* 29, 975–987.e4. doi: 10.1016/j.chom.2021.03.017

Lima, A. A. M., Medeiros, P., and Havt, A. (2018). Enteroaggregative *Escherichia coli* subclinical and clinical infections. *Curr. Opin. Infect. Dis.* 31, 433–439. doi: 10.1097/QCO.0000000000000477

Liu, G., Chen, S., Guan, G., Tan, J., Al-Dhabi, N. A., Wang, H., et al. (2016). Chitosan modulates inflammatory responses in rats infected with enterotoxigenic *Escherichia coli*. *Mediators Inflamm.* 2016:7432845. doi: 10.1155/2016/7432845

Lyimo, B., Buza, J., Subbiah, M., Temba, S., Kipasika, H., Smith, W., et al. (2016). IncF plasmids are commonly carried by antibiotic resistant *Escherichia coli* isolated from drinking water sources in Northern Tanzania. *Int. J. Microbiol.* 2016:3103672. doi: 10.1155/2016/3103672

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Marcadé, G., Deschamps, C., Boyd, A., Gautier, V., Picard, B., Branger, C., et al. (2008). Replicon typing of plasmids in *Escherichia coli* producing extended-spectrum β-lactamases. *J. Antimicrobial Chemotherapy* 63, 67–71. doi: 10.1093/jac/dkn428

Montealegre, M. C., Roy, S., Böni, F., Hossain, M. I., Navab-Daneshmand, T., Caduff, L., et al. (2018). Risk factors for detection, survival, and growth of antibiotic-resistant and pathogenic *escherichia coli* in household soils in rural Bangladesh. *Appl. Environ. Microbiol.* 84:e01978-18. doi: 10.1128/AEM.01978-18

Montealegre, M. C., Talavera Rodríguez, A., Roy, S., Hossain, M. I., Islam, M. A., Lanza, V. F., et al. (2020). High genomic diversity and heterogenous origins of pathogenic and antibiotic-resistant *Escherichia coli* in household settings represent a challenge to reducing transmission in low-income settings. *mSphere* 5:e00704-19. doi: 10.1128/mSphere.00704-19

Moore, A. M., Patel, S., Forsberg, K. J., Wang, B., Bentley, G., Razia, Y., et al. (2013). Pediatric fecal microbiota harbor diverse and novel antibiotic resistance genes. *PLoS One* 8:e78822. doi: 10.1371/journal.pone.0078822

Morales-Erasto, V., García-Sánchez, A., Salgado-Miranda, C., Talavera-Rojas, M., Robles-González, F., Blackall, P., et al. (2011). ERIC-PCR genotyping of emergent serovar C-1 isolates of Avibacterium paragallinarum from Mexico. *Avian. Dis.* 55, 686–688. doi: 10.1637/9670-012711-ResNote.1

Morita, T., Perin, J., Oldja, L., Biswas, S., Sack, R. B., Ahmed, S., et al. (2017). Mouthing of soil contaminated objects is associated with environmental enteropathy in young children. *Trop. Med. Int. Health* 22, 670–678. doi: 10.1111/tmi.12869

Nadimpalli, M. L., Chan, C. W., and Doron, S. (2021). Antibiotic resistance: a call to action to prevent the next epidemic of inequality. *Nat. Med.* 27, 187–188. doi: 10.1038/s41591-020-01201-9

Nowrouzian, F., Hesselmar, B., Saalman, R., Strannegård, I.-L., Åberg, N., Wold, A. E., et al. (2003). *Escherichia coli* in infants' intestinal microflora: colonization rate, strain turnover, and virulence gene carriage. *Pediatr. Res.* 54:8. doi: 10.1203/01.PDR.0000069843.20655.EE

Pärnänen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D. G. J., et al. (2018). Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nat. Commun.* 9:3891. doi: 10.1038/s41467-018-06393-w

Partridge, S. R., and Hall, R. M. (2005). Evolution of transposons containing blaTEM genes. *Antimicrob. Agents Chemother.* 49, 1267–1268. doi: 10.1128/AAC.49.3.1267-1268.2005

Patel, J. B. (2017). *Performance Standards for Antimicrobial Susceptibility Testing*. Wayne, PA: Clinical and laboratory standards institute.

Pightling, A. W., Pettengill, J. B., Luo, Y., Baugher, J. D., Rand, H., and Strain, E. (2018). Interpreting whole-genome sequence analyses of foodborne bacteria for regulatory applications and outbreak investigations. *Front. Microbiol.* 9:1482. doi: 10.3389/fmicb.2018.01482

Poolman, J. T., and Wacker, M. (2016). Extraintestinal pathogenic *Escherichia coli*, a common human pathogen: challenges for vaccine development and progress in the field. *J. Infect. Dis.* 213, 6–13. doi: 10.1093/infdis/jiv429

R Core Team (2013). *R: A Language and Environment for Statistical Computing*. Vienna: R Core Team.

Rantsiou, K., Kathariou, S., Winkler, A., Skandamis, P., Saint-Cyr, M. J., Rouzeau-Szynalski, K., et al. (2018). Next generation microbiological risk assessment: opportunities of whole genome sequencing (WGS) for foodborne pathogen surveillance, source tracking and risk assessment. *Int. J. Food Microbiol.* 287, 3–9. doi: 10.1016/j.ijfoodmicro.2017.11.007

Rottier, W. C., Bamberg, Y. R., Dorigo-Zetsma, J. W., Van Der Linden, P. D., Ammerlaan, H. S., and Bonten, M. J. (2015). Predictive value of prior colonization and antibiotic use for third-generation cephalosporin-resistant *enterobacteriaceae* bacteremia in patients with sepsis. *Clin. Infect. Dis.* 60, 1622–1630. doi: 10.1093/cid/civ121

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J., and Birol, I. (2009). ABySS: a parallel assembler for short read sequence data. *Genome Res.* 19, 1117–1123. doi: 10.1101/gr.089532.108

Talukdar, P. K., Rahman, M., Rahman, M., Nabi, A., Islam, Z., Hoque, M. M., et al. (2013). Antimicrobial resistance, virulence factors and genetic diversity of *Escherichia coli* isolates from household water supply in Dhaka. Bangladesh. *PLoS One* 8:e61090. doi: 10.1371/journal.pone.0061090

Tolmasky, M. E. (2000). Bacterial resistance to aminoglycosides and beta-lactams: the Tn1331 transposon paradigm. *Front. Biosci.* 5:D20–D29. doi: 10.2741/tolmasky

Tran van Nhieu, G., and Collatz, E. (1987). Primary structure of an aminoglycoside 6'-N-acetyltransferase AAC(6')-4, fused *in vivo* with the signal peptide of the Tn3-encoded beta-lactamase. *J. Bacteriol.* 169, 5708–5714. doi: 10.1128/jb.169.12.5708-5714.1987

Treangen, T. J., Ondov, B. D., Koren, S., and Phillippy, A. M. (2014). The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* 15:524. doi: 10.1186/s13059-014-0524-x

US Environmental Protection Agency (2002). *Method 1603: Escherichia coli (E. coli) in Water by Membrane Filtration Using Modified Membrane-Thermotolerant Escherichia coli Agar (Modified MTEC)". EPA-821-R-02–023*. Washington, DC: US Environmental Protection Agency.

Van Aken, S., Lund, N., Ahl, J., Odenholt, I., and Tham, J. (2014). Risk factors, outcome and impact of empirical antimicrobial treatment in extended-spectrum β-lactamase-producing *Escherichia coli* bacteraemia. *Scand. J. Infect. Dis.* 46, 753–762. doi: 10.3109/00365548.2014.937454

Varani, A. M., Siguier, P., Gourbeyre, E., Charneau, V., and Chandler, M. (2011). ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. *Genome Biol.* 12:R30. doi: 10.1186/gb-2011-12-3-r30

Vinué, L., Saenz, Y., Somalo, S., Escudero, E., Moreno, M. A., Ruiz-Larrea, F., et al. (2008). Prevalence and diversity of integrons and associated resistance genes in faecal *Escherichia coli* isolates of healthy humans in Spain. *J. Antimicrobial Chemotherapy* 62, 934–937. doi: 10.1093/jac/dkn331

Woerther, P. L., Burdet, C., Chachaty, E., and Andremont, A. (2013). Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. *Clin. Microbiol. Rev.* 26, 744–758. doi: 10.1128/CMR.00023-13

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* 5, 175–186. doi: 10.1038/nrmicro1614

Wu, G., Zhang, C., Wang, J., Zhang, F., Wang, R., Shen, J., et al. (2016). Diminution of the gut resistome after a gut microbiota-targeted dietary intervention in obese children. *Sci. Rep.* 6:24030. doi: 10.1038/srep24030

Yang, Q. E., Sun, J., Li, L., Deng, H., Liu, B. T., Fang, L. X., et al. (2015). IncF plasmid diversity in multi-drug resistant *Escherichia coli* strains from animals in China. *Front. Microbiol.* 6:964. doi: 10.3389/fmicb.2015.00964

Zhang, L., Kinkelaar, D., Huang, Y., Li, Y., Li, X., and Wang, H. H. (2011). Acquired antibiotic resistance: are we born with it? *Appl. Environ. Microbiol.* 77, 7134–7141. doi: 10.1128/AEM.05087-11

Zhou, Z., Alikhan, N.-F., Mohamed, K., Fan, Y., Achtman, M., Brown, D., et al. (2020). The EnteroBase user's guide, with case studies on *Salmonella* transmissions, Yersinia pestis phylogeny, and *Escherichia* core genomic diversity. *Genome Res.* 30, 138–152. doi: 10.1101/gr.251678.119

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 Amin, Hoque, Roy, Saha, Islam, Julian and Islam. 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 Microbiology

ORIGINAL RESEARCH
published: 01 April 2022
doi: 10.3389/fmicb.2022.838383

Wild Boars as an Indicator of Environmental Spread of ESβL-Producing *Escherichia coli*

Alessandra Mercato[1†], Claudia Cortimiglia[2†], Aseel Abualsha'ar[1], Aurora Piazza[1], Federica Marchesini[1], Giovanni Milani[2], Silvia Bonardi[3], Pier Sandro Cocconcelli[2] and Roberta Migliavacca[1]*

[1] Department of Clinical-Surgical, Diagnostic and Pediatric Sciences, Unit of Microbiology and Clinical Microbiology, University of Pavia, Pavia, Italy, [2] Department for Sustainable Food Processes, Università Cattolica del Sacro Cuore, Piacenza, Italy, [3] Department of Veterinary Science, Unit of Food Inspection, University of Parma, Parma, Italy

Antimicrobial resistance (AMR) represents an increasing issue worldwide, spreading not only in humans and farmed animals but also in wildlife. One of the most relevant problems is represented by Extended-Spectrum Beta-Lactamases (ESβLs) producing *Escherichia coli* because they are the cause of important infections in human. Wild boars (*Sus scrofa*) as a source of ESβLs attracted attention due to their increasing density and their habits that lead them to be at the human-livestock-wildlife interface. The aim of this study was to increase the knowledge about the ESβLs *E. coli* strains carried by wild boars living in a particularly high-density area of Northern Italy. The analysis of 60 animals allowed to isolate 16 ESβL-producing *E. coli* strains (prevalence 23.3%), which were characterised from a phenotypical and molecular point of view. The overall analysis revealed that the 16 isolates were all not only ESβL producers but also multidrug resistant and carried different types of plasmid replicons. The genome analysis performed on a subset of isolates confirmed the heterogeneity observed with pulsed-field gel electrophoresis (PFGE) and highlighted the presence of two pandemic sequence types, ST131 and ST10, with different collections of virulence factors. The genomic context of ESβL genes further evidenced that all of them were surrounded by transposons and insertion sequences, suggesting the possibility to exchange AMR genes. Overall, this study shows the worrying dissemination of ESβL-producing *E. coli* in wild boars in Northern Italy, suggesting the role of these animals as a spreader of AMR and their inclusion in surveillance programmes, to shed light on the "One Health" complex interactions.

Keywords: wild boars, Italy, ESβLs-producing *Escherichia coli*, ST131, ST10

OPEN ACCESS

Edited by:
Ziad Daoud,
Central Michigan University,
United States

Reviewed by:
Alain Hartmann,
Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), France
Alberto Quesada,
University of Extremadura, Spain

***Correspondence:**
Pier Sandro Cocconcelli
pier.cocconcelli@unicatt.it

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *17 December 2021*
Accepted: *02 March 2022*
Published: *01 April 2022*

Citation:
Mercato A, Cortimiglia C, Abualsha'ar A, Piazza A, Marchesini F, Milani G, Bonardi S, Cocconcelli PS and Migliavacca R (2022) Wild Boars as an Indicator of Environmental Spread of ESβL-Producing Escherichia coli. Front. Microbiol. 13:838383. doi: 10.3389/fmicb.2022.838383

INTRODUCTION

The concept of One Health is based on an existing connection between humans, animals, plants, and environmental health (Karesh et al., 2012). In recent years, this concept has been reformulated underlying the role of interconnected ecosystems, i.e., geographically closed systems, in the occurrence of common health traits (van Bruggen et al., 2019). Such interactions between the different domains are supported by the exchange of microbial communities between humans, animals, plants, and the local environment, thus influencing the health conditions of organisms,

communities, and ecosystems (van Bruggen et al., 2019). In this context, despite the importance of microbial communities' distribution across domains, antimicrobial resistance (AMR) is considered the quintessential One Health issue (Robinson et al., 2016).

The emergence and distribution of antimicrobial-resistant bacteria (AMB) between people and animals have been often attributed to abuse/misuse of antimicrobials in husbandry practices (sub-therapeutical doses and long-lasting treatments), which create the ideal conditions for bacteria to develop resistance (Robinson et al., 2016). AMB shed by farmed animals may contaminate agricultural areas through the spreading of manure and reach surface water, thus contributing to contamination of soil, plants, and wild animals (Laxminarayan et al., 2013). In contrast, important sources of AMR bacteria are represented by hospitals, which release their sewage drains and wastewaters in the environment and represent a menace for different ecosystems (Baquero et al., 2008). Even if hospital effluents are treated in wastewater treatment plants (WWTPs), AMR bacteria and their resistant genes can survive (Yang et al., 2016), thus persisting in the environment and circulating in the ecosystems.

It is commonly assumed that this anthropogenically derived pollution of antimicrobials, AMB, and resistance genes from human waste and livestock farms is the source of wildlife contamination (Carroll et al., 2015). As a matter of fact, wildlife commonly carries AMB deriving from contact with anthropogenic sources that pollute the environment with AMB and/or with antimicrobials (Bondo et al., 2016; Furness et al., 2017; Swift et al., 2019). Nevertheless, despite the isolation of AMB, some studies suggest a lack of evidence of direct contact of wild animals with human or livestock sewage, slurry, or faeces, thus questioning clear pathways of AMR transmission (Furness et al., 2017).

Most of the studies in wild animals are focused on the detection of *Escherichia coli* producing Extended-Spectrum Beta-Lactamases (ESβLs), which are known to be emerging in livestock as well as in wildlife, thus escaping from human clinical settings (Guenther et al., 2011; Atterby et al., 2017; Bonardi et al., 2018; Zendri et al., 2020). It has been reported that the occurrence of ESβL-producing *E. coli* in wild animals, especially wild birds, could be a spill-over form of environmental pollution from human and livestock sources (Guenther et al., 2011). As a matter of fact, wild birds have been considered environmental indicators, reservoirs, and even spreaders of AMR (Bonnedahl and Järhult, 2014). However, the role of AMR indicators could also be postulated for wild mammals like wild boars (*Sus scrofa*).

During the last decades, wild boars have been expanding in Europe despite them being one of the most intensively hunted ungulate species (European Food Safety Authority (EFSA), 2014). Due to their high reproductivity rate and omnivorous habits, their population is likely to an overgrowth in many territories (Keuling et al., 2013; European Food Safety Authority (EFSA), 2014). In Italy, wild boars are among the most common wild ungulates, with densities varying from 0.01–0.05 to 2.32–10.5 animals/km^2 across the whole Italian territory (Pittiglio et al., 2018). In the region of the study (Emilia-Romagna region; 22,451 km^2), a density of 1.37–2.31 wild boars/km^2 was estimated, thus consisting of a regional wild boar population ranging between thirty and fifty thousand animals. Since these animals are also used for the production of non-thermally treated foods, such as cured meats and dry fermented sausages, it is possible for bacteria from animals to reach the consumers *via* these foods.

The main aims of the study were the evaluation of (i) prevalence of ESβL-producing *E. coli* among a small group of wild boars hunted in Northern Italy (Emilia-Romagna region); (ii) molecular typing of the isolates to characterise AMR determinants, virulence determinants, and phylogenetic groups; and (iii) comparison with strains isolated from different sources, including human, food, wild, and companion animals.

MATERIALS AND METHODS

Sample Collection and Isolation of ESβL-Producing *Escherichia coli* Strains

From June to December 2018, 60 wild boars (35 females and 25 males) that were hunted in Parma Province, Northern Italy, were tested for the presence of ESβL-producing *E. coli*. Of them, 14 animals (23.3%) belonged to age class 0 (young; <12 months), 17 (28.3%) to age class 1 (subadults; 13–24 months) and 29 (48.3%) to age class 2 (>24 months). For this survey, only animals dead since less than 5 h were selected. Mesenteric lymph nodes (MLNs) and faecal samples were collected immediately after evisceration. MLNs were washed with sterile saline solution and decontaminated using ethyl alcohol before being placed in sterile containers. Faecal samples were collected from the colon and placed in sterile containers. The samples were transported to the laboratory at refrigerated conditions and tested the day of collection.

Mesenteric lymph nodes were cut into small pieces (0.1–0.2 cm) by using sterile scissors. Notably, 5–10 g of MLNs, according to their size, and 10 g of faeces were diluted 1:10 in buffered peptone water (BPW; Oxoid, Basingstoke, United Kingdom) and incubated at 37 ± 1°C for 18–20 h. A 100 μl loopful of the cultures were streaked onto MacConkey (Oxoid) agar plates added with a disk containing cefotaxime (CTX; 5 μg) and incubated at 44 ± 1°C for 21 ± 3 h. Colonies grown in the proximity of the antimicrobial disk were selected to be seeded onto Tryptone Soya Agar (TSA, Oxoid) and Tryptone Soya Broth (TSB, Oxoid) and incubated at 44 ± 1°C for 21 ± 3 h. Indole production was tested by adding James' reagent to TSB cultures. From TSA plates of indole-positive cultures, one well-isolated colony was identified at the species level with the Microgen®GN-A (Biogenetics, Padua, Italy) system. *E. coli* isolates were analysed using the Kirby-Bauer test following EUCAST recommendations (2018) using disks produced by Oxoid containing CTX (5 μg), ceftazidime (CAZ; 10 μg). *E. coli* ATCC 25922 was used as a quality control microorganism. The strains which showed an inhibition zone diameter <17 mm for CTX and <19 mm for CAZ were selected for ESβL testing. Phenotypic identification of ESβL-producing *E. coli* was

performed using the ESβL-Confirm Kit (Rosco Diagnostica, Taastrup, Denmark) following the manufacturer's instructions.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility profiles for the phenotypically identified ESβL-producing *E. coli* isolates were determined using MicroScan Gram-negative MIC/Combo panels and analysed through the semi-automated system MicroScan autoSCAN-4 (Beckman Coulter) following the manufacturer's instructions. Clinical categorisation of the isolates was performed based on the EUCAST 2019 clinical breakpoints.[1]

Molecular Investigation of Resistance Determinants

The presence of ESβL and carbapenemases determinants was investigated by microarray Check-Points CT 103 XL Check-MDR assay (Check-Points Health B.V., Wageningen, Netherlands) and PCR. To determine the exact allelic variant of bla_{CTX-M}, two-directional DNA sequencing was performed. PCR amplicons of 593 bp, obtained using the primer pair Fw: 5′-ATGTGCAGYACCAGTAARGT-3 and Rev: 5′-TGGGTRAARTARGTSACCAGA-3′, were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States). DNA sequencing was performed using the Microsynth services (Microsynth Seqlab, Germany). The alignment between the forward, reverse, and reference DNA sequences was accomplished using ChromasPro software (Technelysium Pty Ltd, South Brisbane, Australia) and analysed using the BLAST software.[2] The PCR assays were performed to assess the presence of resistance determinants against quinolones, namely *qnrB*, *qnrS*, and *aac(6′)-Ib-cr*, and aminoglycosides, like *aadA*, *armA*, *rmtB*, and *rmtC*.

Conjugation Assay and Plasmid Typing

To assess the possible transferability of the bla_{CTX-M} determinants identified, conjugation assays in liquid were performed using three different *E. coli* strains as recipients, including the *E. coli* K12 strain J62 (pro-, his-, trp-, lac-, and Sm-R), J53-2 (met-, pro-, and rif-R), and J53 AzideR. Donor strains in the logarithmic growth phase were mixed with recipients in the early stationary phase in a 1:10 ratio in Mueller Hinton broth (Oxoid, Basingstoke, UK), and the mixture was incubated at 37°C overnight. The transconjugants were selected on MacConkey agar containing cefotaxime (2 mg/L) plus streptomycin (1,000 mg/L), rifampicin (100 mg/L), or sodium azide (100 mg/L) (Tartor et al., 2021). The detection sensitivity of the assay was approximately from eight to ten transconjugants per recipient. At least three possible transconjugant colonies for each recipient were subjected to antimicrobial susceptibility testing and PCR to confirm bla_{CTX-M} gene presence using the primers mentioned above. Plasmids were also typed for both the donors and the transconjugants based on their incompatibility groups using the PCR-based replicon typing scheme PBRT 2.0 Kit (Diatheva, Fano, Italy). Moreover, random amplified polymorphic DNA (RAPD)-PCR was performed for the donor, transconjugants, and recipients using the RAPD-PCR Kit (Amersham BioSciences UK Limited, England) to definitely distinguish transconjugants from donor strains.

Pulsed-Field Gel Electrophoresis

The pulsed-field gel electrophoresis (PFGE) was performed using the *Xba*I restriction enzyme, and the obtained genomic fragments were separated on a CHEF-DR II apparatus (Bio-Rad, Milan, Italy) for 22 h at 14°C. Bacteriophage λ concatenamers were used as DNA size markers. DNA restriction patterns of scanned gel pictures were interpreted following cluster analysis using the Fingerprinting II version 3.0 software (Bio-Rad) using the unweighted pair-group method with arithmetic averages (UPGMA). Only bands larger than 48 kb were considered for the analysis. The Dice correlation coefficient was used with a 1.0% position tolerance to analyse the similarities of the banding patterns, and a similarity threshold of 90% to define clusters. The restriction patterns of the genomic DNA from the isolates were analysed and interpreted according to the criteria of Tenover et al. (1995).

Phylogenetic Group Investigation

Phylogenetic group typing was performed for the 16 ESβL-producing *E. coli* isolates according to Clermont et al. using PCR assays targetting the genes *chuA*, *yjaA*, the DNA fragment TSPE4.C, and *arpA* gene as described previously (Clermont et al., 2013).

Whole-Genome Sequencing and *in silico* Analysis

Notably, five out of the 16 ESβL-producing *E. coli* strains were selected taking into consideration the highest level of antibiotic resistance and different phylogroups and subjected to whole-genome sequencing (WGS) for further investigation (**Figure 1**). The total DNA was extracted from pure overnight culture using the E.Z.N.A.®Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA, United States), following the manufacturer's instructions. The DNA concentration was determined using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and visualised on 0.8% agarose gel to check the DNA integrity. DNA was sequenced by Fasteris (Geneva, Switzerland) using the Illumina Miseq platform (Illumina Inc., San Diego, CA, United States) with 300 paired-end runs.

The quality of raw reads was evaluated using FastQC software. SPAdes tool on the PATRIC website was used to perform the *de novo* assembly, discarding contigs with a length below 400 bp (Wattam et al., 2017), and contigs were annotated using Prokka version 1.13.3 with *e*-value cut-off default (Seemann, 2014). *In silico* investigation of Multilocus Sequence Type (MLST), serotype, fimH subtyping, and phylogroup was investigated using MLST 2.0 for *E. coli* #1 (Larsen et al., 2012), SerotypeFinder 2.0 (Joensen et al., 2015), FimTyper-1.0 (Roer et al., 2017), and default threshold. ResFinder 4.1 (Bortolaia et al., 2020), PlasmidFinder 2.1 (Carattoli and Hasman, 2020), and VirulenceFinder 2.0 (Malberg Tetzschner et al., 2020) were used

[1] http://www.eucast.org

[2] http://blast.ncbi.nlm.nih.gov/Blast.cgi

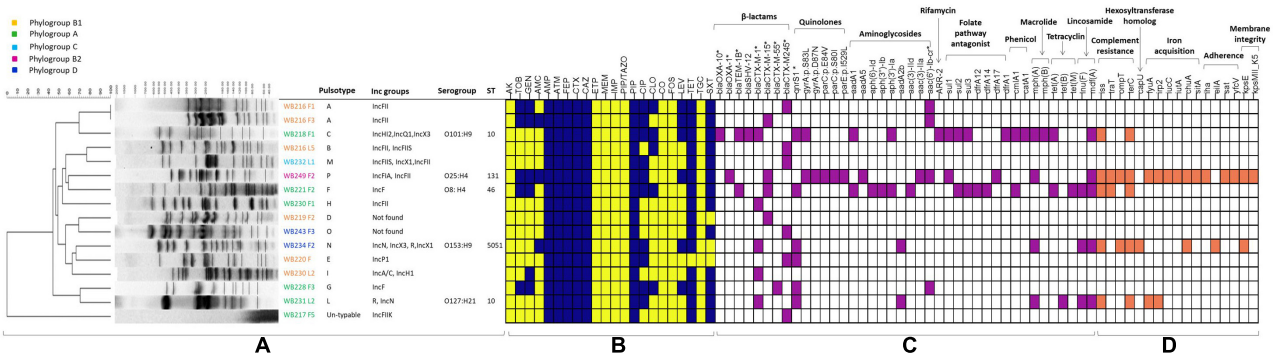


FIGURE 1 | The figure includes all the phenotypical and molecular characterisation, including the analysis performed with whole-genome sequencing (WGS). The part **(A)** is related to the molecular typing by pulsed-field gel electrophoresis (PFGE), with the identification of pulsotypes, phylogroups, and plasmid replicons, for all the extended-spectrum beta-lactamase (ESβL)-producing *Escherichia coli* strains, while STs and serogroups are evidenced for the sequenced strains. The heat map **(B)** shows the phenotypical resistance profile of all ESβL-producing *E. coli*, where yellow identifies susceptible phenotype, while blue identifies the resistance phenotype. The molecular determinants found by PCR and genome analysis are evidenced in the heat map in purple **(C)**, while the virulence genes harboured by the sequenced strains are shown in orange **(D)**. AMR genes and virulence factors are grouped by evidenced categories. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; PIP, piperacillin; ATM, aztreonam; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CXM, cefuroxime; CIP, ciprofloxacin; LEV, levofloxacin; TZP, piperacillin-tazobactam, AK, Amikacin; GM, gentamicin; TOB, tobramycin; CLO, chloramphenicol; ERT, ertapenem; IMI, imipenem; MEM, meropenem; TET, tetracycline; TMS, trimethoprim/sulfamethoxazole. *Determinants detected by PCR.

to detect AMR genes, plasmid replicons, and virulence factors, respectively.

Contigs harbouring ESβL determinants were annotated using Prokka, and the genetic environment of AMR genes was investigated using Geneious Prime version 2021.2.2 and Isfinder (Siguier et al., 2006). In particular, since bla_{CTX-15} is known to be flanked by the transposon element ISEcp1 (AJ242809), a BLASTn search was performed on bla_{CTX-15}-positive genomes.

The phylogeny among wild boar strains was investigated analysing the pan-genome using Roary (Page et al., 2015). Then, the phylogenetic relationship between ST131 and ST10 strains from wild boars and other strains with the same sequence type (ST) and isolated from different sources was investigated.

We explored the genomes deposited in PATRIC and EnteroBase (Zhou et al., 2020), for which information about the ST was available. The data about ST were confirmed using MLST 2.0 for *E. coli* #1 (Larsen et al., 2012). Moreover, metadata about isolation sources (human, animal, and food), isolation country, host health (where applicable), and ESβL genes were also considered. All the selected genomes were re-analysed with ABRicate using the ResFinder database[3] and typed through SerotypeFinder.

Fifty-seven ST131 ESβL-producing *E. coli* genomes with the mentioned features were obtained from PATRIC and EnteroBase (**Supplementary Table 1**). When fastQ files were available, they were assembled with Spades. FASTA files were re-annotated using Prokka version 1.13.3 with *e*-value cut-off default (Seemann, 2014). The GFF files of all the downloaded ST131 and of WB 249 F2 strain were used for the pan and core genome analysis using Roary (Page et al., 2015). The Newick file resulting from Roary analysis was uploaded in iTOL for viewing the relationship based on single nucleotide polymorphism (SNP) detected in the core genome.

The same approach was adopted for the comparison between ST10 wild boar strains and genome sequences of ST10 strains isolated from different sources (**Supplementary Table 2**). Genome sequences of ST10 EsβL-producing *E. coli* were retrieved from the PATRIC database. Sequences were screened confirming the ST group, and a subset of 49 genomes was selected, considering the source (human, companion, and wild animal food) and the positivity for ESβL genes.

RESULTS

Wild Boars Are Carriers of ESβL-Producing and Multidrug Resistant *Escherichia coli*

Notably, 14 wild boars out of 60 (23.3%; 95% CI 14.4–35.4) were found to be positive for the presence of CTX- and CAZ-resistant *E. coli*. A total of 16 *E. coli* isolates were collected from the 14 animals from MLN (5/60; 8.3%) and faeces (11/60; 18.3%) samples, with three wild boars positive both in MLNs and faeces. Positive wild boars were represented by five females (35.7%) and nine males (64.3%) belonging to age class 0 (4/14; 26.6%) and age class 2 (10/29; 34.5%). All the 16 isolates were positive to the ESβL-Confirm Kit.

A wider evaluation of antimicrobial susceptibility profiles of the 16 ESβL-producing *E. coli* showed 100% non-susceptibility to penicillins, third-generation cephalosporins (3GCs) and fourth-generation cephalosporins (4GCs), tetracyclines, monobactams, and tetracycline; 75% to trimethoprim/sulfamethoxazole, 37.5% to chloramphenicol, among fluoroquinolones, 62.5% to ciprofloxacin and 31.25% to levofloxacin, and against aminoglycosides, 31.25% to tobramycin and 37.5% to

[3]https://github.com/tseemann/abricate

gentamycin, and 18.75% to amoxicillin/clavulanate. All isolates resulted fully susceptible to carbapenems, amikacin, tigecycline, fosfomycin, piperacillin/tazobactam, and colistin (**Figure 1**).

Genotypic investigation was then used to determine the identity of genes conferring the phenotypic resistance. The presence of the bla$_{CTX-M}$-type genes was detected by microarray and confirmed using targetted PCR. bla$_{CTX-M-245}$, bla$_{CTX-M-15}$, and bla$_{CTX-M-1}$, were found in 31.3% (n = 5/16), 25% (n = 4/16), 25% (n = 4/16) strains, respectively. Among ESβL-producing strains, fluoroquinolone resistant isolates (56.3%, n = 9/16) harboured either qnrS (55.6%, n = 5/9), aac(6′)Ibcr (33.3%, n = 3/9), or both qnrS and aac(6′)Ibcr (1.1%, n = 1/9) genes, while aminoglycoside resistant isolates (37.5%, n = 6/16) harboured either aadA (33.3%, n = 2/6), aac(6′)Ibcr (50%, n = 3/6), or aadA and aac(6′)Ibcr (16.7%, 1/6) determinants (**Figure 1**).

Plasmid Profile and Conjugation Results

The possible presence of plasmids was assessed for all 16 *E. coli* isolates. IncFII was the prevalent incompatibility group found in the isolates (n = 6/16). Other groups detected included IncN (n = 2/16), IncX1 (n = 2/16), IncX3 (n = 2/16), IncFIIS (n = 2/16), IncFIIK (n = 1/16), IncF (2/16), IncFIA (n = 1/16), IncA/C (n = 1/156), IncH12 (n = 1/16), IncH1 (n = 1/16), IncQ1 (1/16), and IncP1 (n = 1/16). Two isolates resulted negative for all the incompatibility groups searched.

All strains were then subjected to conjugation experiments, and a lateral transfer of resistance genes was observed in 25% (n = 4/16) of them. The resistance profiles of donors and transconjugants confirmed the lateral transfer of 3GC resistance. PCR analysis confirmed the presence of a bla$_{CTX-M}$-type gene in all the transconjugants. Inc group plasmid and RAPD profile analysis performed on all the transconjugants confirmed that the bla$_{CTX-M}$ gene was plasmid-encoded and that plasmids are mobilised by conjugation to the *E. coli* J53 or *E. coli* J62 recipient.

Molecular Typing Reveals High Heterogeneity Among ESβL-Producing *Escherichia coli*

The PFGE analysis of the 16 ESβL-producing *E. coli* isolates showed a high clonal heterogeneity, revealing 14 different pulsotypes, even for the isolates recovered from MLNs and faeces of the same wild boar (**Figure 1**). One *E. coli* isolate resulted not typable with this method.

The assignment to *E. coli* phylogenetic groups of the 16 isolates revealed that the 37.5% (n = 6/16), all CTX-M-producers, belonged to phylogroup A; 37.5% (n = 6/16) belonged to phylogroup B1; 12.5% (n = 2/16) to phylogroup D; 6% (n = 1/16) to phylogroup B1, and 6.25% (n = 1/16) to phylogroup C (**Figure 1**).

In-Depth Genomic Characterisation: Typing, Virulence, Antimicrobial Resistance, and Dissemination Potential

Among the 16 ESβL-producing *E. coli* strains, five of them were selected considering the resistance to a high number of antimicrobials and the phylogroup and sequenced for further deep characterisation. As shown in **Table 1**, the *in silico* analysis evidenced four different STs, and in particular, two strains with ST10, one strain with ST131, one strain with ST46, and one strain with ST5051. The ST10 strains belonged to two different serogroups (i.e., O101:H9 and O127:H21), and the ST131 strain belonged to O25:H4 serogroup; O8:H4 and O153:H9 were detected as serogroup of ST46 and ST505, respectively. While the ST131 strain showed the fimbrial variant fimH30, the other isolates harboured fimH54, except for WB221F2, which carries fimH34.

The analysis of the genome led to a deep characterisation of virulence and AMR content. The analysis of the virulence profile evidenced the absence of Shiga-toxin genes (*stx1* and *stx2*) and intimin gene (*eae*). Some other putative virulence factors were identified. In particular, WB249F2 shows a large collection of genes involved in adherence (*iha* and *yfcV*), complement resistance (*iss*, *traT*, and *ompT*), iron acquisition (*iucC*, *iutA*, *chuA*, *fyuA*, and *irp2*), and membrane integrity (*kpsE* and *kpsMII_K5*). The other strains showed a less enriched panel of virulence factors, some of whom were in common with the WB249F2 strain. Differently from all other strains, WB234F2 carried hexosyltransferase homologue gene (*capU*) and Salmonella hilA homologue (*eilA*).

A more accurate and comprehensive typing of antimicrobial determinants was assessed using the WGS analysis (**Figure 1**). Among ESβL genes, the five sequenced wild boar strains were confirmed to carry the same determinants found by molecular analysis and, moreover, were able to detect other genes. In fact, the screening of the genome revealed that the WB218F1 strain was also a carrier of $bla_{\mathrm{TEM-1B}}$ and $bla_{\mathrm{SHV-12}}$ genes. It is interesting to notice that while all strains were positive for bla_{CTX-M} genes, the WB218F1 also carried all the other EsβL determinants.

Genome analysis of other resistance genes confirmed the most resistance phenotypes against other antimicrobials, identifying determinants directly related to the phenotype. In fact, WB218F1 showed a point mutation in the *gyrA* gene (S83L) linked to the fluoroquinolone phenotype, while the resistance of the WB249F2 strain was due to mutations in *gyrA* (S83L and D87N) gene associated with the *parC* (E84V, S80I) and *parE* (I529L) mutations.

WB218F1 and WB221F2 were positive for the *cmlA1* gene, responsible for the resistance against chloramphenicol together with *catA1* and *flor1*, respectively. The analysis revealed that strains (WB221F2 and WB249F2) phenotypically resistant to

TABLE 1 | Main genome features of five extended-spectrum beta-lactamase (ESβL)-producing *Escherichia coli* selected strains isolated from wild boars.

	WB218 F1	WB221 F2	WB231 L2	WB234 F2	WB249 F2
Genome size (bp)	4917558	4790178	4649545	5051588	5070855
N°contigs	113	164	74	78	73
MLST	10	46	10	5051	131
FimH group	54	34	54	54	30
Serogroup	O101: H9	O8: H4	O127:H21	O153:H9	O25:H4

aminoglycosides such as gentamicin and tobramycin carried not only *aac(6′)-Ib-cr* genes but also other genes belonging to the aminoglycoside-(3)-*N*-acetyl-transferase families, such as *aac(3)-IId* and *aac(3)-IIa*. From a phenotypic point of view, all five strains resulted in resistance to tetracycline; this evidence was confirmed at the genome level only for three strains, which carried *tet(A)*, *tet(B)*, and *tet(M)*. The tetracycline resistance for WB234F2 was related to the presence of *mdf(A)*, a multidrug-resistant (MDR) determinant, while in the case of the WB249F2 strain, no genes were identified. Moreover, the analysis lead to identify the presence of other genes linked to the resistance to trimethoprim-sulfamethoxazole such as *dfrA1*, *dfrA12*, *dfrA17*, *sul3*, *sul2*, and *sul1* in WB218F1, WB221F2, and WB249F2 genomes.

No gene mutations usually linked to levofloxacin were identified, due to the absence of correlation in the ResFinder/PointFinder database (Mahfouz et al., 2020).

The possibility of AMR genes to be mobilised between strains is directly linked to their localisation on mobile genetic elements, i.e., plasmids and transposons. The genome of five strains was screened for plasmid replicons; moreover, this information was linked to the investigation of genome makeup around AMR genes in order to study the possibility of gene sharing through mobile elements.

The analysis with PlasmidFinder matched with the results of PCR (**Table 2**).

The replicon position at the contig level was compared with the position of ESBL determinants, assuming that, if AMR genes were carried by a plasmid, they would be found near the replicons. The analysis revealed that none of them were located on the same contig of plasmid replicons. In particular, the beta-lactamase genes are located on short contigs (**Figure 2**), preventing a complete view of the genomic makeup around them. Anyway, the annotation of these contigs was performed and evidenced that bla_{TEM-1B}, $bla_{CTX-M-1}$, and bla_{SHV-12} genes of WB218F1 strain were surrounded by IS6 and Tn3 family transposase (**Figure 2**), differently from bla_{OXA-1}, which was not enclosed by any transposable elements. The same situation was detected for other strains. $bla_{CTX-M-15}$ and bla_{TEM-1B} genes of WB221F2 strain were bracketed with Tn3-such as transposase and TnpA, as well as $bla_{CTX-M-15}$ of WB249F2; no mobile genetic elements were detected near $bla_{CTX-M-1}$ gene of WB231L2 and WB234F2 strains. Moreover, the Blastn search evidenced the presence of the transposon ISEcp1 near the $bla_{CTX-M-15}$ genes in WB231L2 and WB249F2.

TABLE 2 | Identification of plasmid replicons in the sequenced ESβL-producing *Escherichia coli* strains.

Strain	Plasmid replicon	Contig position	Contig length (bp)
WB218 F1	IncHI2	46	10,181
	IncHI2 2	42	22,264
	IncX3	37	35,167
WB221 F2	IncFIB	92	4,852
		12	104,785
WB231 L2	IncN	26	31,042
	IncR	32	15,115
WB234 F2	IncN	28	31,174
	IncR	33	11,243
	IncX1	25	45,110
WB249 F2	IncFIA	29	17,426
	IncFII	35	8,144

For each strain, the incompatibility group (plasmid replicon) is highlighted with the related number of contig on which the replicon was found and the length of the contig.

Phylogenetic Insight of Wild Boar ESβL-Producing *Escherichia coli* and the Correlation With ST131 and ST10 ESβL-Producing *Escherichia coli* Isolated From Different Sources

To understand how strains were genetically correlated, the pan-genome analysis was carried out. The phylogenetic relationship between wild boar strains is evidenced in **Figure 3**. All the strains shared 3,325 genes (45% of pangenome), which represent the core genome; the remaining non-core 4,085 genes were divided into 1,316 accessory genes (18% of pangenome) shared by 2–4 strains and 2,769 unique genes (cloud genes; 37% of pangenome). The phylogenetic tree built on SNPs of core genes divided strains into three groups, showing a high degree of heterogeneity inside and reflecting the difference in the genetic composition in terms of virulence, AMR profile, ST, and serogroup.

Then, wild boar isolates with ST131 and ST10 were considered for further investigation, with the aim to compare them with other strains with the same ST, isolated from different sources, namely, human, food, wild, and domestic animals. While the phylogenetic tree shows a clear clusterisation of analysed depending on the serotype (**Figure 4A**), including the wild boar ST131 strain in the O25:H4 group, it also reveals a wide distribution of genomes belonging to different sources and countries. The same situation is observed for the comparison among the ST10 strains (**Figure 4B**). Although for the ST10 genomes, a more heterogeneous situation was observed regarding the serogroups, the relationship between wild boar isolates and other strains seems not to follow a strict criterion. Interestingly, the two strains are located far from each other, placing WB231L2 near human and domestic animals, while the WB218F1 is near to food and wild animal isolates.

DISCUSSION

The epidemiology of ESβL-producing microorganisms is quite complicated, including geographical areas, hospitals, communities, hosts, as well as various reservoirs represented by the environment (soil and water), farmed animals, wild animals, and pets. Transmission from food and water, or from direct contact (person-to-person), represents the final step of ESβL-producing bacterial epidemiology (Carattoli, 2008).

The role of wild animals, including wild boars, as maintenance hosts of ESβL-producing *E. coli*, has already been assessed worldwide (Wasyl et al., 2018; Darwich et al., 2021;

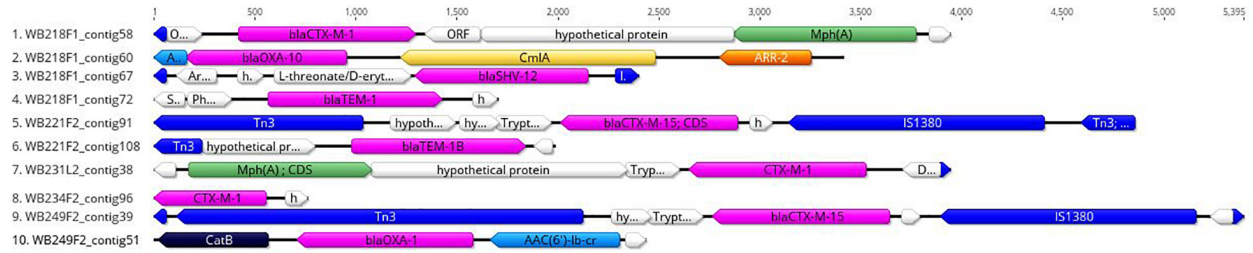


FIGURE 2 | Genomic context of ESβL genes in wild boar isolates. Each line represents a contig harbouring the ESβL determinants, and it is identified by the name of strain and number of the contig. bla_{CTX}, bla_{OXA}, bla_{TEM}, and bla_{SHV} genes are coloured in purple, while the mobile elements are evidenced in blue. Green, yellow, orange, and light blue indicate other AMR genes, while hypothetical or other proteins are white.

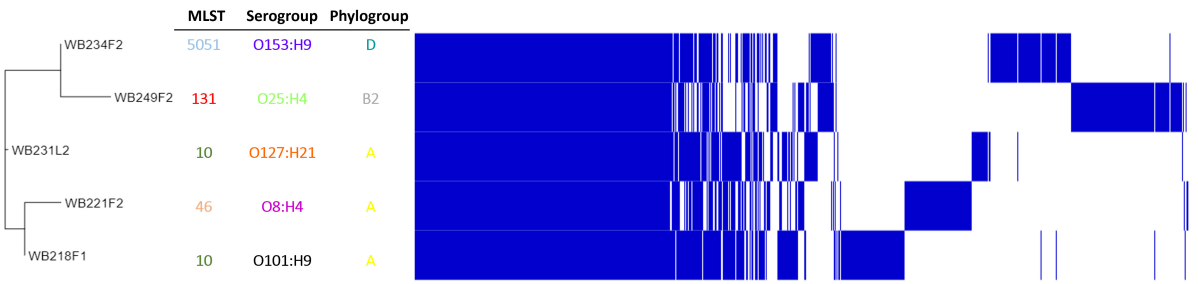


FIGURE 3 | Pangenome analysis of ESβL-producing *Escherichia coli* isolated from wild boars. The figure evidence, on the left, phylogenetic tree, built on SNPs of core genes. Identification of Sequence Type (MLST), serogroup, and phylogroup are also indicated.

Palmeira et al., 2021), with some phylogenetic groups showing clinical relevance (Cristóvão et al., 2017). The acquisition of ESβL-producing *E. coli* by wild boars has been proposed to be related to their proximity to human and other animals' habitats, as well as by their omnivorous habits which may give the animals the possibility to eat human food waste (Poeta et al., 2009), as well as carrions and vegetables contaminated by animal manure.

The major aim of our study was the genomic characterisation of EsβL-producing *E. coli* isolated from wild boars in terms of AMR, virulence, mobile genetic elements, and epidemiology (phylogroups and MLSTs). Since these animals were hunted in mountain areas with limited anthropic influence but can also move in a wider area, they can be considered as indicators of the environmental load of AMR. The comparison with similar studies performed in Northern Italy confirms the high prevalence of ESβL-producing *E. coli*, as 15.96% of ESβL/AmpC *E. coli*-positive wild boars hunted during 2017–2020 in the Lombardy region (Formenti et al., 2021). In our study, where the overall prevalence of ESβL-*E. coli* carriers reached 23.3%, the adults were more frequently positive (35.5%) if compared with the young (26.6%), and the subadults (0.0%), probably because they could move in wider territories and come in contact with different ESβL sources. While young wild boars (0–12 months) were highly exposed to the colonisation due to their impaired immune system, subadults (12–24 months) were probably more reactive in eliminating the microorganisms from the gut.

In this study, all the suspected ESβL-producing *E. coli* showed a MDR profile, with a high percentage (>60%) of fluoroquinolone non-susceptibility level. This represents a particular concern, as this antibiotic class is frequently employed to treat clinical infections. The ESβLs of CTX-M-type resulted in the most spread 3GC/4GC hydrolysing enzymes among the collected isolates. Although this fact is already established and reported in the literature, it is nevertheless interesting to note that the allelic variants identified included the hyper-represented and globally widespread $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$. We also found the $bla_{CTX-M-245}$ variant, neither described earlier in Italy nor in Europe. It was reported only once from a Serratia marcescens isolate collected from a blood sample of a neonate in Iraq (Sahan and Hamed, 2020). This finding suggests that human-animal proximity could also lead to the exchange and circulation of under-detected variants of resistance genes.

The transferability of some resistance plasmids, mainly those ESβL-harbouring, is a further cause for concern. In addition to the ESβL encoding genes, the isolates studied also showed fluoroquinolone and aminoglycoside resistance genes, as *qnrS*, *aac(60)Ibcr*, and *aadA* alone or in combination. Interestingly, the molecular typing showed very high heterogeneity in terms of clonal lineages; isolates collected from different sites (MLNs and faeces) of the same wild boar resulted not clonally related to each other.

Among the strains isolated from wild boars, five were selected in order to be deeper characterised from a genomic point of view. Interestingly, the *in silico* genome analysis highlighted a pandemic clone ST131 isolated from wild boar faeces, belonging to the O25:H4 serogroup and B2 phylogroup. This clone is recognised as a highly virulent group of ExPEC (extraintestinal pathogenic *E. coli*), which is responsible for urinary tract

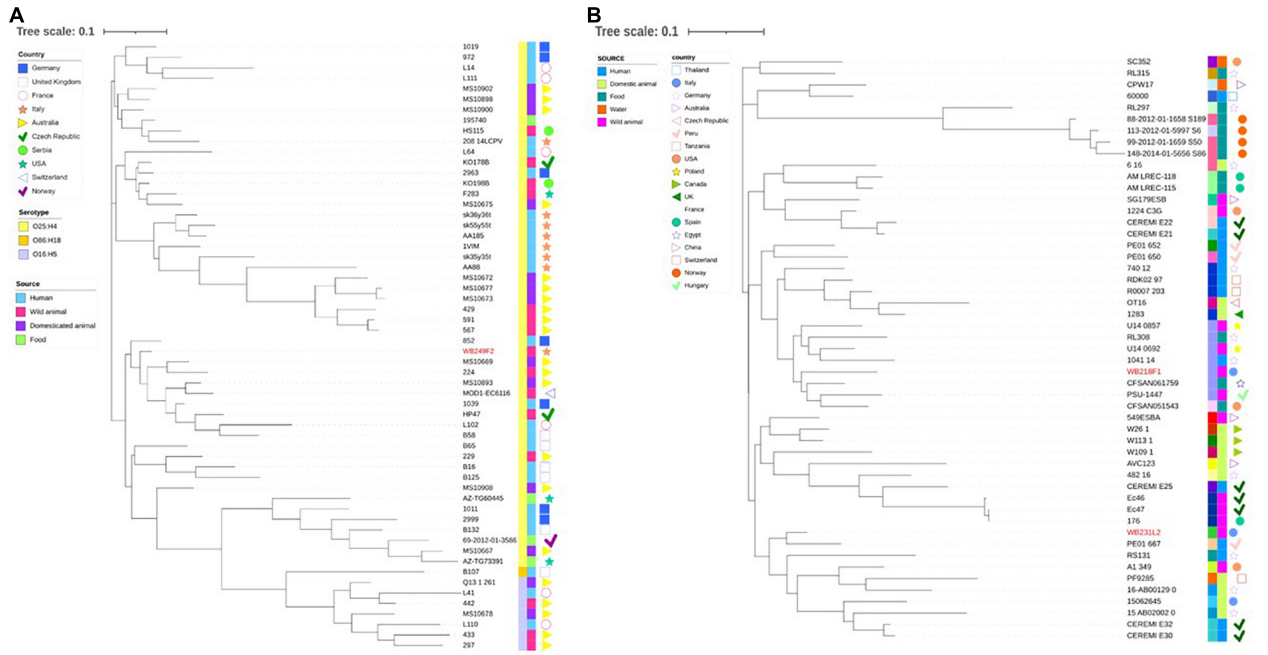


FIGURE 4 | Phylogenetic tree built on the SNPs of core genes in the ST131 **(A)** and ST10 **(B)** ESβL-producing *E. coli* strains analysed. **(A)** WB249F2, the ST131 strain isolated from wild boar, was compared with other 57 strains selected by isolation source and country, and **(B)** the same analysis was performed comparing WB218F1 and WB231L2 with other 49 strains, chosen following the same criteria. The wild boar isolates are highlighted in red in order to better evidence them. Colour-coding is used to report the serotype (first line) and isolation source (second line), while isolation countries are represented by different symbols.

infections, bacteraemia, urinary sepsis, and neonatal sepsis. This strain harbours genomic features typical of ESβLs ExPEC since it shows bla$_{CTX-M-15}$ gene, other than bla$_{OXA-1}$, and virulence pattern which includes genes responsible for improved adherence and ability to survive in the human body (Sarowska et al., 2019). Since the worldwide diffusion of ST131 clones in humans and also in wildlife (Costa et al., 2006; Wang et al., 2017; Sabença et al., 2021), it is not surprising to find this ST in wild boars (Alonso et al., 2017; Holtmann et al., 2021). This fact is supported by the phylogenetic analysis which places WB249F2 near human isolates and also near wild and domestic animals.

While the ST131 clone commonly shows a rich set of virulence genes (Johnson et al., 2010; Blanco et al., 2011), we found other STs with a less extensive virulence pattern. Notably, we isolated two ST10 strains, which are frequently responsible for AMR-resistant human infection (Day et al., 2016; Pietsch et al., 2017). The same ST was detected in wild boars in Germany (Holtmann et al., 2021), and it is known to be common, especially in poultry (Falgenhauer et al., 2018; Ramos et al., 2020). From the analysis of core genome SNPs, the correlation between ST10 wild boar strains and other strains isolated from different origins, especially human and food, from one side, and human and domestic animal from the other side, has been highlighted. These results confirm again the heterogeneity observed among the isolates of this study, suggesting that wild boars could be a carrier and spreader of any type of ESβL-producing *E. coli* clones.

The other STs detected seem not to be widespread. In fact, the ST46 was found rarely in water in Bangladesh (Rashid et al., 2015) and Chinese companion animals (Chen et al., 2019), while no other authors detected the ST5051 clone.

The capability of AMR genes to be shared with other bacteria in the same niche represents a major threat. This possibility is strictly connected to the genome organisation and genomic feature of *E. coli*, in particular with mobile genetic elements, namely, plasmids, transposons, and insertion sequences. With the aim to investigate if the AMR genes could be transferred to other strains, the genomes of five strains were screened. Overall, the ESβL genes were found to be surrounded by different types of transposons, despite the assembly procedure was not able to reconstruct in a definitive way the genomic context. Since the mobility of AMR can be driven not only by the entire plasmid but also by transposable elements, we cannot exclude the exchange of the AMR gene through this mechanism. Further analysis should be performed using long-read sequencing, as suggested by others (Arredondo-Alonso et al., 2017).

CONCLUSION

The main finding of our study is the detection of the ExPEC clone ST131 and the ESβL-MDR ST10 *E. coli* isolates in a restricted wild boar population living in Northern Italy, thus suggesting a circulation of human pathogenic *E. coli* strains among wildlife. Since the concept of One Health is based on the connection between humans, animals, plants, and environmental health, especially in geographically closed systems such as the

area of the study, we can assess that wild animals should be included in each AMR surveillance programme. Their role as indicators of the environmental load of AMR is especially evident in our study since all ESβL-producing *E. coli* strains showed an MDR phenotype, including resistance to highly important antimicrobials.

Given the importance of genome analyses to recognise the different *E. coli* clones and trace their occurrence in humans as well as in other species, we strongly suggest the WGS as the most effective tool to investigate ESβL-producing *E. coli*.

DATA AVAILABILITY STATEMENT

Sequencing data were submitted to the Sequence Read Archive (SRA) under the Project ID PRJNA759409.

AUTHOR CONTRIBUTIONS

SB collected samples and performed the strain isolation. AA, AP, AM, FM, and SB performed phenotypic and molecular analysis. CC and GM carried out the bioinformatic analysis. AP, CC, and SB wrote the manuscript. PC, RM, and SB supervised the experiment design and all the analysis and contributed to the manuscript revision. All authors have read and approved the final draft of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.838383/full#supplementary-material

REFERENCES

Alonso, C. A., González-Barrio, D., Ruiz-Fons, F., Ruiz-Ripa, L., and Torres, C. (2017). High frequency of B2 phylogroup among non-clonally related fecal *Escherichia coli* isolates from wild boars, including the lineage ST131. *FEMS Microbiol. Ecol.* 93, 1–7. doi: 10.1093/femsec/fix016

Arredondo-Alonso, S., Willems, R. J., van Schaik, W., and Schürch, A. C. (2017). On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data. *Microb. Genomics* 3:e000128. doi: 10.1099/mgen.0.000128

Atterby, C., Börjesson, S., Ny, S., Järhult, J. D., Byfors, S., and Bonnedahl, J. (2017). ESBL-producing *Escherichia coli* in Swedish gulls-A case of environmental pollution from humans? *PLoS One* 12:e0190380. doi: 10.1371/journal.pone.0190380

Baquero, F., Martínez, J.-L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265. doi: 10.1016/j.copbio.2008.05.006

Blanco, J., Mora, A., Mamani, R., López, C., Blanco, M., Dahbi, G., et al. (2011). National survey of *Escherichia coli* causing extraintestinal infections reveals the spread of drug-resistant clonal groups O25b:H4-B2-ST131, O15:H1-D-ST393 and CGA-D-ST69 with high virulence gene content in Spain. *J. Antimicrob. Chemother.* 66, 2011–2021. doi: 10.1093/jac/dkr235

Bonardi, S., Cabassi, C. S., Longhi, S., Pia, F., Corradi, M., Gilioli, S., et al. (2018). Detection of Extended- Spectrum Beta-Lactamase producing *Escherichia coli* from mesenteric lymph nodes of wild boars (*Sus scrofa*). *Ital. J. Food Saf.* 7, 213–216. doi: 10.4081/ijfs.2018.7707

Bondo, K. J., Pearl, D. L., Janecko, N., Boerlin, P., Reid-Smith, R. J., Parmley, J., et al. (2016). Epidemiology of antimicrobial resistance in *Escherichia coli* isolates from raccoons (*Procyon lotor*) and the environment on swine farms and conservation areas in Southern Ontario. *PLoS One* 11:e0165303. doi: 10.1371/journal.pone.0165303

Bonnedahl, J., and Järhult, J. D. (2014). Antibiotic resistance in wild birds. *Ups. J. Med. Sci.* 119, 113–116. doi: 10.3109/03009734.2014.905663

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., et al. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* 75, 3491–3500. doi: 10.1093/jac/dkaa345

Carattoli, A. (2008). Animal reservoirs for extended spectrum beta-lactamase producers. *Clin. Microbiol. Infect.* 14(Suppl. 1), 117–123. doi: 10.1111/j.1469-0691.2007.01851.x

Carattoli, A., and Hasman, H. (2020). PlasmidFinder and in silico pMLST: identification and typing of plasmid replicons in whole-genome sequencing (WGS). *Methods Mol. Biol.* 2075, 285–294. doi: 10.1007/978-1-4939-9877-7_20

Carroll, D., Wang, J., Fanning, S., and McMahon, B. J. (2015). Antimicrobial resistance in wildlife: implications for public health. *Zoonoses Public Health* 62, 534–542. doi: 10.1111/zph.12182

Chen, Y., Liu, Z., Zhang, Y., Zhang, Z., Lei, L., and Xia, Z. (2019). Increasing prevalence of ESBL-producing multidrug resistance *Escherichia coli* from diseased pets in Beijing, China from 2012 to 2017. *Front. Microbiol.* 10:2852. doi: 10.3389/fmicb.2019.02852

Clermont, O., Christenson, J. K., Denamur, E., and Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ. Microbiol. Rep.* 5, 58–65. doi: 10.1111/1758-2229.12019

Costa, D., Poeta, P., Sáenz, Y., Vinué, L., Rojo-Bezares, B., Jouini, A., et al. (2006). Detection of *Escherichia coli* harbouring extended-spectrum β-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. *J. Antimicrob. Chemother.* 58, 1311–1312. doi: 10.1093/jac/dkl415

Cristóvão, F., Alonso, C. A., Igrejas, G., Sousa, M., Silva, V., Pereira, J. E., et al. (2017). Clonal diversity of extended-spectrum beta-lactamase producing *Escherichia coli* isolates in fecal samples of wild animals. *FEMS Microbiol. Lett.* 364:fnx039. doi: 10.1093/femsle/fnx039

Darwich, L., Seminati, C., López-Olvera, J. R., Vidal, A., Aguirre, L., Cerdá, M., et al. (2021). Detection of beta-lactam-resistant *Escherichia coli* and toxigenic clostridioides difficile strains in wild boars foraging in an anthropization gradient. *Animals* 11:1585. doi: 10.3390/ani11061585

Day, M. J., Rodríguez, I., van Essen-Zandbergen, A., Dierikx, C., Kadlec, K., Schink, A. K., et al. (2016). Diversity of STs, plasmids and ESBL genes among *Escherichia coli* from humans, animals and food in Germany, the Netherlands and the UK. *J. Antimicrob. Chemother.* 71, 1178–1182. doi: 10.1093/jac/dkv485

European Food Safety Authority (EFSA) (2014). Evaluation of possible mitigation measures to prevent introduction and spread of African swine fever virus through wild boar. *EFSA J.* 12:3616. doi: 10.2903/j.efsa.2014.3616

Falgenhauer, L., Imirzalioglu, C., Oppong, K., Akenten, C. W., Hogan, B., Krumkamp, R., et al. (2018). Detection and characterization of ESBL-producing *Escherichia coli* from humans and poultry in Ghana. *Front. Microbiol.* 9:3358. doi: 10.3389/fmicb.2018.03358

Formenti, N., Calò, S., Parisio, G., Guarneri, F., Birbes, L., Pitozzi, A., et al. (2021). Esbl/ampc-producing *Escherichia coli* in wild boar: epidemiology and risk factors. *Animals* 11, 1–10. doi: 10.3390/ani11071855

Furness, L. E., Campbell, A., Zhang, L., Gaze, W. H., and McDonald, R. A. (2017). Wild small mammals as sentinels for the environmental transmission of antimicrobial resistance. *Environ. Res.* 154, 28–34. doi: 10.1016/j.envres.2016.12.014

Guenther, S., Ewers, C., and Wieler, L. H. (2011). Extended-spectrum beta-lactamases producing e. coli in wildlife, yet another form of environmental pollution? *Front. Microbiol.* 2:246. doi: 10.3389/fmicb.2011.00246

Holtmann, A. R., Meemken, D., Müller, A., Seinige, D., Büttner, K., Failing, K., et al. (2021). Wild boars carry extended-spectrum β-lactamase-and ampc-producing *Escherichia coli*. *Microorganisms* 9:367. doi: 10.3390/microorganisms9020367

Joensen, K. G., Tetzschner, A. M. M., Iguchi, A., Aarestrup, F. M., and Scheutz, F. (2015). Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *J. Clin. Microbiol.* 53, 2410–2426. doi: 10.1128/JCM.00008-15

Johnson, J. R., Johnston, B., Clabots, C., Kuskowski, M. A., and Castanheira, M. (2010). *Escherichia coli* sequence type ST131 as the major cause of serious multidrug-resistant *E. coli* infections in the United States. *Clin. Infect. Dis.* 51, 286–294. doi: 10.1086/653932

Karesh, W. B., Dobson, A., Lloyd-Smith, J. O., Lubroth, J., Dixon, M. A., Bennett, M., et al. (2012). Ecology of zoonoses: natural and unnatural histories. *Lancet* 380, 1936–1945. doi: 10.1016/S0140-6736(12)61678-X

Keuling, O., Baubet, E., Duscher, A., Ebert, C., Fischer, C., Monaco, A., et al. (2013). Mortality rates of wild boar *Sus scrofa* L. in central Europe. *Eur. J. Wildl. Res.* 59, 805–814. doi: 10.1007/s10344-013-0733-8

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 50, 1355–1361. doi: 10.1128/JCM.06094-11

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., et al. (2013). Antibiotic resistance-the need for global solutions. *Lancet. Infect. Dis.* 13, 1057–1098. doi: 10.1016/S1473-3099(13)70318-9

Mahfouz, N., Ferreira, I., Beisken, S., von Haeseler, A., and Posch, A. E. (2020). Large-scale assessment of antimicrobial resistance marker databases for genetic phenotype prediction: a systematic review. *J. Antimicrob. Chemother.* 75, 3099–3108. doi: 10.1093/jac/dkaa257

Malberg Tetzschner, A. M., Johnson, J. R., Johnston, B. D., Lund, O., and Scheutz, F. (2020). In Silico genotyping of *Escherichia coli* isolates for extraintestinal virulence genes by use of whole-genome sequencing data. *J. Clin. Microbiol.* 58, 1–13. doi: 10.1128/JCM.01269-20

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Palmeira, J. D., Cunha, M. V., Carvalho, J., Ferreira, H., Fonseca, C., and Torres, R. T. (2021). Emergence and spread of cephalosporinases in wildlife: a review. *Animals* 11:1765. doi: 10.3390/ani11061765

Pietsch, M., Eller, C., Wendt, C., Holfelder, M., Falgenhauer, L., Fruth, A., et al. (2017). Molecular characterisation of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates from hospital and ambulatory patients in Germany. *Vet. Microbiol.* 200, 130–137. doi: 10.1016/j.vetmic.2015.11.028

Pittiglio, C., Khomenko, S., and Beltran-Alcrudo, D. (2018). Wild boar mapping using population-density statistics: from polygons to high resolution raster maps. *PLoS One* 13:e0193295. doi: 10.1371/journal.pone.0193295

Poeta, P., Radhouani, H., Pinto, L., Martinho, A., Rego, V., Rodrigues, R., et al. (2009). Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. *J. Basic Microbiol.* 49, 584–588. doi: 10.1002/jobm.200900066

Ramos, S., Silva, V., Dapkevicius, M., de, L. E., Caniça, M., Tejedor-Junco, M. T., et al. (2020). *Escherichia coli* as commensal and pathogenic bacteria among food-producing animals: health implications of extended spectrum β-lactamase (ESBL) production. *Animals* 10:2239. doi: 10.3390/ani10122239

Rashid, M., Rakib, M. M., and Hasan, B. (2015). Antimicrobial-resistant and ESBL-producing *Escherichia coli* in different ecological niches in Bangladesh. *Infect. Ecol. Epidemiol.* 5:26712. doi: 10.3402/iee.v5.26712

Robinson, T. P., Bu, D. P., Carrique-Mas, J., Fèvre, E. M., Gilbert, M., Grace, D., et al. (2016). Antibiotic resistance is the quintessential One Health issue. *Trans. R. Soc. Trop. Med. Hyg.* 110, 377–380. doi: 10.1093/trstmh/trw048

Roer, L., Tchesnokova, V., Allesøe, R., Muradova, M., Chattopadhyay, S., Ahrenfeldt, J., et al. (2017). Development of a web tool for *Escherichia coli* subtyping based on fimH Alleles. *J. Clin. Microbiol.* 55, 2538–2543. doi: 10.1128/JCM.00737-17

Sabença, C., Igrejas, G., Poeta, P., Robin, F., Bonnet, R., and Beyrouthy, R. (2021). Multidrug resistance dissemination in *Escherichia coli* isolated from wild animals: bacterial clones and plasmid complicity. *Microbiol. Res.* 12, 123–137. doi: 10.3390/microbiolres12010009

Sahan, Z., and Hamed, S. (2020). Molecular detection of extended-spectrum -lactamases- producer *Serratia marcescens* causing neonatal sepsis in Iraq. *Int. J. Res. Pharmaceuical. Sci.* 11, 5803–5808. doi: 10.26452/ijrps.v11i4.3229

Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G., et al. (2019). Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. *Gut Pathog.* 11:10. doi: 10.1186/s13099-019-0290-0

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Siguier, P., Perochon, J., Lestrade, L., Mahillon, J., and Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res.* 34, 32–36. doi: 10.1093/nar/gkj014

Swift, B. M. C., Bennett, M., Waller, K., Dodd, C., Murray, A., Gomes, R. L., et al. (2019). Anthropogenic environmental drivers of antimicrobial resistance in wildlife. *Sci. Total Environ.* 649, 12–20. doi: 10.1016/j.scitotenv.2018.08.180

Tartor, Y. H., Gharieb, R. M. A., Abd El-Aziz, N. K., El Damaty, H. M., Enany, S., Khalifa, E., et al. (2021). Virulence determinants and plasmid-mediated colistin resistance mcr genes in gram-negative bacteria isolated from bovine milk. *Front. Cell Infect. Microbiol.* 11:761417. doi: 10.3389/fcimb.2021.761417

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., et al. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33, 2233–2239. doi: 10.1128/jcm.33.9.2233-2239.1995

van Bruggen, A. H. C., Goss, E. M., Havelaar, A., van Diepeningen, A. D., Finckh, M. R., and Morris, J. G. J. (2019). One Health - Cycling of diverse microbial communities as a connecting force for soil, plant, animal, human and ecosystem health. *Sci. Total Environ.* 664, 927–937. doi: 10.1016/j.scitotenv.2019.02.091

Wang, J., Ma, Z. B., Zeng, Z. L., Yang, X. W., Huang, Y., and Liu, J. H. (2017). The role of wildlife (wild birds) in the global transmission of antimicrobial resistance genes. *Zool. Res.* 38, 55–80. doi: 10.24272/j.issn.2095-8137.2017.003

Wasyl, D., Zajcac, M., Lalak, A., Skarżyńska, M., Samcik, I., Kwit, R., et al. (2018). Antimicrobial Resistance in *Escherichia coli* isolated from wild animals in Poland. *Microb. Drug Resist.* 24, 807–815. doi: 10.1089/mdr.2017.0148

Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., et al. (2017). Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. *Nucleic Acids Res.* 45, D535–D542. doi: 10.1093/nar/gkw1017

Yang, F., Mao, D., Zhou, H., and Luo, Y. (2016). Prevalence and fate of carbapenemase genes in a wastewater treatment plant in Northern China. *PLoS One* 11:e0156383. doi: 10.1371/journal.pone.0156383

Zendri, F., Maciuca, I. E., Moon, S., Jones, P. H., Wattret, A., Jenkins, R., et al. (2020). Occurrence of ESBL-Producing *Escherichia coli* ST131, Including the H30-Rx and C1-M27 Subclones, Among Urban Seagulls from the United Kingdom. *Microb. Drug Resist.* 26, 697–708. doi: 10.1089/mdr.2019.0351

Zhou, Z., Alikhan, N. F., Mohamed, K., Fan, Y., and Achtman, M. (2020). The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. *Genome Res.* 30, 138–152. doi: 10.1101/gr.251678.119

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 Mercato, Cortimiglia, Abualsha'ar, Piazza, Marchesini, Milani, Bonardi, Cocconcelli and Migliavacca. 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 Microbiology

REVIEW
published: 03 May 2022
doi: 10.3389/fmicb.2022.860436

A 6-Year Update on the Diversity of Methicillin-Resistant *Staphylococcus aureus* Clones in Africa: A Systematic Review

Opeyemi Uwangbaoje Lawal[1†], Olaniyi Ayobami[2], Alaa Abouelfetouh[3,4], Nadira Mourabit[5], Mamadou Kaba[6], Beverly Egyir[7], Shima M. Abdulgader[8] and Adebayo Osagie Shittu[9,10]*

[1] Laboratory of Bacterial Evolution and Molecular Epidemiology, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (ITQB-NOVA), Oeiras, Portugal, [2] Unit for Healthcare-Associated Infections, Surveillance of Antimicrobial Resistance and Consumption, Robert Koch Institute, Berlin, Germany, [3] Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt, [4] Department of Microbiology and Immunology, Faculty of Pharmacy, AlAlamein International University, Alalamein, Egypt, [5] Biotechnology, Environmental Technology and Valorisation of Bio-Resources Team, Department of Biology, Faculty of Sciences and Techniques of Al Hoceima, Abdelmalek Essaadi University, Tetouan, Morocco, [6] Division of Medical Microbiology, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [7] Department of Bacteriology, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Accra, Ghana, [8] Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, DSI-NRF Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Stellenbosch University, Cape Town, South Africa, [9] Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria, [10] Institute of Medical Microbiology, University Hospital Münster, Münster, Germany

OPEN ACCESS

Edited by:
Jean-Marc Rolain,
URMITE CNRS-IRD UMR 6236,
France

Reviewed by:
Grainne Brennan,
National MRSA Reference Laboratory,
St. James's Hospital, Ireland
Ben Pascoe,
University of Bath, United Kingdom

***Correspondence:**
Adebayo Osagie Shittu
bayo_shittu@yahoo.com

†Present address:
Opeyemi Uwangbaoje Lawal,
Department of Food Science,
Canadian Research Institute for Food Safety (CRIFS), University of Guelph,
Guelph, ON, Canada

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 22 January 2022
Accepted: 15 March 2022
Published: 03 May 2022

Citation:
Lawal OU, Ayobami O, Abouelfetouh A, Mourabit N, Kaba M, Egyir B, Abdulgader SM and Shittu AO (2022) A 6-Year Update on the Diversity of Methicillin-Resistant Staphylococcus aureus Clones in Africa: A Systematic Review. Front. Microbiol. 13:860436. doi: 10.3389/fmicb.2022.860436

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of hospital-associated (HA) and community-associated (CA) infections globally. The multi-drug resistant nature of this pathogen and its capacity to cause outbreaks in hospital and community settings highlight the need for effective interventions, including its surveillance for prevention and control. This study provides an update on the clonal distribution of MRSA in Africa.

Methods: A systematic review was conducted by screening for eligible English, French, and Arabic articles from November 2014 to December 2020, using six electronic databases (PubMed, EBSCOhost, Web of Science, Scopus, African Journals Online, and Google Scholar). Data were retrieved and analyzed according to the Preferred Reporting Items for Systematic Review and Meta-Analysis guidelines (registered at PROSPERO: CRD42021277238). Genotyping data was based primarily on multilocus sequence types (STs) and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) types. We utilized the Phyloviz algorithm in the cluster analysis and categorization of the MRSA STs into various clonal complexes (CCs).

Results: We identified 65 studies and 26 publications from 16 of 54 (30%) African countries that provided sufficient genotyping data. MRSA with diverse staphylococcal protein A (*spa*) and SCC*mec* types in CC5 and CC8 were reported across the continent. The ST5-IV [2B] and ST8-IV [2B] were dominant clones in Angola and the Democratic

Republic of Congo (DRC), respectively. Also, ST88-IV [2B] was widely distributed across the continent, particularly in three Portuguese-speaking countries (Angola, Cape Verde, and São Tomé and Príncipe). The ST80-IV [2B] was described in Algeria and Egypt, while the HA-ST239/ST241-III [3A] was only identified in Egypt, Ghana, Kenya, and South Africa. ST152-MRSA was documented in the DRC, Kenya, Nigeria, and South Africa. Panton–Valentine leukocidin (PVL)-positive MRSA was observed in several CCs across the continent. The median prevalence of PVL-positive MRSA was 33% (ranged from 0 to 77%; n = 15).

Conclusion: We observed an increase in the distribution of ST1, ST22, and ST152, but a decline of ST239/241 in Africa. Data on MRSA clones in Africa is still limited. There is a need to strengthen genomic surveillance capacity based on a "One-Health" strategy to prevent and control MRSA in Africa.

Keywords: MRSA – methicillin-resistant *Staphylococcus aureus*, clonal complex (CC), Panton–Valentine leukocidin (PVL), molecular typing, Africa

BACKGROUND

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the important antibiotic-resistant pathogens and a leading cause of hospital-associated (HA) and community-associated (CA) infections worldwide (Lee et al., 2018). Recently, the World Health Organization (WHO) included MRSA as one of the indicators for antimicrobial resistance in the Sustainable Development Goals connected to the health target 3.d (WHO, 2021). MRSA is a major burden in hospital-acquired neonatal infections in sub-Saharan Africa (Okomo et al., 2019). Vancomycin, a glycopeptide, is considered one of the last therapeutic agents for MRSA infections (McGuinness et al., 2017). However, MRSA isolates from clinical samples exhibiting reduced susceptibility to vancomycin have been documented in Africa (Fortuin-de Smidt et al., 2015; Zorgani et al., 2015; Eshetie et al., 2016; Bamigboye et al., 2018; ElSayed et al., 2018). In addition, *mecA*-positive (Lozano et al., 2016), *mecC*-positive MRSA (Dweba and Zishiri, 2019), and vancomycin-resistant (*vanA*, *vanB*-positive) MRSA (Al-Amery et al., 2019) have been identified in food animals on the African continent.

There are varying prevalence rates of MRSA reported in Africa (Wangai et al., 2019), and the epidemiological picture depicts diverse clonal types within regions and countries. We published a systematic review on the molecular epidemiology of MRSA in Africa (Abdulgader et al., 2015). It revealed that the pandemic MRSA clones: sequence type (ST) 5 and ST239/241 were dominant on the continent. However, some clones were limited to specific countries (e.g., ST612 in South Africa) or regions (ST80 in North Africa). Moreover, CA-MRSA (ST8 and ST88) were identified in clinical and non-clinical settings (Abdulgader et al., 2015). Africa is described as a Panton–Valentine leukocidin (PVL) endemic region (Schaumburg et al., 2014). Also, the 2015 review observed a PVL prevalence of 0.3–100% among MRSA identified from humans (carriage and infection) in Africa. Despite these findings, data is still limited, and there are knowledge gaps on the clonal nature of MRSA in Africa.

The epidemiology of MRSA is characterized by the occurrence and dissemination of new and emerging clones leading to constant changes globally (Turner et al., 2019). For instance, a steady increase of ST5 and ST93 as the predominant CA-MRSA clones have been described in Australia (Bloomfield et al., 2020), and ST59 has been replaced by ST239 in China (Li et al., 2018). Furthermore, a decline of ST5 and an increase in ST8 cases have been observed in the United States of America (See et al., 2020) and Canada (Guthrie et al., 2020). Since MRSA is a significant public health problem, understanding the changes in epidemiology through regular monitoring and surveillance is essential to minimize its healthcare and economic burden. Therefore, this review aimed to provide an update describing the clonal characteristics of MRSA in Africa.

METHODS

This systematic review is a 6-year update on the MRSA clonal diversity in Africa. We performed the systematic literature search and analysis according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Page et al., 2021). The study was registered in the PROSPERO database (CRD42021277238). Since this review focused on a narrative description of the eligible studies instead of effect sizes and other related quantitative outcomes, methodological features like sample size, study population, use of appropriate study design were not assessed. Therefore, we did not do a formal risk of bias scoring system.

Literature Search Approach

We used six electronic databases to identify and retrieve relevant information (PubMed, EBSCOhost, Web of Science, Scopus, African Journals Online, and Google Scholar). The search

included articles published in English, French, and Arabic from November 01, 2014, to December 31, 2020. The literature search date was selected to complement the data previously described (Abdulgader et al., 2015). The literature search was also complemented with Publish or perish literature and citation mining algorithm (Harzing, 2007).

Predefined search terms were used (**Supplementary Table 1**), first on a continent-wide basis and then for the 54 African countries. Article titles and abstracts were screened and reviewed independently by two authors (OL, AS), including full-text reviews on all eligible studies.

Identification of Eligible Studies

Studies were eligible on the condition that identification of MRSA was based primarily on the molecular detection of the methicillin resistance (*mecA*) gene (including *mecC*), and the investigations used at least one molecular tool to characterize the isolates. We also included global surveys that involved African countries. All duplicate articles were removed, and data only on phenotypic antibiotic susceptibility testing to identify methicillin-susceptible *Staphylococcus aureus* (MSSA) and *mecA* were excluded. Moreover, African studies that described isolates recovered from humans or animals not resident on the continent were excluded. Sufficient genotyping data was based primarily on multilocus sequence type (MLST) and the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing nomenclature as previously reported (Abdulgader et al., 2015). Also, we included additional data, e.g., staphylococcal protein A (*spa*) types and PVL status. The MRSA STs cluster analysis was performed and categorized into various clonal complexes (CCs) using Phyloviz version 2.0.[1]

Data Extraction and Analyses

We extracted the epidemiological and genotypic data of MRSA from the eligible articles using standardized forms. Publications that described a previously analyzed collection within the period under review were considered as a single study. We determined the PVL rate from eligible studies with a sample size of $\geq$30 MRSA isolates.

Cluster Analysis and Minimum Spanning Tree

The relationship between the MRSA STs described in this review with other common lineages reported worldwide was analyzed as previously described (Abdulgader et al., 2015). Briefly, we downloaded the allelic profiles of the African MRSA STs from the MLST website.[2] Furthermore, 236 randomly selected STs representing the diversity in the database and based on the differences in their allelic profiles were included (**Supplementary Table 2**). The minimum spanning tree was constructed with the goeBURST algorithm using the Phyloviz version 2.0 (see text footnote 1).

[1]http://www.phyloviz.net

[2]https://pubmlst.org/organisms/staphylococcus-aureus

RESULTS

Literature Search and Description of the Articles Included in the Review

The systematic search yielded 3367 articles (**Figure 1**). We screened 314 full-text articles after removing duplicate studies and assessing titles and abstracts. Overall, 65 studies were considered eligible for the qualitative analysis. The data from these studies were obtained from investigations conducted in 22 countries. Most of the single-center studies were from Egypt (n = 9), Nigeria (n = 9), South Africa (n = 8), Algeria (n = 6), and Ghana (n = 5) (**Table 1**). Multicentre studies were from six reports. They included four investigations in Portuguese-speaking African countries: Angola, Cape Verde, and São Tomé and Príncipe (Conceição et al., 2015a,b; Aires-de-Sousa et al., 2018; Rodrigues et al., 2018). Others were one study each from Cameroon and South Africa (Founou et al., 2019), Cote d'Ivoire and the Democratic Republic of Congo (DRC) (Schaumburg et al., 2015).

Identification of *S. aureus* in more than 50% (36/65) of the eligible studies was based on protein profiling (MALDI-TOF) or methods established on PCR detection of conserved (16S rRNA, *nuc*, *tuf*, *gltB*) genes, or the combination of both. The detection of antibiotic resistance and toxin/virulence genes were described only in 37% (24/65) and 83% (54/65) of the studies, respectively (**Table 1**). One study reported *mecC*-positive MRSA from animals (Dweba and Zishiri, 2019). While all the eligible studies characterized MRSA using at least one molecular typing technique, only 40% (26/65) from 16 African countries provided sufficient genotyping data (**Supplementary Table 3**). Furthermore, 12 studies performed whole-genome sequencing (WGS), of which eight carried out adequate analyses to infer MRSA clones (**Table 1**).

Source of Methicillin-Resistant *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* from the eligible studies was classified as either HA, CA, or livestock-associated (LA) based on their source of isolation as provided in the articles. Overall, 40% (26/65) of the studies were on HA-MRSA, while 18% (12/65) each were from the community and animal/livestock settings (**Table 1**). Additionally, 22% (14/65) of studies characterized MRSA from either two (HA-CA: n = 10; HA-LA: n = 2; CA-LA: n = 1) or all the study settings (HA-CA-LA: n = 1). We could not infer the source of isolates in one study.

High Clonal Diversity Among Methicillin-Resistant *Staphylococcus aureus* Isolates Reported in Africa

We observed a high genetic heterogeneity among MRSA in the 26 eligible studies that provided sufficient genotyping data. Based on MLST, they were classified into 39 STs, four of which were unassigned types (**Supplementary Table 3**). The MLST cluster analysis using Phyloviz based on the geoBURST algorithm revealed 15 CCs. They comprised mainly CC1, CC5, CC8, CC22,

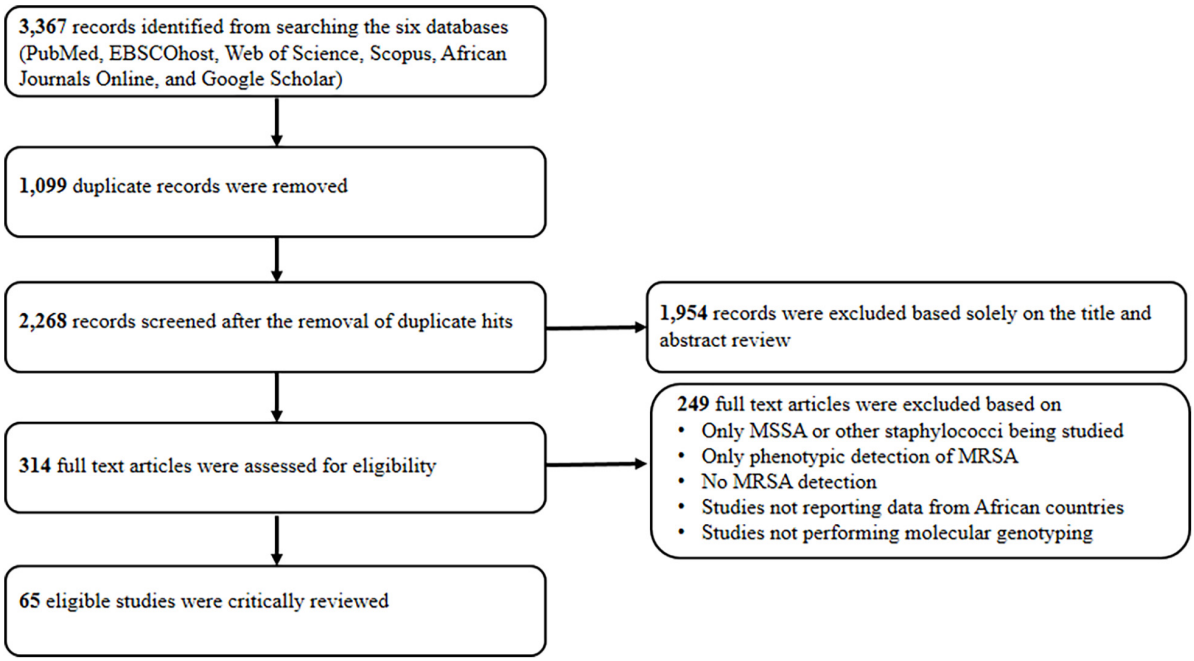


FIGURE 1 | Standard preferred reporting item for systematic reviews. MSSA, methicillin susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*.

CC30, and CC88. Others were CC7, CC15, CC20, CC45, CC80, CC97, CC121, CC152, and CC398 (**Figures 2**, **3**).

Clonal Complex 1

This clone was identified in six countries (**Figure 3**). PVL-positive t590-ST1-V [5C2] was documented from nasal samples both in hospitalized patients and health care workers (HCWs) in São Tomé and Príncipe (Conceição et al., 2015a,b). Another PVL-positive lineage: t657-ST772-V [5C2] (Bengal Bay Clone), was detected from human nasal samples in the community setting in Nigeria (Ogundipe et al., 2020). Moreover, PVL-negative t127-ST1-IV [2B] was described in a nasal sample of a non-hospitalized individual in Morocco (Mourabit et al., 2017), while it's variant (t127-ST1-V [5C2]) was identified from non-human specimens (milk products) in Uganda (Asiimwe et al., 2017b). ST1-V [5C2] and ST913-V [5C2] were recovered from clinical samples in Egypt (Soliman et al., 2020). In South Africa, t465-ST1-I [1B]/IV [2B] was isolated from patients with cystic fibrosis (CF) (Mahomed et al., 2018).

Clonal Complex 5

This lineage was reported in 10 countries (**Figure 3**). The PVL-negative t105-ST5-IV [2B] was the dominant lineage colonizing patients and HCWs (Conceição et al., 2015b; Rodrigues et al., 2018), as well as inanimate surfaces in Angola (Aires-de-Sousa et al., 2018). Also, it was detected in nasal samples of patients and HCWs in São Tomé and Príncipe (Conceição et al., 2015b), and a community patient admitted to a hospital in Algeria (Djoudi et al., 2014). In the DRC, three ST5-IV [2B] variants (t002-ST5-IV [2B], t105-ST5-IV [2B], and PVL-positive t311-ST5-IV [2B]) were described (Lebughe et al., 2017; Vandendriessche et al., 2017). In Kenya, t13150-ST5-II [2A] and t007-ST39-II [2A] were identified from clinical samples (Omuse et al., 2016; Kyany'a et al., 2019). ST5-VI [4B] was reported in a tertiary care hospital in Egypt (Soliman et al., 2020) and Cape Verde (Conceição et al., 2015b). ST5-VII [5C1] was recovered from a patient in the nephrology ward in Algeria (Djoudi et al., 2014). Other reports include ST5-III/V/non-typeable (NT) in South Africa (Abdulgader et al., 2020; Singh-Moodley et al., 2020). The related genotypes such as t6065-ST5/ST2629-V [5C2] in Angola (Conceição et al., 2015a,b), t6065-ST69-V [5C2] in Libya (Khemiri et al., 2017), and t002-ST105-II [2A] in São Tomé and Príncipe (Conceição et al., 2015b) were also noted. One study reported t002/t11469-ST5-V [5C2] in poultry birds (Nworie et al., 2017) in Nigeria (**Supplementary Table 3**).

Clonal Complex 8

ST8-IV [2B] (with diverse *spa* types) was documented in hospitalized patients and HCWs in Angola, Cape Verde, and São Tomé and Príncipe (Conceição et al., 2015a; Rodrigues et al., 2018), and clinical samples in Ghana (Egyir et al., 2015) and Kenya (Omuse et al., 2016). PVL-positive t121-ST8-IV [2B] was identified in Cape Verde (Conceição et al., 2015b), Ghana (Egyir et al., 2015), and São Tomé and Príncipe (Rodrigues et al., 2018). The t451-ST8-V [5C2] was one of the dominant clones among hospitalized patients and HCWs in São Tomé and Príncipe (Conceição et al., 2015a,b; Rodrigues et al., 2018). Also, ST8-V [5C2] was described in hospital settings in Angola (Conceição et al., 2015b; Aires-de-Sousa et al., 2018), Egypt (Soliman et al., 2020), Ghana (Egyir et al., 2015), and Kenya (Omuse et al., 2016).

The PVL-negative ST8-V/VII (largely t1476) was the major clone in the DRC (Lebughe et al., 2017; Vandendriessche et al., 2017), and Angola (Aires-de-Sousa et al., 2018). Two countries, i.e., Morocco (Mourabit et al., 2017) and Nigeria (Ogundipe et al., 2020), described ST8-V [5C2] with different *spa* types (t2231, t2658, and t12236) in non-clinical settings. The t456-ST8-I [1B] was only identified in South Africa (Mahomed et al., 2018). Furthermore, ST239/ST241-III [3A] was noted in hospital settings in Egypt (Soliman et al., 2020), Ghana (Egyir et al., 2015), Kenya (Omuse et al., 2016; Kyany'a et al., 2019), and South Africa (Abdulgader et al., 2020; Singh-Moodley et al., 2020). ST612-IV [2B], which comprised mainly *spa* type t1257, was a major clone in clinical (Singh-Moodley et al., 2020) and non-clinical settings (Amoako et al., 2019) in South Africa. Other related STs include ST72-V [5C2] in Angola (Conceição et al., 2015b; Aires-de-Sousa et al., 2018; Rodrigues et al., 2018) and ST4705-III [3A] in Kenya (Kyany'a et al., 2019).

Clonal Complex 22

ST22-MRSA was identified in six African countries. They include Angola (Conceição et al., 2015b), Algeria (Djoudi et al., 2014), Egypt (Soliman et al., 2020), Kenya (Omuse et al., 2016), and South Africa (Abdulgader et al., 2020; Singh-Moodley et al., 2020). Various *spa* types (t005, t012, t022, t032, t223, t6397, t11293, and t13149) were associated with this lineage that harbored the SCC*mec* IV element (**Supplementary Table 3**). Moreover, it was the major clone recovered from nasal samples of volunteers and outpatients in Tangier, Morocco (Mourabit et al., 2017). Most MRSA isolates from Algeria and Morocco possessed the gene encoding for toxic shock syndrome (*tst*).

Clonal Complex 30

This clone was observed in both human and animal samples. We identified seven *spa* types (t012, t018, t030, t037, t045, t064, and t6278; **Supplementary Table 3**). In South Africa, ST30-II [2A], ST36-II [2A], and ST36-III [3A] were identified from bacteremic patients (Abdulgader et al., 2020; Singh-Moodley et al., 2020), including ST30-I/IV [2B] from CF patients (Mahomed et al., 2018). ST30-V [5C2] was reported in different settings in Angola (Conceição et al., 2015a,b; Rodrigues et al., 2018), and from a chicken meat sample in Tunisia (Chairat et al., 2015). One isolate characterized as t018-ST36-II [2A] was described in Ghana (Egyir et al., 2015) and from the rinsate of processed animals in an abattoir in South Africa (Amoako et al., 2019). Furthermore, the genetically related ST535-IV [2B] was described in a patient in a nephrology ward in Algeria (Djoudi et al., 2014).

Clonal Complex 88

ST88-IV [2B] with diverse *spa* types (t186, t325, t335, t786, t1451, t1603, t1814, t3869, and t12827) was documented in eight studies from seven African countries (**Supplementary Table 3** and **Figure 3**), particularly in Portuguese-speaking nations. It was widely distributed in Angola (Conceição et al., 2015a,b; Aires-de-Sousa et al., 2018; Rodrigues et al., 2018), Cape Verde (Conceição et al., 2015b), and São Tomé and Príncipe (Conceição et al., 2015a,b; Aires-de-Sousa et al., 2018; Rodrigues et al., 2018). Other reports include the DRC (Lebughe et al., 2017; Vandendriessche et al., 2017), and Ghana (Egyir et al., 2015; Wolters et al., 2020). PVL-negative ST88-IV [2B] was recovered from nasal samples of both humans and pigs in Nigeria (Otalu et al., 2018), and ST88-V [5C2] was detected in a blood culture sample in the DRC (Vandendriessche et al., 2017). ST88-MRSA with a NT SCC*mec* was identified in Kenya (Omuse et al., 2016).

Other Clonal Complexes

These include eight clones that belonged to smaller (in number or limited spread across countries) groups (**Supplementary Table 3** and **Figure 3**). They consist of CC7 (ST789-IV [2B]/V [5C2]) (Egyir et al., 2015; Omuse et al., 2016; Ogundipe et al., 2020), CC15 (ST15-V [5C2], and ST1535-V [5C2]) (Nworie et al., 2017; Soliman et al., 2020), and CC20 (ST20-IV [2B]) (Mahomed et al., 2018). CC45 comprising ST45-I [1B], ST45-IV [2B], and ST508-I [1B] was detected in CF patients in South Africa (Mahomed et al., 2018). Also, ST508-V [5C2] associated with CC45 was described in Ghana (Egyir et al., 2015). PVL-positive CC80 (ST80-IV [2B]) was only described in Algeria (Djoudi et al., 2014; Agabou et al., 2017) and Egypt (Soliman et al., 2020). CC152 (mostly PVL-positive) with various *spa* types (t355, t715, t4960, t5691, and t15644) and SCC*mec* types (I, II, IV, V, and VII) were identified in four countries. They include the DRC (Lebughe et al., 2017; Vandendriessche et al., 2017), Kenya (Kyany'a et al., 2019), Nigeria (Ogundipe et al., 2020), and South Africa (Mahomed et al., 2018). ST121-V [5C2] was documented in Egypt (Soliman et al., 2020) and Uganda (Asiimwe et al., 2017b), in addition to PVL-positive isolates in Nigeria (Ogundipe et al., 2020). The LA ST398-IV [2B]/V [5C2] was recovered from the rectal and nasal samples of pigs in Cameroon, South Africa (Founou et al., 2019), and in the nasal sample of a healthy individual in Morocco (Mourabit et al., 2017). Also, ST398-IV [2B] was detected in raw meat samples in Tunisia (Chairat et al., 2015). MRSA with the genotype ST140-IV [2B] (associated with CC398) was recovered from inanimate surfaces in a health care institution in Angola (Aires-de-Sousa et al., 2018).

The Dynamics of Methicillin-Resistant *Staphylococcus aureus* Clones (2014–2020)

We compared MRSA clones reported from a previous study (Abdulgader et al., 2015) and the period under review. New genotyping data were available from Cape Verde, Ethiopia, the DRC, Libya, and Uganda. However, reports on MRSA clones from Senegal, Gabon, and Madagascar in the previous study were absent in the current period under review. Overall, genotyping data from 11 African countries (Angola, Algeria, Cameroon, Egypt, Ghana, Kenya, Tunisia, Morocco, Nigeria, São Tomé and Príncipe, and South Africa) in the two study periods were identified and compared (**Figure 3**). We observed an increase in the number of MRSA clones reported in seven (Angola, Egypt, Kenya, Morocco, Nigeria, São Tomé and Príncipe, and South Africa) of the 11 countries. Specifically, CC1, previously described only in Nigeria and Tunisia (Abdulgader et al., 2015), was identified in clinical and non-clinical settings in six countries

TABLE 1 | Summary of the characteristics of eligible articles on the molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) in Africa.

Country	Study period	Sample type	Host	No of *S. aureus* isolates	*Staphylococcus aureus* ID	No of MRSA	Settings	Molecular typing methods								Detection of genes		References
								coa	*agr*	*spa* typing	PFGE	SCC*mec*	MLST	WGS	PVL	*Virulence	Antibiotic resistance	
Algeria	2010–2012	Nasal swabs	Human	159	NR	9	HA	–	–	–	–	✓	✓	–	✓	✓	–	Djoudi et al., 2014
	2011–2012	NR	Human	NR	NR	99	NR	–	✓	✓	–	–	✓	–	–	✓	✓	Elhani et al., 2015
	2015–2016	Nasal swabs from animals	Camel, horses, sheep, monkeys, cattle	118	MALDI-TOF	6	LA	–	✓	–	–	✓	✓	–	✓	✓	✓	Agabou et al., 2017
	2014–2015	Diverse raw and processed food products	Food	153	MALDI-TOF	26	CA	–	–	–	–	✓	–	–	✓	✓	–	Chaalal et al., 2018
	2014–2015	Raw milk	Cows	69	23 rRNA gene PCR	11	CA	–	–	✓	✓	–	✓	–	✓	✓	✓	Titouche et al., 2019
	2017–2018	Dairy and meat samples	Animal	104	23 rRNA	5	CA	–	–	✓	–	–	✓	–	✓	✓	✓	Titouche et al., 2020
DR Congo	2013–2014	SSTI, UTI, ear-eye-nose-throat infection, blood	Human	186	NR	55	HA	–	✓	✓	–	✓	✓	✓	✓	✓	✓	Lebughe et al., 2017¶
	2009–2012	Blood samples	Human	108	NR	27	HA	–	–	✓	–	✓	✓	–	✓	✓	✓	Vandendriessche et al., 2017
Egypt	2010–2012	Human: pus, sputum, urine, cerebrospinal fluid, swabs, mastitic cow milk	Human, mastitic cow	133	*nuc* gene PCR	30	HA/LA	✓	–	✓	–	✓	–	–	✓	–	–	Abd El-Hamid and Bendary, 2015
	2011	Nasal swabs	Human	54	*nuc* gene PCR	33	CA	–	–	✓	–	✓	–	–	✓	–	–	Abou Shady et al., 2015
	2014	Diabetic foot, nasal discharge, boils, abscesses, sputum, urine, wounds, burns, vaginal smear	Human	136	NR	85	HA	✓	✓	–	–	✓	–	–	–	✓	–	El-baz et al., 2017
	2013	Nasal swabs of health care workers, hospital environmental surfaces	Human	112	16S rRNA gene PCR	34	HA	–	–	✓	–	✓	–	–	✓	✓	✓	Khairalla et al., 2017

(Continued)

TABLE 1 | (Continued)

Country	Study period	Sample type	Host	No of *S. aureus* isolates	*Staphylococcus aureus* ID	No of MRSA	Settings	Molecular typing methods								Detection of genes		References
								coa	*agr*	*spa* typing	PFGE	SCC*mec*	MLST	WGS	PVL	*Virulence	Antibiotic resistance	
	2016–2017	Human: pus, blood, cerebrospinal fluid, pericardial fluid, sputum, urine, swabs from human; Sheep and cow: pus, meat, and milk from mastitic animals	Human, sheep, and cows	65	16S rRNA and *nuc* gene PCR	65	HA [20] LA [22], CA [23]	✓	✓	✓	–	✓	✓	–	✓	✓	✓	Abd El-Hamid et al., 2019
	NR	Clinical and milk samples from mastitic cow	Cows	17	*nuc* gene PCR	5	LA	–	–	–	–	–	✓	–	–	–	–	Oreiby et al., 2019
	2014–2016	Blood, sputum, and pus	Human	NR	*nuc* gene PCR	120	HA [80], CA [40]	–	–	–	–	✓	–	–	✓	–	–	Shehata et al., 2019
	2017–2018	Milk from mastitic cow	Cows	42	MALDI-TOF	12	LA	–	✓	✓	–	–	–	–	✓	✓	✓	El-Ashker et al., 2020
	2017–2018	Diverse samples from ICU	Human	NR	NR	18	HA	–	–	–	–	✓	✓	✓	✓	✓	✓	Soliman et al., 2020¶
Ethiopia	2016–2017	Nasal swabs from workers and cow udder	Farm workers/ cows	70	*nuc* gene PCR	1	LA	–	–	✓	–	✓	–	–	–	–	–	Kalayu et al., 2020
	2014–2018	Blood, wound lesions	Human	80	MALDI-TOF, 16S rRNA PCR	1	HA	–	–	–	–	✓	✓	–	✓	✓	–	Verdú-Expósito et al., 2020
Gabon	2012–2013	Throat swabs, skin lesions	Human	103	NR	3	CA	–	–	–	–	✓	–	–	✓	✓	✓	Okuda et al., 2016
Ghana	2010–2013	Clinical samples, nasal swabs	Human	24	Microarray	24	HA, CA	✓	✓	✓	–	✓	✓	–	✓	–	–	Egyir et al., 2015
	2007–2012	Blood, sputum, and pus	Human	56	MALDI-TOF, *nuc* gene PCR	1	HA	–	–	✓	–	–	–	–	✓	–	–	Dekker et al., 2016
	2014–2015	Nasal swabs	Human	123	NR	2	HA	–	–	✓	–	–	✓	–	✓	✓	–	Eibach et al., 2017
	NR	Nasal swabs from cattle, pigs, goats, sheep, and handlers	Human/ animals	25	MALDI-TOF	2	CA	–	–	✓	–	–	✓	✓	✓	✓	✓	Egyir et al., 2020

(Continued)

TABLE 1 | (Continued)

Country	Study period	Sample type	Host	No of *S. aureus* isolates	*Staphylococcus aureus* ID	No of MRSA	Settings	Molecular typing methods								Detection of genes		References
								coa	*agr*	*spa* typing	PFGE	SCC*mec*	MLST	WGS	PVL	*Virulence	Antibiotic resistance	
	2016	Wound	Human	28	NR	8	HA	–	–	–	–	✓	✓	✓	✓	–	✓	Wolters et al., 2020¶
Kenya	NR	Nasal swabs, pus, blood, tracheal aspirate, axillary swab, ear swab, sputum, vulva swabs	Human	93	NR	32	HA	–	–	✓	✓	✓	✓	–	–	–	–	Omuse et al., 2016
	2015–2018	NR	Human	32	VITEK 2	8	HA	–	–	✓	–	✓	✓	✓	–	✓	✓	Kyany'a et al., 2019¶
Libya	2008, 2014	Swabs; nose, ears, wounds, throat; pus, sputum, urine	Human	NR	NR	95	HA/CA	–	–	✓	–	–	✓	–	✓	–	–	Ahmed et al., 2017
	2013	Wound	Human	NR	*nuc* gene PCR	32	HA	–	–	–	✓	✓	✓	–	✓	✓	✓	Khemiri et al., 2017
Madagascar	NR	Nasal swabs	Human	171	*nuc* gene PCR	20	HA [14], CA [6]	–	–	✓	–	–	–	–	✓	✓	–	Hogan et al., 2016
Morocco	2012–2013	Nasal swabs	Human	400	16S rRNA and *nuc* gene PCR	17	CA	–	–	✓	✓	✓	✓	–	✓	✓	–	Mourabit et al., 2017
Nigeria	2013	Clinical samples	Human	156	API 20	66	HA	–	–	–	–	✓	–	–	✓	✓	–	Alli et al., 2015
	2010–2011	Nasal swabs, wounds, vaginal discharge, blood, urine, sputum	Human	290	*nuc* gene PCR	7	HA [5], CA [2]	–	–	✓	–	–	–	–	✓	–	–	Ayepola et al., 2015
	NR	Cloacal samples from birds	Birds	247	Staph Latex Agglutination	15 (subsampled 8 MRSA isolates)	LA	–	–	✓	–	✓	✓	–	✓	–	✓	Nworie et al., 2017
	NR	Blood, urine, wound, sputum	Human	92	VITEK 2	12	HA	–	–	–	–	✓	–	–	✓	–	–	Enwuru et al., 2018
	NR	Nasal swabs from food animals and abattoir workers and environmental samples	Human and animals	109	MALDI-TOF and *tuf* gene PCR	18	LA	–	–	✓	–	–	–	–	–	–	–	Odetokun et al., 2018

(Continued)

TABLE 1 | (Continued)

Country	Study period	Sample type	Host	No of *S. aureus* isolates	*Staphylococcus aureus* ID	No of MRSA	Settings	Molecular typing methods								Detection of genes		References
								coa	*agr*	*spa* typing	PFGE	SCC*mec*	MLST	WGS	PVL	*Virulence	Antibiotic resistance	
	2013–2015	Nasal swabs	Pigs/human	NR	MALDI-TOF	38	LA [26], CA [12]	–	–	✓	–	✓	✓	✓	✓	✓	–	Otalu et al., 2018¶
	NR	Diverse samples from humans, animals, and animal products	Human, animals, and chicken in a poultry farm	61	MALDI-TOF	56 (subsampled 30 MRSA isolates)	LA	–	–	✓	–	✓	✓	✓	✓	✓	✓	Ogundipe et al., 2020¶
	NR	Intestine	Flies	275	*nuc* gene PCR, MALDI-TOF	4	CA	–	–	✓	–	–	✓	✓	✓	✓	✓	Onwugamba et al., 2020
	2015–2016	Fomites	Inanimate materials	14	*nuc* gene PCR, MALDI-TOF	3	CA	–	–	✓	–	–	✓	✓	✓	–	–	Shittu et al., 2020b
Rwanda	2013–2014	Clinical samples	Human	138	NR	39	HA	–	–	–	–	✓	–	–	–	–	–	Masaisa et al., 2018
South Africa	2010–2012	Clinical samples	Human	2709	*nuc* gene PCR	1160	HA	–	–	✓	–	✓	✓	–	–	–	–	Perovic et al., 2015
	2015	Nasal, blood, pus, central venous catheter, sputum, wound	Human	NR	VITEK and MALDI-TOF	27	HA	–	–	–	✓	–	–	–	✓	–	✓	Amoako et al., 2016
	2013–2016	Diverse clinical samples	Human	1914	VITEK 2	482	HA [449], CA [33]	–	–	✓	–	✓	✓	–	–	–	–	Perovic et al., 2017
	2013–2014	Sputum	Human	33	MALDI-TOF	17	HA	–	–	✓	✓	✓	✓	–	✓	✓	–	Mahomed et al., 2018
	NR	Nasal and hands swabs, litter, transport truck, carcass, cecal samples, retail point meats	Farm workers, animals, and slaughterhouse environment	145	API Staph kit	12	LA	–	–	✓	–	✓	✓	✓	–	–	✓	Amoako et al., 2019¶
	2013–2016	Blood samples	Human	2164	API Staph/MALDI-TOF	484	HA/CA	–	–	–	–	✓	–	–	✓	–	–	Singh-Moodley et al., 2019
	2015–2017	Blood	Human	199	VITEK 2	54	HA	–	✓	✓	–	✓	✓	–	–	–	–	Abdulgader et al., 2020
	2010–2017	Blood culture	Human	5820	VITEK/MALDI-TOF and *nuc* gene PCR	2019 (subsampled 48 MRSA isolates)	HA/CA	–	–	✓	–	✓	✓	–	–	–	–	Singh-Moodley et al., 2020
Tanzania	2013–2015	Clinical samples	Human	30	NR	10	HA	–	–	–	–	–	✓	✓	✓	✓	✓	Kumburu et al., 2018
	2015	Raw milk	Raw milk	48	*gltB* gene PCR	3	CA	–	–	✓	–	–	–	–	–	–	–	Mohammed et al., 2018

(Continued)

TABLE 1 | (Continued)

Country	Study period	Sample type	Host	No of *S. aureus* isolates	*Staphylococcus aureus* ID	No of MRSA	Settings	Molecular typing methods								Detection of genes		References
								coa	*agr*	*spa* typing	PFGE	SCC*mec*	MLST	WGS	PVL	*Virulence	Antibiotic resistance	
	2014–2015	Nasal swab, wound swab	Human	158	VITEK	10	HA, CA	–	–	✓	–	–	✓	–	✓	–	–	Moremi et al., 2019
Tunisia		Raw meat	Chicken	43	*nuc* gene PCR	2	LA	–	–	✓	✓	✓	✓	–	✓	✓	✓	Chairat et al., 2015
	2013–2014	Milk from mastitic cow	Cows	15	*nuc* gene PCR	3	LA	–	–	✓	–	✓	✓	–	–	✓	✓	Klibi et al., 2018
	2008–2009	Device-related infection, pus, blood, biological fluid	Human	87	NR	32	HA	–	✓	–	✓	✓	–	–	✓	✓	–	Mesrati et al., 2018
Uganda	2013	Animals	Milk samples	41	NR	23	LA	–	–	✓	✓	✓	–	–	✓	✓	–	Asiimwe et al., 2017b
	2013	Nasal swabs	Human	73	NR	48	CA	–	–	✓	✓	✓	–	–	✓	–	–	Asiimwe et al., 2017a
	2011	Nasopharyngeal samples	Children <5 years	144	NR	45	CA	–	–	✓	–	✓	–	–	✓	–	–	Kateete et al., 2019a,b
Zambia	2009–2012	Pus, blood	Human	NR	NR	32	HA	–	–	✓	–	✓	–	–	✓	–	–	Samutela et al., 2017
Multicentre studies																		
Angola, Sao Tome and Principe and Cape Verde	2010–2014	Diverse clinical samples and nasal swabs from health care workers and healthy individual	Human	454	*nuc* gene PCR	162	HA	–	–	✓	✓	✓	✓	–	✓	✓	–	Conceição et al., 2015b
Angola-Sao Tome Principe	2010–2014	Nasal swabs	Human	164	NR	29	HA	–	–	✓	✓	✓	✓	–	✓	✓	–	Conceição et al., 2015a
	2017	Hospital surfaces	Environmental samples	23	NR	16	HA	–	–	✓	✓	✓	✓	–	✓	–	–	Aires-de-Sousa et al., 2018
	2017	Nasal swabs	Human	110	*nuc* gene PCR	33	HA/CA	–	–	✓	✓	✓	✓	–	✓		–	Rodrigues et al., 2018
Cameroon-South Africa	2016	Nasal and rectal swabs and hand swabs from human	Pigs/human	NR	VITEK 2	5	LA	–	–	✓	–	✓	✓	✓	✓	✓	✓	Founou et al., 2019¶
DR Congo-Cote d'Ivoire	2010–2013	Nares swabs	Human and animals	495	*nuc* gene PCR	19	HA/LA	–	–	✓	–	–	✓	–	✓	✓	–	Schaumburg et al., 2015

*NR, not reported; HA, hospital-associated; CA, community-associated; LA, livestock-associated; –, not determined; *, other toxin/virulence associated genes; ¶, studies that provided sufficient genotyping data based on whole genome sequencing (WGS).*

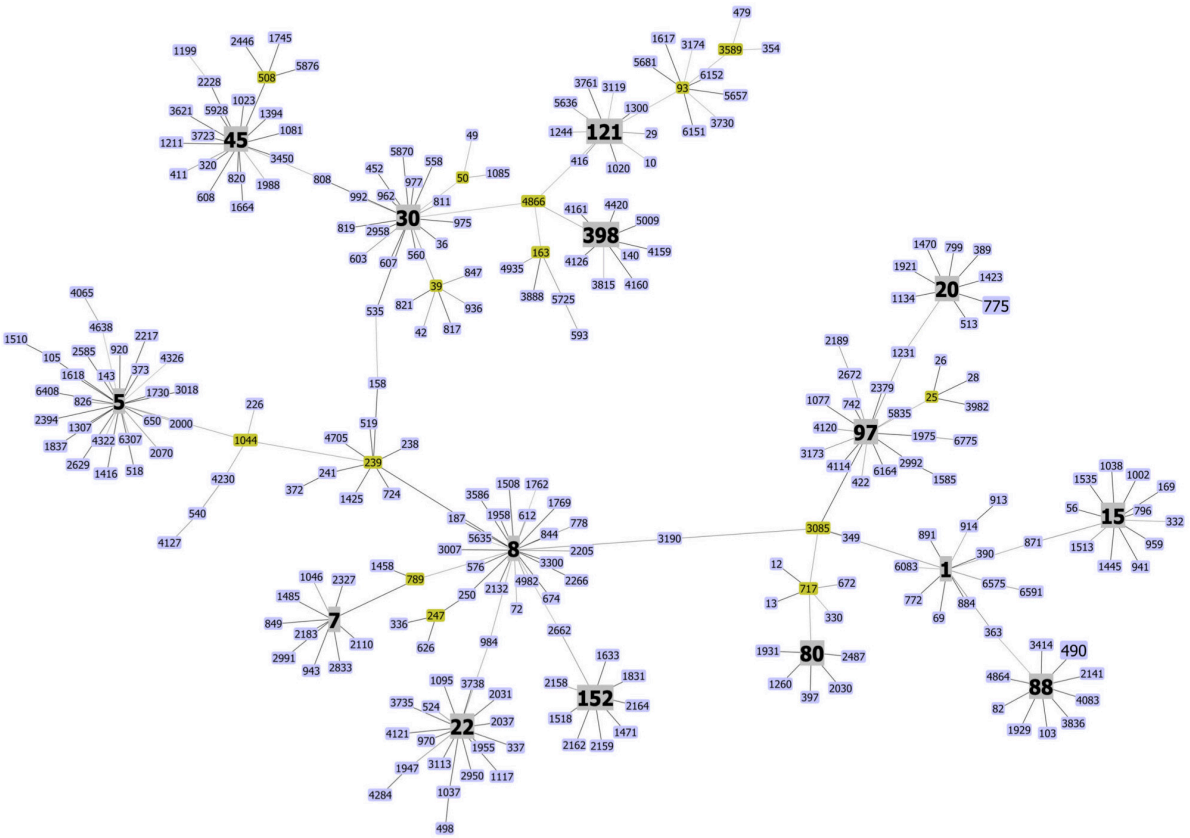


FIGURE 2 | Clonal diversity of methicillin-resistant *Staphylococcus aureus* in Africa. The minimum spanning tree was constructed with Phyloviz software version 2.0 hosted on http://www.phyloviz.net. The allelic profiles were obtained from the MLST database hosted on (https://pubmlst.org/organisms/staphylococcus-aureus) that included the sequence types of the MRSA described in this review, and 236 randomly selected STs based on the differences in their allelic profiles and representative of the MRSA diversity worldwide. Each node depicts an ST, and nodes centrally located and bearing different colors correspond to a group founder or sub-founder. Clonal complexes (CCs) reported in this study are colored in gray.

(Egypt, Morocco, Nigeria, São Tomé and Príncipe, South Africa, and Uganda). ST22-IV [2B] (CC22), previously documented only in South Africa (Abdulgader et al., 2015), was described in Angola, Algeria, Egypt, Kenya, Morocco, and South Africa. Also, CC152-MRSA identified only in Nigeria (Abdulgader et al., 2015) was reported in the DRC, Kenya, Nigeria, and South Africa. In contrast, the HA Brazilian/Hungarian clone (ST239/241-III [3A]), which was previously described as a major clone on the continent, was noted only in four countries (Egypt, Ghana, Kenya, and South Africa). Some MRSA clones were still limited to specific countries and regions. ST80-IV [2B] (CC80) and ST612-IV [2B] (CC8) were identified only in North African countries and South Africa, respectively. Overall, CC5-MRSA, CC8-MRSA, and CC88-MRSA remained widely distributed across the continent (**Figure 3**).

Panton–Valentine Leukocidin-Positive Methicillin-Resistant *Staphylococcus aureus* and Clonal Population in Africa

Methicillin-resistant *Staphylococcus aureus* carriage of the PVL gene was investigated in 50 of the 65 eligible studies. PVL-positive isolates were reported in 26 studies in 11 countries (**Table 1** and **Supplementary Table 3**). The lineages and countries were: CC1 (Egypt, São Tomé and Príncipe, and Nigeria), CC5 (Algeria, Angola, and DRC), CC8 (Cape Verde, DRC, Ghana, São Tomé and Príncipe, and South Africa), CC22 (Angola), and CC30 (Angola and South Africa). Others are CC80 (Algeria and Egypt), CC121 (Nigeria, Egypt, and Uganda), CC152 (DRC and Nigeria), and CC398 (Cameroon). The prevalence of PVL-positive MRSA ranged from 0% (Otalu et al., 2018) to 77% (23/30) (Ogundipe et al., 2020), with a median of 33% (**Table 2**).

DISCUSSION

This systematic review provided an update on the diversity of MRSA clones in Africa for the past 6 years (2014–2020). We observed a slight increase in the number of studies and countries that provided sufficient genotyping data. Diverse MRSA clones were distributed across human, environmental, and animal settings. CC5, CC8, and CC88 were the major clones identified in Africa. Various *spa* types and SCC*mec* elements characterized CC5-MRSA. It was postulated that the African ST5-MRSA

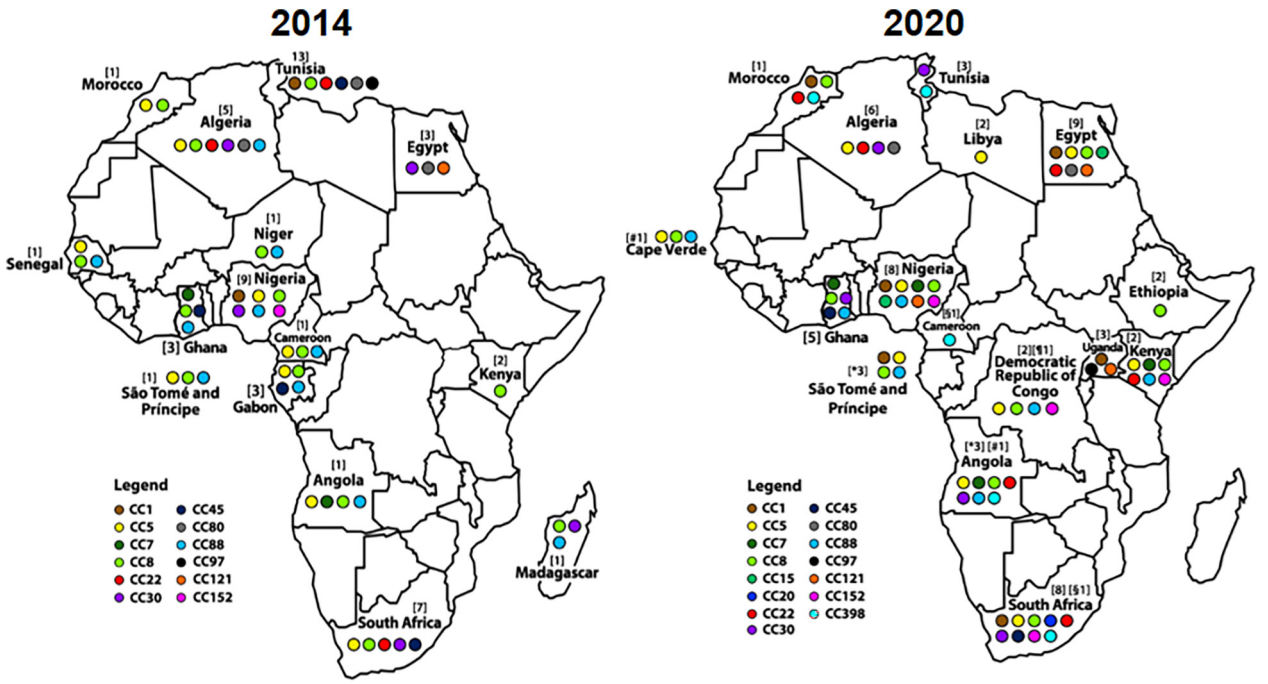


FIGURE 3 | Distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) clones in Africa as reported (Abdulgader et al., 2015) and this current study (2020). Each clonal complex (CC) is represented with colored oval shape and eligible studies carried out in each country is indicated in parenthesis. The symbols (#, *, §, or ¶) on the right side (2020) depict data that were extracted from the same multicentre study.

evolved from ST5-MSSA through acquiring the SCC*mec* element (Schaumburg et al., 2014). Its capacity and higher propensity to acquire various SCC*mec* elements could play a significant role in its increased dissemination and adaptation to different environments in Africa. However, the phylogeny, origin, and features for the spread of CC5-MRSA remain unclear in Africa.

Five SCC*mec* types and 11 *spa* types were associated with CC8-MRSA suggesting its high diversity in Africa. The CC8 is comprised of the hospital (Archaic [ST250], Iberian [ST247], and Brazilian/Hungarian/EMRSA-1 [ST239]) and CA (USA300 [t008-ST8], USA500 [t064-ST8]) clones (Bowers et al., 2018). ST239 was described as a major clone on the continent (Abdulgader et al., 2015) but has declined in the current period under review. It was identified only in four countries. ST239-MRSA evolved from recombination events between ST8 and ST30, in addition to the acquisition of antibiotic resistance and virulence determinants that contribute to its pathogenic capabilities (Robinson and Enright, 2004; Gill et al., 2021). However, this clone's low competitive potential relative to ST8 and ST30 could contribute to its gradual decline in different continents (Dai et al., 2019; Gill et al., 2021).

USA300 isolates harbor the SCC*mec* type IVa element, PVL-positive, with the arginine catabolic mobile element (ACME). These factors are lacking in USA500 isolates. A phylogenomic study provided some insights on the origin and the features for the spread of ST8-MRSA in Africa (Strauß et al., 2017). First, the heterogeneity of SCC*mec* types suggests the different introduction of these genetic elements to the ST8 genetic background. Secondly, African USA300 isolates formed a monophyletic group within the North American Epidemic (NAE) USA300 clade. This observation suggests a single introduction episode of this clone to the African continent followed by an extensive spread in the population (Strauß et al., 2017). However, it should be noted that the African USA300 isolates analyzed in the investigation were PVL-positive, unlike most of the MRSA (PVL-negative) identified in our study (**Supplementary Table 3**). Also, a phylogenetic analysis of t1476-ST8-IV-MRSA isolates (PVL, ACME-negative) from HIV-infected patients in Tanzania (Manyahi et al., 2021) revealed that they were unrelated to NAE USA300 and the African USA300 previously described in Gabon and East Africa. We hypothesize that t1476-ST8-MRSA from Tanzania, Angola, DRC, and Kenya (**Supplementary Table 3**) may have acquired different SCC*mec* elements despite sharing common genetic characteristics. Further studies are needed to unravel the origin and nature of CC8-MRSA in Africa.

CC88-MRSA is regarded as an "African" clone due to its wide distribution in West, Central, and East Africa (Schaumburg et al., 2014). It is noteworthy that CC88-MRSA was widely distributed in Portuguese-speaking African countries (Angola, Cape Verde, and São Tomé and Príncipe). The reasons for this observation are unclear. However, we postulate that demographic and cultural relationships could play a significant role in establishing this clone in these African countries. We observed an expansion of CC1-MRSA, CC22-MRSA, and CC152-MRSA in Africa. Unlike the European CC1-MRSA, which is mainly t127-ST1-IV [2A],

TABLE 2 | Prevalence of Panton–Valentine leukocidin (PVL) gene reported in eligible studies with ≥30 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.

Country	No of MRSA	No of PVL-positive MRSA	% Prevalence	References
Angola	127	2	2	Conceição et al., 2015b
DR Congo	55	5	9	Lebughe et al., 2017
	27	2	7	Vandendriessche et al., 2017
	30	22	73	Abd El-Hamid and Bendary, 2015
	34	5	15	Khairalla et al., 2017
	65	30	46	Abd El-Hamid et al., 2019
	120	40	33	Shehata et al., 2019
Libya	95	32	34	Ahmed et al., 2017
Nigeria	66	6	9	Alli et al., 2015
	38	0	0	Otalu et al., 2018
	30	23	77	Ogundipe et al., 2020
South Africa	484 (subsampled 108 MRSA isolates)	27	25	Singh-Moodley et al., 2019
Uganda	48	25	52	Asiimwe et al., 2017a
	45	19	42	Kateete et al., 2019a
Zambia	32	3	9	Samutela et al., 2017

the African CC1-MRSA (identified in six countries) comprised *spa* types t127, t465, and t590, and most of them harbored the SCC*mec* V element. ST22-IV [2B] (CC22), which is tagged epidemic MRSA-15 (EMRSA-15), was previously documented only in South Africa (Abdulgader et al., 2015), but now in six African countries. The CC152 lineage is a successful MSSA clone in Africa that is mainly PVL-positive. CC152-MRSA was previously noted in Nigeria (Abdulgader et al., 2015), but it is now described in four countries. The increasing trend of CC152-MRSA with diverse *spa* types and SCC*mec* elements in Africa is also noteworthy. This observation supports the evidence of multiple introductions among MSSA isolates in sub-Saharan Africa as the basis for the evolution of this clone (Baig et al., 2020). Recent studies have also reported CC152-MRSA from humans (Egyir et al., 2020, 2021) and animals (Shittu et al., 2021), including fomites (Shittu et al., 2020b) in Africa. The emergence of PVL-positive CC152-MRSA is of public health concern. Hence, there is a need to understand the dynamics for introducing and acquiring the *mecA* gene by CC152-MSSA isolates in Africa.

ST80-IV [2B] (CC80) was limited to North African countries and ST612-IV [2B] (CC8) in South Africa, as described previously (Abdulgader et al., 2015). However, MRSA in various STs (ST80, ST728, ST1931, ST2030, ST3247, and ST5440) assigned to CC80 was recently described in environmental samples associated with livestock in South Africa (Ramaite et al., 2021). ST612-IV [2B] has been detected in wound patients in Tanzania (Moremi et al., 2019). Also, it has been described in a poultry farm and workers in South Africa, raising concerns about its spread across the poultry food chain (Amoako et al., 2019). There is still a paucity of data on the molecular epidemiology of MRSA in animals in Africa. Hence, their role in the dissemination of MRSA remains unclear. Nonetheless, we observed diverse clones (ST1, ST5, ST8, ST36, and ST88) with various SCC*mec* types associated with the hospital and community settings recovered from livestock and their surroundings. Our findings suggest human to animal transmission and adaptation in poultry and food animals, which warrants further investigations. These observations somewhat indicate the changing epidemiological landscape and highlight the need for a "One-Health" approach to understanding MRSA epidemiology in Africa.

Panton–Valentine leukocidin is a pore-forming protein consisting of two sub-units (lukF-PV, lukS-PV) that target human granulocytes, monocytes, and macrophages (Holzinger et al., 2012). It is mainly associated with skin and soft tissue infection (SSTI) (Friesen et al., 2020), and in particular, pyomyositis in developing countries (Shittu et al., 2020a). This study identified PVL-positive MRSA from nine CCs in 10 countries. Africa is regarded as a PVL-endemic region (Schaumburg et al., 2015). The high prevalence (median: 33%) of PVL-positive MRSA, particularly among nasal samples of hospitalized patients and non-hospitalized individuals in Africa (**Supplementary Table 3**), is of public health concern. Recurrent SSTIs are associated with *S. aureus* carriers colonized with PVL-positive *S. aureus* (Rentinck et al., 2021). So far, the burden of PVL-positive *S. aureus* is not well known despite its high prevalence in Africa. Knowledge on factors that contribute to the high prevalence of PVL in Africa could help unravel the pathogenic role of PVL and develop strategies against PVL-related diseases.

Genomic epidemiology is a powerful tool to provide valuable information on the emergence of high-risk pandemic clones, antibiotic resistance mechanisms, and virulence determinants (Baker et al., 2018). The characterization of MRSA using conventional molecular typing techniques (e.g., *spa* typing, MLST) describes only a fraction of the entire *S. aureus* genome (Price et al., 2013). WGS offers a better opportunity to expand our knowledge about clinical and epidemiologic aspects of MRSA infection and colonization, including transmission patterns, evolution, and guide on appropriate interventions (Humphreys and Coleman, 2019). Our data showed that 12 of the 26 studies utilized WGS. However, eight provided sufficient genotyping data. Understanding the epidemiology of MRSA based on WGS is still in its infancy in Africa. Nonetheless, international scientific cooperation efforts support genomic sequencing capacity building on the continent, e.g., the Fleming Fund, SEQAFRICA. It is expected that these initiatives will provide quality genotyping data that will assist in MRSA surveillance in Africa. However, these efforts will require complementary local investment to ensure quality and representative genotyping data and sustainability.

In August 2017, two independent consortia converged to form the StaphNet Africa. This consortium was co-convened by the corresponding author and Dr. Beverly Egyir (Ghana).

The first kick-off meeting took place at the Noguchi Memorial Institute for Medical Research, University of Ghana. The conference, sponsored by the Wellcome Trust-Cambridge Centre for Global Health Research, brought together biomedical scientists and physicians with a research focus on *S. aureus* from 10 African countries (Nigeria, Ghana, Egypt, Gabon, Kenya, Mozambique, South Africa, Uganda, Kenya, and the Gambia), and the United Kingdom. Although the network's activities have been hampered by funding, one of its resolutions was to provide regular updates on the epidemiology of *S. aureus* in Africa. This systematic review is an affirmation of this resolution. Also, an African version of the biennial International Symposium on Staphylococci and Staphylococcal Infections (ISSSI), known as the African Symposium on Staphylococci and Staphylococcal Infections (ASSSI), was adopted for implementation. The symposium is to provide a platform for researchers to network and share current research work on *S. aureus* in Africa. We anticipate that this initiative, with others, will provide periodic data on MRSA surveillance in Africa.

CONCLUSION

We have provided an update on the clonal diversity of MRSA in Africa in the past 6 years. Nonetheless, there is still a paucity of data as sufficient genotyping data were available in only 16 of 54 (30%) countries. This systematic review did not investigate antibiotic resistance and virulence gene repertoire of the various African MRSA clones and their level of transmissibility. The origin and features underlying the spread of MRSA clones in Africa are not clear. Identifying human-associated lineages in food animals and products provides evidence to adopt a "One-Health" approach to understand the epidemiology of MRSA in Africa. There is a need to develop robust local capacity in genotyping, including WGS technologies, to determine the genetic factors that contribute to the evolution and adaptation of various African MRSA clones. Lastly, an active continent-wide antimicrobial resistance surveillance program and data exchange across One-Health sectors and professionals are required to monitor the clonal dissemination and emergence of new MRSA clones in Africa.

AUTHOR CONTRIBUTIONS

AS, BE, SA, and AA initiated the update on the diversity of MRSA in Africa. OL retrieved the data from the various databases and was jointly reviewed by AS. AS and OL wrote the initial manuscript. All authors reviewed and agreed on the final version of the manuscript before submission for peer review.

FUNDING

This study received support from the Deutsche Forschungsgemeinschaft (SCHA 1994/5-1), the Alexander von Humboldt Foundation ("Georg Forster-Forschungsstipendium"), and the Westfälische Wilhelms-Universität Münster Fellowship granted to AS.

ACKNOWLEDGMENTS

We appreciate the kind assistance of Akintunde Sallam.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.860436/full#supplementary-material

Supplementary Table 1 | Search strings used to identify eligible studies available in six electronic databases.

Supplementary Table 2 | Sequence types and corresponding allele profiles used for clustering analysis.

Supplementary Table 3 | Summary of the methicillin-resistant *Staphylococcus aureus* (MRSA) clones reported in 26 eligible studies.

REFERENCES

Abd El-Hamid, M., and Bendary, M. (2015). Comparative phenotypic and genotypic discrimination of methicillin resistant and susceptible *Staphylococcus aureus* in Egypt. *Cell. Mol. Biol.* 61, 101–112. doi: 10.14715/cmb/2015.61.4.17

Abd El-Hamid, M. I. A., Bendary, M. M., Merwad, A. M. A., Elsohaby, I., Ghaith, D. M., and Alshareef, W. A. (2019). What is behind phylogenetic analysis of hospital-, community- and livestock-associated methicillin-resistant *Staphylococcus aureus*? *Transbound. Emerg. Dis.* 66, 1506–1517. doi: 10.1111/tbed.13170

Abdulgader, S., van Rijswijk, A., Whitelaw, A., and Newton-Foot, M. (2020). The association between pathogen factors and clinical outcomes in patients with *Staphylococcus aureus* bacteraemia in a tertiary hospital, Cape Town. *Int. J. Infect. Dis.* 91, 111–118. doi: 10.1016/j.ijid.2019.11.032

Abdulgader, S. M., Shittu, A. O., Nicol, M. P., and Kaba, M. (2015). Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Africa: a systematic review. *Front. Microbiol.* 6:348. doi: 10.3389/fmicb.2015.00348

Abou Shady, H. M. A., Bakr, A. E. A., Hashad, M. E., Alzohairy, M. A., Shady, H. M. A., Bakr, A. E. A., et al. (2015). *Staphylococcus aureus* nasal carriage among outpatients attending primary health care centers: a comparative study of two cities in Saudi Arabia and Egypt. *Braz. J. Infect. Dis.* 19, 68–76. doi: 10.1016/j.bjid.2014.09.005

Agabou, A., Ouchenane, Z., Ngba Essebe, C., Khemissi, S., Chehboub, M. T. E., Chehboub, I. B., et al. (2017). Emergence of nasal carriage of ST80 and ST152 PVL+ *Staphylococcus aureus* isolates from livestock in Algeria. *Toxins* 9:303. doi: 10.3390/toxins9100303

Ahmed, M. O., Baptiste, K. E., Daw, M. A., Elramalli, A. K., Abouzeed, Y. M., and Petersen, A. (2017). Spa typing and identification of pvl genes of meticillin-resistant *Staphylococcus aureus* isolated from a Libyan hospital in Tripoli. *J. Glob. Antimicrob. Resist.* 10, 179–181. doi: 10.1016/j.jgar.2017.06.004

Aires-de-Sousa, M., Rodrigues, S., Conceição, T., and de Lencastre, H. (2018). Evaluation of different screening methodologies for the detection of methicillin-resistant *Staphylococcus aureus* from environmental surfaces: swabs, gauzes, and polywipes. *Microb. Drug Resist.* 24, 585–589. doi: 10.1089/mdr.2017.0349

Al-Amery, K., Elhariri, M., Elsayed, A., El-Moghazy, G., Elhelw, R., El-Mahallawy, H., et al. (2019). Vancomycin-resistant *Staphylococcus aureus* isolated from camel meat and slaughterhouse workers in Egypt. *Antimicrob. Resist. Infect. Control* 8, 1–8. doi: 10.1186/s13756-019-0585-4

Alli, O. A. T., Ogbolu, D. O., Shittu, A. O., Okorie, A. N., Akinola, J. O., and Daniel, J. B. (2015). Association of virulence genes with mecA gene in *Staphylococcus aureus* isolates from Tertiary Hospitals in Nigeria. *Indian J. Pathol. Microbiol.* 58:464. doi: 10.4103/0377-4929.168875

Amoako, D. G., Bester, L. A., Somboro, A. M., Baijnath, S., Govind, C. N., and Essack, S. Y. (2016). Plasmid-mediated resistance and virulence mechanisms in the private health sector in KwaZulu-Natal, South Africa: an investigation of methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates collected during a three month period. *Int. J. Infect. Dis.* 46, 38–41. doi: 10.1016/j.ijid.2016.03.019

Amoako, D. G., Somboro, A. M., Abia, A. L. K., Allam, M., Ismail, A., Bester, L., et al. (2019). Genomic analysis of methicillin-resistant *Staphylococcus aureus* isolated from poultry and occupational farm workers in umgungundlovu District, South Africa. *Sci. Total Environ.* 670, 704–716. doi: 10.1016/j.scitotenv.2019.03.110

Asiimwe, B. B., Baldan, R., Trovato, A., and Cirillo, D. M. (2017b). Prevalence and molecular characteristics of *Staphylococcus aureus*, including methicillin resistant strains, isolated from bulk can milk and raw milk products in pastoral communities of South-West Uganda. *BMC Infect. Dis.* 17:422. doi: 10.1186/s12879-017-2524-4

Asiimwe, B. B., Baldan, R., Trovato, A., and Cirillo, D. M. (2017a). Molecular epidemiology of panton-valentine leukocidin-positive community-acquired methicillin resistant *Staphylococcus aureus* isolates in pastoral communities of rural south western Uganda. *BMC Infect. Dis.* 17:24. doi: 10.1186/s12879-016-2124-8

Ayepola, O. O., Olasupo, N. A., Egwari, L. O., Becker, K., and Schaumburg, F. (2015). Molecular characterization and antimicrobial susceptibility of *Staphylococcus aureus* isolates from clinical infection and asymptomatic carriers in southwest Nigeria. *PLoS One* 10:e0137531. doi: 10.1371/journal.pone.0137531

Baig, S., Rhod Larsen, A., Martins Simões, P., Laurent, F., Johannesen, T. B., Lilje, B., et al. (2020). Evolution and population dynamics of clonal complex 152 community-associated methicillin-resistant *Staphylococcus aureus*. *mSphere* 5, e226–e220. doi: 10.1128/mSphere.00226-20

Baker, S., Thomson, N., Weill, F.-X., and Holt, K. E. (2018). Genomic insights into the emergence and spread of antimicrobial-resistant bacterial pathogens. *Science* 360, 733–738. doi: 10.1126/science.aar3777

Bamigboye, B. T., Olowe, O. A., and Taiwo, S. S. (2018). Phenotypic and molecular identification of vancomycin resistance in clinical *Staphylococcus aureus* Isolates in Osogbo, Nigeria. *Eur. J. Microbiol. Immunol.* 8:25. doi: 10.1556/1886.2018.00003

Bloomfield, L. E., Coombs, G. W., Tempone, S., and Armstrong, P. K. (2020). Marked increase in community-associated methicillin-resistant *Staphylococcus aureus* infections, Western Australia, 2004–2018. *Epidemiol. Infect.* 148:e153. doi: 10.1017/S0950268820000849

Bowers, J. R., Driebe, E. M., Albrecht, V., Mcdougal, L. K., Granade, M., Roe, C. C., et al. (2018). Improved subtyping of *Staphylococcus aureus* clonal complex 8 strains based on whole-genome phylogenetic analysis. *mSphere* 3, e00464–17. doi: 10.1128/mSphere.00464-17

Chaalal, W., Chaalal, N., Bourafa, N., Kihal, M., Diene, S. M., and Rolain, J.-M. (2018). Characterization of *Staphylococcus aureus* isolated from food products in western Algeria. *Foodborne Pathog. Dis.* 15, 353–360. doi: 10.1089/fpd.2017.2339

Chairat, S., Gharsa, H., Lozano, C., Gómez-Sanz, E., Gómez, P., Zarazaga, M., et al. (2015). Characterization of *Staphylococcus aureus* from raw meat samples in tunisia: detection of clonal lineage ST398 from the African continent. *Foodborne Pathog. Dis.* 12, 686–692. doi: 10.1089/fpd.2015.1958

Conceição, T., Coelho, C., de Lencastre, H., and Aires-de-Sousa, M. (2015a). Frequent occurrence of oxacillin-susceptible mecA-positive *Staphylococcus aureus* (OS-MRSA) strains in two African countries. *J. Antimicrob. Chemother.* 70, 3200–3204. doi: 10.1093/jac/dkv261

Conceição, T., Coelho, C., Silva, I. S., de Lencastre, H., and Aires-de-Sousa, M. (2015b). *Staphylococcus aureus* in former Portuguese colonies from Africa and the far east: missing data to help fill the world map. *Clin. Microbiol. Infect.* 21, 842.e1–842.e10. doi: 10.1016/j.cmi.2015.05.010

Dai, Y., Liu, J., Guo, W., Meng, H., Huang, Q., He, L., et al. (2019). Decreasing methicillin-resistant *Staphylococcus aureus* (MRSA) infections is attributable to the disappearance of predominant MRSA ST239 clones, Shanghai, 2008–2017. *Emerg. Microbes Infect.* 8, 471–478. doi: 10.1080/22221751.2019.1595161

Dekker, D., Wolters, M., Mertens, E., Boahen, K. G., Krumkamp, R., Eibach, D., et al. (2016). Antibiotic resistance and clonal diversity of invasive *Staphylococcus aureus* in the rural Ashanti region. *Ghana. BMC Infect. Dis.* 16:720. doi: 10.1186/s12879-016-2048-3

Djoudi, F., Benallaoua, S., Aleo, A., Touati, A., Challal, M., Bonura, C., et al. (2014). Descriptive epidemiology of nasal carriage of *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* among patients admitted to two healthcare facilities in Algeria. *Microb. Drug Resist.* 21, 218–223. doi: 10.1089/mdr.2014.0156

Dweba, C. C., and Zishiri, O. T. (2019). Isolation and molecular identification of virulence, antimicrobial and heavy metal resistance genes in *Staphylococcus aureus*. *Pathogens* 8, 1–21.

Egyir, B., Bentum, J., Attram, N., Fox, A., Obeng-Nkrumah, N., Appiah-Korang, L., et al. (2021). Whole genome sequencing and antimicrobial resistance of *Staphylococcus aureus* from surgical site infections in Ghana. *Pathogens* 10:196. doi: 10.3390/pathogens10020196

Egyir, B., Guardabassi, L., Monecke, S., Addo, K. K., Newman, M. J., and Larsen, A. R. (2015). Methicillin-resistant *Staphylococcus aureus* strains from Ghana include USA300. *J. Glob. Antimicrob. Resist.* 3, 26–30. doi: 10.1016/j.jgar.2014.11.006

Egyir, B., Hadjirin, N. F., Gupta, S., Owusu, F., Agbodzi, B., Adogla-Bessa, T., et al. (2020). Whole-genome sequence profiling of antibiotic-resistant *Staphylococcus aureus* isolates from livestock and farm attendants in Ghana. *J. Glob. Antimicrob. Resist.* 22, 527–532. doi: 10.1016/j.jgar.2020.03.029

Eibach, D., Nagel, M., Hogan, B., Azuure, C., Krumkamp, R., Dekker, D., et al. (2017). Nasal Carriage of *Staphylococcus aureus* among children in the Ashanti region of Ghana. *PLoS One* 12:e0170320. doi: 10.1371/journal.pone.0170320

El-Ashker, M., Gwida, M., Monecke, S., El-Gohary, F., Ehricht, R., Elsayed, M., et al. (2020). Antimicrobial resistance pattern and virulence profile of S. *aureus* isolated from household cattle and buffalo with mastitis in Egypt. *Vet. Microbiol.* 240:108535. doi: 10.1016/j.vetmic.2019.108535

El-baz, R., Rizk, D. E., Barwa, R., and Hassan, R. (2017). Virulence characteristics and molecular relatedness of methicillin resistant *Staphylococcus aureus* harboring different staphylococcal cassette chromosome mec. *Microb. Pathog.* 113, 385–395. doi: 10.1016/j.micpath.2017.11.021

Elhani, D., Gharsa, H., Kalai, D., Lozano, C., Gómez, P., Boutheina, J., et al. (2015). Clonal lineages detected amongst tetracycline-resistant meticillin-resistant *Staphylococcus aureus* isolates of a Tunisian hospital, with detection of lineage ST398. *J. Med. Microbiol.* 64, 623–629. doi: 10.1099/jmm.0.000066

ElSayed, N., Ashour, M., and Amine, A. E. K. (2018). Vancomycin resistance among *Staphylococcus aureus* isolates in a rural setting, Egypt. *Germs* 8:134. doi: 10.18683/germs.2018.1140

Enwuru, N. V., Adesida, S. A., Enwuru, C. A., Ghebremedhin, B., Mendie, U. E., and Coker, A. O. (2018). Genetics of bi-component leukocidin and drug resistance in nasal and clinical *Staphylococcus aureus* in Lagos, Nigeria. *Microb. Pathog.* 115, 1–7. doi: 10.1016/j.micpath.2017.12.030

Eshetie, S., Tarekegn, F., Moges, F., Amsalu, A., Birhan, W., and Huruy, K. (2016). Methicillin resistant *Staphylococcus aureus* in Ethiopia: a meta-analysis. *BMC Infect. Dis.* 16:689. doi: 10.1186/s12879-016-2014-0

Fortuin-de Smidt, M. C., Singh-Moodley, A., Badat, R., Quan, V., Kularatne, R., Nana, T., et al. (2015). *Staphylococcus aureus* bacteraemia in Gauteng academic hospitals, South Africa. *Int. J. Infect. Dis.* 30, 41–48. doi: 10.1016/j.ijid.2014.10.011

Founou, L. L., Founou, R. C., Allam, M., Ismail, A., Djoko, C. F., and Essack, S. Y. (2019). Genome analysis of methicillin-resistant *Staphylococcus aureus* isolated from pigs: detection of the clonal lineage ST398 in Cameroon and South Africa. *Zoonoses Public Health* 66, 512–525. doi: 10.1111/zph.12586

Friesen, J., Neuber, R., Fuhrmann, J., Kietzmann, H., Wenzel, T., Schaumburg, F., et al. (2020). Panton-valentine leukocidin–positive *Staphylococcus aureus* in skin and soft tissue infections from primary care patients. *Clin. Microbiol. Infect.* 26, 1416.e1–1416.e4. doi: 10.1016/j.cmi.2020.06.029

Gill, J. L., Hedge, J., Wilson, D. J., and MacLean, R. C. (2021). Evolutionary processes driving the rise and fall of *Staphylococcus aureus* ST239, a dominant hybrid Pathogen. *mBio* 12:e0216821. doi: 10.1128/mBio.02168-21

Guthrie, J. L., Teatero, S., Hirai, S., Fortuna, A., Rosen, D., Mallo, G. V., et al. (2020). Genomic epidemiology of invasive methicillin-resistant *Staphylococcus aureus*

infections among hospitalized individuals in Ontario, Canada. *J. Infect. Dis.* 222, 2071–2081. doi: 10.1093/infdis/jiaa147

Harzing, A. (2007). *Publish or Perish; Harzing Resources*. Available online at: https://harzing.com/resources/publish-or-perish (accessed May 4, 2021).

Hogan, B., Rakotozandrindrainy, R., Al-Emran, H., Dekker, D., Hahn, A., Jaeger, A., et al. (2016). Prevalence of nasal colonisation by methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* among healthcare workers and students in Madagascar. *BMC Infect. Dis.* 16:420. doi: 10.1186/s12879-016-1733-6

Holzinger, D., Gieldon, L., Mysore, V., Nippe, N., Taxman, D. J., Duncan, J. A., et al. (2012). *Staphylococcus aureus* Panton-valentine leukocidin induces an inflammatory response in human phagocytes via the NLRP3 inflammasome. *J. Leukoc. Biol.* 92, 1069–1081. doi: 10.1189/jlb.0112014

Humphreys, H., and Coleman, D. C. (2019). Contribution of whole-genome sequencing to understanding of the epidemiology and control of meticillin-resistant *Staphylococcus aureus*. *J. Hosp. Infect.* 102, 189–199. doi: 10.1016/j.jhin.2019.01.025

Kalayu, A. A., Woldetsadik, D. A., Woldeamanuel, Y., Wang, S.-H., Gebreyes, W. A., and Teferi, T. (2020). Burden and antimicrobial resistance of S. *aureus* in dairy farms in Mekelle, Northern Ethiopia. *BMC Vet. Res.* 16:20. doi: 10.1186/s12917-020-2235-8

Kateete, D. P., Asiimwe, B. B., Mayanja, R., Mujuni, B., Bwanga, F., Najjuka, C. F., et al. (2019a). Nasopharyngeal carriage, spa types and antibiotic susceptibility profiles of *Staphylococcus aureus* from healthy children less than 5 years in Eastern Uganda. *BMC Infect. Dis.* 19:1023. doi: 10.1186/s12879-019-4652-5

Kateete, D. P., Bwanga, F., Seni, J., Mayanja, R., Kigozi, E., Mujuni, B., et al. (2019b). CA-MRSA and HA-MRSA coexist in community and hospital settings in Uganda. *Antimicrob. Resist. Infect. Control* 8:94. doi: 10.1186/s13756-019-0551-1

Khairalla, A. S., Wasfi, R., and Ashour, H. M. (2017). Carriage frequency, phenotypic, and genotypic characteristics of methicillin-resistant *Staphylococcus aureus* isolated from dental health-care personnel, patients, and environment. *Sci. Rep.* 7:7390. doi: 10.1038/s41598-017-07713-8

Khemiri, M., Akrout Alhusain, A., Abbassi, M. S., El Ghaieb, H., Santos Costa, S., Belas, A., et al. (2017). Clonal spread of methicillin-resistant *Staphylococcus aureus*-t6065-CC5-SCCmecV-agrII in a Libyan hospital. *J. Glob. Antimicrob. Resist.* 10, 101–105. doi: 10.1016/j.jgar.2017.04.014

Klibi, A., Jouini, A., Gómez, P., Slimene, K., Ceballos, S., Torres, C., et al. (2018). Molecular characterization and clonal diversity of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates of milk of cows with clinical mastitis in Tunisia. *Microb. Drug Resist.* 24, 1210–1216. doi: 10.1089/mdr.2017.0278

Kumburu, H. H., Sonda, T., Leekitcharoenphon, P., van Zwetselaar, M., Lukjancenko, O., Alifrangis, M., et al. (2018). Hospital epidemiology of methicillin-resistant *Staphylococcus aureus* in a tertiary care hospital in Moshi, Tanzania, as determined by whole genome sequencing. *Biomed Res. Int.* 2018:2087693. doi: 10.1155/2018/2087693

Kyany'a, C., Nyasinga, J., Matano, D., Oundo, V., Wacira, S., Sang, W., et al. (2019). Phenotypic and genotypic characterization of clinical *Staphylococcus aureus* isolates from Kenya. *BMC Microbiol.* 19:245. doi: 10.1186/s12866-019-1597-1

Lebughe, M., Phaku, P., Niemann, S., Mumba, D., Peters, G., Muyembe-Tamfum, J.-J., et al. (2017). The Impact of the *Staphylococcus aureus* virulome on infection in a developing country: a cohort study. *Front. Microbiol.* 8:1662. doi: 10.3389/fmicb.2017.01662

Lee, A. S., de Lencastre, H., Garau, J., Kluytmans, J., Malhotra-Kumar, S., Peschel, A., et al. (2018). Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Primer* 4:18033. doi: 10.1038/nrdp.2018.33

Li, S., Sun, S., Yang, C., Chen, H., Yin, Y., Li, H., et al. (2018). The changing pattern of population structure of *Staphylococcus aureus* from bacteremia in China from 2013 to 2016: ST239-030-MRSA replaced by ST59-t437. *Front. Microbiol.* 9:332. doi: 10.3389/fmicb.2018.00332

Lozano, C., Gharsa, H., Slama, K. B., Zarazaga, M., and Torres, C. (2016). *Staphylococcus aureus* in animals and food: methicillin resistance, prevalence and population structure. a review in the African continent. *Microorganisms* 4:12. doi: 10.3390/microorganisms4010012

Mahomed, G. T., Kock, M. M., Masekela, R., Hoosien, E., and Ehlers, M. M. (2018). Genetic relatedness of *Staphylococcus aureus* isolates obtained from cystic fibrosis patients at a tertiary academic hospital in Pretoria, South Africa. *Sci. Rep.* 8:12222. doi: 10.1038/s41598-018-30725-x

Manyahi, J., Moyo, S. J., Aboud, S., Langeland, N., and Blomberg, B. (2021). Predominance of PVL-negative community-associated methicillin-resistant *Staphylococcus aureus* sequence type 8 in newly diagnosed HIV-infected adults, Tanzania. *Eur. J. Clin. Microbiol. Infect. Dis.* 40, 1477–1485. doi: 10.1007/s10096-021-04160-2

Masaisa, F., Kayigi, E., Seni, J., Bwanga, F., and Muvunyi, C. M. (2018). Antibiotic resistance patterns and molecular characterization of methicillin-resistant *Staphylococcus aureus* in clinical settings in Rwanda. *Am. J. Trop. Med. Hyg.* 99, 1239–1245. doi: 10.4269/ajtmh.17-0554

McGuinness, W. A., Malachowa, N., and DeLeo, F. R. (2017). Vancomycin resistance in *Staphylococcus aureus*. *Yale J. Biol. Med.* 90, 269–281.

Mesrati, I., Saidani, M., Jemili, M., Ferjeni, S., Slim, A., and Boubaker, I. B.-B. (2018). Virulence determinants, biofilm production and antimicrobial susceptibility in *Staphylococcus aureus* causing device-associated infections in a Tunisian hospital. *Int. J. Antimicrob. Agents* 52, 922–929. doi: 10.1016/j.ijantimicag.2018.05.004

Mohammed, J., Ziwa, M. H., Hounmanou, Y. M. G., Kisanga, A., and Tuntufye, H. N. (2018). Molecular typing and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* isolated from bovine milk in Tanzania. *Int. J. Microbiol.* 2018:e4287431. doi: 10.1155/2018/4287431

Moremi, N., Claus, H., Vogel, U., and Mshana, S. E. (2019). The role of patients and healthcare workers *Staphylococcus aureus* nasal colonization in occurrence of surgical site infection among patients admitted in two centers in Tanzania. *Antimicrob. Resist. Infect. Control* 8:102. doi: 10.1186/s13756-019-0554-y

Mourabit, N., Arakrak, A., Bakkali, M., and Laglaoui, A. (2017). Nasal carriage of sequence type 22 MRSA and livestock-associated ST398 clones in Tangier, Morocco. *J. Infect. Dev. Ctries.* 11, 536–542. doi: 10.3855/jidc.9235

Nworie, A., Onyema, A. S., Okekpa, S. I., Elom, M. O., Umoh, N. O., Usanga, V. U., et al. (2017). A novel methicillin-resistant *Staphylococcus aureus* t11469 and a poultry endemic strain t002 (ST5) are present in chicken in Ebonyi State, Nigeria. *Biomed Res. Int.* 2017:2936461. doi: 10.1155/2017/2936461

Odetokun, I. A., Ballhausen, B., Adetunji, V. O., Ghali-Mohammed, I., Adelowo, M. T., Adetunji, S. A., et al. (2018). *Staphylococcus aureus* in two municipal abattoirs in Nigeria: risk perception, spread and public health implications. *Vet. Microbiol.* 216, 52–59. doi: 10.1016/j.vetmic.2018.01.022

Ogundipe, F. O., Ojo, O. E., Feßler, A. T., Hanke, D., Awoyomi, O. J., Ojo, D. A., et al. (2020). Antimicrobial resistance and virulence of methicillin-resistant *Staphylococcus aureus* from human, chicken and environmental samples within live bird markets in three Nigerian cities. *Antibiotics* 9:588. doi: 10.3390/antibiotics9090588

Okomo, U., Akpalu, E. N. K., Doare, K. L., Roca, A., Cousens, S., Jarde, A., et al. (2019). Aetiology of invasive bacterial infection and antimicrobial resistance in neonates in sub-Saharan Africa: a systematic review and meta-analysis in line with the STROBE-NI reporting guidelines. *Lancet Infect. Dis.* 19, 1219–1234. doi: 10.1016/S1473-3099(19)30414-1

Okuda, K. V., Toepfner, N., Alabi, A. S., Arnold, B., Bélard, S., Falke, U., et al. (2016). Molecular epidemiology of *Staphylococcus aureus* from Lambaréné, Gabon. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 1963–1973. doi: 10.1007/s10096-016-2748-z

Omuse, G., Van Zyl, K. N., Hoek, K., Abdulgader, S., Kariuki, S., Whitelaw, A., et al. (2016). Molecular characterization of *Staphylococcus aureus* isolates from various healthcare institutions in Nairobi, Kenya: a cross sectional study. *Ann. Clin. Microbiol. Antimicrob.* 15:51. doi: 10.1186/s12941-016-0171-z

Onwugamba, F. C., Mellmann, A., Nwaugo, V. O., Süselbeck, B., and Schaumburg, F. (2020). Antimicrobial resistant and enteropathogenic bacteria in 'filth flies': a cross-sectional study from Nigeria. *Sci. Rep.* 10:16990. doi: 10.1038/s41598-020-74112-x

Oreiby, A, Khalifa, H., Eid, A., Ahmed, A., Shimamoto, T., and Shimamoto, T. (2019). *Staphylococcus aureus* and bovine mastitis: molecular typing of methicillin resistance and clinical description of infected quarters. *J. Hell. Vet. Med. Soc.* 70, 1511–1516. doi: 10.12681/jhvms.20956

Otalu, O. J., Kwaga, J. K. P., Okolocha, E. C., Islam, M. Z., and Moodley, A. (2018). High Genetic Similarity of MRSA ST88 isolated from pigs and humans in Kogi State, Nigeria. *Front. Microbiol.* 9:3098. doi: 10.3389/fmicb.2018.03098

Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., et al. (2021). The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* 372:n71. doi: 10.1136/bmj.n71

Perovic, O., Iyaloo, S., Kularatne, R., Lowman, W., Bosman, N., Wadula, J., et al. (2015). Prevalence and trends of *Staphylococcus aureus* bacteraemia in hospitalized patients in South Africa, 2010 to 2012: laboratory-based surveillance mapping of antimicrobial resistance and molecular epidemiology. *PLoS One* 10:e0145429. doi: 10.1371/journal.pone.0145429

Perovic, O., Singh-Moodley, A., Govender, N. P., Kularatne, R., Whitelaw, A., Chibabhai, V., et al. (2017). A small proportion of community-associated methicillin-resistant *Staphylococcus aureus* bacteraemia, compared to healthcare-associated cases, in two South African provinces. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 2519–2532. doi: 10.1007/s10096-017-3096-3

Price, J. R., Didelot, X., Crook, D. W., Llewelyn, M. J., and Paul, J. (2013). Whole genome sequencing in the prevention and control of *Staphylococcus aureus* infection. *J. Hosp. Infect.* 83, 14–21. doi: 10.1016/j.jhin.2012.10.003

Ramaite, K., Ekwanzala, M. D., Dewar, J. B., and Momba, M. N. B. (2021). Human-associated methicillin-resistant *Staphylococcus aureus* clonal complex 80 isolated from cattle and aquatic environments. *Antibiotics* 10:1038. doi: 10.3390/antibiotics10091038

Rentinck, M.-N., Krüger, R., Hoppe, P.-A., Humme, D., Niebank, M., Pokrywka, A., et al. (2021). Skin infections due to Panton-Valentine leukocidin (PVL)-producing S. *aureus*—cost effectiveness of outpatient treatment. *PLoS One* 16:e0253633. doi: 10.1371/journal.pone.0253633

Robinson, D. A., and Enright, M. C. (2004). Evolution of *Staphylococcus aureus* by large chromosomal replacements. *J. Bacteriol.* 186, 1060–1064. doi: 10.1128/JB.186.4.1060-1064.2004

Rodrigues, S., Conceição, T., Silva, I. S., de Lencastre, H., and Aires-de-Sousa, M. (2018). Frequent MRSA nasal colonization among hospitalized children and their parents in Angola and São Tomé and Príncipe. *J. Hosp. Infect.* 100, 344–349. doi: 10.1016/j.jhin.2018.05.015

Samutela, M. T., Kalonda, A., Mwansa, J., Lukwesa-Musyani, C., Mwaba, J., Mumbula, E. M., et al. (2017). Molecular characterisation of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated at a large referral hospital in Zambia. *Pan Afr. Med. J.* 26:108. doi: 10.11604/pamj.2017.26.108.10982

Schaumburg, F., Alabi, A. S., Peters, G., and Becker, K. (2014). New epidemiology of *Staphylococcus aureus* infection in Africa. *Clin. Microbiol. Infect.* 20, 589–596. doi: 10.1111/1469-0691.12690

Schaumburg, F., Pauly, M., Anoh, E., Mossoun, A., Wiersma, L., Schubert, G., et al. (2015). *Staphylococcus aureus* complex from animals and humans in three remote African regions. *Clin. Microbiol. Infect* 21, 345.e1–8. doi: 10.1016/j.cmi.2014.12.001

See, I., Mu, Y., Albrecht, V., Karlsson, M., Dumyati, G., Hardy, D. J., et al. (2020). Trends in incidence of methicillin-resistant *Staphylococcus aureus* bloodstream infections differ by strain type and healthcare exposure, united states, 2005–2013. *Clin. Infect. Dis.* 70, 19–25. doi: 10.1093/cid/ciz158

Shehata, M. M. K., Radwan, S. M., and Ali, S. A. M. (2019). Effects of gamma-irradiation on antibiotic resistance and diagnostic molecular markers of methicillin-resistant *Staphylococcus aureus* in Egyptian cancer patients. *Int. J. Radiat. Biol.* 95, 1728–1743. doi: 10.1080/09553002.2019.1664785

Shittu, A. O., Mellmann, A., and Schaumburg, F. (2020b). Molecular characterization of *Staphylococcus aureus* complex from fomites in Nigeria. *Infect. Genet. Evol.* 85:104504. doi: 10.1016/j.meegid.2020.104504

Shittu, A. O., Deinhardt-Emmer, S., Vas Nunes, J., Niemann, S., Grobusch, M. P., and Schaumburg, F. (2020a). Tropical pyomyositis: an update. *Trop. Med. Int. Health* 25, 660–665. doi: 10.1111/tmi.13395

Shittu, A. O., Taiwo, F. F., Froböse, N. J., Schwartbeck, B., Niemann, S., Mellmann, A., et al. (2021). Genomic analysis of *Staphylococcus aureus* from the West African Dwarf (WAD) goat in Nigeria. *Antimicrob. Resist. Infect. Control* 10:122. doi: 10.1186/s13756-021-00987-8

Singh-Moodley, A., Lowe, M., Mogokotleng, R., and Perovic, O. (2020). Diversity of SCCmec elements and spa types in South African *Staphylococcus aureus* mecA-positive blood culture isolates. *BMC Infect. Dis.* 20:816. doi: 10.1186/s12879-020-05547-w

Singh-Moodley, A., Strasheim, W., Mogokotleng, R., Ismail, H., and Perovic, O. (2019). Unconventional SCCmec types and low prevalence of the Panton-valentine leukocidin exotoxin in South African blood culture *Staphylococcus aureus* surveillance isolates, 2013-2016. *PLoS One* 14:e0225726. doi: 10.1371/journal.pone.0225726

Soliman, M. S., Soliman, N. S., El-Manakhly, A. R., ElBanna, S. A., Aziz, R. K., and El-Kholy, A. A. (2020). Genomic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) by high-throughput sequencing in a tertiary care Hospital. *Genes* 11:1219. doi: 10.3390/genes11101219

Strauß, L., Stegger, M., Akpaka, P. E., Alabi, A., Breurec, S., Coombs, G., et al. (2017). Origin, evolution, and global transmission of community-acquired *Staphylococcus aureus* ST8. *PNAS* 114, 10596–10604. doi: 10.1073/pnas.1702472114

Titouche, Y., Hakem, A., Houali, K., Meheut, T., Vingadassalon, N., Ruiz-Ripa, L., et al. (2019). Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) ST8 in raw milk and traditional dairy products in the Tizi Ouzou area of Algeria. *J. Dairy Sci.* 102, 6876–6884. doi: 10.3168/jds.2018-16208

Titouche, Y., Houali, K., Ruiz-Ripa, L., Vingadassalon, N., Nia, Y., Fatihi, A., et al. (2020). Enterotoxin genes and antimicrobial resistance in *Staphylococcus aureus* isolated from food products in Algeria. *J. Appl. Microbiol.* 129, 1043–1052. doi: 10.1111/jam.14665

Turner, N. A., Sharma-Kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., et al. (2019). Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nat. Rev. Microbiol.* 17, 203–218. doi: 10.1038/s41579-018-0147-4

Vandendriessche, S., De Boeck, H., Deplano, A., Phoba, M.-F., Lunguya, O., Falay, D., et al. (2017). Characterisation of *Staphylococcus aureus* isolates from bloodstream infections, Democratic Republic of the Congo. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 1163–1171. doi: 10.1007/s10096-017-2904-0

Verdú-Expósito, C., Romanyk, J., Cuadros-González, J., TesfaMariam, A., Copa-Patiño, J. L., Pérez-Serrano, J., et al. (2020). Study of susceptibility to antibiotics and molecular characterization of high virulence *Staphylococcus aureus* strains isolated from a rural hospital in Ethiopia. *PLoS One* 15:e0230031. doi: 10.1371/journal.pone.0230031

Wangai, F. K., Masika, M. M., Maritim, M. C., and Seaton, R. A. (2019). Methicillin-resistant *Staphylococcus aureus* (MRSA) in East Africa: red alert or red herring? *BMC Infect. Dis.* 19:596. doi: 10.1186/s12879-019-4245-3

WHO (2021). *Global Antimicrobial Resistance And Use Surveillance System (GLASS) Report: 2021*. Geneva: World Health Organization.

Wolters, M., Frickmann, H., Christner, M., Both, A., Rohde, H., Oppong, K., et al. (2020). Molecular characterization of *Staphylococcus aureus* isolated from chronic infected wounds in rural Ghana. *Microorganisms* 8:2052. doi: 10.3390/microorganisms8122052

Zorgani, A. A., Elahmer, O., Abaid, A., Elaref, A., Elamri, S., Aghila, E., et al. (2015). Vancomycin susceptibility trends of methicillin-resistant *Staphylococcus aureus* isolated from burn wounds: a time for action. *J. Infect. Dev. Ctries.* 9, 1284–1288. doi: 10.3855/jidc.6976

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 Lawal, Ayobami, Abouelfetouh, Mourabit, Kaba, Egyir, Abdulgader and Shittu. 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 Microbiology

ORIGINAL RESEARCH
published: 16 May 2022
doi: 10.3389/fmicb.2022.882422

High Prevalence of bla_{CTXM-1}/IncI1-Iγ/ST3 Plasmids in Extended-Spectrum β-Lactamase-Producing *Escherichia coli* Isolates Collected From Domestic Animals in Guadeloupe (French West Indies)

*Gaëlle Gruel[1], David Couvin[1], Stéphanie Guyomard-Rabenirina[1], Guillaume Arlet[2], Jean-Christophe Bambou[3], Matthieu Pot[1], Xavier Roy[4], Antoine Talarmin[1], Benoit Tressieres[5], Séverine Ferdinand[1] and Sébastien Breurec[1,5,6]**

[1] Transmission, Reservoir and Diversity of Pathogens Unit, Pasteur Institute of Guadeloupe, Pointe-à-Pitre, France, [2] U1135-Cimi-Paris, Sorbonne University, Paris, France, [3] INRAE, ASSET, Petit-Bourg, France, [4] Veterinary Clinic, Baie-Mahault, France, [5] INSERM 1424, Center for Clinical Investigation, University Hospital Center of Guadeloupe, Pointe-à-Pitre, France, [6] Faculty of Medicine Hyacinthe Bastaraud, University of the Antilles, Pointe-à-Pitre, France

Extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-E) have been classified in the group of resistant bacteria of highest priority. We determined the prevalence of ESBL-E collected in feces from household and shelter pets in Guadeloupe (French West Indies). A single rectal swab was taken from 125 dogs and 60 cats between June and September 2019. The prevalence of fecal carriage of ESBL-E was 7.6% (14/185, 95% CI: 4.2-12.4), within the range observed worldwide. The only risk factor associated with a higher prevalence of ESBL-E rectal carriage was a stay in a shelter, suggesting that refuges could be hotspots for their acquisition. All but one (*Klebsiella pneumoniae* from a cat) were *Escherichia coli*. We noted the presence of a $bla_{CTX-M-1}$/IncI1-Iγ/sequence type (ST3) plasmid in 11 ESBL-producing *E. coli* isolates belonging to ST328 (n = 6), ST155 (n = 4) and ST953 (n = 1). A $bla_{CTX-M-15}$ gene was identified in the three remaining ESBL-E isolates. The $bla_{CTX-M-1}$ and most of the antimicrobial resistance genes were present in a well-conserved large conjugative IncI1-Iγ/ST3 plasmid characterized by two accessory regions containing antibiotic resistance genes. The plasmid has been detected worldwide in *E. coli* isolates from humans and several animal species, such as food-producing animals, wild birds and pets, and from the environment. This study shows the potential role of pets as a reservoir of antimicrobial-resistant bacteria or genes for humans and underlines the importance of basic hygiene measures by owners of companion animals.

Keywords: **Enterobacteriaceae, *Escherichia coli*, ESBL, pets, shelter, nanopore, plasmid**

OPEN ACCESS

Edited by:
Ziad Daoud,
Central Michigan University,
United States

Reviewed by:
Michael Brouwer,
Wageningen University and Research,
Netherlands
Jean-Yves Madec,
Agence Nationale de Sécurité
Sanitaire de l'Alimentation,
de l'Environnement et du Travail
(ANSES), France

****Correspondence:***
Sébastien Breurec
sbreurec@gmail.com

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *23 February 2022*
Accepted: *07 April 2022*
Published: *16 May 2022*

Citation:
Gruel G, Couvin D, Guyomard-Rabenirina S, Arlet G, Bambou J-C, Pot M, Roy X, Talarmin A, Tressieres B, Ferdinand S and Breurec S (2022) High Prevalence of bla_{CTXM-1}/IncI1-Iγ/ST3 Plasmids in Extended-Spectrum β-Lactamase-Producing Escherichia coli Isolates Collected From Domestic Animals in Guadeloupe (French West Indies). Front. Microbiol. 13:882422. doi: 10.3389/fmicb.2022.882422

INTRODUCTION

Currently, resistance to antibiotics is one of the most urgent public health threats in the world and could be responsible for more deaths than cancer by 2050 (i.e., 10 million deaths per year) (O'Neill, 2016). This complex problem involves humans, animals, and the environment. Recently, the World Health Organization published a global priority list of antibiotic-resistant bacteria, on which third-generation cephalosporin (3GC)-resistant Enterobacteriaceae were in priority 1 group ((World Health Organization, 2020). This type of resistance is mediated mainly by acquired extended-spectrum β-lactamase (ESBL) genes located on plasmids (Bush and Bradford, 2020). ESBL enzymes can confer resistance to all β-lactams except for carbapenems and cephamycins and are frequently associated with genes that confer resistance to a wide variety of antimicrobial agents.

During the past 30 years, the number of companion animals in developed countries has been increasing. For example, in 2020, approximately 45% of households in the United States had one or more dogs and 26% had one or more cats, the highest rates since measurement began in 1982 (AMVM, 2020; AMVM, 2021). In the same year, the estimated number of households in Europe that owned at least one companion animal was about 88 million, most of which were dogs and cats, representing 89.8 and 110.1 million animals, respectively (The European Pet Food Industry, 2021). To our knowledge, no data on dog and cat populations in Guadeloupe are available.

Since the first report of an ESBL-producing uropathogenic *Escherichia coli* isolate from a dog in Spain in 1998 (Teshager et al., 2000), more reports of ESBL-producing Enterobacteriaceae (ESBL-E) in animals have been published globally (Bush and Bradford, 2020; Salgado-Caxito et al., 2021), raising concern that animals are possible sources and reservoirs of ESBLs for humans (Dupouy et al., 2019). Close contact between pets and owners provides opportunities for transmission of antimicrobial-resistant bacteria. Feeding with raw pet food was found as risk factor for ESBL-E colonization in cats (Baede et al., 2017).

Guadeloupe, a French overseas territory located in the Caribbean, was considered a very high-resource country in 2013 (Human Development Report, 2013). Data on resistance to antibiotics in Guadeloupe are scarce and recent. The University Hospital of Guadeloupe faced the emergence of carbapenemase-producing Enterobacteriaceae isolates and a very high incidence of nosocomial ESBL-E infections (Bastian et al., 2015; Breurec et al., 2015; Le Terrier et al., 2021). A moderate prevalence of ESBL-E (around 5%) was observed in studies on community human urinary tract infections (Guyomard-Rabenirina et al., 2016), on fecal carriage by domestic animals and livestock (Gruel et al., 2021) and on wild fauna (Guyomard-Rabenirina et al., 2020). Guadeloupe is an ideal place to study the circulation of multi-resistant bacteria and resistance genes because of its insularity and small population (395,700 inhabitants) and area (1,436 km^2). The primary objective of our study was to determine the prevalence of ESBL-E in feces from household and shelter pets in Guadeloupe. The secondary objectives were to determine the risk factors associated with ESBL-E colonization in pets and to use whole-genome sequencing (WGS) to identify the genetic background of ESBL-E collected from pets and the genetic basis for resistance to 3GC.

MATERIALS AND METHODS

Sampling

Between June 2019 and September 2019, single rectal swabs were taken from 125 dogs and 60 cats. Of the 185 pets investigated, 15 were from the main animal shelter of Guadeloupe and 170 from seven veterinary clinics that provide preventive health services, vaccination or medical consultation in 18 cities throughout Guadeloupe and Les Saintes, a small nearby island. None of the animals displayed clinical signs of diarrhea and had received antibiotic treatment in the 3 months before inclusion.

For each animal examined, a questionnaire eliciting information on factors such as age, municipality of residence, general health and lifestyle (indoor or free roaming outdoors, close contact with other animals or not) was completed by each veterinarian based on the owners' declarations. Samples were stored in the veterinary clinics at + 4°C until shipment to the microbiology laboratory of the Pasteur Institute of Guadeloupe (within 48 h), where they were immediately analyzed. The project was approved by the Committee for Ethics in animal experiments of the French West Indies and Guyana (reference HC_2020_1). The animals were cared for and used according to French decree No. 2013-118 of 1 February 2013 on the protection of animals, which meets European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Enterobacteriaceae: Isolation, Identification and Antibiotic Susceptibility Testing

The bacterial populations of interest were enriched and preselected by incubating each rectal swab specimen in 9 mL of Luria-Bertani (LB) broth for 16–20 h at + 37°C; of this, 100 μL were inoculated on CHROMagar^{TM} CCA supplemented with ceftriaxone (4 mg/L) (Guyomard-Rabenirina et al., 2020). Presumptive Enterobacteriaceae colonies were identified on selective media on morphological criteria (blue, smooth, round colonies were presumed to be *E. coli*, and pink, smooth, round colonies were presumed to be members of *Klebsiella, Enterobacter,* or *Serratia* spp. (KES group), isolated randomly and identified in the Api 20E test (BioMérieux, Marcy-l'Etoile, France). Three colonies were randomly identified for each identical morphology.

The susceptibility of the Enterobacteriaceae isolates to antibiotics was assessed with the disk diffusion method on Mueller-Hinton agar according to the 2020 guidelines of CA-SFM/EUCAST (CA-SFM/EUCAST, 2020). The antimicrobial agents tested were ampicillin 10 μg, amoxicillin/clavulanic acid 20/10 μg, ticarcillin 75 μg, cefoxitin 30 μg, cefotaxime 5 μg, ceftazidime 10 μg, cefepim 30 μg, temocillin 30 μg, ertapenem 10 μg, nalidixic acid 30 μg, ciprofloxacin 5 μg, fosfomycin

200 μg, amikacin 30 μg, gentamicin 10 μg, tetracycline 30 μg, tigecycline 15 μg and trimethoprime/sulfamethoxazole 1.25/23.75 μg. Growth inhibition diameters were measured in the automated Adagio system (Bio-Rad). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as control strains. Isolates with a resistant or intermediate phenotype were classified together for the analysis. If more than one Enterobacteriaceae isolate within the same species with the same antibiotic susceptibility pattern was isolated from the same animal, only one randomly chosen isolate was analyzed. A double-disk synergy test was performed for all isolates (Breurec et al., 2013).

Core Genome Phylogenetic Analyses and Antibiotic Resistance Genes

WGS was performed on 14 ESBL-E isolates (13 *E. coli* and 1 *K. pneumoniae*) collected from the pets included in this study at the "Plateforme de microbiologie mutualisée" of the Pasteur International Bioresources network (Institut Pasteur, Paris, France). DNA was extracted with a DNA minikit (Qiagen, Hilden, Germany). The libraries were prepared with a Nextera XT kit (Illumina), and sequencing was performed with the NextSeq 500 system (Illumina), generating 150 bp paired-end reads, for a mean 48-fold coverage. The reads were deposited in the NCBI-SRA public archives under the project's accession number PRJNA798606. They were trimmed and filtered with AlienTrimmer (Criscuolo and Brisse, 2014).

Genomes were assembled with SPAdes software v3.9.0 (Bankevich et al., 2012). The quality of the assemblies was checked with QUAST software (Gurevich et al., 2013), giving a mean N50 of 112,604 (minimum, 61,542; maximum, 134,844). For ESBL-producing *E. coli* (ESBL-Ec) isolates, a core genome was extracted with Roary software (Page et al., 2015). Recombination sequences were identified and removed from the global core genome alignment with ClonalFrameML software (Didelot and Wilson, 2015), giving a global alignment of 3,367,757 bp, 3,429 genes shared by 100% of the bacterial isolates, and 1,254 polymorphic sites. Maximum likelihood (ML) phylogenetic reconstruction was performed with RAxML version 8.2.8 (Stamatakis, 2014), the GTR-CAT model, and 100 bootstrap replicates, and the tree was drawn with iTOL (Letunic and Bork, 2021). *In silico* multilocus sequence typing (MLST) was done with MLST software[1] against the PubMLST database (Jolley et al., 2018). *E. coli* was phylotyped with the EzClermont web application (Waters et al., 2020). ABRicate[2] was also used with the default parameter to assess the content of plasmids and antibiotic resistance genes associated with PlasmidFinder and ResFinder databases, respectively (Zankari et al., 2012; Carattoli et al., 2014).

In silico Detection of Plasmid and Syntenic Analysis

To investigate the presence of an IncI1-Iγ/ST3 signature plasmid in *E. coli* susceptible to 3GC (3GCS-Ec), 51 3GCS-Ec were isolated from frozen stools collected from 51 randomly chosen pets included in this study. A previously described PCR method was used to screen for isolates carrying an IncI1 plasmid (Carattoli et al., 2005). Four IncI1-Iγ positive *E. coli* isolates were identified. WGS was performed as described above. An IncI1-Iγ/ST3 signature was recovered in one isolate (pEC345).

We performed nanopore sequencing (Oxford Nanopore Technology®[3]) to fully sequence IncI1-Iγ/ST3 plasmids in two ESBL-Ec isolates representative of the genomic diversity of our set of ESBL-Ec, namely EC405 and EC440, and on 3GCS-Ec, namely pEC345, as previously described (accession numbers pEC405: CP094200; pEC440: CP094199; pEC345: CP094198) (Guyomard-Rabenirina et al., 2020). MinION reads were base-called with Guppy software v3.6.0 and further de-barcoded and screened for quality (resulting in mean q > 11) in EPI2ME v2020.2.10 "Fastq Barcoding workflow." A hybrid assembly was then performed from both high-quality Illumina and nanopore reads through the Unicycler pipeline (Wick et al., 2017). PlasmidFinder and pMLST were used to identify plasmids with an IncI1/ST3 signature. Antibiotic resistance was further confirmed by ResFinder. The $bla_{CTX-M-1}$/IncI1/ST3 profile has been described in three previously assembled and annotated plasmids carried by *E. coli* from Guadeloupe, pEC38 (GenBank accession number CP053677) associated with a bloodstream infection in a patient living in Guadeloupe, pEC1 from a bird (CP053560) and pEc7 (CP053679) from a rat (Guyomard-Rabenirina et al., 2020). Open reading frames and pseudogenes were predicted by RAST 2.0 (Brettin et al., 2015) and were further annotated in BLASTP/BLASTN against the UniProtKB/Swiss-Prot (Boutet et al., 2016). Mobile elements and resistance genes were annotated with ISfinder (Siguier et al., 2012), ResFinder (Zankari et al., 2012) and PlasmidFinder (Carattoli et al., 2014) in online databases. Multiple and paired sequences were compared with MAUVE (Darling et al., 2004) and Easyfig (Sullivan et al., 2011). The nucleotide sequence of pEC405 was searched with Mash Screen (Ondov et al., 2019) in the PLSDB database (a resource containing 34,513 plasmid sequences collected from the NCBI nucleotide database) (Galata et al., 2019).

Conjugation Efficiency of bla_{CTXM-1}/IncI1-Iγ/ST3 Plasmids

Conjugation in LB was performed with *E. coli* K12 J5 resistant to sodium azide as the recipient strain, as described previously (Wang et al., 2008). We used the 4 $bla_{CTX-M-1}$ positive *E. coli* isolates (pEC38, pEC405, pEC440, pEC1, and pEC7) described above as plasmid donors. The donor and recipient isolates were grown separately in 10 mL of fresh LB broth during 6 h at 37°C with shaking. Optical densities were measured at 600 nm to ensure that cultures of donor and recipient were in logarithmic phase. The donor and recipient strains were mixed in equal volume (1 mL) in 8 mL of fresh LB broth and incubated overnight with shaking. Transconjugants were selected on Drigalski agar (Bio-Rad) supplemented with sodium azide (500 mg/L; Sigma Chemical Co., United States) and cefotaxime (4 mg/L; Sigma Chemical Co., United States). Conjugation experiments were performed at a donor/recipient ratio of 2/1. All conjugation

[1]https://github.com/tseemann/mlst

[2]https://github.com/tseemann/abricate

[3]https://nanoporetech.com/

experiments were performed in triplicate. Each donor strain and recipient isolate were sub-cultured on media with cefotaxime and/or sodium azide as controls. After 24 h, the plates were counted, and five colonies were selected from each agar plate for verification by PCR of the presence of an IncI1 plasmid, as described above. The approximate conjugation efficiency was determined as the ratio of the number of transconjugants obtained to the number of donor cells.

Statistical Analysis

Quantitative variables were summarized as medians with interquartile ranges (IQRs), and categorical data were expressed as percentages. In bivariate analyses, the χ^2 test (or Fisher's exact test when appropriate) was used to compare categorical variables. A logistic regression model was used to identify factors associated with the presence of ESBL-E in cats and dogs and to calculate crude and adjusted odds ratios and their 95% confidence intervals (95% CI). Multivariate analyses were not performed due to the low number of cases of ESBL-E in cats (6 cases) and in dogs (8 cases). We considered $P < 0.05$ to be significant. Microsoft Access 2003 was used for data entry. Statistical analyses were performed with Stata Version 10 and SPSS (V21, IBM SPSS Statistics, Chicago, IL, United States).

RESULTS

Prevalence and Risk Factors of ESBL-E

During the first sampling campaign, between June and September 2019, a total of 185 pets (80 males, 105 females; median age, 18 months [IQR (7–48)] were included. The prevalence of fecal carriage of ESBL-E was 7.6% (14/185, 95% CI: 4.2–12.4): 6 cats (10.0%) and 8 dogs (6.4%) (**Table 1**). No simultaneous carriage of ESBL-E isolates belonging to different species was detected. A total of 14 ESBL-E were recovered, all but one of which (*K. pneumoniae* from a cat) were *E. coli*. They were resistant to the β-lactams tested, except for cefoxitin (0% resistance), ertapenem (0% resistance), and temocillin (7% resistance). In addition, they had high rates of resistance to tetracycline (86%) and a high rate of susceptibility to trimethoprime/sulfamethoxazole (79%), nalidixic acid (86%), ciprofloxacin (86%), fosfomycin (93%), tigecycline (100%), amikacin (100%), and gentamicin (100%). The double-disk synergy test was positive for all isolates. If we included only pets from veterinary clinics, the prevalence of ESBL-E was 4.7% (95% CI: 2.1–9.1). Most of them were seen for vaccination ($n = 73$, 42.9%), surgery ($n = 31$, 18.2%), preventive health visit ($n = 26$, 15.3%) or skin and soft tissue infection ($n = 12$, 7.1%).

A stay in the shelter for cats (OR = 62.5, 95% CI: 5.8–672.8, $p < 0.001$) and chronical disease for dogs (OR = 12.1, 95% CI, 2.0–72.5, $p = 0.005$) were significantly associated with ESBL-E fecal carriage (**Table 1**).

Genetic Background and Antimicrobial Resistance Genes of ESBL-Enterobacteriaceae Isolates

All the ESBL-Ec isolates belonged to phylogenetic groups associated with commensalism, including A ($n = 2$, 15%) and B1 ($n = 11$, 85%). Separation into three branches was observed on the phylogenetic tree based on the 13 ESBL-*E. coli* isolates and constructed with RAxML (**Figure 1**). The first branch consisted only of ST328 isolates ($n = 6$) isolated from five cats and one dog collected at the animal shelter. They were nearly identical at core-genome level, with sequences separated by only 0–5 single nucleotide polymorphisms (SNPs) (**Supplementary Table 1**).

TABLE 1 | Risk factors for extended-spectrum beta-lactamase producing-*Enterobacteriaceae* rectal carriage in cats (*N* = 60) and dogs (*N* = 125) from Guadeloupe (French West Indies).

	Cats (*N* = 60)						Dogs (*N* = 125)					
	Cats	**ESBL-E[a] carriage**	**%**	**p**	**OR**	**95% CI**	**Dogs**	**ESBL-E carriage**	**%**	**p**	**OR**	**95% CI**
Sex												
Female	27	1	3.7	0.209	1		75	3	3.8	0.151	1	
Male	33	5	15.2		4.7.	0.5–41.7	47	5	10.6		3.0	0.7–13.2
Age ≥ 20 months												
No	37	6	16.2	0.075	1		55	1	1.8	0.070	1	
Yes	22	0	0.0		nc[b]		66	7	10.6		6.4	0.8–53.8
Shelter stay												
No	51	1	2.0	< 0.001	1		119	7	5.9	0.333	1	
Yes	9	5	55.6		62.5	5.8–672.8	6	1	16.7		3.2	0.3–31.2
Chronic disease												
No	55	6	10.9	1.000	1		118	5	4.2	0.005	1	
Yes	5	0	0.0		nc		7	3	42.9		17.0	3.0–97.0
Antibiotic administration ≤ 3 months												
No	52	6	11.5	0.585	1		106	6	5.7	0.350	1	
Yes	8	0	0.0		nc		19	2	10.5		2.0	0.4–10.5

[a]*Extended-spectrum β-lactamase Enterobacteriaceae.* [b]*nc, not calculable.*

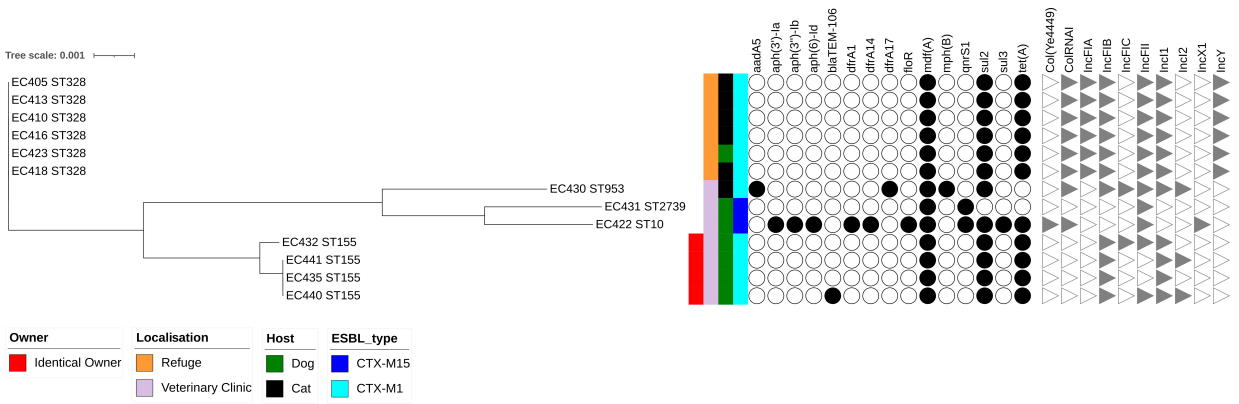


FIGURE 1 | Maximum likelihood phylogenetic tree of 13 extended-spectrum β-lactamase-producing *Escherichia coli* isolates collected in Guadeloupe based on multiple sequence alignments of the 3,429 core genome loci. Sequence type (ST) is indicated for each isolate. Hosts, ESBL genes, site of inclusion, and an identical owner are indicated by vertical colored strips. Other antibiotic resistance genes characterized by ResFinder and Inc. incompatibility group characterized by PlasmidFinder are indicated by black dots and gray triangles, respectively.

A second branch contained two isolates (EC431 and EC422) assigned to ST10 and ST2739, respectively, from dogs. The last branch consisted of four ST155 isolates collected from dogs. We found 0–1 SNP among three closely related isolates (EC435, EC440, and EC441) belonging to the same owner, whereas 70 SNPs separated them from isolate EC432. All the dogs had attended the same veterinary clinic. The ESBL-producing *K. pneumoniae* was assigned to ST17.

Two types of ESBL gene were identified, $bla_{CTX-M-15}$ (3/14) and $bla_{CTX-M-1}$ (11/14) (**Figure 1** and **Supplementary Table 2**). All 11 isolates carrying $bla_{CTX-M-1}$ were assigned to *E. coli* spp. and belonged to three STs: ST328 (n = 6), ST155 (n = 4), and ST953 (n = 1). The $bla_{CTX-M-1}$ gene, systematically associated with *sul2* gene, conferring resistance to sulfonamides. An IncI1-Iγ/ST3 signature was detected in all *E. coli* isolates carrying an $bla_{CTX-M-1}$ gene.

One $bla_{CTX-M-15}$ was associated with a *K. pneumoniae* isolate (ST17) collected from a cat. It also harbored two genes coding for β-lactamases, $bla_{SHV-187}$ and bla_{TEM-1B}, and also genes coding for *aph*(3″)-*Ib*, *aph*(6)-*Id*, *dfrA14*, *fosA6*, *mdf*(*A*), *qnrB1*, *sul2* and *tet*(*A*) genes, conferring resistance to aminoglycosides, trimethoprim, fosfomycin, macrolides, quinolones and tetracyclines, respectively (**Supplementary Table 2**).

Sequence Analyses and Synteny of IncI1-Iγ/ST3 Plasmids

Four IncI1-Iγ positive *E. coli* isolates were identified by a specific PCR among 51 3GCS-Ec isolated from frozen stools collected from 51 randomly chosen pets included in this study. An IncI1-Iγ/ST3 signature was recovered in one isolate (pEC345), the three others being assigned to ST7. The long-read sequencing approach was used to determine the full sequence of the previous IncI1-Iγ/ST3 plasmid and that of 2 $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmids, representative of the genomic diversity of our set of ESBL-Ec isolates, namely, pEC405 and pEC440 (**Figure 1** and **Table 2**). To better characterize the plasmids circulating in Guadeloupe, three $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmids previously isolated in Guadeloupe from *E. coli* isolates associated with a human (pEC38), a bird (pEC1) and a rat (pEC7) were compared (Guyomard-Rabenirina et al., 2020). Plasmid pEC405 shared 98% of its sequence with pEC440 and pEC38, 94% with pEC1 and pEc7, 87% with pEC345. Nucleotide identity was > 99%. The plasmids can be divided into four functional modules consisting of antimicrobial resistance-encoding loci with plasmid replication, plasmid transfer, and plasmid maintenance functions. The *sul2* gene was present in both plasmids, $bla_{CTX-M-1}$ being recovered in all ESBL-Ec (**Table 2**). No gene associated with resistance to heavy metal ions was identified. A type II toxin–antitoxin system consisting of YacA and YacB was present in all plasmids, and they encoded a colicin.

Sequence alignment of the six IncI1-Iγ/ST3 signature plasmids showed the presence of two accessory regions containing antibiotic resistance genes (**Figure 2**). Smaller variations were observed at other locations on these plasmids. The first accessory region was delimited by *repA* (1–1,077 bp according to the reference plasmid pEC405) and by the *glmM* gene (11,875–12,177 bp) (**Figure 3**). The alignment was similar in ESBL-Ec plasmids from pets (pEC405 and pEC440) and from a human bloodstream infection (pEC38). The antimicrobial resistance genes *sul2* and *tet*(*A*) were present, as well as a non-functional truncated transposase belonging to the Tn3 family. A large section of the accessory module was different in pEC1 (bird) and pEC7 (rat) plasmids from previous plasmids (from 4,838 to 10,047 bp according to the reference plasmid pEC1). *aadA5*, *dfrA17*, and *sul2*, encoding resistance to streptomycin, trimethoprim, and sulfonamides, respectively, and a transposase belonging to the Tn3 family were present. This hypervariable region (from 2,401 to 14,462 bp according to the reference plasmid pEC345) was different in the plasmid associated with the 3GCS-*E. coli* (pEC345). A complete Tn3 transposon-containing *tnpA* encoding a Tn3 transposase, *tnpR* encoding a Tn3 resolvase, *dfrA1* coding for resistance to trimethoprim, *satA* conferring

resistance to streptothricin, *aadA1* coding for resistance to streptomycin and spectinomycin, and $bla_{TEM-1-B}$ encoding a β-lactamase were found. A *sul2* gene was detected outside the transposon as well as a truncated IS91 (**Figure 3**).

The *traABC* regulatory gene cluster was located at the distal end of the type IV pili locus region in IncI1-Iγ/ST3 plasmids. The shufflon consisted of several invertible DNA segments (**Table 2**) and was located at the proximal end of the type IV pili locus region. For 3GCR *E. coli*, $bla_{CTX-M-1}$ was linked with ISEcp1 and inserted into the shufflon. It was delimited by the *rci* gene encoding a shufflon-specific recombinase and by the *pilV* gene encoding a membrane protein involved in fimbrial biogenesis. The plasmid pEC405 displayed an insertion of two copies of IS21 (from 103,656 to 105,457 pb) at proximity of the *traABC* cluster (**Figure 2**). This insertion was not present in the other plasmids.

The nucleotide sequence of our reference plasmid pEC405 was compared with the plasmid records stored in the PLSDB database by Mash with a maximal *P* of 0.1 and a distance threshold set at 0.01. A total of 55 IncI1-Iγ/ST3 plasmids (minimum 98,119 bp, maximum 123,053 bp, median 108,737 bp) were obtained (**Supplementary Table 3**), 52 (95%) displaying a $bla_{CTX-M-1}$. The plasmids included in the hits were assigned to *E. coli* spp., except for one *Salmonella enterica*. They were isolated from a wide range of hosts, including animals [chicken (n = 21), pig (n = 8), turkey (n = 3), bird (n = 2), bovine (n = 2), dog (n = 1), duck (n = 1), rat (n = 1), wild boar (n = 1)], humans (n = 6) and the environment (n = 6) between 2002 and 2017, most having been extracted from samples collected in 2014 (n = 15). The isolates were mainly from Europe (n = 48), especially France (n = 10), North America (n = 4), Asia (n = 2) and Australia (n = 1). By computing pairwise distances with Mash with a distance cutoff of 0.00123693 (at least 950 of 1,000 shared hashes), we identified eight *E. coli* isolates harboring quasi-similar $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmids with the *tet(A)/sul2* region. According to the NCBI, they were found in four river samples from France, in fecal samples from two chickens and one pig from France and also in one human blood sample from Guadeloupe. The previous isolate was described above as pEC38. Syntenic analyses revealed that the previously described plasmid pEC38 shared 99% of its sequence with pEC405 while pEC440 was almost identical. Nucleotide identity was > 99.6%.

Conjugation efficiencies were substantially similar for pEC38, pEC405, pEC440, pEC1, and pEC7, between $3.1.10^{-2}$ and $8.34.10^{-2}$ (**Table 2**).

DISCUSSION

The overall prevalence of ESBL-E rectal carriage in pets (4.7%, 95% CI: 2.1–9.1) was moderate, in agreement with observations of fecal carriage among pigs (7.3%), beef cattle (14.7%) and humans (4.4%) in Guadeloupe (Gruel et al., 2021; Le Terrier et al., 2021). The prevalence of ESBL-E carriage was in the range observed worldwide (average, 6.9%) in a meta-analysis, the highest level being reported in dogs in Africa (16.5%) and in cats in Asia (16.8%) (Salgado-Caxito et al., 2021). The observed discrepancies may be due to the different populations

TABLE 2 | Characteristics associated with IncI1-Iγ/ST3 plasmids from six *Escherichia coli* isolates collected in Guadeloupe.

Isolate name	Year of isolation	Host	Biological sample	MLST	Plasmid name	pMLST	Estimated plasmid size (bp)	Antibiotic resistance genes	Shuffon regions	Virulence determinants	Conjugation efficiency Mean[a] (SD)	Accession number
EC405	2019	Cat	Feces	ST328	pEC405	ST3	109,284	$bla_{CTX-M-1}$, *tet(A)*, *sul2*	CC′BB′AA′	Colicin	0.057 (0,008)	CP094200
EC440	2019	Dog	Feces	ST155	pEC440	ST3	108,832	$bla_{CTX-M-1}$, *tet(A)*, *sul2*	C′BB′CAA′	Colicin	0.081 (0.010)	CP094199
EC38	2013	Human	Blood	ST349	pEC38	ST3	108,442	$bla_{CTX-M-1}$, *tet(A)*, *sul2*	CC′BB′AA′	Colicin	0.083 (0.003)	CP053677
EC1	2013	Bird	Feces	ST196	pEC1	ST3	114,029	*aadA5*, $bla_{CTX-M-1}$, *dfrA17*, *sul2*	B′BCC′AA′	Colicin	0.065 (0.017)	CP053560
EC7	2013	Rat	Feces	ST196	pEC7	ST3	114,029	*aadA5*, $bla_{CTX-M-1}$, *dfrA17*, *sul2*	B′BCC′AA′	Colicin	0.031 (0.014)	CP053679
EC345	2019	Dog	Feces	Unknown	pEC345	ST3	109,373	*aacA1*, bla_{TEM-1B}, *dfrA1*, *satA*, *sul2*	B′CA′	Colicin	Not done	CP094198

[a]*Conjugation experiments were performed in triplicate. All isolates produced an extended-spectrum β-lactamase ($bla_{CTX-M-1}$), except for pEC345.*

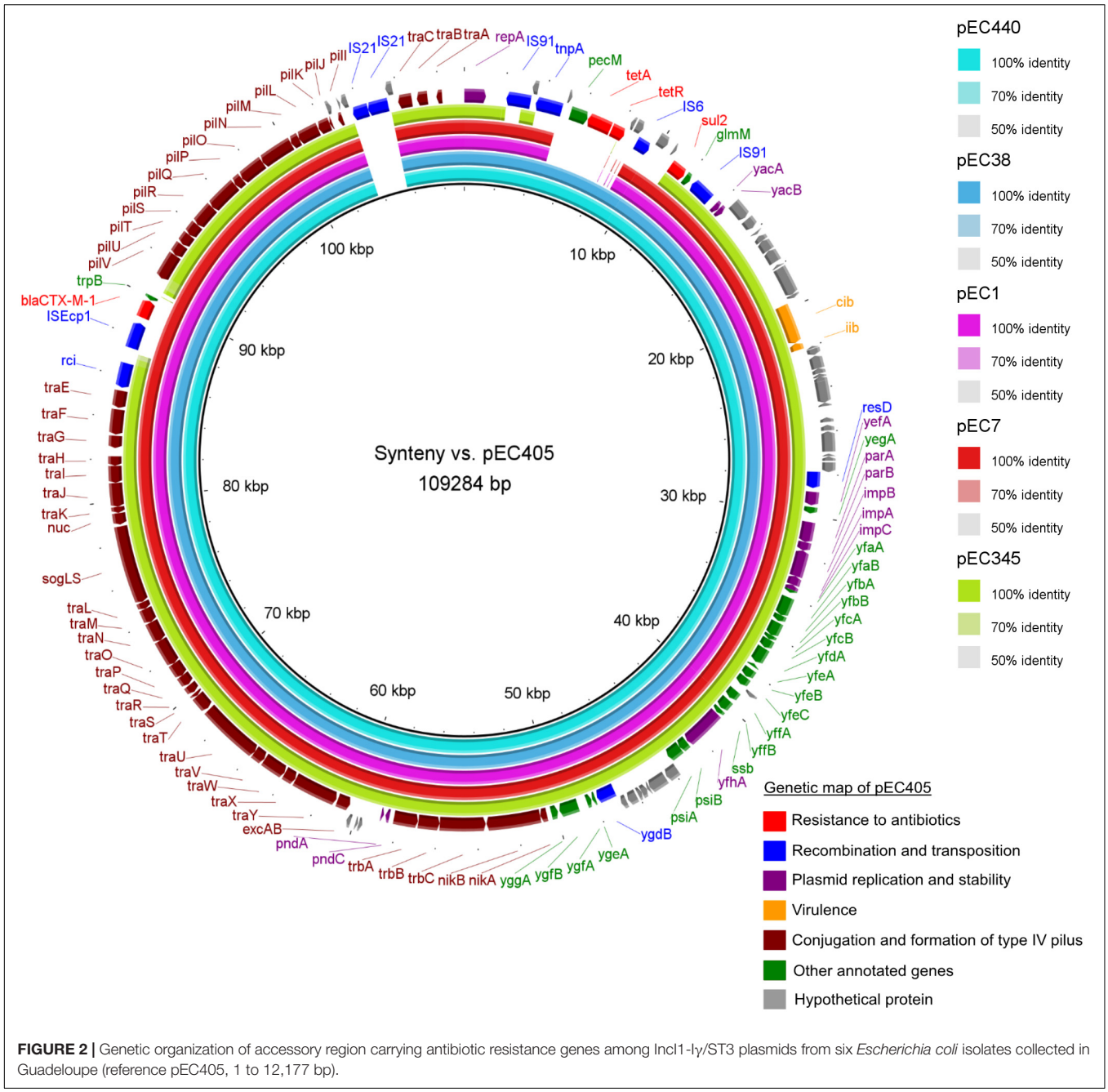


FIGURE 2 | Genetic organization of accessory region carrying antibiotic resistance genes among IncI1-Iγ/ST3 plasmids from six *Escherichia coli* isolates collected in Guadeloupe (reference pEC405, 1 to 12,177 bp).

investigated (e.g., healthy or ill individuals, geographic setting, species) or in sampling (clinical or non-clinical samples) and detection strategies.

A stay in a shelter was significantly associated with a higher prevalence of ESBL-E rectal carriage among cats, suggesting that refuges may be hotspots for their acquisition. To the best of our knowledge, this is the first time that shelters have been described as reservoirs of ESBL-E. It should be noted that domestic animals in shelters in Guadeloupe are often intended for adoption in mainland France, which would contribute to the geographic spread of ESBL-E. The ESBL-Ec isolates collected from pets living in the refuge were genetically almost identical (0–1 cgMLST allelic mismatches) and belonged to ST328, indicating spread of a single clone among pets. In view of the high ESBL-E prevalence among pets in the shelter, new hygiene and animal separation measures were implemented by veterinarians to prevent transmission. A second sampling campaign carried out in the refuge 1 year later on 15 pets that had not been sampled during the first campaign did not show the presence of ESBL-E rectal carriage (data not shown). Therefore, conditions such as overcrowding and lack of hygiene may have led to the infection. Chronic disease was

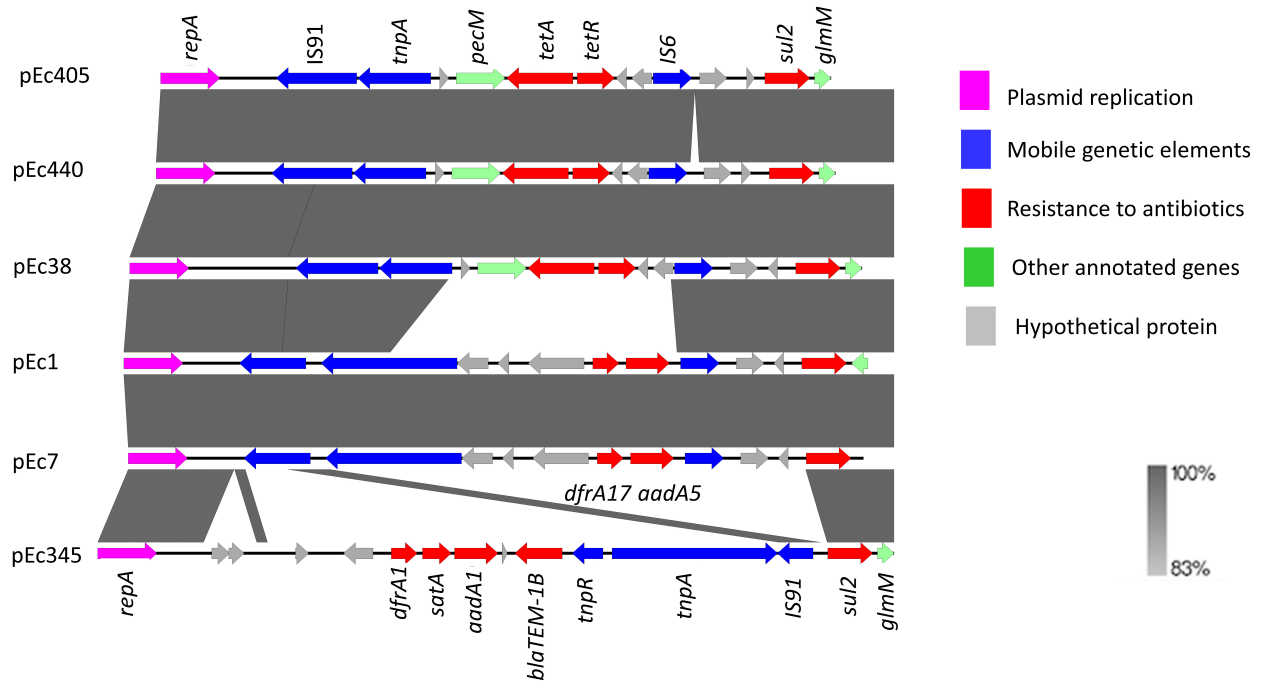


FIGURE 3 | Syntenic analysis of 6 IncI1-Iγ/ST3 plasmids from six *Escherichia coli* isolates collected in Guadeloupe. All isolates produced an extended-spectrum β-lactamase ($bla_{CTX-M-1}$) except for pEC345. The innermost black ring 1 represents the reference sequence of pEC405. The subsequent rings correspond to pairwise comparisons with pEC405: ring 2 represents pEC440; ring 3, pEC38; ring 4, pEC1; ring 5, pEc7; ring 6, pEC345. The last two rings represent a genetic map of pEC405.

significantly associated with a higher prevalence of ESBL-E rectal carriage among dogs, as previously described in humans (Hu et al., 2020).

Five STs were recovered, of which two were internationally successful clones (ST10 and ST155), both isolated from dogs. These two major clones have been described extensively in humans (Rafai et al., 2015), in domestic animals worldwide (Salgado-Caxito et al., 2021) and also in numerous other animal species, such as healthy poultry (Bortolaia et al., 2011) and migratory avian species (Mohsin et al., 2017). They were also isolated in two horses in Guadeloupe (Sadikalay et al., 2018), and ST10 was isolated from an iguana (Guyomard-Rabenirina et al., 2020). These cases highlight the potential role of companion animals in the transmission of ESBL-E to humans and vice versa. To the best of our knowledge, other genetic backgrounds such as ST328, ST953 and ST2739 have never or rarely been associated with pets. No $bla_{CTX-M-15}$-producing *E. coli* ST131 was isolated in our study, even though the successful pandemic clone *E. coli* O25b:H4-ST131 is a serious concern worldwide (Coque et al., 2008; Rogers et al., 2011). Three ESBL-Ec ST155 isolates (EC435, EC440, and EC441) were separated by 0–1 SNPs. They were isolated from three dogs in the same household, indicating clonal transmission. Another isolate belonging to ST155 was separated from the three previous isolates by 70 SNPs and was isolated from another dog that had lived for 6 months in the same household as the other three dogs 18 months previously, indicating horizontal transmission. The only ESBL-producing *K. pneumoniae* was assigned to ST17, a successful human clone that has emerged as an important vehicle for worldwide dissemination of antibiotic-resistance genes (Huynh et al., 2020).

Most of ESBL-Ec isolated in this study (86%) carried $bla_{CTX-M-1}$, in agreement with previous studies in Guadeloupe among healthy food-producing animals and wild fauna (Guyomard-Rabenirina et al., 2020; Gruel et al., 2021). $bla_{CTX-M-1}$ is considered to be the most common ESBL in livestock and domestic animals (European Food Safety Authority and European Centre for Disease Prevention and Control, 2018). In our study, the $bla_{CTX-M-1}$ gene from our ESBL-*Ec* isolates was systematically associated with an IncI1-Iγ/ST3 plasmid. Two plasmids representative of the global genomic diversity of our set of ESBL-Ec isolates from pets were sequenced and found to be closely related to previously described $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmids found in three *E. coli* isolates from Guadeloupe associated with a human bloodstream infection and with feces from a rat and a bird (Guyomard-Rabenirina et al., 2020), although they were isolated 5 years apart. Two accessory modules carrying antimicrobial resistance genes were observed, in accordance with previous findings (Mo et al., 2020). In addition, shorter variations were observed at different positions of the plasmid, confirming that IncI1-Iγ can integrate accessory genes at different positions.

The $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmid is epidemiologically relevant, as it has been recovered in *E. coli* isolates from numerous

animal and human sources in several countries in Europe, Asia and North America (Zurfluh et al., 2015; Baron et al., 2020; Moffat et al., 2020), indicating its significant role in the worldwide spread of $bla_{CTX-M-1}$ genes (Carattoli et al., 2021). Furthermore, $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmids quasi similar to those found in *E. coli* from Guadeloupe (reference pEC405) were described in *E. coli* isolates from France recovered from four river samples and two chicken fecal samples, demonstrating its fitness, which contributes to its persistence in *E. coli* communities and its geographical spread. Its higher frequency in animals than in humans suggests an animal contribution to the CTX-M-1 reservoir in humans through the spread of this specific $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmid. To increase knowledge on its emergence, we investigated the prevalence of IncI1-Iγ/ST3 plasmids in 3GCS *E. coli* in domestic animals. Although it is rare (only one isolate), its presence should be considered in the perspective of the ubiquitous character of *E. coli*. This group of plasmids is present, suggesting that they have acquired resistance to 3GC by insertion of $bla_{CTX-M-1}$ into existing plasmids in animals rather than by wide spread of previously uncommon plasmids. The use of antibiotics in animals has probably favored their spread.

Although plasmids are abundant in bacterial populations, these genetic elements are generally responsible for a decrease in fitness due to physiological alterations in their bacterial hosts (Alonso-Del Valle et al., 2021). The only potential advantages associated with acquisition of $bla_{CTX-M-1}$/IncI1-Iγ/ST3 are associated with the presence of antibiotic resistance genes. In an *in vivo* model in chickens, competitive exclusion of *E. coli* carrying an $bla_{CTX-M-1}$/IncI1-Iγ/ST7 could decrease the levels of bacteria carrying this plasmid through differences in growth rate compared to resident bacteria already present in the microbiome (Fischer et al., 2019). Different strategies can be proposed to explain the persistence of plasmids in bacterial populations over long periods in the absence of direct selection pressure. *In vitro* studies have shown that IncI1 plasmids harboring $bla_{CTX-M-1}$ show no or negligible fitness loss in ESBL-Ec strains and that these plasmids can persist for long periods in the absence of antimicrobial selection (Fischer et al., 2014). All these plasmids display a toxin/antitoxin system that is beneficial for stable inheritance of the plasmids and encode a colicin that represent a selective advantage (Majeed et al., 2011), as it can successfully compete with related bacteria in the gut without colicin production. In addition, *in vivo* and *in vitro* studies demonstrate that horizontal transfer of the ESBL gene by conjugation can outweigh competition by growth, in the absence of antibiotics, resulting in expansion and persistence of IncI1 plasmids carrying $bla_{CTX-M-1}$, in agreement with the high conjugation efficiency of our IncI1-Iγ/ST3 plasmids (Fischer et al., 2019). This is worrisome, as, if this hypothesis is confirmed, a reduction in antibiotic use is probably insufficient to reverse the trend, and new strategies will be required to inhibit conjugation (Lopatkin et al., 2017).

In conclusion, we found a moderate prevalence of ESBL-E fecal carriage in healthy dogs and cats in Guadeloupe (7.6%). Well-conserved $bla_{CTX-M-1}$/IncI1-Iγ/ST3 plasmids are spread widely among domestic animals and humans in Guadeloupe. The findings stress the importance of basic hygiene measures for owners in close proximity to companion animals.

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: NCBI, PRJNA798606, PRJNA600948, and PRJNA798907.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for Ethics in animal experiments of the French West Indies and Guyana. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SG-R, J-CB, SF, and SB conceived and designed the study. GG, SG-R, and SF collected biological samples, isolates, and epidemiological data. GG, DC, GA, and SB analyzed the data. BT performed the statistical analyses. GG, DC, and SB wrote the manuscript. All authors critically revised the manuscript and read and approved the final manuscript.

FUNDING

This work was supported by a FEDER grant financed by the European Union and Guadeloupe Region (Program Opérationnel FEDER-Guadeloupe-Conseil Régional 2014-2020, grant number 2015-FED-192).

ACKNOWLEDGMENTS

We would like to thank all the technicians and students involved in this work at the Institut Pasteur of Guadeloupe, particularly Paola Claude. We would also like to thank E. Heseltine for editorial assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.882422/full#supplementary-material

Supplementary Table 1 | Comparison of differential SNPs detected in 13 extended-spectrum β-lactamase-producing *Escherichia coli* isolates collected in Guadeloupe (French West Indies).

Supplementary Table 2 | Molecular characteristics of 13 extended-spectrum β-lactamase-producing *Escherichia coli* isolates from pets in Guadeloupe (French West Indies).

Supplementary Table 3 | Metadata on IncI1-Iγ/ST3 plasmids in the PLSDB database that share at least 800 of 1000 hashes with pEC405.

REFERENCES

Alonso-Del Valle, A., Leon-Sampedro, R., Rodriguez-Beltran, J., Delafuente, J., Hernandez-Garcia, M., Ruiz-Garbajosa, P., et al. (2021). Variability of plasmid fitness effects contributes to plasmid persistence in bacterial communities. *Nat. Commun.* 12:2653. doi: 10.1038/s41467-021-22849-y

AMVM (2020). *Pet Ownership is on the Rise. American Veterinary Medical Association*. Available online at: https://www.avma.org/blog/pet-ownership-rise (accessed February 22, 2022).

AMVM (2021). *Pet Population Still on the Rise, with Fewer Pets Per Household. American Veterinary Medical Association*. Available online at: https://www.avma.org/javma-news/2021-12-01/pet-population-still-rise-fewer-pets-household (accessed January 22, 2022).

Baede, V. O., Broens, E. M., Spaninks, M. P., Timmerman, A. J., Graveland, H., Wagenaar, J. A., et al. (2017). Raw pet food as a risk factor for shedding of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in household cats. *PLoS One* 12:e0187239. doi: 10.1371/journal.pone.0187239

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Baron, S., Le Devendec, L., Lucas, P., Larvor, E., Jove, T., and Kempf, I. (2020). Characterisation of plasmids harbouring extended-spectrum cephalosporin resistance genes in *Escherichia coli* from French rivers. *Vet. Microbiol.* 243:108619. doi: 10.1016/j.vetmic.2020.108619

Bastian, S., Nordmann, P., Creton, E., Malpote, E., Thiery, G., Martino, F., et al. (2015). First case of NDM-1 producing *Klebsiella pneumoniae* in Caribbean islands. *Int. J. Infect. Dis.* 34, 53–54. doi: 10.1016/j.ijid.2015.03.002

Bortolaia, V., Larsen, J., Damborg, P., and Guardabassi, L. (2011). Potential pathogenicity and host range of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates from healthy poultry. *Appl. Environ. Microbiol.* 77, 5830–5833. doi: 10.1128/AEM.02890-10

Boutet, E., Lieberherr, D., Tognolli, M., Schneider, M., Bansal, P., Bridge, A. J., et al. (2016). UniProtKB/Swiss-Prot, the manually annotated section of the uniprot knowledgebase: how to use the entry view. *Methods Mol. Biol.* 1374, 23–54. doi: 10.1007/978-1-4939-3167-5_2

Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., et al. (2015). RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep.* 5:8365. doi: 10.1038/sre08365

Breurec, S., Bastian, S., Cuzon, G., Bernabeu, S., Foucan, T., Galanth, S., et al. (2015). Emergence of OXA-48-producing *Escherichia coli* in the Caribbean islands. *J. Glob. Antimicrob. Resist.* 3, 217–218. doi: 10.1016/j.jgar.2015.04.004

Breurec, S., Guessennd, N., Timinouni, M., Le, T. A., Cao, V., Ngandjio, A., et al. (2013). *Klebsiella pneumoniae* resistant to third-generation cephalosporins in five African and two Vietnamese major towns: multiclonal population structure with two major international clonal groups, CG15 and CG258. *Clin. Microbiol. Infect.* 19, 349–355. doi: 10.1111/j.1469-0691.2012.03805.x

Bush, K., and Bradford, P. A. (2020). Epidemiology of beta-lactamase-producing pathogens. *Clin. Microbiol. Rev.* 33, e47–e19. doi: 10.1128/CMR.00047-19

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods* 63, 219–228. doi: 10.1016/j.mimet.2005.03.018

Carattoli, A., Villa, L., Fortini, D., and Garcia-Fernandez, A. (2021). Contemporary IncI1 plasmids involved in the transmission and spread of antimicrobial resistance in *Enterobacteriaceae*. *Plasmid* 118:102392. doi: 10.1016/j.plasmid.2018.12.001

Carattoli, A., Zankari, E., Garcia-Fernandez, A., Voldby Larsen, M., Lund, O., Villa, L., et al. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14

CA-SFM/EUCAST (2020). *Comité de l'antibiogramme de la Société Française de Microbiologie: Recommandations 2020*. Available online at: https://www.sfm-microbiologie.org/wp-content/uploads/2020/04/CASFM2020_Avril2020_V1.1.pdf (accessed January 22, 2022).

Coque, T. M., Novais, A., Carattoli, A., Poirel, L., Pitout, J., Peixe, L., et al. (2008). Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. *Emerg. Infect. Dis.* 14, 195–200. doi: 10.3201/eid1402.070350

Criscuolo, A., and Brisse, S. (2014). AlienTrimmer removes adapter oligonucleotides with high sensitivity in short-insert paired-end reads. commentary on turner (2014) assessment of insert sizes and adapter content in FASTQ data from NexteraXT libraries. *Front. Genet.* 5:130. doi: 10.3389/fgene.2014.00130

Darling, A. C., Mau, B., Blattner, F. R., and Perna, N. T. (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 14, 1394–1403. doi: 10.1101/gr.2289704

Didelot, X., and Wilson, D. J. (2015). ClonalFrameML: efficient inference of recombination in whole bacterial genomes. *PLoS Comput. Biol.* 11:e1004041. doi: 10.1371/journal.pcbi.1004041

Dupouy, V., Abdelli, M., Moyano, G., Arpaillange, N., Bibbal, D., Cadiergues, M. C., et al. (2019). Prevalence of beta-lactam and quinolone/fluoroquinolone resistance in *Enterobacteriaceae* from dogs in France and Spain-characterization of ESBL/pAmpC isolates, genes, and conjugative plasmids. *Front. Vet. Sci.* 6:279. doi: 10.3389/fvets.2019.00279

European Food Safety Authority and European Centre for Disease Prevention and Control (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. *EFSA J.* 16:e05500. doi: 10.2903/j.efsa.2018.5500

Fischer, E. A., Dierikx, C. M., Van Essen-Zandbergen, A., Van Roermund, H. J., Mevius, D. J., Stegeman, A., et al. (2014). The IncI1 plasmid carrying the blaCTX-M-1 gene persists in *in vitro* culture of a *Escherichia coli* strain from broilers. *BMC Microbiol.* 14:77. doi: 10.1186/1471-2180-14-77

Fischer, E.a.J, Dierikx, C. M., Van Essen-Zandbergen, A., Mevius, D., Stegeman, A., Velkers, F. C., et al. (2019). Competition between *Escherichia coli* populations with and without plasmids carrying a gene encoding extended-spectrum beta-lactamase in the broiler chicken gut. *Appl. Environ. Microbiol.* 85:e00892–19. doi: 10.1128/AEM.00892-19

Galata, V., Fehlmann, T., Backes, C., and Keller, A. (2019). PLSDB: a resource of complete bacterial plasmids. *Nucleic Acids Res.* 47, D195–D202. doi: 10.1093/nar/gky1050

Gruel, G., Sellin, A., Riveiro, H., Pot, M., Breurec, S., Guyomard-Rabenirina, S., et al. (2021). Antimicrobial use and resistance in *Escherichia coli* from healthy food-producing animals in Guadeloupe. *BMC Vet. Res.* 17:116. doi: 10.1186/s12917-021-02810-3

Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 29, 1072–1075. doi: 10.1093/bioinformatics/btt086

Guyomard-Rabenirina, S., Malespine, J., Ducat, C., Sadikalay, S., Falord, M., Harrois, D., et al. (2016). Temporal trends and risks factors for antimicrobial resistant *Enterobacteriaceae* urinary isolates from outpatients in Guadeloupe. *BMC Microbiol.* 16:121. doi: 10.1186/s12866-016-0749-9

Guyomard-Rabenirina, S., Reynaud, Y., Pot, M., Albina, E., Couvin, D., Ducat, C., et al. (2020). Antimicrobial resistance in wildlife in Guadeloupe (French West Indies): distribution of a single blaCTX-M-1/IncI1/ST3 plasmid among humans and wild animals. *Front. Microbiol.* 11:1524. doi: 10.3389/fmicb.2020.01524

Hu, Y., Matsui, Y., and Lee, W. R. (2020). Risk factors for fecal carriage of drug-resistant *Escherichia coli*: a systematic review and meta-analysis. *Antimicrob. Resist. Infect. Control* 9:31. doi: 10.1186/s13756-020-0691-3

Human Development Report (2013). *The Rise of the South: Human Progress in a Diverse World*. Available online at: http://hdr.undp.org/sites/default/files/reports/14/hdr2013_en_complete.pdf (accessed January 22, 2022).

Huynh, B. T., Passet, V., Rakotondrasoa, A., Diallo, T., Kerleguer, A., Hennart, M., et al. (2020). *Klebsiella pneumoniae* carriage in low-income countries: antimicrobial resistance, genomic diversity and risk factors. *Gut Microbes* 11, 1287–1299. doi: 10.1080/19490976.2020.1748257

Jolley, K. A., Bray, J. E., and Maiden, M. C. J. (2018). Open-access bacterial population genomics: bigsdb software, the PubMLST.org website and their applications. *Wellcome Open Res.* 3:124. doi: 10.12688/wellcomeopenres.14826.1

Le Terrier, C., Vinetti, M., Bonjean, P., Richard, R., Jarrige, B., Pons, B., et al. (2021). Impact of a restrictive antibiotic policy on the acquisition of extended-spectrum

beta-lactamase-producing *Enterobacteriaceae* in an endemic region: a before-and-after, propensity-matched cohort study in a Caribbean intensive care unit. *Crit. Care* 25:261. doi: 10.1186/s13054-021-03660-z

Letunic, I., and Bork, P. (2021). Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. doi: 10.1093/nar/gkab301

Lopatkin, A. J., Meredith, H. R., Srimani, J. K., Pfeiffer, C., Durrett, R., and You, L. (2017). Persistence and reversal of plasmid-mediated antibiotic resistance. *Nat. Commun.* 8:1689. doi: 10.1038/s41467-017-01532-1

Majeed, H., Gillor, O., Kerr, B., and Riley, M. A. (2011). Competitive interactions in *Escherichia coli* populations: the role of bacteriocins. *ISME J.* 5, 71–81. doi: 10.1038/ismej.2010.90

Mo, S. S., Telke, A. A., Osei, K. O., Sekse, C., Slettemeas, J. S., Urdahl, A. M., et al. (2020). blaCTX-M-1/IncI1-Igamma plasmids circulating in *Escherichia coli* from norwegian broiler production are related, but distinguishable. *Front. Microbiol.* 11:333. doi: 10.3389/fmicb.2020.00333

Moffat, J., Chalmers, G., Reid-Smith, R., Mulvey, M. R., Agunos, A., Calvert, J., et al. (2020). Resistance to extended-spectrum cephalosporins in *Escherichia coli* and other enterobacterales from Canadian turkeys. *PLoS One* 15:e0236442. doi: 10.1371/journal.pone.0236442

Mohsin, M., Raza, S., Schaufler, K., Roschanski, N., Sarwar, F., Semmler, T., et al. (2017). High prevalence of CTX-M-15-Type ESBL-producing *Escherichia coli* from migratory avian species in Pakistan. *Front. Microbiol.* 8:2476. doi: 10.3389/fmicb.2017.02476

Ondov, B. D., Starrett, G. J., Sappington, A., Kostic, A., Koren, S., Buck, C. B., et al. (2019). Mash screen: high-throughput sequence containment estimation for genome discovery. *Genome Biol.* 20:232. doi: 10.1186/s13059-019-1841-x

O'Neill, J. (2016). *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations*. Available online at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf (accessed January 22, 2022).

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Rafai, C., Frank, T., Manirakiza, A., Gaudeuille, A., Mbecko, J. R., Nghario, L., et al. (2015). Dissemination of IncF-type plasmids in multiresistant CTX-M-15-producing *Enterobacteriaceae* isolates from surgical-site infections in Bangui, Central African Republic. *BMC Microbiol.* 15:15. doi: 10.1186/s12866-015-0348-1

Rogers, B. A., Sidjabat, H. E., and Paterson, D. L. (2011). *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. *J. Antimicrob. Chemother.* 66, 1–14. doi: 10.1093/jac/dkq415

Sadikalay, S., Reynaud, Y., Guyomard-Rabenirina, S., Falord, M., Ducat, C., Fabre, L., et al. (2018). High genetic diversity of extended-spectrum beta-lactamases producing *Escherichia coli* in feces of horses. *Vet. Microbiol.* 219, 117–122. doi: 10.1016/j.vetmic.2018.04.016

Salgado-Caxito, M., Benavides, J. A., Adell, A. D., Paes, A. C., and Moreno-Switt, A. I. (2021). Global prevalence and molecular characterization of extended-spectrum beta-lactamase producing-*Escherichia coli* in dogs and cats - a scoping review and meta-analysis. *One Health* 12:100236. doi: 10.1016/j.onehlt.2021.100236

Siguier, P., Varani, A., Perochon, J., and Chandler, M. (2012). Exploring bacterial insertion sequences with ISfinder: objectives, uses, and future developments. *Methods Mol. Biol.* 859, 91–103. doi: 10.1007/978-1-61779-603-6_5

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039

Teshager, T., Dominguez, L., Moreno, M. A., Saenz, Y., Torres, C., and Cardenosa, S. (2000). Isolation of an SHV-12 beta-lactamase-producing *Escherichia coli* strain from a dog with recurrent urinary tract infections. *Antimicrob. Agents Chemother.* 44, 3483–3484. doi: 10.1128/AAC.44.12.3483-3484.2000

The European Pet Food Industry (2021). *Annual Report*. Available online at: https://fediaf.org/images/FINAL_FEDIAF_AR_2021.pdf (accessed January 22, 2022).

Wang, A., Yang, Y., Lu, Q., Wang, Y., Chen, Y., Deng, L., et al. (2008). Presence of qnr gene in *Escherichia coli* and *Klebsiella pneumoniae* resistant to ciprofloxacin isolated from pediatric patients in China. *BMC Infect. Dis.* 8:68. doi: 10.1186/1471-2334-8-68

Waters, N. R., Abram, F., Brennan, F., Holmes, A., and Pritchard, L. (2020). Easy phylotyping of *Escherichia coli via* the ezclermont web app and command-line tool. *Access Microbiol.* 2:acmi000143. doi: 10.1099/acmi.0.000143

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13:e1005595. doi: 10.1371/journal.pcbi.1005595

World Health Organization (2020). *Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics*. Available online at: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed January 22, 2022).

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zurfluh, K., Nuesch-Inderbinen, M., Morach, M., Zihler Berner, A., Hachler, H., and Stephan, R. (2015). Extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* isolated from vegetables imported from the Dominican Republic, India, Thailand, and Vietnam. *Appl. Environ. Microbiol.* 81, 3115–3120. doi: 10.1128/AEM.00258-15

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 Gruel, Couvin, Guyomard-Rabenirina, Arlet, Bambou, Pot, Roy, Talarmin, Tressieres, Ferdinand and Breurec. 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 Microbiology

ORIGINAL RESEARCH
published: 18 May 2022
doi: 10.3389/fmicb.2022.846116

High-Frequency Detection of *fosA3* and $bla_{CTX-M-55}$ Genes in *Escherichia coli* From Longitudinal Monitoring in Broiler Chicken Farms

*Maísa Fabiana Menck-Costa[1], Ana Angelita Sampaio Baptista[2], Luiz Eduardo de Souza Gazal[1], Larissa Justino[2], Matheus Silva Sanches[1], Marielen de Souza[2], Erick Kenji Nishio[1], Beatriz Queiroz dos Santos[2], Victor Dellevedove Cruz[1], João Vitor Monteiro Berbert[2], Bruna Carolina Gonçalves[1], Galdino Andrade[1], Eliana Carolina Vespero[3], Gerson Nakazato[1] and Renata Katsuko Takayama Kobayashi[1]**

[1] Department of Microbiology, Biological Sciences Center, State University of Londrina, Londrina, Brazil, [2] Department of Preventive Veterinary Medicine, Avian Medicine Laboratory, State University of Londrina, Londrina, Brazil, [3] Department of Pathology, Clinical Analysis and Toxicology, Health Sciences Center, State University of Londrina, Londrina, Brazil

OPEN ACCESS

Edited by:
Ziad Daoud,
Central Michigan University,
United States

Reviewed by:
Dandan He,
Henan Agricultural University, China
Stephanie Lo,
Wellcome Sanger Institute (WT),
United Kingdom

***Correspondence:**
Renata Katsuko Takayama Kobayashi
kobayashirkt@uel.br

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 30 December 2021
Accepted: 11 April 2022
Published: 18 May 2022

Citation:
Menck-Costa MF, Baptista AAS, Gazal LES, Justino L, Sanches MS, de Souza M, Nishio EK, Queiroz dos Santos B, Cruz VD, Berbert JVM, Gonçalves BC, Andrade G, Vespero EC, Nakazato G and Kobayashi RKT (2022) High-Frequency Detection of fosA3 and $bla_{CTX-M-55}$ Genes in Escherichia coli From Longitudinal Monitoring in Broiler Chicken Farms. Front. Microbiol. 13:846116. doi: 10.3389/fmicb.2022.846116

Considering the worrying emergence of multidrug resistance, including in animal husbandry and especially in food-producing animals, the need to detect antimicrobial resistance strains in poultry environments is relevant, mainly considering a One Health approach. Thus, this study aimed to conduct longitudinal monitoring of antimicrobial resistance in broiler chicken farms, with an emphasis on evaluating the frequency of resistance to fosfomycin and β-lactams. *Escherichia coli* was isolated from broiler chicken farms (cloacal swabs, meconium, poultry feed, water, poultry litter, and *Alphitobius diaperinus*) in northern Paraná from 2019 to 2020 during three periods: the first period (1st days of life), the second period (20th to 25th days of life), and third period (40th to 42nd days of life). Antibiogram tests and the detection of phenotypic extended-spectrum β-lactamase (ESBL) were performed, and they were confirmed by seaching for genes from the bla_{CTX-M} group. The other resistance genes searched were *mcr-1* and *fosA3*. Some ESBL $bla_{CTX-M-1}$ group strains were selected for ESBL identification by sequencing and enterobacterial repetitive intergenic consensus-polymerase chain reaction analysis. To determine the transferability of the $bla_{CTX-M-1-}$ and *fosA3*-carrying plasmids, strains were subjected to conjugation experiments. A total of 507 *E. coli* were analyzed: 360 from cloacal swabs, 24 from meconium samples, 3 from poultry feed samples, 18 from water samples, 69 from poultry litter samples, and 33 from *A. diaperinus* samples. Among the strain isolate, 80% (406/507) were multidrug-resistant (MDR), and 51% (260/507) were ESBL-positive, with the $bla_{CTX-M-1}$ group being the most frequent. For the *fosA3* gene, 68% (344/507) of the strains isolated were positive, deserves to be highlighted *E. coli* isolated from day-old chickens (OR 6.34,

CI 2.34–17.17), when compared with strains isolated from other origins (poultry litter, *A. diaperinus*, water, and poultry feed). This work alerts us to the high frequency of the *fosA3* gene correlated with the CTX-M-1 group (OR 3.57, CI 95% 2.7–4.72, $p < 0.05$), especially the $bla_{CTX-M-55}$ gene, in broiler chickens. This profile was observed mainly in day-old chicken, with a high percentage of *E. coli* that were MDR. The findings emphasize the importance of conducting longitudinal monitoring to detect the primary risk points during poultry production.

Keywords: $bla_{CTX-M-55}$ gene, *fosA3* gene, broiler chicken farms, multidrug resistance, fosfomycin, longitudinal monitoring, ESBL (extended spectrum beta lactamase)

INTRODUCTION

Antimicrobial resistance is a global public health threat, as infections with multiresistant bacteria are predicted to become the leading cause of death 30 years from now (O'Neill, 2014; European Commission, 2018). Approximately 33,000 deaths/year are estimated to occur in the European Union due to bacterial resistance to antibiotics (Cassini et al., 2019). Currently, strains with a high resistance profile (Avershina et al., 2021a,b; Zalewska et al., 2021), which could be aggravated by the increased use of antimicrobials during the SARS Cov-2 pandemic, are being increasingly detected (Abelenda-Alonso et al., 2020; Huttner et al., 2020; Lobie et al., 2021; Nieuwlaat et al., 2021).

The One Health concept allows for a greater understanding of the scope of this issue (Thanner et al., 2016; Aslam et al., 2021; Gazal et al., 2021), given the connection that exists between human, animal, and environmental health (Aslam et al., 2018; Aarestrup et al., 2021). Through this, the World Health Organization (WHO), together with the United Nations Food and Agriculture Organization and the World Organization for Animal Health, have promoted the monitoring and surveillance of resistance to antimicrobials, aiming to detect the possible causes and main points responsible for the selection of multiresistant strains (Poirel et al., 2018). Therefore, establishing programs to monitor resistance and control the use of antimicrobials are strategies that can minimize the rapid spread of antimicrobial resistance genes (Schnall et al., 2019).

Some studies have already shown that the poultry environment (Cunha et al., 2017; Saharan et al., 2020; Gazal et al., 2021) and its products (Koga et al., 2015; Davis et al., 2018; Cyoia et al., 2019) are responsible for harboring and transmitting multidrug-resistant (MDR) strains that produce extended-spectrum β-lactamase (ESBL) or other mechanisms with resistance to antimicrobials, that are considered the last resort in human medicine, such as fosfomycin and colistin (WHO, 2018, 2019).

Among the microorganisms present in the poultry environment, *Escherichia coli* stands out and is even considered a bioindicator of antimicrobial resistance (Poirel et al., 2018; Manges et al., 2019; Büdel et al., 2020). MDR strains facilitate the spread of resistance genes and enhance the phenomenon of antimicrobial resistance (Cazares et al., 2020). Among the genes that encode ESBL, the CTX-M group is predominant and the most widespread (Paterson and Bonomo, 2005; Laube et al., 2013; De Oliveira et al., 2020; Song et al., 2020). The $bla_{CTX-M-1}$ group is the most prevalent gene detected from the CTX-M group, and the CTX-M-55 enzyme has already been described in animal production in countries from Europe, China, and Brazil (Cunha et al., 2017; Lukman et al., 2017; Lupo et al., 2018; Park et al., 2019; Gazal et al., 2021; Lay et al., 2021).

Fosfomycin is used in human medicine in cases of urinary tract infections caused by MDR strains in Brazil (Huttner et al., 2018; Camposda et al., 2020) and cases of bacterial prostatitis (Kwan and Beahm, 2020), with the *fosA3* gene being the most frequently associated with fosfomycin resistance.

Brazil is the world's largest exporter and the second-largest chicken meat producer. Paraná is the most productive state, and when its production is added to the other states of the southern region of Brazil, it represents 60% of the country's chicken meat production (Aquino, 2021). In the state, the broiler breeding system works by integrators. Companies provide food, birds, and technical assistance, and farmers provide labor and installations, such as poultry sheds (Frost et al., 2003; Arikan et al., 2017). Thus, common poultry nuclei supply day-old broilers to large geographic regions, constituting the broiler production pyramid (Zurfluh et al., 2014).

Due to high productivity, there is heterogeneity in the size of the farms, the type of poultry sheds, and the number of birds housed, with an average of 14 birds/m^2 (Frost et al., 2003; Arikan et al., 2017; De Oliveira Sidinei et al., 2021).

Therefore, this study aimed to conduct longitudinal monitoring of antimicrobial resistance in broiler chicken farms, emphasizing the frequency of resistance to fosfomycin and β -lactams.

MATERIALS AND METHODS

This project was approved by the Ethics Committee on the Use of Animals of the State University of Londrina – CEUA/UEL, processing number 13142.2019.51.

Poultry Farm Characteristics

Samples (cloacal swabs, meconium, water, poultry feed, poultry litter, and *Alphitobius diaperinus*) were collected from four broiler farms in the northern region of Paraná – from March 2019 to July 2020, no farms were sampled simultaneously.

The all-in/all-out system was adopted in all farms evaluated, with an interval between batches of 7 to 12 days and a housing capacity from 10,000 to 30,000 chickens. Some sheds were technified (dark house), and others were conventional sheds. The poultry litter (composed of shavings) was reused for up to seven cycles, with fermentation conducted by canvas covering between them (De Oliveira Sidinei et al., 2021).

The animals evaluated came from three different hatcheries, but all were from the same agroindustry.

All farms adopted the use of organic acids in water within 20–25 days of life (dol), and on farms 1, 2, and 3, the supply of antimicrobials was reported, such as tiamulin (before the prohibition of its use) and enrofloxacin (EN; Brasil, 2020).

Regarding water, all the farms provided chlorinated water (1–3 ppm), and the feed was provided by the integrator company, respecting the stages of chicken rearing.

Poultry Farm Samples

Sample collections (cloacal swabs, meconium, water, poultry feed, poultry litter, and *A. diaperinus*) were collected at three different periods: the first period (1st dol or day-old), the second period (20th to 25th dol), and the third period (40th to 42nd dol).

The cloacal swabs were collected on the first day of life before housing the birds, and meconium was collected from the bottom of the transport boxes. Thirty cloacal swabs were collected per farm, placed in Cary Blair medium (Absorb), and sent under refrigeration for processing. Swabs (n = 30) were grouped into five pools, totaling 60 samples.

From the environment, samples were collected from poultry litter (n = 12) with sterile boot swabs (Brasil, 2016) using two pairs of props, each sampling 50% of the shed. Feed samples (2 kg/silo per moment, totaling 12 samples) and *A. diaperinus* (100 adult beetles per house, totaling 12 samples) were collected. Water samples were collected at the beginning (initial water) and end (final water) of the drinking fountain line for a total of 24 samples. In total, 124 samples of materials from different sources were processed.

Processing and Selection of the Isolates

The water samples were analyzed by a multiple-tube fermentation technique according to Eckner (1998) and (Brasil, 2013). After incubation in buffered peptone water, at 37°C for 18–24 h, the other samples were seeded on MacConkey agar without supplementation (MC) and supplemented with the antimicrobials, ciprofloxacin (CIP), cefotaxime (CTX), and ciprofloxacin + cefotaxime (CIP/CTX), at a final concentration of 8 μg/ml. The lactose-fermenting colonies were subjected to identification by biochemical screening using triple-sugar iron agar, indole production, Simmons citrate, urease production, lysine decarboxylation, and sorbitol and cellobiose fermentation tests (Merck, Darmstadt, Alemanha; Awogbemi et al., 2018; Moeinizadeh and Shaheli, 2021; Thomrongsuwannakij et al., 2021).

Up to three *E. coli* colonies were selected from each culture medium and stored at -20°C in brain heart infusion broth (Himedia Laboratories Pvt. Ltd., Mumbai, Índia) supplemented with 20% glycerol until processing. For the subsequent analyses, six bacterial isolates from each origin were used, per farm, per period.

Antimicrobial Resistance and Extended-Spectrum β-Lactamase Production

Antimicrobial sensitivity was determined using the disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI, 2018). Nineteen antimicrobials belonging to seven different classes were used, β-lactams: amoxicillin-clavulanic acid (AMC, 10/20 μg), ampicillin (AMP, 10 μg), cefazolin (CFZ, 30 μg), cefoxitin, (CFO, 30 μg), ceftiofur (CTF, 30 μg), ceftriaxone (CRO, 30 μg), ceftazidime (CAZ, 30 μg), CTX (30 μg), CRO (30 μg), aztreonam (ATM, 30 μg), and imipenem (IPM, 30 μg); quinolones: CIP (5 μg), EN (10 μg), and nalidixic acid (NAL, 30 μg); sulfonamides: sulfamethoxazol + trimethoprim (SXT, 1.25/23.75 μg); tetracyclines: tetracycline (TET, 30 μg); aminoglycosides: gentamicin (CN, 10 μg); amphenicols: chloramphenicol (C, 30 μg); and fosfomycin: fosfomycin/trometamol (FOT, 200 μg; Oxoid Ltd., Basingstoke, Hants, Reino Unido, United Kingdom). *E. coli* ATCC 25922 was used as a standard control for the antibiogram test. The results were interpreted according to CLSI (2019) and BRCAST (2019) except for CTF and EN, which followed CLSI (2008).

For ESBL detection, the double synergism technique was performed (Jarlier et al., 1988) with an AMC disk (10/20 μg), placed at 20 mm disks of ATM (30 μg), CAZ (30 μg), CTF (30 μg), and cefepime (FEP, 30 μ g).

Antimicrobial Resistance Survey

Bacterial DNA was extracted using the Pure Link Genomic DNA Mini Kit (Invitrogen).

We searched for genes that conferred resistance to β-lactams ($bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-25}$; Woodford et al., 2006), colistin resistance coding genes (*mcr-1*; Liu et al., 2016), and fosfomycin (*fosA3*; Sato et al., 2013).

All PCR amplicons were visualized on 1.5% agarose gels stained with GelRed (Biotium, Hayward, CA, United States). After gel electrophoresis, the images were captured using Image Capture Systems (LPixImageHE).

Analysis of the Genetic Similarity Profile by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction

The genetic similarity profile was evaluated by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) analysis, in accordance with Versalovic et al. (1991). The PCR products were subjected to 2% agarose gel electrophoresis for 4 h in TBE buffer, stained with ethidium bromide (0.5 μg/mL), and visualized in a UV transilluminator (Vilbert Loumart). The similarity dendrogram was constructed using Gel J 2.0 software (Heras et al., 2015), using the unweighted pair group method with arithmetic mean (UPGMA) and the data similarity coefficient for cluster

analysis (Jaccard), with a tolerance index of 1.0. The standard cutoff level to define the clusters was 85% (Daga et al., 2019).

Sequencing

Sequencing was performed from the PCR product amplified for IS*Ecp* (Zurita et al., 2016). When negative for this insertion sequence, the amplified product for the gene using $bla_{CTX-M-1}$ (Fei Tian et al., 2011; Cantón et al., 2012; Dierikx et al., 2013) was characterized for bidirectional Sanger sequencing on ABI-PRISM 3500 XL (Applied Biosystems) following the manufacturer's recommendations. For the interpretation and alignment of the sequencing results, Chromas was used to evaluate the electropherogram, and ClustalW was used to achieve alignment of the sequences. Using BLAST, the sequences were compared with the NCBI database.

Conjugation Experiment

Conjugation was performed to assess the horizontal transfer capacity of the $bla_{CTX-M-55}$ gene and *fosA3* (Gonçalves et al., 2021). Two *E. coli* $bla_{CTX-M-55}$ and *fosA3* from Group B1 (negative for *chuA* and *YjaA* and positive for TspE4 *and ArpA*) were used as donors, and one *E. coli* from Group D (negative for TspE4 *and YjaA* and positive for *chuA and ArpA*) resistant to gentamicin was used as the recipient. All isolates were from day-old chickens (Clermont et al., 2013).

Statistical Evaluation

Statistical analysis was performed using R version 3.5.1. To assess the relationship between the studied variables, multivariate logistic regression analysis was used. The odds ratio (OD) calculation of the prediction model ($\alpha = 5\%$) was used to identify the set of information that best explained the relationship of risk factors associated with the occurrence of *fosA3*- and ESBL-producing *E. coli* (Menard, 2002).

RESULTS

Escherichia coli Phenotypic and Genotypic Profile

A total of 507 *E. coli* were isolated: 360 from cloacal swabs, 24 from meconium, 3 from poultry feed, 18 from water, 69 from poultry litter, and 33 from *A. diaperinus* (**Table 1**).

Among the 507 *E. coli* isolated, fosfomycin (41% – 202/507) and AMP (68% – 347/507) are the antimicrobials that deserve the most attention due to their high percentage of resistance. Therefore, more than 80.1% (406/507) were MDR, and more than 51% (260/507) were ESBL producers. Regarding resistance to β-lactams, the $bla_{CTX-M-1}$ group, was present in 40% (202/507) of the strains, the most frequent bla_{CTX-M} group, followed by the $bla_{CTX-M-2}$ group at 17% (86/507; **Table 2**). The strains that presented some gene from the bla_{CTX-M} group had greater resistance to CAZ (98.9%, 268/271), CFZ (94.5%, 256/271), and AMP (92.3%, 250/271).

Comparing the resistance profiles of the strains, no significant difference was observed between the sampled periods regarding the presence of MDR and *fosA3* and the occurrence of ESBL. For the simultaneous presence of the *fosA3* and bla_{CTX-M} genes, the strain that had *fosA3* was 3.57 (CI 2.7–4.72) times more likely to also have a gene in the $bla_{CTX-M-1}$ group.

Escherichia coli Isolated From Cloacal Swab and Meconium Samples

Twenty-four *E. coli* samples from meconium and 360 *E. coli* samples from cloacal swabs were isolated, totaling 384 *E. coli* from chickens (**Table 1**). On all farms surveyed and, in all periods, *E. coli* ESBL-production was detected.

In the first period, *E. coli* resistant to CTX (70% – 101/144) and fosfomycin (47.9% – 69/144) was isolated from meconium and cloacal swab samples, and the first period was 1.64 (CI 1.01–2.65) times more likely to harbor the *fosA3* gene, than other periods. In the second period, the highest percentage of MDR was observed (90% – 108/120). In the third period, there was a decrease in strains resistant to some antimicrobials, such as fosfomycin (34% – 41/120), quinolones, and third and fourth generation cephalosporins, and it was the period with the lowest number of strains harboring the *fosA3* gene (OR 0.49, CI 0.31–0.77), compared to the first and second periods (**Figure 1**).

Strains isolated from chickens (meconium + cloacal swabs) were 1.62 (CI 1.19–2.2) times more likely to be ESBL producers and present the *fosA3* gene than the other samples. Cloacal swab samples were 2.53 (CI 1.61–3.97) times more likely to be resistant to three or more classes of antimicrobials.

None of the strains isolated were positive for the *mcr-1* gene or $bla_{CTX-M-9}$ and $bla_{CTX-M-25}$ groups. However, five strains isolated from cloacal swabs had the $bla_{CTX-M-8}$ group, and they were from different periods and cloacal swab samples but belonged to the same farm.

Escherichia coli Isolated From Poultry Litter and *A. diaperinus* Samples

A total of 69 *E. coli* isolated from poultry litter were obtained (**Table 1**). In one of the farms, no strains isolated from poultry litter were resistant to CIP or CTX in the first period. In the second period, the frequency of the isolation of ESBL-producing *E. coli* was higher than that of the others, so the MDR profile prevailed in this evaluation period (83.3% – 20/24). Notably, a gradual increase in resistance to fosfomycin and CTX was found from the first period to the second (**Figure 2**). Strains isolated from poultry litter were more likely to be ESBL producers (OR 2.07, CI 1.31–3.27); additionally, they were more likely (OR 1.64, CI 1.08–2.48) to have the *fosA3* and $bla_{CTX-M-1}$ genes than other samples.

Regarding, only one strain was positive (5% – 1/21) to the *mcr-1* gene, and it came from the first period; in this strain, the presence of the *fosA3* and $bla_{CTX-M-2}$ genes was also observed (**Table 2**).

TABLE 1 | Number of *Escherichia coli* isolates by periods and samples.

Periods	Farms	Poultry farm 1	Poultry farm 2	Poultry farm 3	Poultry farm 4	Total
	Samples	*(Number of isolates)*				
First period (1st day of life)	*Meconium*	6	6	6	6	24
	First pool of cloacal swab	6	6	6	6	24
	Second pool of cloacal swab	6	6	6	6	24
	The third pool of cloacal swab	6	6	6	6	24
	The fourth pool of cloacal swab	6	6	6	6	24
	The fifth pool of cloacal swab	6	6	6	6	24
	Poultry feed	0	0	3	0	3
	Initial water	0	3	0	3	6
	Final water	0	3	3	3	9
	Poultry litter	3	6	6	6	21
	Alphitobius diaperinus	NA	NA	NA	NA	0
Second period (20th to 25th days of life)	*First pool of cloacal swab*	6	6	6	6	24
	Second pool of cloacal swab	6	6	6	6	24
	The third pool of cloacal swab	6	6	6	6	24
	The fourth pool of cloacal swab	6	6	6	6	24
	The fifth pool of cloacal swab	6	6	6	6	24
	Poultry feed	0	0	0	0	0
	Initial water	0	0	0	0	0
	Final water	0	0	0	0	0
	Poultry litter	6	6	6	6	24
	Alphitobius diaperinus	0	6	0	3	9
Third period (40th to 42nd days of life)	*First pool of cloacal swab*	6	6	6	6	24
	Second pool of cloacal swab	6	6	6	6	24
	The third pool of cloacal swab	6	6	6	6	24
	The fourth pool of cloacal swab	6	6	6	6	24
	The fifth pool of cloacal swab	6	6	6	6	24
	Poultry feed	0	0	0	0	0
	Initial water	0	0	0	0	0
	Final water	0	0	3	0	3
	Poultry litter	6	6	6	6	24
	Alphitobius diaperinus	6	6	6	6	24

A total of 33 *E. coli* strains isolated from *A. diaperinus* were collected in the second and third periods (**Table 1**). The highest percentage of MDR- and ESBL-producing isolates was observed in the third period (**Table 2**). *A. diaperinus* was not collected in the first period since these beetles were not present on the farms.

Escherichia coli Isolated From Poultry Feed and Water Samples

In the feed samples, *E. coli* were isolated on only one farm (farm 3 – **Table 1**), which showed 100% resistance to NAL, CIP, and CFZ, and were sensitive to some antimicrobials, such as fosfomycin. Poultry feed-isolated strains tended not to be MDR-, ESBL- or *fosA3*-positive (OR < 0.01).

Regarding the water samples, 18 strains were obtained, and a more significant recovery of *E. coli* occurred in the first period (**Table 1**).

None of the strains isolated from the water were MDR or ESBL positive, nor did they present any resistance genes studied. Strains isolated from start water and end water tended not to be MDR-, ESBL-, or *fosA3*-positive (OR < 0.01).

Enterobacterial Repetitive Intergenic Consensus Sequence (ERIC-PCR) and Sequencing

Forty-three bacterial isolates from poultry litter, cloacal swabs, and meconium, positive for the $bla_{CTX-M-1}$ and *fosA3* genes, were selected for ERIC-PCR analysis and $bla_{CTX-M-1}$ sequencing. The sequenced isolates carried the enzyme $bla_{CTX-M-55}$.

Regarding ERIC-PCR, ten clonal groups with more than 85% similarity were observed. Some of these groups were represented by *E. coli* isolated from cloacal swabs in the first periods but from different farms (clonal group III), *E. coli* isolated from the same farm but at different periods (clonal group I), and *E. coli* isolated from different farms, periods, and sources (clonal groups I, VI, VIII, and IX), as shown in **Figure 3**.

TABLE 2 | Percentage (%) of isolates with MDR, ESBL, $bla_{CTX-M-1}$ group, $bla_{CTX-M-2}$ group, or *fosA3* gene from the cloacal swab, meconium, poultry litter, and *A. diaperinus* samples, per period.

	Periods/Samples	First period (1st days of life) %	Second period (20th to 25th days of life) %	Third period (40th to 42nd days of life) %
		(Positive samples/total samples)		
MDR	Cloacal Swab	83.3 (100/120)	90 (108/120)	81.7 (98/120)
	Meconium	83.3 (20/24)	Not sampled	Not sampled
	Poultry litter	81 (17/21)	83.3 (20/24)	79.2 (19/24)
	A. diaperinus	Not sampled	55.6 (5/9)	75 (18/24)
ESBL	Cloacal Swab	59.2 (71/120)	54.2 (65/120)	51.7 (62/120)
	Meconium	54.2 (13/24)	Not sampled	Not sampled
	Poultry litter	42.9 (2/21)	58.3 (14/24)	50 (12/24)
	A. diaperinus	Not sampled	33.3 (3/9)	45.8 (11/24)
$bla_{CTX-M-1}$	Cloacal Swab	55 (66/120)	48.3 (58/120)	24.2 (29/120)
	Meconium	37.5 (9/24)	Not sampled	Not sampled
	Poultry litter	38.1 (8/21)	45.8 (11/24)	50 (12/24)
	A. diaperinus	Not sampled	66.7 (6/9)	12.5 (3/24)
$bla_{CTX-M-2}$	Cloacal Swab	21.7 (21/120)	15.8 (19/120)	17.5 (21/120)
	Meconium	8.3 (2/24)	Not sampled	Not sampled
	Poultry litter	23.8 (5/21)	12.5 (3/24)	8.3 (2/24)
	A. diaperinus	Not sampled	11.1 (1/9)	29.2 (7/24)
fosA3	Cloacal Swab	78 (92/120)	74.2 (89/120)	61.7 (74/120)
	Meconium	87.5 (21/24)	Not sampled	Not sampled
	Poultry litter	66.7 (14/21)	75 (18/24)	66.7 (16/24)
	A. diaperinus	Not sampled	66.7 (6/9)	58.3 (14/24)

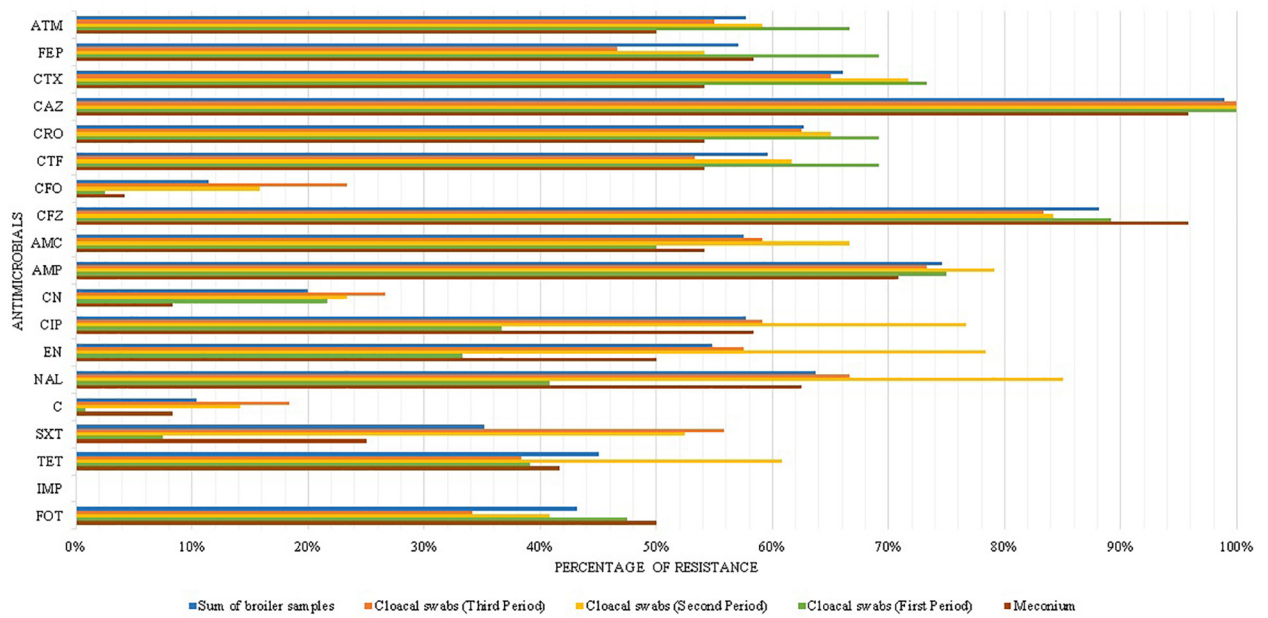


FIGURE 1 | Phenotypic resistance profile of broiler samples by periods. FOT, Fosfomycin-trometamol; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; C, chloramphenicol; CN, gentamicin; CIP, ciprofloxacin; NAL, nalidixic acid; EN, enrofloxacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CFZ, cefazolin; CFO, cefoxitin; CTF, ceftiofur; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ATM, aztreonam; and IMP, imipenem.

Conjugation Experiment Results

Two transconjugants were obtained in the conjugation assay; these isolates belonged to phylogenetic group D, confirming that they are the recipient strains and were positive for the $bla_{CTX-M-55}$ and *fosA3* groups, demonstrating the horizontal transfer capacity of these genes.

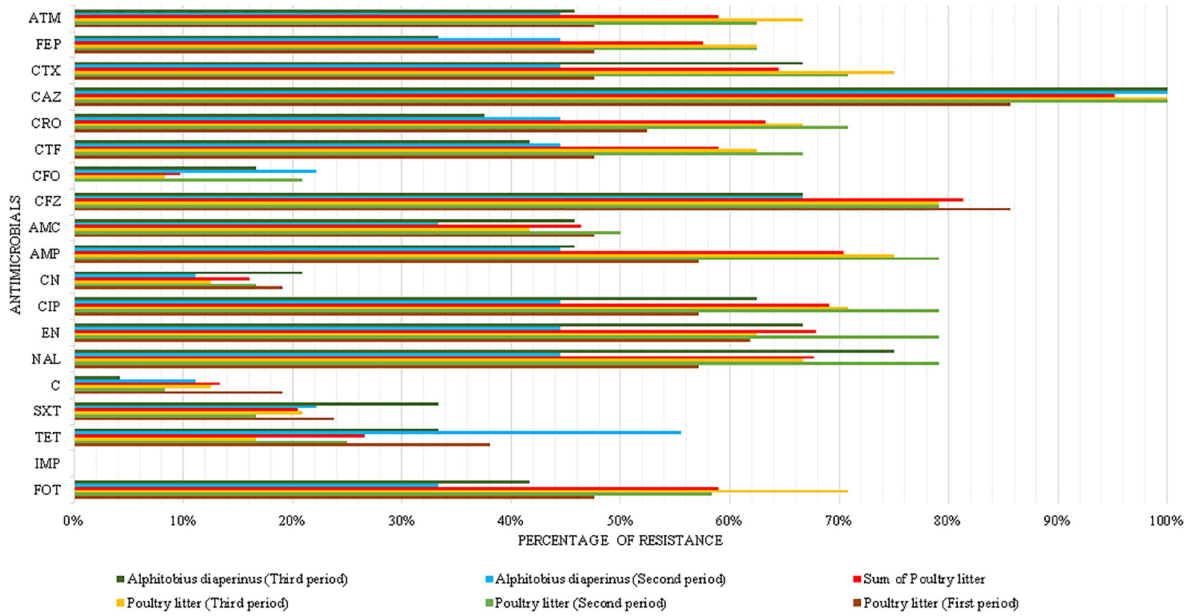


FIGURE 2 | Phenotypic resistance profile of poultry litter and *A. diaperinus* by periods. FOT, Fosfomycin-trometamol; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; C, chloramphenicol; CN, gentamicin; CIP, ciprofloxacin; NAL, nalidixic acid; EN, enrofloxacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CFZ, cefazolin; CFO, cefoxitin; CTF, ceftiofur; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ATM, aztreonam; and IMP, imipenem.

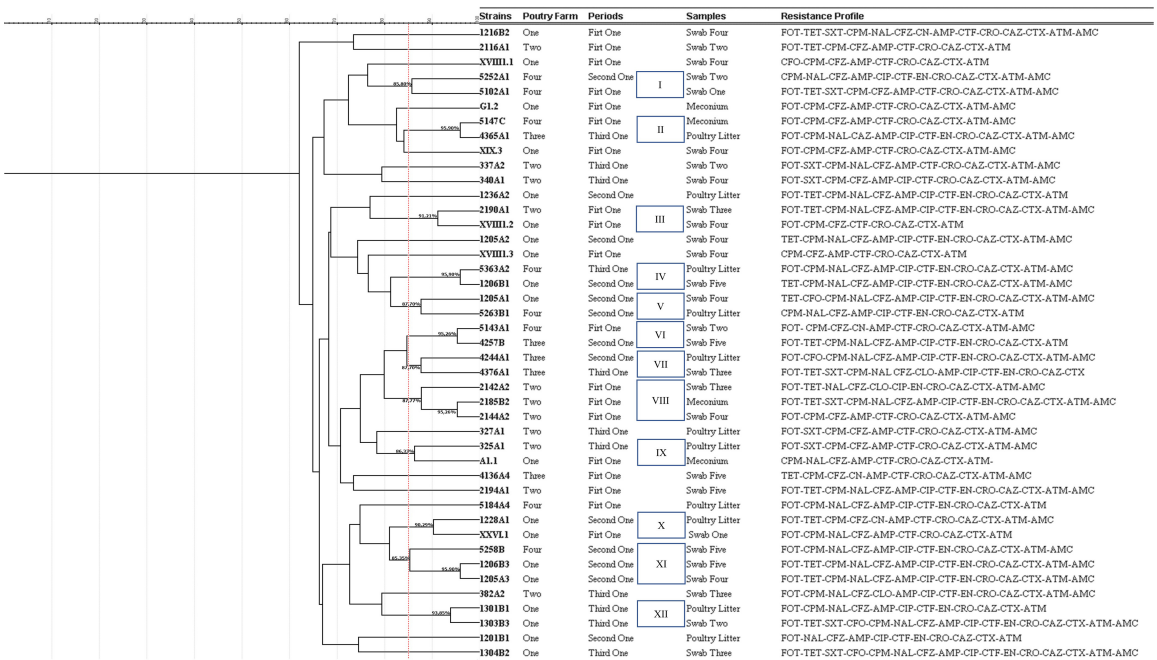


FIGURE 3 | Dendrogram with the characteristics of *E. coli* harboring *fosA3*/CTX-M-55 by ERIC-PCR. FOT, Fosfomycin-trometamol; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; C, chloramphenicol; CN, gentamicin; CIP, ciprofloxacin; NAL, nalidixic acid; EN, enrofloxacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CFZ, cefazolin; CFO, cefoxitin; CTF, ceftiofur; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ATM, aztreonam; and IMP, imipenem.

DISCUSSION

Emerging resistance to antimicrobials is a concern and needs to be investigated. According to PanBr-Agro, broiler farms are important places for monitoring resistance to antimicrobials (Brasil, 2018) due to the sanitary challenges in the poultry production system and the consequent use of antimicrobials for different purposes. Thus, poultry production is one of

the segments responsible for the selection of isolates resistant to antimicrobials (Cuong et al., 2019; Hedman et al., 2020; Zalewska et al., 2021), and the control of some antimicrobials can minimize zoonotic risks of multiresistant transmission bacteria from animals to humans (Magnusson, 2020). Our study carried out a longitudinal monitoring, researching multiresistant bacteria from different samples in three life periods of broilers, to investigate possible risk points in the spread of antimicrobial resistance.

Our results demonstrate that the main critical point for the spread of antimicrobial resistance in broiler chicken farms was the chickens, which arrived colonized with *E. coli* harboring the *fosA3* and $bla_{CTX-M-1}$ genes, especially the $bla_{CTX-M-55}$. We detected 68% (344/507) of the isolates positive for the *fosA3* gene. Our *fosA3* positive strains were from cloacal swab, meconium, *A. diaperinus*, and poultry litter samples.

According to the WHO, fosfomycin is in the AWaRe classification group, and it acts against strains resistant to carbapenems (Martirosov and Lodise, 2016; Pauwels et al., 2021). Cottell and Webber (2017) analyzed 676 *E. coli* isolated from urine in a hospital in the United Kingdom, and 12% were resistant to fosfomycin. The authors demonstrated that *E. coli* isolated from infections in humans have a low resistance to fosfomycin and highlighted the importance of keeping this antimicrobial for human use only. Derington et al. (2020) tested fosfomycin as a therapeutic option for treating complicated urinary tract infections, leading to the resolution of the evaluated conditions. Based on these results, they, and other authors report that fosfomycin is an essential human therapeutic option (Wachino et al., 2010; Neuner et al., 2012; Jean et al., 2016; Cao et al., 2017; Abbott et al., 2020; Loras et al., 2020; Seok et al., 2020), against urinary infections for example, the second most important bacterial infection affecting humans (Moeinizadeh and Shaheli, 2021).

It is an antimicrobial that should only be used against multiresistant microorganisms (WHO, 2019); therefore, its use should be restricted to human medicine. Notably, Brazil does not have a ban on using this antimicrobial in animal production, being a country that uses fosfomycin in poultry production (Roth et al., 2019). The use of antimicrobials leads to selective pressure and consequently the selection of resistant strains (WHO, 2018, 2019); thus, the monitoring and surveillance of antimicrobials in animal production should promote the rational use of the same.

We report that both meconium and cloacal swab samples from day-old chickens (first period) had a 1.64 (CI 1.01–2.65) times greater chance of harboring the *fosA3* gene than those from other periods. Vertical transmission can occur at any point in poultry production before the chickens reach the farms. In a longitudinal study, Poulsen et al. (2017) demonstrated that broiler breeders could transmit microorganisms to broilers. Although vertical transmission of *E. coli* to day-old chickens has already been shown by other authors (Lu et al., 2003; Dierikx et al., 2013; Fischer et al., 2014; Zurfluh et al., 2014; Osman et al., 2018; Apostolakos et al., 2019, 2020; Dame-Korevaar et al., 2019, Dame-Korevaar et al., 2020; Zhao et al., 2019), this is the first report of the presence of fosfomycin-resistant *E. coli* in day-old chickens.

Enterobacterial repetitive intergenic consensus-polymerase chain reaction detected clonal groups formed by strains from different farms isolated from day-old chickens (first period) and groups with *E. coli* isolated from the same farm but at different periods (clonal group I), suggesting possible vertical transmission and the perpetuation of *fosA3*/CTX-M-55 samples, respectively. This finding serves as a warning about the potential spread of resistance within the broiler production chain.

We also showed that strains isolated from poultry litter were more likely to be ESBL producers (OR 2.07, CI 1.31–3.27) than other samples, an important point in disseminating and perpetuating ESBL strains in poultry farming. Curiously in our research, poultry litter did not have the high number of resistance when compared with day-old chicken sample. The use of the same poultry litter in subsequent production cycles, when subjected to a sanitary vacuum and a good fermentation process, may result in a low microbial load (Wei et al., 2013; Vieira et al., 2015; Waziri and Kaltungo, 2017; Gurmessa et al., 2021; Moffo et al., 2021; Yévenes et al., 2021). In Brazil, poultry litter is reused for more than one consecutive batch, and this practice is allowed if sanitary problems have not occurred with the batches housed on the poultry litter to be reused (Waziri and Kaltungo, 2017).

Gazal et al. (2021) sampled broiler farms and found that the main sources of ESBL dissemination were poultry litter and *Alphitobius* sp. samples, which were detected from day one in farms sampled. Our study did not isolate MDR and ESBL-producing *E. coli* from all farms in the first period from poultry litter. One of the broiler farms analyzed reported having performed efficient poultry litter management, demonstrated by the low resistance profile of the strains isolated. In this and in other poultry farms it was also possible to observe a reduction or elimination of *A. diaperinus* in the first periods, possibly due to the poultry litter treatment process carried out between batches. Siller et al. (2020) reported that even when poultry litter undergoes a fermentation process, other events can interfere with the number of microorganisms present, regardless of how many times the litter has already been used. Therefore, it is essential to point out that poultry litter is indeed a sample that presents a potential risk factor inside poultry houses.

In our study, when we subjected *E. coli* strains harboring *fosA3* and $_{bla}CTX-M-55$ to conjugation experiments with a strain sensitive to fosfomycin and CTX, we observed that they were able to transmit both resistance genes. With the spread of these resistant strains, an increase in therapeutic failures in treating ESBL-producing microorganisms is expected. The use of fosfomycin risks the selection of ESBL coproducers since CTX-M, and *fosA3* genes have already been confirmed to colocalize on plasmids (Sarker et al., 2019). Another important point to highlight about conjugation is that the recipient strain belongs to phylogenetic group D, and the donor strain belongs to phylogenetic Group B1, which demonstrates an exchange of genetic material between commensal microorganisms and microorganisms with potential pathogenicity (Clermont et al., 2013).

Regarding the *E. coli* isolated from water and poultry feed, these strains were not identified as MDR microorganisms or ESBL producers. This finding characterizes the samples as not

critical points for the spread of antimicrobial resistance in broiler production (Miles et al., 2006; Rossato et al., 2019; Gazal et al., 2021).

In the present work, 4.33% (25/507) of the strains isolated had more than one gene from the bla_{CTX-M} group, which should be highlighted regardless of the low percentage due to the potential for the development of other enzyme recombinants by these strains in the future (Yin et al., 2020; Leão et al., 2021).

Colistin is an antimicrobial indicated as a last resort to treat infections in humans. The first report of the *mcr-1* gene, one of the genes that confers resistance to this antimicrobial, was in China by Liu et al. (2016), and *mcr-1* has been correlated with the emergence of pandrug-resistant microorganisms (McGann et al., 2016). The *mcr-1* gene is more frequently detected in farm animals than in humans (Elbediwi et al., 2019) but can cause serious infectious diseases such as pyelonephritis in humans (Birgy et al., 2018). Ahmed et al. (2020) found in Bangladesh that 25% (300/1200) of *E. coli* strains isolated from broiler chicken farms were positive for the *mcr-1* gene. Out of 507 *E. coli* isolates, we detected only one positive *mcr-1* gene in our work. This is possibly due to the local prohibition of using this antimicrobial as a growth promoter. This strain is a fourth-generation cephalosporin-resistant, producer of ESBL ($bla_{CTX-M-2}$ group) and harbors the *fosA3* gene.

We used culture media supplemented with antimicrobials to select isolates in the present work. This technique can favor the selection of strains with a higher resistance profile and more significant gene variability (Ceccarelli et al., 2019). We used preselection, CTX, an indicator of the CTX-M group (Paterson and Bonomo, 2005), and CIP antimicrobial with coselection to the ESBL enzyme (Jiang et al., 2008; Hassan et al., 2012; Seo and Lee, 2019). In this way, we were able to detect strains with a high resistance profile, avoiding underreporting of the antimicrobial-resistant profile.

Hence, in this work, a high frequency of *E. coli* harboring the *fosA3*/CTX-M-55 strains was detected in poultry farming, from day-old chickens to preslaughter chickens, and in different samples (cloacal swab + meconium, chicken litter, and beetles), with these genes transferable by conjugation. Therefore, considering the importance of fosfomycin to human medicine and the fact that Brazil is one of the largest exporters of chicken meat in the world (ABPA, 2021) and one of the few countries that use this antimicrobial in poultry production, we reinforce the need to ban its use in the poultry sector.

CONCLUSION

In conclusion, this study demonstrated that the spread of *fosA3*-mediated fosfomycin resistance was correlated with the presence of the CTX-M-1 group, especially the $bla_{CTX-M-55}$ gene, in broiler chickens. This profile was observed mainly in day-old chickens, with a high profile of *E. coli* strains multidrug resistant to antimicrobials. The findings emphasize the importance of conducting longitudinal monitoring to detect the main risk points in poultry production and thus intervene, prevent drug resistance, and promote the rational use of antimicrobials.

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: NCBI GenBank – OM326827–OM326869.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of State University of Londrina (CEUA/UEL). Processing number: 13142.2019.51.

AUTHOR CONTRIBUTIONS

MM-C contributed to developing experimental research, data analysis, and writing the manuscript. AB, LG, LJ, MdS, MS, BQ, VC, JB, GA, and BG contributed to the development of experimental research. EN contributed to the statistical analysis. RK, GN, AB, EV, LG, and GA contributed to and assisted in the design of the work and preparation of the article and critically reviewed the manuscript. All authors have participated in this study and commented on the manuscript.

FUNDING

Postgraduate Program supported this study in Microbiology of Universidade Estadual de Londrina and in part by the National Council for Scientific and Technological Development – CNPq (433656/2018-2 Chamada MCTIC/CNPqNo 28/2018 and 313305/2019-6 to RK) and (315435/2018-6 to GN). Maísa Fabiana Menck Costa was funded by a Ph.D. scholarship from Coordination for the Improvement of Higher Education Personnel (CAPES) – Finance Code 001. Additional support was provided by the Bill and Melinda Gates Foundation's Grand Challenges Explorations Brazil – New Approaches to Characterize the Global Burden of Antimicrobial Resistance (number OPP1193112).

ACKNOWLEDGMENTS

We would also like to thank Bill and Melinda Gates Foundation's (OPP1193112) for the great help in publishing the newspaper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.846116/full#supplementary-material

REFERENCES

Aarestrup, F. M., Bonten, M., and Koopmans, M. (2021). Pandemics–One Health preparedness for the next. *Lancet. Reg. Health. Eur.* 9:100210. doi: 10.1016/j.lanepe.2021.100210

Abbott, I. J., van Gorp, E., Wijma, R. A., Meletiadis, J., Mouton, J. W., and Peleg, A. Y. (2020). Evaluation of pooled human urine and synthetic alternatives in a dynamic bladder infection in vitro model simulating oral fosfomycin therapy. *J. Microbiol. Methods* 171:105861. doi: 10.1016/j.mimet.2020.105861

Abelenda-Alonso, G., Padullés, A., Rombauts, A., Gudiol, C., Pujol, M., Alvarez-Pouso, C., et al. (2020). Antibiotic prescription during the COVID-19 pandemic: a biphasic pattern. *Infect. Control Hosp. Epidemiol.* 41, 1371–1372. doi: 10.1017/ice.2020.381

ABPA (2021). *Annual Report of Brazilian Poultry Association*. São Paulo: Brazilian Poultry Union.

Ahmed, S., Das, T., Islam, M. Z., Herrero-Fresno, A., Biswas, P. K., and Olsen, J. E. (2020). High prevalence of mcr-1-encoded colistin resistance in commensal *Escherichia coli* from broiler chicken in Bangladesh. *Scientific Rep.* 10:18637. doi: 10.1038/s41598-020-75608-2

Apostolakos, I., Feudi, C., Eichhorn, I., Palmieri, N., Fasolato, L., Schwarz, S., et al. (2020). High-resolution characterisation of ESBL/pAmpC-producing *Escherichia coli* isolated from the broiler production pyramid. *Sci. Rep.* 10:11123. doi: 10.1038/s41598-020-68036-9

Apostolakos, I., Mughini-Gras, L., Fasolato, L., and Piccirillo, A. (2019). Assessing the occurrence and transfer dynamics of ESBL/pAmpC-producing *Escherichia coli* across the broiler production pyramid. *PLoS One* 14:e0217174. doi: 10.1371/journal.pone.0217174

Aquino, C. (2021). *Poultry and Products Annual Brazil*. Available online at: https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Poultry%20and%20Products%20Annual_Brasilia_Brazil_09-01-2021.pdf (accessed March 17, 2022).

Arikan, M. S., Akin, A. C., Akcay, A., Aral, Y., Sariozkan, S., Cevrimli, M. B., et al. (2017). Effects of transportation distance, slaughter age, and seasonal factors on total losses in broiler chickens. Brazilian. *J. Poult. Sci.* 19, 421–428. doi: 10.1590/1806-9061-2016-0429

Aslam, B., Khurshid, M., Arshad, M. I., Muzammil, S., Rasool, M., Yasmeen, N., et al. (2021). Antibiotic resistance: one health one world outlook. *Front. Cell. Infect. Microbiol.* 11:1153. doi: 10.3389/fcimb.2021.771510

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., et al. (2018). Antibiotic resistance: a rundown of a global crisis. *Infect. Drug Res.* 11, 1645–1658. doi: 10.2147/IDR.S173867

Avershina, E., Shapovalova, V., and Shipulin, G. (2021a). Fighting antibiotic resistance in hospital-acquired infections: current state and emerging technologies in disease prevention, diagnostics and therapy. *Front. Microbiol.* 12:2044. doi: 10.3389/fmicb.2021.707330

Avershina, E., Sharma, P., Taxt, A. M., Singh, H., Frye, S. A., Paul, K., et al. (2021b). AMR-Diag: neural network based genotype-to-phenotype prediction of resistance towards β-lactams in *Escherichia coli* and *Klebsiella pneumoniae*. *Comput. Struct. Biotechnol. J.* 19, 1896–1906. doi: 10.1016/j.csbj.2021.03.027

Awogbemi, J., Moses, A., and Akinkunmi, E. O. (2018). A Survey of antimicrobial agents usage in poultry farms and antibiotic resistance in *Escherichia coli* and Staphylococci Isolates from the poultry in ile-ife, nigeria. *J. Infect.Diseases Epidemiol.* 4, 1–8. doi: 10.23937/2474-3658/1510047

Birgy, A., Madhi, F., Hogan, J., Doit, C., Gaschignard, J., Caseris, M., et al., (2018). CTX-M- 55-, MCR- 1-, and FosA-Producing multidrug-resistant *Escherichia coli* infection in a child in France. *Antimicrob. Agents Chemother.* 62(4), e00127-e118. doi: 10.1128/AAC.00127-18

Brasil (2013). *Fundação Nacional de Saúde. Manual prático de análise de água / Fundação Nacional de Saúde – 4. ed. – Brasília*: Funasa.Bengaluru

Brasil (2016). *Instrução normativa No 20, DE 21 de Outubro de 2016*. Brasília: Ministério da Saúde

Brasil (2018). *Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância das Doenças Transmissíveis. Plano de ação nacional de prevenção e controle da resistência aos antimicrobianos no âmbito da saúde única 2018-2022 (PAN-BR) / Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância das Doenças Transmissíveis*. – Brasília: Ministério da Saúde.

Brasil (2020). *Instrução Normativa no 1, de 13 de janeiro de 2020—DOU – Imprensa Nacional*. South America:AG, Brasil

BRCAST (2019). *Redefinição das categorias dos testes de Sensibilidade S, I e R*. Shanghai.BRCAST

Büdel, T., Kuenzli, E., Campos-Madueno, E. I., Mohammed, A. H., Hassan, N. K., Zinsstag, J., et al., (2020). On the island of zanzibar people in the community are frequently colonized with the same MDR Enterobacterales found in poultry and retailed chicken meat. *J. Antimicrob. Chemother.* 75(9), 2432–2441. doi: 10.1093/jac/dkaa198

Campos, A. C. da C., Andrade, N. L., Couto, N., Mutters, N. T., de Vos, M., Rosa, A. C. de P., et al.,. (2020). Characterization of fosfomycin heteroresistance among multidrug-resistant *Escherichia coli* isolates from hospitalized patients in Rio de Janeiro. *Brazil. J. Glob. Antimicrob. Resist.* 22, 584–593. doi: 10.1016/j.jgar.2020.04.026

Cantón, R., Gonzalez-Alba, J. M., and Galán, J. C. (2012). CTX-M Enzymes: Origin and Diffusion. *Front. Microbiol.* 3:110. doi: 10.3389/fmicb.2012.00110

Cao, X.L., Shen, H., Xu, Y.Y., Xu, X.J., Zhang, Z.F., Cheng, L., et al., (2017). High prevalence of fosfomycin resistance gene fosA3 in blaCTX-M-harbouring *Escherichia coli* from urine in a Chinese tertiary hospital during 2010–2014. *Epidemiol. Infect.* 145(4), 818–824. doi: 10.1017/S0950268816002879

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., et al.,. (2019). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* 19(1), 56–66. doi: 10.1016/S1473-3099(18)30605-4

Cazares, A., Moore, M. P., Hall, J. P. J., Wright, L. L., Grimes, M., Emond-Rhéault, J.-G., et al., (2020). A megaplasmid family driving dissemination of multidrug resistance in *Pseudomonas*. *Nature Commun.* 11(1):1370. doi: 10.1038/s41467-020-15081-7

Ceccarelli, D., Kant, A., van Essen-Zandbergen, A., Dierikx, C., Hordijk, J., Wit, B., et al., (2019). Diversity of plasmids and genes encoding resistance to extended spectrum cephalosporins in commensal *Escherichia coli* from dutch livestock in 2007–2017. *Front. Microbiol.* 10:76. doi: 10.3389/fmicb.2019.00076

Clermont, O., Christenson, J. K., Denamur, E., and Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Env.Microbiol.Rep.* 5(1), 58–65. doi: 10.1111/1758-2229.12019

CLSI (2008). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 5th ed. CLSI standard VET01 (ISBN 978-1-68440-008-9 [Print]; ISBN 978-1-68440- 009-6 [Electronic])*. Clinical and Laboratory Standards Institute, Pennsylvania US. 2018.

CLSI (2018). *Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Fifth Informational Supplement CLSI Document M100*. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2019). *Suggested Grouping of US-FDA Approved Antimicrobial Agents That Should Be Considered for Routine Testing and Reporting on Nonfastidious Organisms by Clinical Laboratories. 29ed. CLSI guideline M100-S29*. Wayne, PA: Clinical and Laboratory Institute.

Cottell, J. L., and Webber, M. A. (2017). Prevalence, mechanisms and comparison of detection methods of fosfomycin resistance in E. coli from urinary tract infections. *BioRxiv* 234–435. doi: 10.1101/234435

Cunha, M. P. V., Lincopan, N., Cerdeira, L., Esposito, F., Dropa, M., Franco, L. S., et al., (2017). Coexistence of CTX-M-2, CTX-M-55, CMY-2, FosA3, and QnrB19 in Extraintestinal Pathogenic *Escherichia coli* from Poultry in Brazil. *Antimicrob. Agents Chemother.* 61(4), e02474-e2416. doi: 10.1128/AAC.02474-16

Cuong, N. V., Phu, D. H., Van, N. T. B., Dinh Truong, B., Kiet, B. T., Hien, B. V., et al., (2019). High-Resolution monitoring of antimicrobial consumption in vietnamese small-scale chicken farms highlights discrepancies between study metrics. *Front. Vet. Sci.* 6:174. doi: 10.3389/fvets.2019.00174

Cyoia, P. S., Koga, V. L., Nishio, E. K., Houle, S., Dozois, C. M., de Brito, K. C. T., et al., (2019). Distribution of ExPEC Virulence Factors, blaCTX-M, fosA3, and mcr-1 in *Escherichia coli* isolated from commercialized chicken carcasses. *Front. Microbiol.* 9:3254. doi: 10.3389/fmicb.2018.03254

Daga, A. P., Koga, V. L., Soncini, J. G. M., de Matos, C. M., Perugini, M. R. E., Pelisson, M., et al., (2019). *Escherichia coli* bloodstream infections in patients at

a university hospital: virulence factors and clinical characteristics. *Front. Cell. Infect. Microbiol.* 9:191. doi: 10.3389/fcimb.2019.00191

Dame-Korevaar, A., Fischer, E. A. J., van der Goot, J., Stegeman, A., and Mevius, D. (2019). Transmission routes of ESBL/pAmpC producing bacteria in the broiler production pyramid, a literature review. *Prev. Vet. Med.* 162, 136–150. doi: 10.1016/j.prevetmed.2018.12.002

Dame-Korevaar, A., Kers, J. G., van der Goot, J., Velkers, F. C., Ceccarelli, D., Mevius, D. J., et al., (2020). Competitive exclusion prevents colonization and compartmentalization reduces Transmission of ESBL-Producing *Escherichia coli* in Broilers. *Front. Microbiol.* 11:2853. doi: 10.3389/fmicb.2020.566619

Davis, G. S., Waits, K., Nordstrom, L., Grande, H., Weaver, B., Papp, K., et al., (2018). Antibiotic-resistant *Escherichia coli* from retail poultry meat with different antibiotic use claims. *BMC Microbiol.* 18(1):174. doi: 10.1186/s12866-018-1322-5

De Oliveira Sidinei, M. E. A., Marcato, S. M., Perez, H. L., and Bánkuti, F. I. (2021). Biosecurity, environmental sustainability, and typological characteristics of broiler farms in Paraná State, Brazil. *Prev. Vet. Med.* 194:105426. doi: 10.1016/j.prevetmed.2021.105426

De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., et al., (2020). Antimicrobial Resistance in ESKAPE pathogens. *Clin. Microbiol. Rev.* 33(3), e00181-e119. doi: 10.1128/CMR.00181-19

Derington, C. G., Benavides, N., Delate, T., and Fish, D. N. (2020). Multiple-dose oral fosfomycin for treatment of complicated urinary tract infections in the outpatient setting. *Open For. Infect. Dis.* 7:ofaa034. doi: 10.1093/ofid/ofaa034

Dierikx, C. M., Goot, J. A., van der, Smith, H. E., Kant, A., and Mevius, D. J. (2013). Presence of ESBL/AmpC -Producing *Escherichia coli* in the broiler production pyramid: a descriptive study. *PLoS One*, 8(11):e79005. doi: 10.1371/journal.pone.0079005

Eckner, K. F. (1998). Comparison of membrane filtration and multiple-tube fermentation by the colilert and enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and enterococci used in drinking and bathing water quality monitoring in southern sweden. *Appl. Env. Microbiol.* 64(8), 3079–3083. doi: 10.1128/AEM.64.8.3079-3083.1998

Elbediwi, M., Li, Y., Paudyal, N., Pan, H., Li, X., Xie, S., et al., (2019). Global burden of colistin-resistant bacteria: mobilized colistin resistance genes study (1980–2018). *Microorganisms* 7:461 doi: 10.3390/microorganisms7100461

European Commission (2018). *A European One Health Action Plan Against Antimicrobial Resistance (AMR); 2017/C 212/01*. Available online at https://ec.europa.eu/health/antimicrobial-resistance/eu-action-on-antimicrobial-resistance_en [accessed November 11, 2021]

Fei Tian, S., Zhuo Chu, Y., yi Chen, B., Nian, H., and Shang, H. (2011). ISEcp1 element in association with blaCTX-M genes of E. coli that produce extended-spectrum β-lactamase among the elderly in community settings. *Enferm. Infecc. Microbiol. Clín.* 29, 731–734. doi: 10.1016/j.eimc.2011.07.011

Fischer, J., Rodríguez, I., Baumann, B., Guiral, E., Beutin, L., Schroeter, A., et al., (2014). Blactx-M-15-carrying *Escherichia coli* and *Salmonella* isolates from livestock and food in Germany. *J. Antimicrob. Chemother.* 69(11), 2951–2958. doi: 10.1093/jac/dku270

Frost, A. R., Parsons, D. J., Stacey, K. F., Robertson, A. P., Welch, S. K., Filmer, D., Fothergill, A.et al., (2003). Progress towards the development of an integrated management system for broiler chicken production. *Comput. Electron. Agric.* 39(3), 227–240. doi: 10.1016/S0168-1699(03)00082-6

Gazal, L. E. de S., Medeiros, L. P., Dibo, M., Nishio, E. K., Koga, V. L., Gonçalves, B. C. et al., (2021). Detection of ESBL/AmpC-Producing and Fosfomycin-Resistant *Escherichia coli* from different sources in poultry production in southern brazil. *Front. Microbiol.* 11:604544. doi: 10.3389/fmicb.2020.604544

Gonçalves, B. C., Dibo, M., Gazal, L. E.,de S., Medeiros, L. P., Costa, M. F. M., Kobayashi, R. K. T., et al., (2021). "Obtaining transconjugant *Escherichia coli* costs isolated from chicken litter and chicks" "obtenção de cepas transconjugantes de *Escherichia coli* isoladas de cama de frango e pintainhos". *Braz. J. Dev.* 7(2), 18518–18525. doi: 10.34117/bjdv7n2-474

Gurmessa, B., Ashworth, A. J., Yang, Y., Savin, M., Moore, P. A., Ricke, S. C., et al., (2021). Variations in bacterial community structure and antimicrobial resistance gene abundance in cattle manure and poultry litter. *Env. Res.* 197:111011. doi: 10.1016/j.envres.2021.111011

Hassan, W., Hashim, A., and Domany, R. (2012). Plasmid mediated quinolone resistance determinants qnr, aac(6')-Ib-cr, and qep in ESBL-producing *Escherichia coli* clinical isolates from Egypt. *Indian J. Med. Microbiol.* 30(4), 442–447. doi: 10.4103/0255-0857.103766

Hedman, H. D., Vasco, K. A., and Zhang, L. (2020). A Review of antimicrobial resistance in poultry farming within low-resource settings. *Animals (Basel)* 10:1264. doi: 10.3390/ani10081264

Heras, J., Domínguez, C., Mata, E., Pascual, V., Lozano, C., Torres, C., et al., (2015). GelJ – a tool for analyzing DNA fingerprint gel images. *BMC Bioinformatics*, 16(1), 270. doi: 10.1186/s12859-015-0703-0

Huttner, A., Kowalczyk, A., Turjeman, A., Babich, T., Brossier, C., Eliakim-Raz, N.,et al., (2018). Effect of 5-Day Nitrofurantoin vs single-dose fosfomycin on clinical resolution of uncomplicated lower urinary tract infection in women: a randomized clinical trial. *JAMA* 319(17), 1781–1789. doi: 10.1001/jama.2018.3627

Huttner, B. D., Catho, G., Pano-Pardo, J. R., Pulcini, C., and Schouten, J. (2020). COVID-19: Don't neglect antimicrobial stewardship principles!. *Clin. Microbiol. Infect.* 26(7), 808–810. doi: 10.1016/j.cmi.2020.04.024

Jarlier, V., Nicolas, M.-H., Fournier, G., and Philippon, A. (1988). Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev. Infect. Dis.* 10(4), 867–878. doi: 10.1093/clinids/10.4.867

Jean, S.-S., Coombs, G., Ling, T., Balaji, V., Rodrigues, C., Mikamo, H., Kim, M.-J.,et al.,. (2016). Epidemiology and antimicrobial susceptibility profiles of pathogens causing urinary tract infections in the Asia-Pacific region: results from the study for monitoring antimicrobial resistance trends (SMART), 2010–2013. *Int. J.Antimicrob. Agents*. 47(4), 328–334. doi: 10.1016/j.ijantimicag.2016.01.008

Jiang, Y., Zhou, Z., Qian, Y., Wei, Z., Yu, Y., Hu, S., Li, L.,et al., (2008). Plasmid-mediated quinolone resistance determinants qnr and aac(6')-Ib-cr in extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in China. *J. Antimicrob. Chemother.* 61(5), 1003–1006. doi: 10.1093/jac/dkn063

Koga, V. L., Rodrigues, G. R., Scandorieiro, S., Vespero, E. C., Oba, A.,et al., (2015). Evaluation of the Antibiotic Resistance and Virulence of *Escherichia coli* Strains Isolated from Chicken Carcasses in 2007 and 2013 from Paraná. *Brazil Foodborne Pathogens Dis.* 12(6), 479–485. doi: 10.1089/fpd.2014.1888

Kwan, A. C. F., and Beahm, N. P. (2020). Fosfomycin for bacterial prostatitis: a review. *Int. J.Antimicrob. Agents* 56(4):106106. doi: 10.1016/j.ijantimicag.2020.106106

Laube, H., Friese, A., von Salviati, C., Guerra, B., Käsbohrer, A., Kreienbrock, L., et al., (2013). Longitudinal monitoring of extended-spectrum-beta-lactamase/ampc-producing *Escherichia coli* at german broiler chicken fattening farms. *Appl. Env. Microbiol.* 79(16), 4815–4820. doi: 10.1128/AEM.00856-13

Lay, K. K., Jeamsripong, S., Sunn, K. P., Angkititrakul, S., Prathan, R., Srisanga, S., and Chuanchuen, R. (2021). Colistin Resistance and ESBL Production in *Salmonella* and *Escherichia coli* from pigs and pork in the thailand, cambodia, lao pdr, and myanmar border area. *Antibiotics* 10(6):657. doi: 10.3390/antibiotics10060657

Leão, C., Clemente, L., Guerra, V., Botelho, A., and Amaro, A. (2021). Occurrence of a rare multidrug resistant *Escherichia coli* coharboring blaCTX-M-32 and blaCTX-M-2 Genes in a bovine. *Micro. Drug Res.* 27(8), 1155–1157. doi: 10.1089/mdr.2020.0520

Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., et al., (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet. Infect. Dis.* 16(2), 161–168. doi: 10.1016/S1473-3099(15)00424-7

Lobie, T. A., Roba, A. A., Booth, J. A., Kristiansen, K. I., Aseffa, A., Skarstad, K.,et al., (2021). Antimicrobial resistance: a challenge awaiting the post-COVID-19 era. *Int. J. Infect. Dis.* 111, 322–325. doi: 10.1016/j.ijid.2021.09.003

Loras, C., Mendes, A. C., Peixe, L., Novais, Â., and Alós, J.-I. (2020). *Escherichia coli* resistant to fosfomycin from urinary tract infections: detection of the fosA3 gene in Spain. *J. Glob. Antimicrob. Res.* 21, 414–416. doi: 10.1016/j.jgar.2020.01.023

Lu, J., Sanchez, S., Hofacre, C., Maurer, J. J., Harmon, B. G., and Lee, M. D. (2003). Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. *Appl. Env.Microbiol.* 69(2), 901–908. doi: 10.1128/aem.69.2.901-908.2003

Lukman, D., Sudarwanto, M., Purnawarman, T., Latif, H., Pisestyani, H., Sukmawinata, E.,Akineden, Ö. et al., (2017). CTX-M-1 and CTX-M-55 Producing *Escherichia coli* isolated from broiler feces in poultry slaughterhouse, bogor, west java province. *Glob. Adv.Res.J. Med. Med. Sci.* 5, 287–291.

Lupo, A., Saras, E., Madec, J.-Y., and Haenni, M. (2018). Emergence of blaCTX-M-55 associated with fosA, rmtB and mcr gene variants in *Escherichia coli* from various animal species in France. *J. Antimicrob. Chem.* 73(4), 867–872. doi: 10.1093/jac/dkx489

Magnusson, U. (2020). Prudent and effective antimicrobial use in a diverse livestock and consumer's world. *J. Animal Sci.* 98, S4-S8. doi: 10.1093/jas/skaa148

Manges, A. R., Geum, H. M., Guo, A., Edens, T. J., Fibke, C. D., and Pitout, J. D. (2019). Global Extraintestinal Pathogenic *Escherichia coli* (ExPEC) Lineages. *Clin. Microbiol. Rev.* 32(3), e135-e118. doi: 10.1128/CMR.00135-18

Martirosov, D. M., and Lodise, T. P. (2016). Emerging trends in epidemiology and management of infections caused by carbapenem-resistant *Enterobacteriaceae*. *Diagn. Microbiol. Infect. Dis.* 85(2), 266–275. doi: 10.1016/j.diagmicrobio.2015.10.008

McGann, P., Snesrud, E., Maybank, R., Corey, B., Ong, A. C., Clifford, R., et al., (2016). *Escherichia coli* Harboring mcr-1 and blaCTX-M on a Novel IncF Plasmid: First report of mcr-1 in the united states. *Antimicrob. Agents Chem.* 60(7), 4420–4421. doi: 10.1128/AAC.01103-16

Menard, S. (2002) Applied logistic regression analysis. Sage, England, US

Miles, T. D., McLaughlin, W., and Brown, P. D. (2006). Antimicrobial resistance of *Escherichia coli* isolates from broiler chickens and humans. *BMC Vet. Res.* 2:7. doi: 10.1186/1746-6148-2-7

Moeinizadeh, H., and Shaheli, M. (2021). Frequency of hlyA, hlyB, hlyC and hlyD genes in uropathogenic *Escherichia coli* isolated from UTI patients in shiraz. *GMS Hygiene Infect. Control* 16:Doc25. doi: 10.3205/dgkh000396

Moffo, F., Mouiche, M. M. M., Djomgang, H. K., Tombe, P., Wade, A., Kochivi, F. L., et al., (2021). Poultry litter contamination by *Escherichia coli* resistant to critically important antimicrobials for human and animal use and risk for public health in cameroon. *Antibiotics* 10(4):402. doi: 10.3390/antibiotics10040402

Neuner, E. A., Sekeres, J., Hall, G. S., and van Duin, D. (2012). Experience with fosfomycin for treatment of urinary tract infections due to multidrug-resistant organisms. *Antimicrob. Agents Chemother.* 56(11), 5744–5748. doi: 10.1128/AAC.00402-12

Nieuwlaat, R., Mbuagbaw, L., Mertz, D., Burrows, L. L., Bowdish, D. M. E., Moja, L., et al., (2021). Coronavirus Disease 2019 and antimicrobial resistance: parallel and interacting health emergencies. *Clin. Infect. Dis.* 72(9), 1657–1659. doi: 10.1093/cid/ciaa773

O'Neill, J. (2014). *The Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*. Available online at https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf (accessed November 20, 2021).

Osman, K. M., Kappell, A. D., Elhadidy, M., ElMougy, F., El-Ghany, W. A. A., Orabi, A.,et al., (2018). Poultry hatcheries as potential reservoirs for antimicrobial-resistant *Escherichia coli*: a risk to public health and food safety. *Sci. Rep.* 8(1):5859. doi: 10.1038/s41598-018-23962-7

Park, H., Kim, J., Ryu, S., and Jeon, B. (2019). Predominance of blaCTX-M-65 and blaCTX-M-55 in extended-spectrum β-lactamase-producing *Escherichia coli* from raw retail chicken in South Korea. *J. Glob. Antimicrob. Res.* 17, 216–220. doi: 10.1016/j.jgar.2019.01.005

Paterson, D. L., and Bonomo, R. A. (2005). Extended-Spectrum β-Lactamases: a clinical update. *Clin. Microbiol. Rev.* 18(4), 657–686. doi: 10.1128/CMR.18.4.657-686.2005

Pauwels, I., Versporten, A., Drapier, N., Vlieghe, E., Goossens, H., and Saleem, Z. (2021). Hospital antibiotic prescribing patterns in adult patients according to the WHO access, watch and reserve classification (AWaRe): Results from a worldwide point prevalence survey in 69 countries. *J. Antimicrob. Chemother.* 76, 1614-1624. doi: 10.1093/jac/dkab050

Poirel, L., Madec, J.-Y., Lupo, A., Schink, A.-K., Kieffer, N., Nordmann, P., and Schwarz, S. (2018). Antimicrobial resistance in *Escherichia coli*. *Microbiol. Spectr.* 6, 289–316. doi: 10.1128/microbiolspec.ARBA-0026-2017

Poulsen, L. L., Thøfner, I., Bisgaard, M., Christensen, J. P., Olsen, R. H., and Christensen, H. (2017). Longitudinal study of transmission of *Escherichia coli* from broiler breeders to broilers. *Vet. Microbiol.* 207, 13–18. doi: 10.1016/j.vetmic.2017.05.029

Rossato, J. M., Brito, B. G., Kobayashi, R. K. T., Koga, V. L., Sarmiento, J. J. P., Nakazato, G., et al. (2019). Antimicrobial resistance, diarrheagenic and avian pathogenic virulence genes in *Escherichia coli* from poultry feed and the ingredients. *Arq. Bras. Med. Vet. Zootec.* 71, 1968–1976. doi: 10.1590/1678-4162-10355

Roth N., Kasbohrer A., Mayrhofer S., Zitz U., Hofacre C.,and Domig K. J. (2019). The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: a global overview. *Poul. Sci.* 98, 1791–1804. 10.3382/ps/pey539 doi: 10.3382/ps/pey539

Saharan, V. V., Verma, P., and Singh, A. P. (2020). *Escherichia coli*, *Salmonella* spp., and Staphylococcus aureus susceptibility to antimicrobials of human and veterinary importance in poultry sector of India. *J. Food Safety* 40(1):e12742. doi: 10.1111/jfs.12742

Sarker, Md. Mannan, MS., Ali MY., Bayzid M., Ahad A., Bupasha ZB..et al., (2019). Antibiotic resistance of *Escherichia coli* isolated from broilers sold at live bird markets in Chattogram, Bangladesh. *J Adv Vet Anim Res.* 6, 272–277. doi: 10.5455/javar.2019.f344

Sato, N., Kawamura, K., Nakane, K., Wachino, J.-I., and Arakawa, Y. (2013). First detection of fosfomycin resistance gene fosA3 in CTX-M-producing *Escherichia coli* isolates from healthy individuals in Japan. *Micro. Drug Res.* 19(6), 477–482. doi: 10.1089/mdr.2013.0061

Schnall, J., Rajkhowa, A., Ikuta, K., Rao, P., and Moore, C. E. (2019). Surveillance and monitoring of antimicrobial resistance: Limitations and lessons from the GRAM project. *BMC Med.* 17(1);176. doi: 10.1186/s12916-019-1412-8

Seo, K. W., and Lee, Y. J. (2019). Characterization of plasmid mediated quinolone resistance determinants in ciprofloxacin resistant-*Escherichia coli* from chicken meat produced by integrated broiler operations in Korea. *Int. J. Food Microbiol.* 307:108274. doi: 10.1016/j.ijfoodmicro.2019.108274

Seok, H., Choi, J. Y., Wi, Y. M., Park, D. W., Peck, K. R., and Ko, K. S. (2020). Fosfomycin resistance in *escherichia coli* isolates from south korea and in vitro activity of fosfomycin alone and in combination with other antibiotics. *Antibiotics* 9(3):112. doi: 10.3390/antibiotics9030112

Siller, P., Dähre, K., Thiel, N., Nübel, U., and Rösler, U. (2020). Impact of short-term storage on the quantity of extended-spectrum beta-lactamase–producing *Escherichia coli* in broiler litter under practical conditions. *Poul. Sci.* 99.2125-2135. doi: 10.1016/j.psj.2019.11.043

Song, J., Oh, S.-S., Kim, J., Park, S., and Shin, J. (2020). Clinically Relevant Extended-Spectrum β-Lactamase–Producing *Escherichia coli* isolates from food animals in South Korea. *Front. Microbiol.* 11:604. doi: 10.3389/fmicb.2020.00604

Thanner, S., Drissner, D., and Walsh, F. (2016). Antimicrobial resistance in agriculture. *MBio* 7(2), e2227-e2215. doi: 10.1128/mBio.02227-15

Thomrongsuwannakij, T., Narinthorn, R., Mahawan, T., and Blackall, P. J. (2021). Molecular and phenotypic characterization of avian pathogenic *Escherichia coli* isolated from commercial broilers and native chickens. *Poul. Sci.* 101:101527. doi: 10.1016/j.psj.2021.101527

Versalovic, J., Koeuth, T., and Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic Acids Res.* 19, 6823–6831. doi: 10.1093/nar/19.24.6823

Vieira, M. de F. A., Tinoco, I. de F. F., Santos, B. M. dos Inoue, K. R. A., Mendes, M. A. et al., (2015). Sanitary quality of broiler litter reused. *Eng. Agrícola* 35(5), 800–807. doi: 10.1590/1809-4430-Eng.Agric.v35n5p800-807/2015

Wachino, J., Yamane, K., Suzuki, S., Kimura, K., and Arakawa, Y. (2010). Prevalence of fosfomycin resistance among ctx-m-producing *Escherichia coli* clinical isolates in japan and identification of novel plasmid-mediated Fosfomycin-Modifying Enzymes. *Antimicrob. Agents Chemother.* 54(7), 3061–3064. doi: 10.1128/AAC.01834-09

Waziri, M. I., and Kaltungo, B. Y. (2017). Poultry litter selection, management and utilization in the tropics. *Poul. Sci.* 6, 44-55. doi: 10.5772/65036

Wei, S., Morrison, M., and Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. *Poul. sci.* 92, 671–683. doi: 10.3382/ps.2012-02822

WHO (2018). *WHO Report On Surveillance Of Antibiotic Consumption: 2016-2018 Early Implementation*. Geneva: World Health Organization.

WHO. (2019). *The 2019 WHO AWaRe Classification Of Antibiotics For Evaluation And Monitoring Of Use*. Available online at https://apps.who.int/iris/handle/10665/327957 [Accessed January 11, 2021]

Woodford, N., Fagan, E. J., and Ellington, M. J. (2006). Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *J. Antimicrob. Chemother.* 57(1), 154–155. doi: 10.1093/jac/dki412

Yévenes, K., Pokrant, E., Trincado, L., Lapierre, L., Galarce, N., Martín, B. S., et al., (2021). Detection of antimicrobial residues in poultry litter: monitoring a risk through a selective and sensitive HPLC–MS/MS method. *Animals* 11(5), 1399. doi: 10.3390/ani11051399

Yin, M., Hu, G., Shen, Z., Fang, C., Zhang, X., Li, D., et al., (2020). In vivo evolution of ctx-m-215, a novel narrow-spectrum β-lactamase in an *Escherichia coli* clinical isolate conferring resistance to mecillinam. *Antimicrob. Agents Chemother.* 64(11), e00562-e520. doi: 10.1128/AAC.00562-20

Zalewska, M., Błażejewska, A., Czapko, A., and Popowska, M. (2021). Antibiotics and antibiotic resistance genes in animal manure – consequences of its application in agriculture. *Front. Microbiol.* 12:640. doi: 10.3389/fmicb.2021.610656

Zhao, S., Wang, C.L., Chang, S.K., Tsai, Y.L., and Chou, C.H. (2019). Characterization of *Escherichia coli* Isolated from day-old chicken fluff in taiwanese hatcheries. *Avian Dis.* 63(1), 9–16. doi: 10.1637/11935-072318-Reg.1

Zurfluh, K., Wang, J., Klumpp, J., Nüesch-Inderbinen, M., Fanning, S., and Stephan, R. (2014). Vertical transmission of highly similar blaCTX-M-1-harboring IncI1 plasmids in *Escherichia coli* with different MLST types in the poultry production pyramid. *Front. Microbiol.* 5:519.

Zurita, J., Ortega-Paredes, D., and Barba, P. (2016). First Description of *Shigella* sonnei Harboring blaCTX-M-55 outside Asia. *J. Microbiol. Biotechnol.* 26(12), 2224–2227. doi: 10.4014/jmb.1605.05069

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 Menck-Costa, Baptista, Gazal, Justino, Sanches, de Souza, Nishio, Queiroz dos Santos, Cruz, Berbert, Gonçalves, Andrade, Vespero, Nakazato and Kobayashi. 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 Microbiology

ORIGINAL RESEARCH
published: 20 May 2022
doi: 10.3389/fmicb.2022.847449

Assessment of Antibacterial and Anti-biofilm Effects of Vitamin C Against *Pseudomonas aeruginosa* Clinical Isolates

Wedad M. Abdelraheem[1], Marwa M. M. Refaie[2], Rehab Kamal Mohamed Yousef[3], Aliaa S. Abd El Fatah[4], Yosra M. Mousa[5] and Rabab Rashwan[1]*

[1] Department of Medical Microbiology and Immunology, Faculty of Medicine, Minia University, Minya, Egypt, [2] Department of Pharmacology, Faculty of Medicine, Minia University, Minya, Egypt, [3] Department of Pathology, Faculty of Medicine, Minia University, Minya, Egypt, [4] Internal Medicine Department, Faculty of Medicine, Minia University, Minya, Egypt, [5] Chest Department, Faculty of Medicine, Minia University, Minya, Egypt

There is a persistent need to look for alternative therapeutic modalities to help control the pandemic of antimicrobial resistance. Assessment of antibacterial and anti-biofilm effects of vitamin C (ascorbic acid) was the aim of the current study. The micro-dilution method determined the minimal inhibitory concentration (MIC) of ascorbic acid or antibiotics alone and in combinations against *Pseudomonas aeruginosa* (*P. aeruginosa*) clinical isolates. The micro-titer plate method monitored the effect of ascorbic acid on the biofilm-producing isolates of *P. aeruginosa*. The effect of ascorbic acid on the differential expression of different antibiotic-resistant genes and biofilm encoding genes of *P. aeruginosa* isolates were also tested using real-time polymerase chain reaction (PCR). For *in vivo* assessment of the antibacterial effects of ascorbic acid alone or combined with an antibiotic, rats were infected with *P. aeruginosa* clinical isolate followed by different treatment regimens. MICs of ascorbic acid among *P. aeruginosa* isolates were in the range of 156.2–1,250 μg/ml, while MIC50 and MIC90 were 312.5 and 625 μg/ml, respectively. At sub-inhibitory concentrations (19.5–312.5 μg/ml), ascorbic acid had 100% biofilm inhibitory effect. Furthermore, ascorbic acid-treated bacteria showed downregulation of genes underpinning biofilm formation and antibiotic resistance. *In vivo* assessment of vitamin C and ceftazidime in rats showed that administration of both at a lower dose for treatment of pseudomonas infection in rats had a synergistic and more powerful effect. Vitamin C shows excellent *in vitro* results as an antibacterial and anti-biofilm agent. Vitamin C should be routinely prescribed with antibiotics to treat bacterial infections in the clinical setting.

Keywords: antimicrobial and anti-biofilm effects of vitamin C, anti-biofilm strategies, synergism, MDR, biofilm former *P. aeruginosa* strains

OPEN ACCESS

Edited by:
Ziad Daoud,
Central Michigan University,
United States

Reviewed by:
Veronica Lazar,
University of Bucharest, Romania
Murugan Kasi,
Manonmaniam Sundaranar University,
India

****Correspondence:***
Wedad M. Abdelraheem
wedad.abdelreheam@mu.edu.eg;
altaqwa.2012@yahoo.com

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *02 January 2022*
Accepted: *12 April 2022*
Published: *20 May 2022*

Citation:
Abdelraheem WM, Refaie MMM, Yousef RKM, Abd El Fatah AS, Mousa YM and Rashwan R (2022) Assessment of Antibacterial and Anti-biofilm Effects of Vitamin C Against Pseudomonas aeruginosa Clinical Isolates. Front. Microbiol. 13:847449. doi: 10.3389/fmicb.2022.847449

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is a versatile bacteria characterized by multiple resistance to various antimicrobial agents. It is one of the top causes of opportunistic infections and a primary causative agent of hospital-acquired infections (Stover et al., 2000). The emergence of multi-drug resistant (MDR) *P. aeruginosa* is a potential public health risk and may compromise

effective antibiotic therapy. Biofilm formation is an essential cause of antibiotic resistance among *P. aeruginosa* clinical isolates. There are a list of factors considered to be responsible for biofilm resistance, which include restricted penetration of antimicrobials into a biofilm barrier, decreased growth rate of biofilm persister cells, and expression of possible biofilm-specific resistance genes (Lewis, 2001). Biofilm formation is leading to another form of resistance, also called tolerance or phenotypical resistance of biofilm embedded cells. Biofilm-forming *P. aeruginosa* has the ability to convert to an antibiotic-resistant phenotype after antibiotic exposure with enhanced antibiotic tolerance (Drenkard and Ausubel, 2002).

There is considerable concern about the pandemic increase in antibiotic-resistant bacteria leading to treatment failure (Hancock and Speert, 2000). Vitamin C (ascorbic acid) represents one such alternative, and many studies have demonstrated its antibacterial action. It has a strong growth inhibitory effect on *Staphylococcus aureus*, *Enterococcus faecalis* (Isela et al., 2013), *Helicobacter pylori*, *Campylobacter jejuni* (Zhang et al., 1997), and *Mycobacterium tuberculosis* (Vilchèze et al., 2013), and even on fungi such as *Aspergillus* (Gupta and Guha, 1941). At first, it was hypothesized that the antimicrobial effect of vitamin C was due to its pH-lowering effect. However, another study proved the potent antimicrobial properties of vitamin C directed against *Streptocccus pyogenes*, even in pH-neutral conditions. Also, vitamin C is a powerful antioxidant agent produced against free radicals and reactive oxygen species (Slade and Knox, 1950).

Vitamin C is cheap, available, and has few or no side effects. Vitamin C is frequently prescribed as a nutritional supplement, has known antioxidant effects, and has been used as an adjuvant in cancer chemotherapy. Vitamin C is a gluconic acid lactone derived from glucuronic acid and water-soluble ketolactone with two ionizable hydroxyl groups (Sorice et al., 2014). In nature, there are two isomeric molecules of vitamin C present in equal parts, the reduced form (D-ascorbic acid) and the chemically active and oxidized form (L-ascorbic acid) (Hong et al., 2016), which are mutually interchangeable. The natural supplies of vitamin C are citrus fruits, kiwi, mango, strawberries, papaya, tomatoes, green leafy vegetables, and broccoli (Farris, 2014). This study investigated the effect of ascorbic acid alone and in combination with antibiotics on the growth and biofilm-forming potential of clinical isolates of *P. aeruginosa in vitro*. We also examined the effect of ascorbic acid alone and in combination with an antibiotic on treating *P. aeruginosa* infection in rats.

MATERIALS AND METHODS

Microorganisms

Previously identified *P. aeruginosa* clinical isolates collected from patients with burn or infected wounds, at the surgery department of Minia University Hospital, were included in this study. The identification of the isolates was done according to morphological characteristics and biochemical reactions using API Test Kits (bioMerieux, France). We selected 50 biofilm-producing *P. aeruginosa* from all other isolates to perform this study.

Determination of Minimal Inhibitory Concentration of Ascorbic Acid or Antimicrobials Alone and in Combinations

The minimal inhibitory concentration (MIC) of ascorbic acid or antibiotics alone and in combinations against all isolates was determined by micro-dilution method, according to the clinical and laboratory standard institute (CLSI) guidelines [Clinical and Laboratory Standard Institute [CLSI], 2019]. A stock solution of ascorbic acid was prepared by dissolving commercially purchased ascorbic acid (L-ascorbic acid, Sigma) in sterile distilled water (20 mg/ml). Stock solutions of the following antibiotics: piperacillin, piperacillin/tazobactam, ceftazidime, ciprofloxacin, and gentamicin were prepared by dissolving commercially purchased antibiotics in sterile distilled water (1 mg/ml). Müeller-Hinton broth (MHB) (Difco, United States) was used as a basal medium. Serial dilutions of ascorbic acid or antibiotics alone and in combinations were prepared in 100 μl volume of MHB in micro-titer plate wells. A quantity of 10 μl of bacterial suspension of each isolate with 0.5 McFarland turbidity was then inoculated in the wells. Wells containing a basal medium with and without bacterial suspension, but free of antibiotic and ascorbic acid, and wells containing the antibiotic or ascorbic acid were included in each assay as growth control. The plates were incubated for 24 h at 37°C. The lowest concentration of antibiotics separately or combined with ascorbic acid, which prevented the growth, was regarded as the MIC. The MIC results were interpreted according to CLSI guidelines [Clinical and Laboratory Standard Institute [CLSI], 2019].

Determination of Fractional Inhibitory Concentration

Fractional inhibitory concentration (FIC) was used to interpret the MIC results as follows (Mackay et al., 2000): FIC of antibiotic = MIC of the antibiotic combined with ascorbic acid/MIC of antibiotic alone. FIC $\leq$ 0.5 means syngergism, FIC $>$ 0.5–4 means indifference, and FIC $>$ 4 means antagonism.

Time-Kill Kinetics Assay

Vitamin C was tested to detect the time-kill kinetics. Briefly, grown culture of MHB with pure bacterial colonies (1.0×10^6 CFU/ml) was supplemented with ascorbic acid (625 μg/ml). Serial dilution was performed at each time point (0, 2, 4, 6, 12, and 24 h), and subculture on Muller Hinton agar plates was incubated at 37°C. Positive control test was performed for the tested strains without ascorbic acid. A graph of log CFU versus time was created. Each experiment was performed in triplicates (Appiah et al., 2017).

Effect of Ascorbic Acid on Biofilm Production

The effect of ascorbic acid on the biofilm-producing isolates of *P. aeruginosa* was assessed by the microtiter plate method according to the instructions by Samet et al., 2013. Initially, 190 μl of bacterial suspension equivalent to 0.5 McFarland in

Luria Bertani broth (LB) was inoculated in 96 microtiter plates. Sub-MIC of ascorbic acid was added to each well, excluding the positive and negative control wells. Plates were incubated at 37°C for 24 h. After incubation, the content of the wells were gently removed. The wells were washed with phosphate-buffered saline solution to remove free-unattached bacteria. Biofilms formed by adherent bacteria were air- and heat-fixed at 60°C for 1 h, and then stained with crystal violet (0.1%). Excess stain was rinsed off and the wells were washed with water. Ethanol 95% was added to the wells, and after 15 min, the optical densities (ODs) of the stained bacteria were determined with an ELISA reader (model CS, Biotec) at 590 nm. These OD values were considered an index of bacteria-forming biofilms. Experiments were performed in triplicate, and the data were then averaged.

Effect of Ascorbic Acid on Relative Genes Expression

Pseudomonas aeruginosa isolates were tested to express different antibiotic-resistant genes and biofilm encoding genes before and after treating the isolates with ascorbic acid, using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) according to the steps described below.

RNA Extraction

Isolated bacteria were inoculated in two tubes containing 2 ml LB broth with and without sub-MIC of ascorbic acid. The tubes were incubated at 37°C with shaking at 200 runs per minute (rpm) for 24 h. Bacterial RNA was extracted by the Direct-zol RNA extraction kit (CORP, Australia) according to the kit protocol. Absorbance was measured by a spectrophotometer (Genova, United States), and the ratio of absorbance at 260 nm and 280 nm was used to evaluate the purity of the extracted RNA. A result within the range of 1.8–2 was considered as acceptable purity. The quality of the extracted RNA was assessed *via* gel electrophoresis at 100 V for 60 min.

Real-Time Polymerase Chain Reaction

Quantitative RT-PCR was done using one-step SYBR green kits (SensiFAST SYBR Lo-ROX Kit, United Kingdom) in an ABI 7500 instrument (Applied Biosystems, United States). The RT-PCR reaction was prepared with a final volume of 20 μl (master mix: 10 μl, forward primer: 1 μl, reverse primer: 1 μl, reverse transcriptase: 0.2 μl, RNase inhibitor: 0.4 μl, water up to 16 μl, and template: 4 μl). Different genes and primers are listed in **Table 1**. Negative control samples containing deionized water instead of template, one for each gene, were included in the same PCR run.

We analyzed the PCR results with relative quantification to *16srRNA* as a reference gene. We calculated the fold changes of mRNA levels using the comparative cycle threshold (ΔΔCt). ΔCt = mean Ct of gene of interest – mean Ct reference gene. ΔΔCt = ΔCT of the test sample –Δ CT of the control sample. The relative quantity (RQ) = $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The relative expression was then analyzed by using free data analysis tools. PCR products were examined by gel electrophoresis to exclude any unspecific products that may be present.

TABLE 1 | Sequence of primers used for RT-PCR.

Gene	Primer	References
ampC	F:TCG CCT GAA GGC ACT GGT R: GGT GGC GGT GAA GGT CTT G	Konings et al., 2013
bla_{SHV}	F: CGCCTGTGTATTATCTCCCT R: CGAGTAGTCCACCAGATCCT	Abdelraheem, 2019
bla_{TEM}	F: TTTCGTGTCGCCCTTATTCC R: ATCGTTGTCAGAAGTAAGTTGG	
gyrA	F: GTGTGCTTTATGCCATGAG R: GGTTTCCTTTTCCAGGTC	Gorgani et al., 2009
aacC1	F: ATGGGCATCATTCGCACATGTAGG R: TTAGGTGGCGGTACTTGGGTC	Aliakbarzade et al., 2014
lasR	F: AAGTGGAAAATTGGAGTGGAG R: GTAGTTGCCGACGATGAAG	Abdelraheem and Mohamed, 2021
lecA	F: CACCATTGTGTTTCCTGGCGTTCA R: AGAAGGCAACGTCGACTCGTTGAT	
pelA	F: AAGAACGGATGGCTGAAGG R: TTCCTCACCTCGGTCTCG	
16s rRNA	F:ACG CAA CTG ACGAGT GTG AC R: GAT CGC GAC ACCGAA CTA AT	Abdelraheem et al., 2020

F, forward; R, reverse.
16s rRNA is the reference gene for P. aeruginosa.

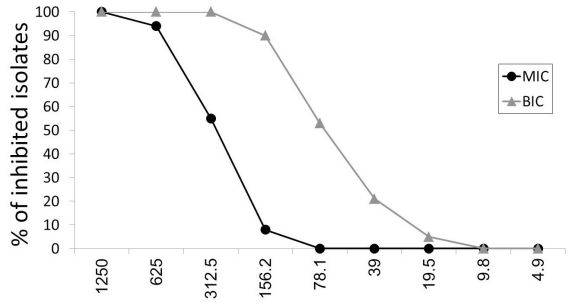


FIGURE 1 | Antibacterial and anti-biofilm effect of ascorbic acid. MIC, minimal inhibitory concentration; BIC, biofilm inhibitory concentration.

In vivo Assessment of the Antibacterial Effect of Ascorbic Acid

The experiment was conducted according to the standard practices concerning the ethics and animal procedures of the Institutional Research Ethics Committee.

Rats

Forty male Wistar rats weighing 250–280 g were obtained from National Research Center (Giza, Egypt). The 40 rats were divided into four groups (10 rats each), group 1 (Pneumonia; PN), group 2 (Vitamin C; VIT C), group 3 (Antibiotic; AB), and group 4 (VIT C+AB). The rats in groups 1, 2, 3, and 4 were intraperitoneally injected with *P. aeruginosa* clinical isolate. Three hours after injection, five rats were culled from group 1 to ensure that pneumonia had been established. Group 1 did not receive any treatment. Treatment options, given by intraperitoneal injection, were started 3 h after inoculation for groups 2, 3, and 4 for 72 h. Group 2 was treated with ascorbic acid (400 mg/kg/day), group 3 was treated with ceftazidime antibiotic (80 mg/kg/day),

TABLE 2 | The results of ascorbic acid /antimicrobial combinations.

Antibiotic	MIC[1]	MIC[2]	Number of isolates	FIC
Piperacillin	512	256	14	0.5
	128	64	11	
	32	16	11	
	16	8	7	
	8	4	5	
	4	2	2	
Ceftazidime	128	32	11	0.25
	64	16	9	
	16	4	11	
	4	1	14	
	2	0.5	3	
	1	0.25	2	
Ciprofloxacin	256	32	10	0.125
	32	4	12	
	16	2	10	
	4	0.5	4	
	1	0.125	6	
	0.5	0.06	8	
Gentamicin	128	16	16	0.125
	64	8	4	
	16	2	11	
	8	1	9	
	2	0.25	4	
	1	0.125	6	
Pepracillin/tazobactam	64/4	32/4	5	0.5
	32/4	16/4	16	
	16/4	8/4	8	
	8/4	4/4	12	
	2/4	1/4	6	
	1/4	0.5/4	3	

[1]MIC of antibiotic alone,[2]MIC of antimicrobial with sub-MIC of ascorbic acid.

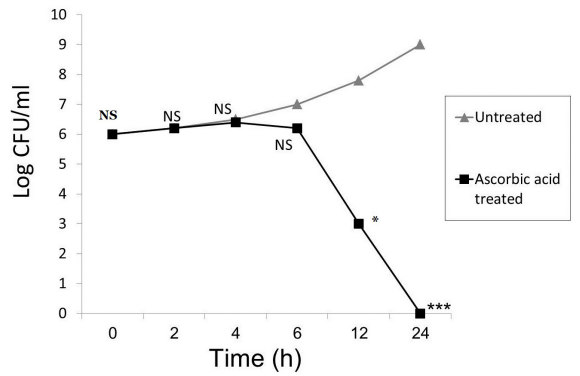


FIGURE 2 | Time-kill kinetics of ascorbic acid. Mean from 3 replicates plotted for all panels; (* means significant, *p*- value* < 0.05, *** < 0.001, and NS means not significant).

and group 4 was treated with ceftazidime (20 mg/kg/day) and ascorbic acid (200 mg/kg/day). The dose was calculated according to MIC results and rat pharmacokinetics.

Intraperitoneal *P. aeruginosa* Inoculation and Sample Collections

For intraperitoneal (*i.p*) inoculation in rats, the fresh cultured broth was prepared from isolated colonies of *P. aeruginosa*. Rats were injected with *P. aeruginosa* in 1,000 μl saline. The actual inoculum was quantitated by plating 10-fold serial dilutions on agar plates on brain-heart infusion (BHI). The clinical appearance of the rats was followed up. At the end of our experiment, the rats were culled. Initially, the rats were anesthetized, and then the peritoneal cavity and trachea were lavaged with 3 ml of phosphate-buffered saline. Blood samples were collected from the abdominal aorta for bacterial culture or serum separation. The lungs were aseptically removed, divided, and used for bacteriological, biochemical, and histological examination.

Bacterial Count

The lungs were aseptically homogenized in sterile saline using tissue homogenizers. A volume of 100 μl of 10-fold serial dilutions of the tissue homogenate, whole blood, bronchoalveolar lavage, and peritoneal lavage fluid were cultured on solid media. The bacterial count was calculated per 1 ml of blood and lavage fluid or per gram of tissue.

Quantification of Oxidative Stress Parameters

Blood was centrifuged at 3,000 rpm for 10 min (T30 centrifuge, Germany). Sera were kept frozen at −80°C for further analysis. The lungs were washed with saline and then divided. Specimens were homogenized in ice-cold phosphate buffer, centrifuged for 15 min at 5,000 rpm, and the supernatant was kept frozen at −80°C for further examination. The index of lipid peroxidation is the malondialdehyde (MDA) level, which was determined following the method of Buege and Aust, 1978. Glutathione (GSH) (Moron et al., 1979) and total antioxidant capacity (TAC) were evaluated using colorimetric kits and following the manufacturer's guidelines.

Statistical Analyses

Statistical analyses were performed using the Graph Pad Prism, Version 8.0 for Windows (Graph Pad Software, San Diego, CA, United States). Data are presented as means ± standard error of the mean (SEM). The quantitative measurements were assessed by Student's *t*-test or one-way ANOVA followed by Turkey's multiple comparison tests, when appropriate. The difference was considered significant when p-value < 0.05.

RESULTS

In vitro Antibacterial and Anti-biofilm Effect of Ascorbic Acid

Minimal inhibitory concentrations of ascorbic acid among *P. aeruginosa* isolates were 156–1,250 μg/ml, as presented in **Figure 1**. The MIC50 and MIC90 of ascorbic acid, which inhibit the growth of 50% and 90% of isolates, were 312.5 and 625 μg/ml, respectively.

At sub-inhibitory concentrations (19.5–312.5 μg/ml), ascorbic acid had 100% biofilm inhibitory effect as presented in **Figure 1**. BIC50 and BIC90, biofilm inhibitory concentrations of ascorbic acid that inhibits the biofilm formation in 50% and 90% of isolates, were 78.1 and 156.2 μg/ml, respectively.

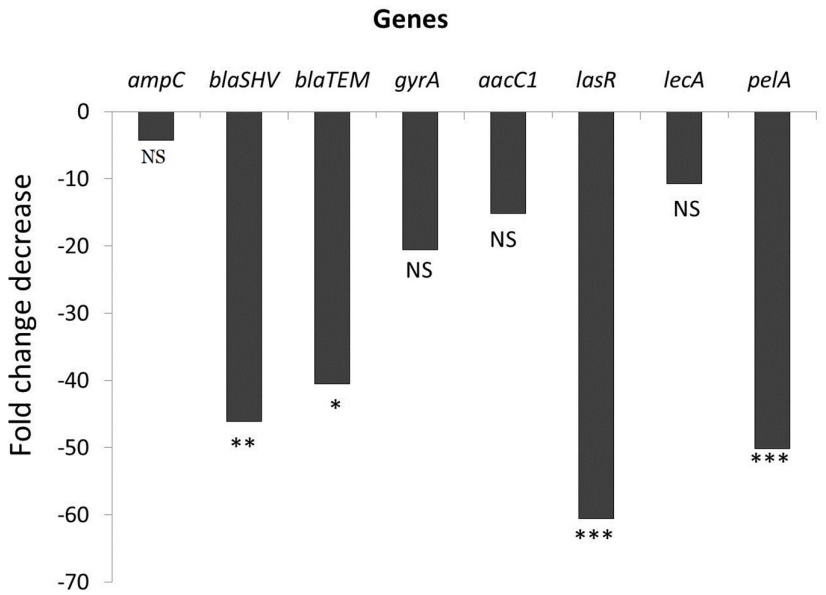


FIGURE 3 | Effect of ascorbic acid on gene expression (* means significant, *p*- value* < 0.05, value** < 0.01, *** < 0.001).

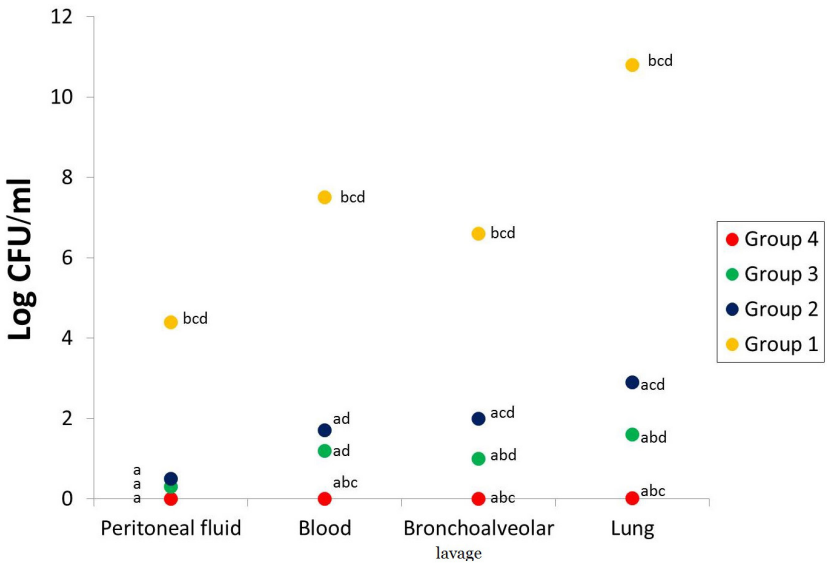


FIGURE 4 | Bacterial count in the peritoneal lavage, blood, bronchoalveolar lavage, and lungs of different rat groups. [a]Significant difference compared to PN group. [b]Significant difference compared to VIT C group. [c]Significant difference compared to AB group. [d]Significant difference compared to VIT C+AB group. PN, pneumonia; VIT C, vitamin C; AB, antibiotic.

The Results of Ascorbic Acid /Antimicrobial Combinations

The results of the combination studies are shown in **Table 2**. Synergy was detected in the five antimicrobial–ascorbic acid combinations tested.

Piperacillin and piperacillin/tazobactam MIC showed onefold decrease when combined with ascorbic acid (FIC = 0.5). Ceftazidime MIC showed twofold reductions when combined with ascorbic acid (FIC = 0.25). Ciprofloxacin and gentamicin MIC showed threefold decreases when combined with ascorbic acid (FIC = 0.125).

Time-Kill Kinetics Study

The time-kill kinetics study of ascorbic acid against the tested *P. aeruginosa* isolates significantly reduced the number of viable bacterial cells over the first 12, and 24 h, respectively, as shown in **Figure 2**.

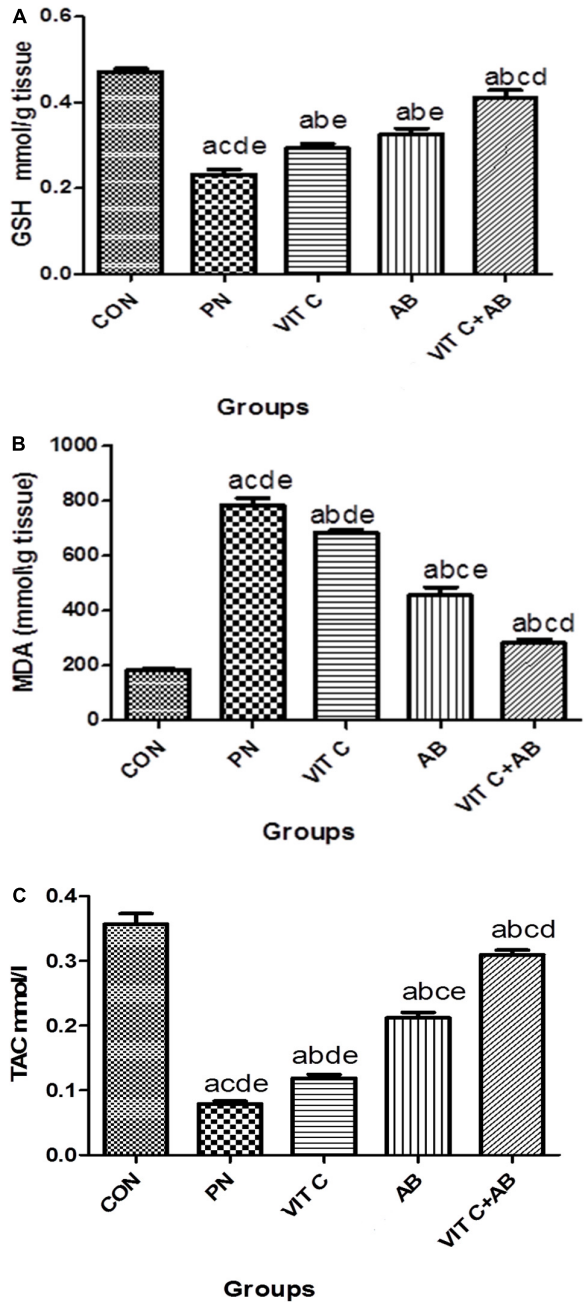


FIGURE 5 | Oxidative stress parameters of different rat groups. **(A)** Glutathione (GSH) in lung tissue, **(B)** malondialdehyde (MDA) in lung tissue, **(C)** total antioxidant capacity (TAC) in serum. [a]Significant difference compared to PN group. [b]Significant difference compared to VIT C group. [c]Significant difference compared to AB group. [d]Significant difference compared to VIT C+AB group. PN, pneumonia group; VIT C, vitamin C; AB, antibiotic.

Effect of Ascorbic Acid on Gene Expression

The results showed that the expression levels of all tested antimicrobial resistance genes (*ampC, bla*$_{SHV}$*, bla*$_{TEM}$*, gyrA,* and *aacC1*) and biofilm-associated gnes (*lasR, lecA,* and *pelA*) were down-regulated as shown in **Figure 3**. We observed a 46.1- and 40.5-fold decrease in the expression of the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes, respectively, under ascorbic acid-treated condition (p value < 0.05). Results also confirmed that there was a significant fold change decrease (60.6 and 50.1) in transcription of biofilm-associated genes, *lasR* and *pelA,* respectively, (p value < 0.05) compared to untreated culture, thus confirming the anti-biofilm effect of ascorbic acid.

In vivo Assessment of the Antibacterial Effect of Ascorbic Acid

All remaining rats in group 1 died after 24 h, and then all the rats were culled after 72 h (6 h after the last dose of treatment).

Bacterial Count

The five rats culled at the start of the experiment (3 h after inoculation of *P. aeruginosa*) presented bilateral pneumonia with a mean *P. aeruginosa* count of $\log_{10}$; 4.4, 7.5, 6.6, and 10.8 for peritoneal fluid, blood, bronchoalveolar lavage, and lung, respectively (**Figure 4**). The viable count after treatment was calculated in peritoneal fluid, blood, bronchoalveolar lavage, and lung, and the mean is presented in **Figure 4**. VIT C+AB group shows complete eradication of *P. aeruginosa* infection. A significant reduction of the bacterial count was seen in the AB group and VIT C group, respectively, in comparison with the untreated PN group (p value < 0.05).

Oxidative Stress Parameters

VIT C alone, AB alone, and VIT C+AB groups showed a significant decrease in MDA with a substantial increase in GSH and TAC compared to the control group. Co-administration of VIT C + AB showed more improvement and normalization of oxidative stress parameters compared to VIT C or AB groups, as shown in **Figures 5A–C**.

Histological Examination

Comparison of the histological picture of rat lung tissues of different groups is presented in **Figures 6A–D**. The five rats killed at the start of the experiment (PN group) showed a histological picture of bilateral pneumonia. Improvement of the histological picture in all treatment groups was detected but with different degrees. The VIT C+AB group showed nearly complete improvement of the signs of pneumonia. Signs of pneumonia were still present in VIT C and AB groups, respectively, but less than PN group.

DISCUSSION

This study is the first insight wherein we evaluated antimicrobial and anti-biofilm effect of vitamin C against *P. aeruginosa* clinical isolates *in vitro* and *in vivo*.

The antimicrobial effect of vitamin C exists against the all the *P. aeruginosa* clinical isolates tested. To our surprise, vitamin C inhibited the growth of *P. aeruginosa* clinical isolates at lower concentration ranges of 156.2–1,250 μg/ml, while MIC50 and MIC90 were 312.5 and 625 μg/ml, respectively.

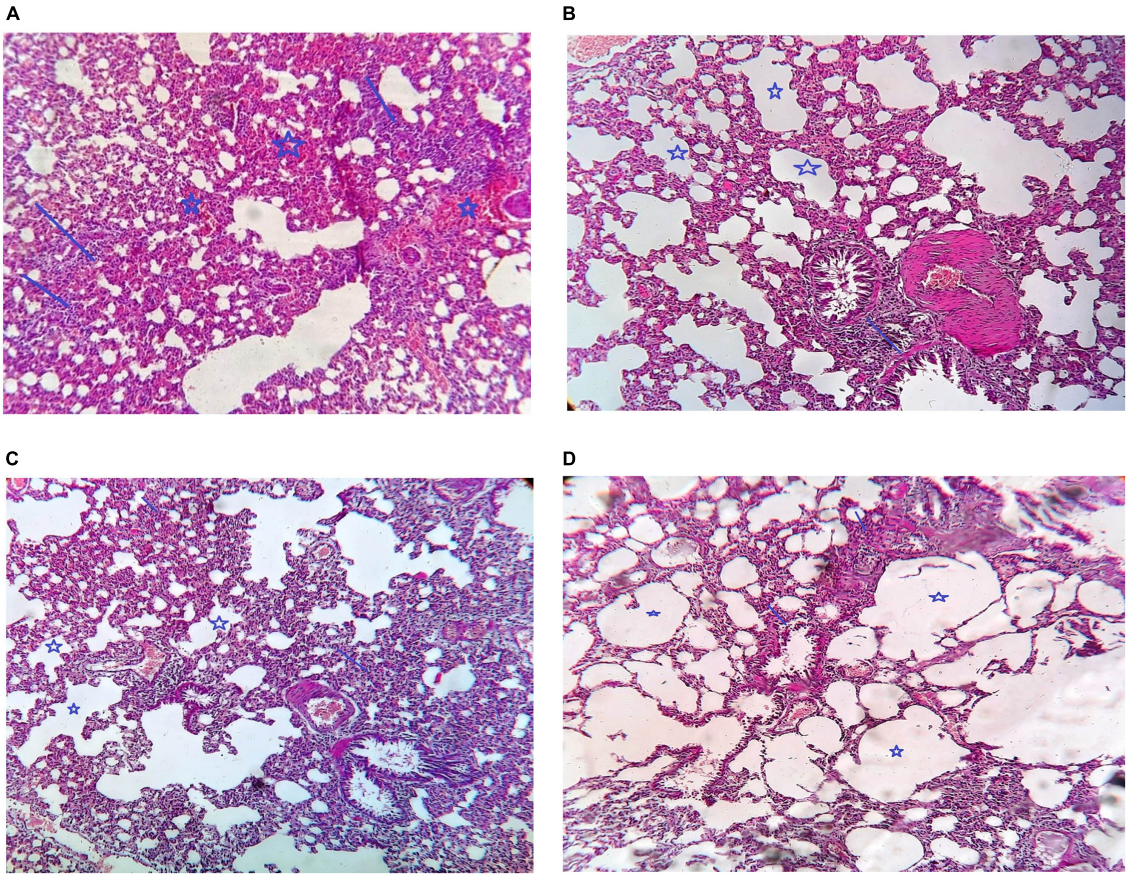


FIGURE 6 | Histological examination of different groups of rat lungs. **(A)** PN group, Arrow show multiple foci of moderate inflammatory infiltrate. Star showed marked intraalveolar congestion, **(B)** VIT C group: Arrow showed that there is alveoli are still congested and contain inflammatory infiltrate but less than that in case of pneumonia only. Star showed that there is some alveoli are cleared from congestion and inflammatory infiltrate, **(C)** AB group: Arrow show moderate peribroncheal inflammatory infiltrate. Star show alveoli cleared from congestion and inflammatory infiltrate, **(D)** VIT C+ AB group: Arrow showed there is no peribronchial inflammatory infiltrate. Star showed cleared alveoli with no congestion or inflammation.

The antimicrobial effect of vitamin C corroborates with the previous results by Golonka et al. (2017), which depict that the MIC 310 μg/mL of vitamin C could inhibit *P. aeruginosa* growth *in vitro*. In addition, vitamin C at low concentration (150 μg/ml) was shown to inhibit the growth of *S. aureus* and *E. faecalis* (Verghese et al., 2017). Further studies reported the antibacterial effect of vitamin C against *E. coli* and Klebsiella (Abd El-Baky and El-Gebaly, 2012).

This is in contrast to the findings reported by Pandit et al., 2017. They reported that vitamin C at low concentrations did not exhibit any inhibitory effect against planktonic bacteria. They suggested that vitamin C enhances the antibiotic bacterial susceptibility without demonstrating direct bactericidal activity.

With regard to the reports mentioned above, the differences of low or no antibacterial effect of vitamin C are probably attributed to the variations in the methodologies attributed during the study, i.e., differences in the bacterial strains, bacterial cell density, culture media composition, and concentration of vitamin C.

Moving on to the biofilm production, we found an anti-biofilm effect of vitamin C on the same isolates that were examined, based on the absorbance results that we obtained from the ELISA reader. Biofilm inhibitory concentrations of ascorbic acid among *P. aeruginosa* isolates were 19.5–312.5 μg/ml, with BIC50 and BIC90 being 78.1 and 156.2 μg/ml, respectively. In this aspect, Abd El-Baky and El-Gebaly (2012) reported that vitamin C at a concentration of 100 mg/ml could inhibit biofilm production on the catheter surface. There was up to a 92% reduction in biofilm production. Likewise, Sendamangalam et al. (2011) and Eydou et al. (2020) reported 2 mg/ml and 5.61 mg/ml, respectively, as the biofilm inhibitory concentration of vitamin C against *S. mutans*.

Furthermore, vitamin C could effectively counteract biofilm production by methicillin-resistant *S. aureus* (MRSA) at a lower concentration range of 8–16 μg/ml (Ali Mirani et al., 2018).

A synergistic effect was detected in the five antimicrobial–ascorbic acid combinations tested; piperacillin and piperacillin/tazobactam (FIC = 0.5), ceftazidime (FIC = 0.25), ciprofloxacin and gentamicin (FIC = 0.125). The enhancement of antibiotic activity or the reversal of antibiotic resistance by non-traditional antibiotics support the classification of vitamin C as a modifier of antibiotic activity. Similar synergistic effects

between antimicrobials and ascorbic acid were previously reported by Cursino et al. (2005) and Abd El-Baky and El-Gebaly (2012).

The synergistic effect of ascorbic acid with antibiotics may be due to its effect on certain metabolic activities associated with protein synthesis inside bacterial cells, making the bacterial cells more permeable to antibiotics through its effect on the cytoplasmic membrane, or it could be due to the effect of hydrogen peroxide produced by the oxidation of ascorbic acid, which causes antibiotics to have a higher potency (Kramarenko et al., 2007). Certain antibiotics increase the production of reactive oxygen species (free radicals) in various bacterial species, suggesting that ascorbic acid may serve as a free radical scavenger (Albesa et al., 2004). Also, the synergistic effect of ascorbic acid with antibiotics may be due to down regulation of antibiotic-resistant genes.

Biofilm-forming *P. aeruginosa* strains demonstrated higher expression levels of *lasR, lecA*, and *pelA* genes (Abdelraheem et al., 2020).

Further, to confirm the anti-biofilm effect of vitamin C and the corresponding synergistic effect with antibiotics against *P. aeruginosa* tested isolates, a transcriptional response was analyzed in the presence of Vitamin C. The results showed that all tested biofilm-associated gene expression levels vital to *P. aeruginosa* biofilm formation (*lasR, lecA,* and *pelA*) were down-regulated after being treated with ascorbic acid, with significant decrease in *lasR and pelA* (p value < 0.05). Therefore, it was suggested that the anti-biofilm activity of vitamin C was due to the inhibition of biofilm-forming genes expression.

Also, the expression levels of all tested genes (*ampC, bla$_{SHV}$, bla$_{TEM}$, aacC1, and gyrA*) are essential for resistance to piperacillin, piperacillin/tazobactam, ceftazidime, ciprofloxacin and gentamicin were down-regulated after treating *P. aeruginosa* isolates with ascorbic acid, the expression levels of all tested genes (ampC, bla$_{SHV}$, bla$_{TEM}$, aacC1, and gyrA), which are essential for resistance to piperacillin, piperacillin/tazobactam, ceftazidime, ciprofloxacin and gentamicin, were down-regulated, with a significant decrease in bla$_{SHV}$ and bla$_{TEM}$ (P value < 0.05).

In our *in vivo* investigation, the efficacy of vitamin C treatment in rats after infection with *P. aeruginosa* showed that the administration of vitamin C in the infected animal partially resolved the disease and lowered the bacterial count. *In vivo,* the rats' vitamin C and antibiotic assessment showed that antibiotic alone has a better antibacterial effect than vitamin C alone. Still, the administration of both at a lower dose for treating pseudomonas infection in rats has a synergistic and more powerful impact. Vitamin C shows excellent *in vitro* results as an antibacterial and anti-biofilm agent. Still, a combination of vitamin C and antibiotics is necessary to eradicate the bacterial infection *in vivo.* Vitamin C has a synergistic effect with antibiotics *in vitro* and *in vivo*. Vitamin C should be routinely prescribed with antibiotics to treat pseudomonas infections in clinical settings as this combination will shorten the antibiotic course, decrease the dose of the antibiotic, and reduce the development of bacterial resistance. However, further *in vivo* studies are needed before generalizing the concept about the effectiveness of ascorbic acid–antibiotics combined therapy. Future research on ascorbic acid–antimicrobial interactions is required to control MDR bacteria.

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 Ethical Committee of Minia University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Research Ethics Committee.

AUTHOR CONTRIBUTIONS

WA, RR, AA, and YM: concept and study design. WA, MR, and RY: experiments and results analysis. All authors drafted and revised the manuscript and read and approved the final manuscript.

ACKNOWLEDGMENTS

We would like to thank the technicians at Pharmacology Department, Faculty of Medicine, Minia University, who helped us deal with mice.

REFERENCES

Abd El-Baky, R., and El-Gebaly, E. (2012). Effect of levofloxacin and Vitamin C on bacterial adherence and preformed biofilm on urethral catheter surfaces. *J. Microb. Biochem. Technol.* 4, 131–136. doi: 10.4172/1948-5948.1000083

Abdelraheem, W. M. (2019). Fresh garlic extract has a synergistic effect with antibiotics on ESBLs producing *E. coli* urinary isolates. *J. Adv. Microbiol.* 14, 1–9.

Abdelraheem, W. M., and Mohamed, E. S. (2021). The effect of Zinc Oxide nanoparticles on *Pseudomonas aeruginosa* biofilm formation and virulence genes expression. *J. Infect. Dev. Ctries* 15, 826–832. doi: 10.3855/jidc.13958

Abdelraheem, W., Abdelkader, A., Mohamed, E., and Mohammed, M. (2020). Detection of biofilm formation and assessment of biofilm genes expression in different *Pseudomonas aeruginosa* clinical isolates. *Meta Gene* 23:100646.

Albesa, I., Becerra, M. C., Battan, P. C., and Paes, P. L. (2004). Oxidative stress involved in the antibacterial action of different antibiotics. *Biochem. Biophys. Res. Commun.* 317, 605–609. doi: 10.1016/j.bbrc.2004.03.085

Ali Mirani, Z., Khan, M. N., Siddiqui, A., Khan, F., Aziz, M., Naz, S., et al. (2018). Ascorbic acid augments colony spreading by reducing biofilm formation of

methicillin-resistant *Staphylococcus aureus*. *Iran. J. Basic Med. Sci.* 21, 175–180. doi: 10.22038/IJBMS.2018.20714.5398

Aliakbarzade, K., Farajnia, S., KarimiNik, A., Zarei, F., and Tanomand, A. (2014). Prevalence of aminoglycoside resistance genes in *Acinetobacter baumannii* isolates. *Jundishapur J. Microbiol.* 7:e11924.

Appiah, T., Boakye, Y. D., and Agyare, C. (2017). Antimicrobial activities and time-kill kinetics of extracts of selected ghanaian mushrooms. *Evid. Based Complement. Altern. Med.* 2017:4534350. doi: 10.1155/2017/4534350

Buege, J., and Aust, S. (1978). Microsomal lipid peroxidation. *Methods Enzymol.* 52, 302–310.

Clinical and Laboratory Standard Institute [CLSI] (2019). *Performance Standards for Antimicrobial Susceptibility Testing.* CLSI Supplement M100, 29th Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Cursino, L., Chartone-Souza, E., Nascimento, A., and Andréa Maria, A. (2005). Synergic interaction between ascorbic acid and antibiotics against *Pseudomonas aeruginosa*. *Braz. Arch. Biol. Technol.* 48, 379–384.

Drenkard, E., and Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 416, 740–743. doi: 10.1038/416740a

Eydou, Z., Jad, B. N., Elsayed, Z., Ismail, A., Magaogao, M., and Hossain, A. (2020). Investigation on the effect of vitamin C on growth & biofilm-forming potential of Streptococcus mutans isolated from patients with dental caries. *BMC Microbiol.* 20:231. doi: 10.1186/s12866-020-019 14-4

Farris, K. (2014). "Cosmeceutical vitamins: vitamin C," in *Cosmeceuticals*, 3rd Edn. Vol. 2, ed. Z. D. Draelos (Amsterdam, Elsevier), 37–42.

Golonka, I., Oleksy, M., Junka, A., Matera-Witkiewicz, A., Bartoszewicz, M., and Musiał, W. (2017). Selected physicochemical and biological properties of EthylAscorbic acid compared to ascorbic acid. *Biol. Pharm. Bulletin.* 40, 1199–1206. doi: 10.1248/bpb.b16-00967

Gorgani, N., Ahlbrand, S., Patterson, A., and Pourmand, N. (2009). Detection of point mutations associated with antibiotic resistance in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 34, 414–418. doi: 10.1016/j.ijantimicag. 2009.05.013

Gupta, G., and Guha, B. (1941). The effect of Vitamin C and certain other substances on the growth of microorganisms. *Ann. Biochem. Exp. Med.* 1, 14–26.

Hancock, R., and Speert, D. (2000). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug. Resist. Updat.* 3, 247–255.

Hong, J., Kim, H., Kang, S., Lee, W., and Hwang, I. (2016). Vitamin C is taken up by human T cells *via* sodium-dependent vitamin C transporter 2 (SVCT2) and exerts inhibitory effects on the activation of these cells in vitro. *Anat. Cell Biol.* 49, 88–98. doi: 10.5115/acb.2016.49.2.88

Isela, S., Sergio, N., Jose, M., Rene, H., and Claudio, C. (2013). Ascorbic acid on oral microbial growth and biofilm formation. *Pharma Innov.* 28, 104–109.

Konings, A., Martin, L., Sharples, K., Roddam, L., Latham, R., Reid, D., et al. (2013). *Pseudomonas aeruginosa* uses multiple pathways to acquire iron during chronic infection in cystic fibrosis lungs. *Infect. Immun.* 81, 2697–2704. doi: 10.1128/IAI.00418-13

Kramarenko, G. G., Hummel, S. G., Martin, S. M., and Buettner, G. R. (2007). Ascorbate reacts with singlet oxygen to produce hydrogen peroxide. *Photochem. Photobiol.* 82, 1634–1637. doi: 10.1562/2006-01-12-RN-774

Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* 45, 999–1007. doi: 10.1128/AAC.45.4.999-1007.2001

Livak, K., and Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta DeltaC(T)) Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001. 1262

Mackay, M. L., Milne, K., and Gould, I. M. (2000). Comparison of methods for assessing synergic antibiotic interactions. *Int. J. Antimicrob. Agents* 15, 125–129. doi: 10.1016/s0924-8579(00)00149-7

Moron, M. S., Depierre, J. W., and Mannervik, B. (1979). Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim. Biophys. Acta* 582, 67–78. doi: 10.1016/0304-4165(79)90289-7

Pandit, S., Ravikumar, V., Abdel-Haleem, A. M., Derouiche, A., Mokkapati, V. R., Sihlbom, C., et al. (2017). Low concentrations of VitC reduce the synthesis of extracellular polymers and destabilize bacterial biofilms. *Front. Microbiol.* 8:2599. doi: 10.3389/fmicb.2017.02599

Samet, M., Ghaemi, E., Jahanpur, S., and Jamalli, A. (2013). Evaluation of biofilm-forming capabilities of urinary *Escherichia coli* isolates in microtiter plate using two different culture media. *Int. J. Mol. Clin. Microbiol.* 3, 244–247.

Sendamangalam, V., Choi, O. K., Kim, D., and Seo, Y. (2011). The anti-biofouling effect of polyphenols against Streptococcus mutans. *Biofouling* 27, 13–19. doi: 10.1080/08927014.2010.535897

Slade, H., and Knox, G. (1950). Nutrition and the role of reducing agents in the formation of streptolysin O by a group A hemolytic streptococcus. *J. Bacteriol.* 60:301. doi: 10.1128/jb.60.3.301-310.1950

Sorice, A., Guerriero, E., Capone, F., Colonna, G., Castello, G., and Costantini, S. (2014). Ascorbic acid: its role in immune system and chronic inflammation diseases. *Mini Rev. Med. Chem.* 14, 444–452. doi: 10.2174/1389557514666140428112602

Stover, C., Pham, X., Erwin, A., Mizoguchi, S., Warrener, P., Hickey, M., et al. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964. doi: 10.1038/3502 3079

Verghese, R. J., Mathew, S. K., and David, A. (2017). Antimicrobial activity of vitamin C demonstrated on uropathogenic *Escherichia coli* and *Klebsiella pneumoniae*. *J. Curr. Res. Sci. Med.* 3, 88–93. doi: 10.4103/jcrsm.jcrsm_35_17

Vilchèze, C., Hartman, T., Weinrick, B., and Jacobs, J. (2013). Mycobacterium tuberculosis is extraordinarily sensitive to killing by a Vitamin C-induced Fenton reaction. *Nat. Commun.* 4:1881. doi: 10.1038/ncomms 2898

Zhang, H., Wakisaka, N., Maeda, O., and Yamamoto, T. (1997). Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: *Helicobacterpylori*. *Cancer* 80, 1897–1903.

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 Abdelraheem, Refaie, Yousef, Abd El Fatah, Mousa and Rashwan. 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 Microbiology

ORIGINAL RESEARCH
published: 10 June 2022
doi: 10.3389/fmicb.2022.915375

Genomic Analysis of a Highly Virulent NDM-1-Producing *Escherichia coli* ST162 Infecting a Pygmy Sperm Whale (*Kogia breviceps*) in South America

Fábio P. Sellera[1,2,3†], Brenda Cardoso[2,4†], Danny Fuentes-Castillo[2,5], Fernanda Esposito[2,6], Elder Sano[2,4], Herrison Fontana[2,6], Bruna Fuga[2,4,6], Daphne W. Goldberg[7], Lourdes A. V. Seabra[3], Marzia Antonelli[7], Sandro Sandri[7], Cristiane K. M. Kolesnikovas[7] and Nilton Lincopan[2,4,6*]*

[1] Department of Internal Medicine, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil, [2] One Health Brazilian Resistance Project (OneBR), São Paulo, Brazil, [3] School of Veterinary Medicine, Metropolitan University of Santos, Santos, Brazil, [4] Department of Microbiology, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil, [5] Departamento de Patología y Medicina Preventiva, Facultad de Ciencias Veterinarias, Universidad de Concepción, Chillán, Chile, [6] Department of Clinical Analysis, School of Pharmacy, University of São Paulo, São Paulo, Brazil, [7] R3 Animal, Florianópolis, Brazil

Carbapenemase-producing Enterobacterales are rapidly spreading and adapting to different environments beyond hospital settings. During COVID-19 lockdown, a carbapenem-resistant NDM-1-positive *Escherichia coli* isolate (BA01 strain) was recovered from a pygmy sperm whale (*Kogia breviceps*), which was found stranded on the southern coast of Brazil. BA01 strain belonged to the global sequence type (ST) 162 and carried the bla_{NDM-1}, besides other medically important antimicrobial resistance genes. Additionally, genes associated with resistance to heavy metals, biocides, and glyphosate were also detected. Halophilic behavior (tolerance to > 10% NaCl) of BA01 strain was confirmed by tolerance tests of NaCl minimal inhibitory concentration, whereas halotolerance associated genes *katE* and *nhaA*, which encodes for catalase and Na^+/H^+ antiporter cytoplasmic membrane, respectively, were *in silico* confirmed. Phylogenomics clustered BA01 with poultry- and human-associated ST162 lineages circulating in European and Asian countries. Important virulence genes, including the *astA* (a gene encoding an enterotoxin associated with human and animal infections) were detected, whereas *in vivo* experiments using the *Galleria mellonella* infection model confirmed the virulent behavior of the BA01 strain. WHO critical priority carbapenemase-producing pathogens in coastal water are an emerging threat that deserves the urgent need to assess the role of the aquatic environment in its global epidemiology.

Keywords: carbapenems, NDM carbapenemases, nosocomial bacteria, one health, wildlife, aquatic environment

OPEN ACCESS

Edited by:
Ziad Daoud, Central Michigan University, United States

Reviewed by:
Ahmed Mahrous Soliman, Kafrelsheikh University, Egypt
Andres Felipe Opazo-Capurro, University of Concepcion, Chile

***Correspondence:**
Fábio P. Sellera
fsellera@usp.br
Nilton Lincopan
lincopan@usp.br

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *07 April 2022*
Accepted: *18 May 2022*
Published: *10 June 2022*

Citation:
Sellera FP, Cardoso B, Fuentes-Castillo D, Esposito F, Sano E, Fontana H, Fuga B, Goldberg DW, Seabra LAV, Antonelli M, Sandri S, Kolesnikovas CKM and Lincopan N (2022) Genomic Analysis of a Highly Virulent NDM-1-Producing Escherichia coli ST162 Infecting a Pygmy Sperm Whale (Kogia breviceps) in South America. Front. Microbiol. 13:915375. doi: 10.3389/fmicb.2022.915375

INTRODUCTION

The rapid and global spread of carbapenemase-producing Enterobacterales has triggered an unprecedented public health crisis due to the lack of novel clinically effective antibiotics (Queenan and Bush, 2007; Nordmann et al., 2011a; Lee et al., 2022). Carbapenems are broad-spectrum β-lactam antibiotics that have been administered as a last-line resort, being generally reserved to treat life-threatening infections caused by multidrug-resistant (MDR) Gram-negative bacterial infections (Papp-Wallace et al., 2011; Lee et al., 2022). The production of carbapenemases by certain Enterobacterales and non-fermentative bacteria, can threaten the efficacy of these antimicrobials making them useless (Queenan and Bush, 2007; Nordmann et al., 2011a; Lee et al., 2022).

Due to their clinical impacts in human medicine, carbapenemase-producing bacteria were recent classified as critical priority pathogens by the World Health Organization (WHO) (Tacconelli et al., 2018). Particularly, the emergence of metallo-β-lactamase NDM-1-producing bacteria has been a phenomenon of global interest (Nordmann et al., 2011a,b; Dortet et al., 2014). Of epidemiological concern, although the successful spread of plasmids encoding bla_{NDM}-type genes has been primarily related to nosocomial settings (Wu et al., 2019), there is growing evidence of their occurrence beyond the human medicine context (Mills and Lee, 2019; Ranjan and Thatikonda, 2021). Indeed, NDM-1-producing bacteria have been increasingly reported in environmental samples (mostly in anthropogenic-impacted aquatic environments) (Ranjan and Thatikonda, 2021). More critically, NDM-1-positive bacteria have begun to be documented in wild animals (Fischer et al., 2013; Liao et al., 2019; Mairi et al., 2020), which could indicate, in part, that these critical-priority bacteria can spill over into natural ecosystems, and then further spread in wildlife (Dolejska and Literak, 2019; Mills and Lee, 2019; Cohen et al., 2020; Mairi et al., 2020).

In this study, we report the identification of an NDM-1-positive *Escherichia coli* strain belonging to the international clone sequence type (ST) 162 in a pygmy sperm whale (*Kogia breviceps*), highlighting negative clinical and ecological implications related to the dissemination of WHO critical priority pathogens in the marine environment.

MATERIALS AND METHODS

Bacterial Isolation, Identification, and Antimicrobial Susceptibility Testing

During a surveillance study conducted to investigate the occurrence and genomic features of critical priority Gram-negative pathogens circulating at the marine ecosystems in Brazil, part of the Grand Challenges Explorations—New Approaches to Characterize the Global Burden of Antimicrobial Resistance program, we characterized a multidrug-resistant *E. coli* recovered from a pygmy sperm whale (*Kogia breviceps*), during the COVID-19 lockdown.

The animal (**Supplementary Figure 1**) was received at CEPRAM/R3 Animal (Florianópolis, Santa Catarina state, Southern Brazil), as part of the Santos Basin Beach Monitoring Project, licensed by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) of the Brazilian Ministry of Environment under ABIO N° 755/2016. The whale was found stranded alive on October 19th, 2020, in a beach (−28.1663385: −48.6577602), located on the city of Imbituba, in Santa Catarina state, Southern Brazil. The animal was immediately monitored in the water by the Santos Basin Beach Monitoring Project team. External examination revealed multiple signs of interspecific interaction in the ventral and lateral regions of the body and a deep circular lesion consistent with bite marks from cookiecutter sharks (*Isistius* spp.). The whale was carefully monitored during transport, receiving benzodiazepines (Diazepam, 0.1 mg/kg, IM) and bronchodilators (Aminophylline, 4 mg/kg, IM, SID) to dilate the lungs' airways. Upon arrival at Associação R3 Animal, in Florianópolis, the whale was transferred to a 60,000 L tank under continuous supervision. To replace the animal's hydration, 1.5 L of water were initially administered through a 17 mm equine nasogastric tube and later the volume was increased to 3 L every 3 h and antibiotic therapy was started (Enrofloxacin, 5 mg/kg, IM, BID). On October 21, the animal begun to show signs of agitation, swimming erratically, jumping, and bending its body, even after being sedated. The veterinary staff observed prolonged periods of apnea and bradycardia. Cardiac massage and administration of emergency drugs were attempted, but the animal succumbed to death.

At necropsy, besides other gross alterations, the prescapular lymph nodes were clearly swollen. A yellowish purulent material was collected from the lymph nodes and placed in Stuart transport medium, and immediately sent to a microbiology laboratory for bacterial culture and antimicrobial susceptibility testing. The *E. coli* strain (BA01) was recovered, being identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Antimicrobial susceptibility was performed and interpreted according to the Clinical and Laboratory Standards Institute recommendations (CLSI, 2020). In this respect, human and veterinary antibiotics were tested, including amoxicillin-clavulanic acid, ceftazidime, cefotaxime, ceftriaxone, ceftiofur, cefepime, cefoxitin, aztreonam, ertapenem, meropenem, imipenem, ciprofloxacin, levofloxacin, trimethoprim/sulfamethoxazole, fosfomycin, gentamicin, tetracycline, and amikacin. A drug-susceptible *E. coli* (ATCC 25922) was included as a control strain. Moreover, to determinate whether BA01 strain could survive in the marine environment, tolerance test of NaCl minimal inhibitory concentration (MIC) was performed using 0.1–15% NaCl solutions (Fernandes et al., 2020a).

Whole Genome Sequence Analysis

The whole genomic DNA of *E. coli* BA01 was extracted (PureLink™; Invitrogen) and used to prepare a library that was sequenced using the NextSeq550 platform (2 × 75-bp paired-end) (Illumina). Raw sequencing data were quality filtered to remove low-quality bases (Phred20 quality score)

using Trimmomatic v0.32.[1] The sequence reads were assembled *De novo* using default parameters of Unicycler v0.4.8.[2] Draft genome sequence was automatically annotated using the NCBI Prokaryotic Genome Annotation Pipeline v.3.2.[3] BA01 circular genome map (**Figure 1**) was performed using the Proksee platform[4] and BLASTN.

Multilocus sequence type (MLST), plasmid replicons, resistome, virulome, type fimbrial, and serotype were performed *in silico* using MLST v2.0, PlasmidFinder v2.0, ResFinder 4.0, VirulenceFinder 2.0, FimTyper v1.0, and SerotypeFinder v2.0, respectively; available from the centre for Genomic Epidemiology.[5] In addition, ABRicate v0.9.8[6] was used to screen putative virulence factors through VFDB database.[7]

[1]https://github.com/timflutre/trimmomatic
[2]https://github.com/rrwick/Unicycler
[3]www.github.com/tseemann/prokka
[4]https://beta.proksee.ca/
[5]http://genomicepidemiology.org/
[6]https://github.com/tseemann/abricate
[7]https://github.com/haruosuz/vfdb

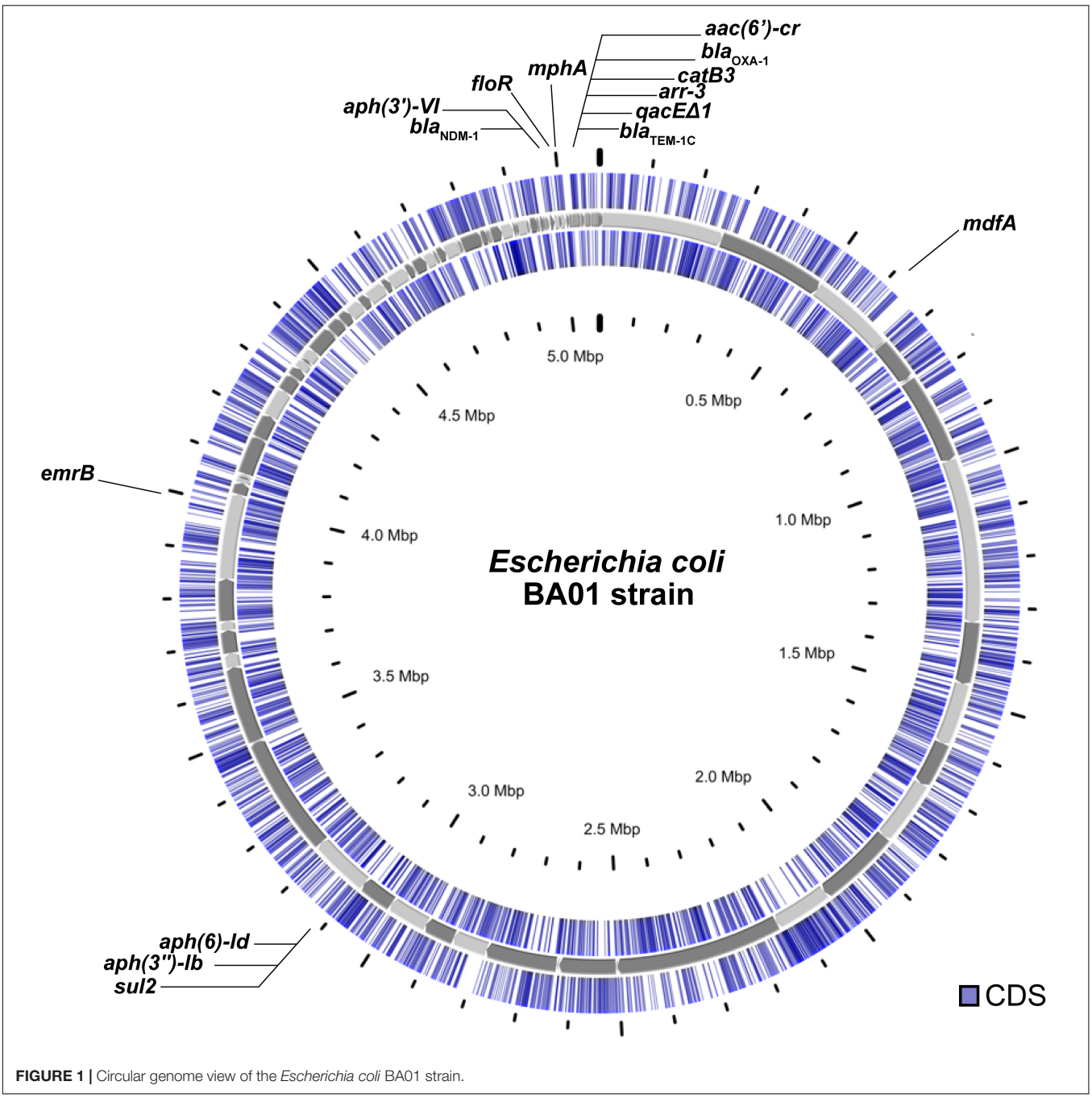


FIGURE 1 | Circular genome view of the *Escherichia coli* BA01 strain.

The presence of heavy metal (HM) genes was predicted by comparison with the BacMet—Antibacterial Biocide and Metal Resistance Genes Database,[8] whereas for detection of mercury, arsenic, and disinfectant resistance genes DRG (quaternary ammonium compounds), we performed alignment of sequenced reads against our in-house database. Moreover, the presence of halotolerance-associated genes (*katE* and *nhaA*) was *in silico* investigated using BLASTN. A ≥ 90% identity threshold was used as a filter for all accessed databases.

Phylogenetic Analysis

In order to compare BA01 with other *E. coli* strains, we performed a search for *E. coli* ST162 on *Escherichia*/*Shigella* database in Enterobase.[9] For phylogenetic analysis purpose, FastANI v1.32[10] was used to select the 30 genomes with highest average nucleotide identity (ANI) to BA01 among 542 genome assemblies of strains with data for country, year, and source of isolation downloaded from Enterobase. CSI phylogeny v1.4[11] was used with default settings to generate a maximum-likelihood phylogenetic tree with BA01 and the 30 selected genomes. *E. coli* ST162 strain W2-5 chromosome sequence (RefSeq accession number NZ_CP032989.1) was used as reference. ABRicate v1.0.1 (see text footnote 6) was used with ResFinder and PlasmidFinder databases to screen the genomes for antimicrobial resistance and plasmid replicons. Identity and coverage were set to 98 and 100%, respectively. Mutations in quinolone resistance-determining regions were assessed using CGE PointFinder pipeline.[12] iTOL v6[13] was used to root the tree at midpoint and to annotate the tree with Enterobase and ABRicate data.

In vivo Virulence Assays in the *Galleria mellonella* Infection Model

To evaluate the virulence potential of strains, an *in vivo* experiment was carried out with the *Galleria mellonella* infection model (Tsai et al., 2016; Moura et al., 2018). *G. mellonella* larvae, of nearly 250–350 mg, were inoculated with 10^5 CFU of each strain and survival analysis was evaluated each hour, for 96 h. For each strain, groups of *G. mellonella* containing five larvae were evaluated. *E. coli* strain ATCC 25922 was used as non-virulent control, whereas hypervirulent meningitis/sepsis-associated K1 *E. coli* MNEC RS218 strain was used as hypervirulent control samples (Fuentes-Castillo et al., 2021a). Data were analyzed by the log rank test, with P of 0.05 indicating statistical significance (Graph Pad Software, San Diego, CA, United States).

Plasmid Conjugation

To evaluate the transferability of the bla_{NDM-1} gene, conjugation experiments were carried out. Plasmid conjugation was assessed by mating-out assay using *E. coli* BA01 and sodium azide-resistant *E. coli* C600 (lactose-negative) as donor and recipient strains, respectively. Transconjugants were obtained from MacConkey agar plates supplemented with ertapenem (4 μg/mL) and sodium azide (100 μg/mL).

RESULTS AND DISCUSSION

The *E. coli* strain BA01 strain displayed a MDR profile to amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, cefepime, cefoxitin, ceftiofur, ertapenem (MIC = 16 mg/L), imipenem (MIC = 16 mg/L), meropenem (MIC = 16 mg/L), amikacin, ciprofloxacin, enrofloxacin, levofloxacin, chloramphenicol, and tetracycline (Magiorakos et al., 2012), remaining susceptible to aztreonam, gentamicin, sulfamethoxazole/trimethoprim, and fosfomycin. Additionally, BA01 strain displayed NaCl tolerance (>10%), confirming its ability to survive in the marine environment.

TABLE 1 | Genomic and epidemiological data of *E. coli* strain BA01 isolated from a pygmy sperm whale (*Kogia breviceps*) in Brazil.

Strain	BA01
Genome size (Mbp)	5.7
No. of CDS[a]	4,744
tRNA (*n*)	56
rRNA (*n*)	71
Non-coding RNA (*n*)	11
Pseudogenes	136
CRISPR	2
MLST (ST)[b]	162
Resistome	
β-lactams	bla_{NDM-1}, bla_{TEM-1C}, bla_{OXA-1}
Aminoglycosides	*aph(6)-Id, aph(3'')-Ib, aph(3')-VI*
Fluoroquinolones	*aac(6')-Ib-cr, qnrB6, gyrA* (S83F, D87A), *parC* (S80I)
Tetracyclines	*tet(A)*
Rifamycins	*arr-3*
Phenicols	*catB3*, *floR*
Sulphonamides	*sul1*, *sul2*
Macrolides	*ermB, mdf, mphA*
Heavy metal and Biocides	*acrEF, arsBCR, emrDK, mdtEFKN, mvrC, phnCDGHIJKLMNOP, tehAB, tolC, yjiO*
Halotolerance genes	*katE, nhaA*
Virulome	*astA, entA, entC, entE, entB, entD, entF, entS, csgB, csgD, csgF, csgG, espX4, espX5, fdeC, fepA, fepB, fepC, fepD, fepG, fes, espL1, espR1, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, gspC, gspD, gspE, gspF, gspG, gspH, gspI, gspJ, gspK, gspL, gspM, espX1, iroB, iroC, iroD, iroE, iroN, iucA, iucB, iucC, iucD, iutA, ompA, ykgK/ecpR, yagZ/ecpA, yagY/ecpB, yagX/ecpC, yagW/ecpD, yagV/ecpE*
Plasmidome	IncC-ST3, IncFIB [F18:A-:B1]
GenBank accession number	JAENJJ000000000
OneBR ID	ONE128

[a]*CDSs, coding sequences.*
[b]*MLST, Multilocus sequence type. ST, sequence type.*

[8]http://bacmet.biomedicine.gu.se
[9]https://enterobase.warwick.ac.uk
[10]https://github.com/ParBLiSS/FastANI
[11]https://cge.cbs.dtu.dk/services/CSIPhylogeny/
[12]https://bitbucket.org/genomicepidemiology/pointfinder
[13]https://itol.embl.de

Genomic analysis revealed a broad resistome, with genes conferring resistance to β-lactams (bla_{NDM-1}, bla_{TEM-1C}, and bla_{OXA-1}), aminoglycosides [*aph(6)-Id*, and *aph(3″)-Ib*, *aph(3′)-VI*], macrolide, (*ermB*, *mdfA*, and *mphA*), rifamycin (*arr-3*), quinolones [*aac(6′)-Ib-cr*, and *qnrB6*], phenicols (*catB3* and *floR*), sulfonamide (*sul1* and *sul2*), and tetracycline (*tetA*) (**Table 1**). Additionally, chromosomal point mutations in ParC (S80I) and GyrA (S83L and D87N) were detected, which may justify the fluoroquinolone-resistant profile. Furthermore, plasmid replicons IncFIB and IncA/C2 were also detected (**Table 1**).

Halotolerance associated genes *katE* and *nhaA*, which encodes for catalase and Na + /H + antiporter cytoplasmic membrane, respectively, were *in silico* predicted (Rimon et al., 2007; Prodhan et al., 2008). Furthermore, genes conferring resistance to heavy metals [i.e., arsenic resistance (*arsBCR*), tellurite (*tehAB*)] and biocides [i.e., quaternary ammonium compounds (*acrEF*, *emrK*, *mdtEFKN, mvrC, tolC, yjiO*) and glyphosate (*phnCDEFGHIJKLMNOP*)] were also detected (**Table 1**).

The bla_{NDM-1} gene was located on the IncC plasmid and was successfully transferred to the *E. coli* C600 strain, being confirmed by PCR-based replicon typing of transconjugant (Carattoli et al., 2005). The transconjugant *E. coli* displayed resistance to amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, cefepime, cefoxitin, ceftiofur, ertapenem, imipenem, meropenem, amikacin, ciprofloxacin, enrofloxacin, levofloxacin, chloramphenicol, and tetracycline, remaining susceptible to aztreonam, gentamicin, sulfamethoxazole/trimethoprim, and fosfomycin. However, due to limitations of short-read sequencing technology, it was not possible to obtain complete nucleotide sequences of this plasmid. Further analysis revealed that the aminoglycoside 3′-phosphotransferase [*aph(3′)-VI*] and the carbapenemase-encoding bla_{NDM-1} genes were located downstream of IS*Aba125* and IS*Aba14* mobile genetic elements, respectively, along with the bleomycin resistance protein (ble_{MBL}), N-(5′-phosphoribosyl)anthranilate isomerase (*iso*) and twin-arginine translocation pathway signal protein (*tat*) being harbored by a Tn*125*-like transposon (**Figure 2A**) identified in a 8,630-bp contig highly similar (100% nucleotide identity; 100% query coverage) to that found on *Klebsiella pneumoniae* plasmids (Genbank accession number: LR697132.1; LR697099.1; CP021961.1) and close related (100% nucleotide identity; 70% query coverage) to pAB17 plasmid (Genbank accession number: MT002974.1) identified in a nosocomial lineage of *Acinetobacter baumannii* in Brazil (Rossi et al., 2021). In addition, we also identified a class 1 integron carrying an integron-integrase gene (*intI1*) along with other genes encoding antimicrobial resistance, including aminoglycoside-6′-N-acetyltransferase-Ib [*aac(6′)Ib-cr*], class D beta-lactamase OXA-1 (bla_{OXA-1}), chloramphenicol O-acetyltransferase (*catB3*), rifampin ADP-ribosyl transferase (*arr-3*), and quaternary ammonium compound (*qacEΔ1*) (**Figure 2B**). In this respect, there is a growing concern about the spread of biocides contaminating aquatic environments, especially QACs, since these compounds are widely used in domiciliary and hospital setting, including disinfectants formulations (Zubris et al., 2017). As a consequence, ecosystems impacted by heavy metal and biocides could favor the selection and persistence of MDR bacteria harboring broad resistomes (Baker-Austin et al., 2006; Kim et al., 2018).

E. coli ST162 is a pandemic lineage that has been isolated from multiple sources including clinical, environmental, and domestic and wild animal samples (Fuentes-Castillo et al., 2020). When compared with BA01, the 30 selected *E. coli* ST162 genomes for the phylogenetic tree had ANI ranging between 99.7994 and 99.8948%. Among the 31 genomes analyzed, SNP counts variated between 0 and 1,343 (**Supplementary Table 1**).

Phylogenetic analysis revealed that BA01 is closely related to two strains isolated in 2018 from poultry in Hungary, differing from both strains by 59 SNPs (**Figure 3** and **Supplementary Table 1**). While these two strains from Hungary share the same resistome, BA01 has several resistance genes that are absent in these strains [bla_{NDM-1}, bla_{OXA-1}, bla_{TEM-1C}, *aac(6′)-Ib-cr*, *aph(3′)-VI*, *sul1*, *catB3*, *erm(B)*, *mph(A),* and *qnrB6*], as well as an IncC-type plasmid.

Virulome of BA01 strain, included genes/operons that encodes to enteroaggregative EAST-1 heat-stable toxin (*astA*), iron

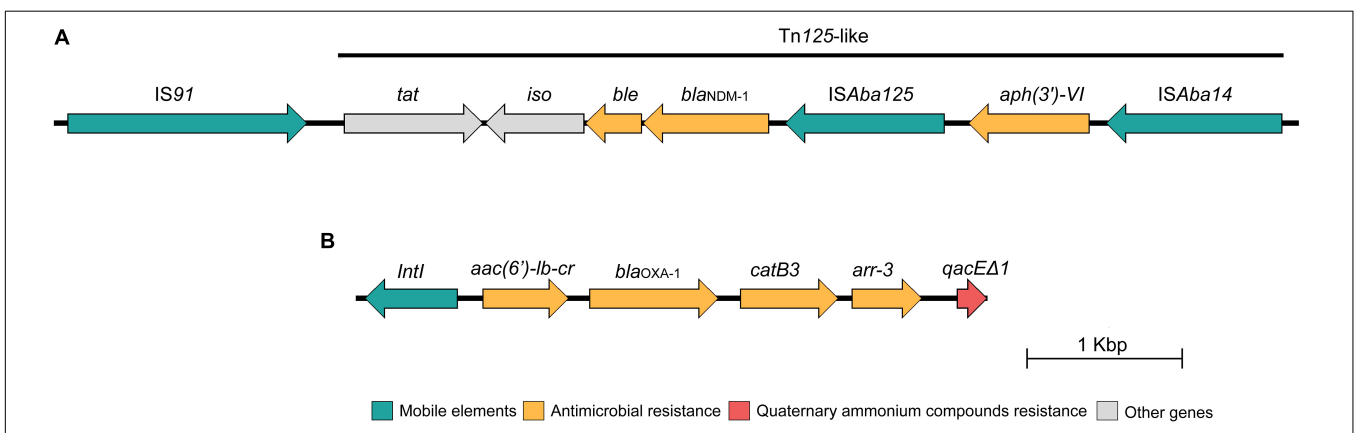


FIGURE 2 | Schematic presentation of the genetic environment context of the bla_{NDM-1} gene **(A)** and the class I integron **(B)** identified in the *Escherichia coli* BA01 strain. Arrows indicate protein-coding sequences and are colored by function.

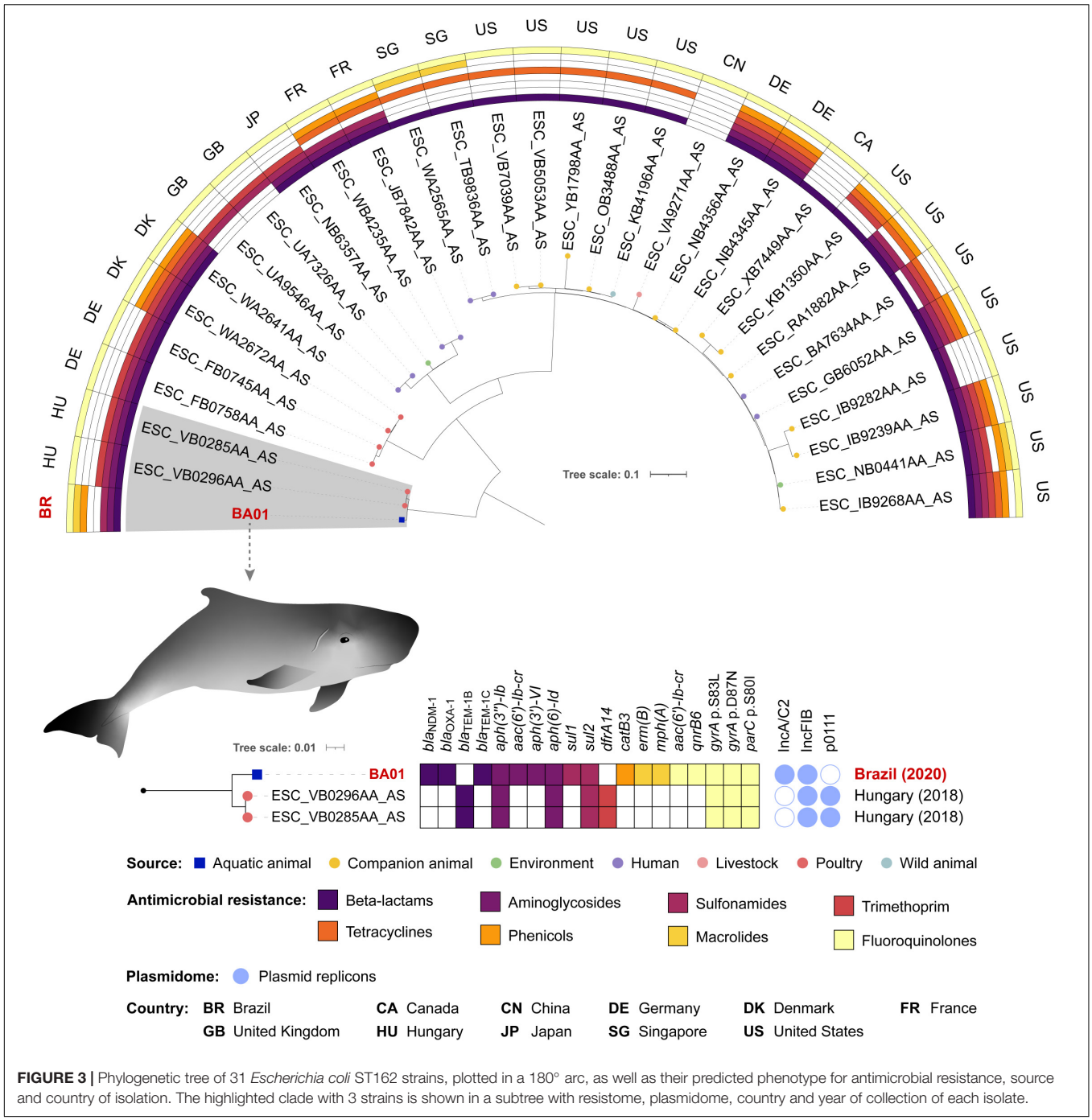


FIGURE 3 | Phylogenetic tree of 31 *Escherichia coli* ST162 strains, plotted in a 180° arc, as well as their predicted phenotype for antimicrobial resistance, source and country of isolation. The highlighted clade with 3 strains is shown in a subtree with resistome, plasmidome, country and year of collection of each isolate.

acquisition systems (*entACEDFS*, *fepABCDG*, *fes*, *iroBCDEN*, *iucABCD*, and *iutA*), adherence factors (*fdeC*, *ecpRABCDE*, *csgBDFG*, and *fimABCDEFGHI*), secretion systems components (*espL1*, *espR1*, *espX1*, *espX4*, *espX5,* and *gspCDEFGHIJKLM*) and outer membrane protein A (*ompA*). Of note, the *astA* virulence factor has been commonly found in *E. coli* strains associated with extra-intestinal disease in animals and outbreaks of diarrhea in humans and animals worldwide (Zajacova et al., 2012; Silva et al., 2014; Maluta et al., 2016; Ochi et al., 2017; Dubreuil, 2019). Indeed, the presence of *astA* gene along with other virulent associated genes detected in the BA01 genome (i.e., genes encoding for adherence factors and iron acquisition systems) could favor the virulent behavior of this strain (Fuentes-Castillo et al., 2020, Fuentes-Castillo et al., 2021a), which was supported by *in vivo* experiments using *G. mellonella* larvae. In this respect, the *E. coli* BA01 strain and the hypervirulent meningitis/sepsis-associated K1 *E. coli* MNEC RS218 strain killed 70 and 100% of the *G. mellonella* larvae within 80 h post-infection, respectively, presenting higher mortality rates than the non-virulent *E. coli* ATCC 25922 strain ($P < 0.05$) (**Figure 4**).

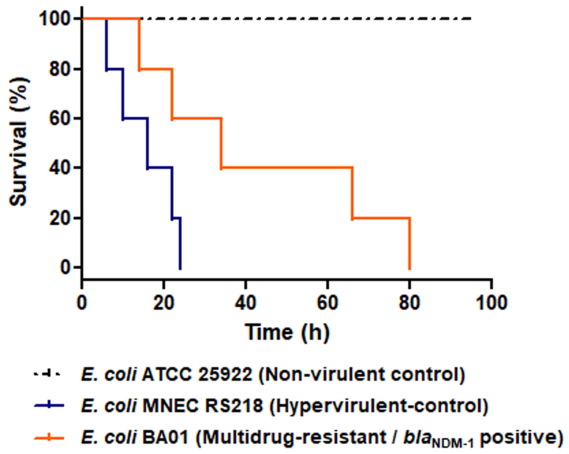


FIGURE 4 | Virulent behavior of NDM-1-positive *Escherichia coli* isolated from a pygmy sperm whale (*Kogia breviceps*), in Brazil. Kaplan-Meier survival curves of *G. mellonella* infected with 10^5 CFU/larvae of the NDM-1-positive *E. coli* BA01 strain (orange line), the non-virulent *E. coli* ATCC 25922 strain (dashed black line) and the hypervirulent meningitis/sepsis associated *E. coli* strain MNEC RS218 strain (blue line). The *E. coli* BA01 strain and the hypervirulent meningitis/sepsis-associated K1 *E. coli* MNEC RS218 strain killed 70 and 100% of the *G. mellonella* larvae within 80 h post-infection, respectively, leading to higher mortality rates than the non-virulent *E. coli* ATCC 25922 strain ($P < 0.05$). For each strain, groups of *G. mellonella* containing five larvae were evaluated in three separate experiments.

The $bla_{\mathrm{NDM-1}}$ gene was firstly reported in *Klebsiella pneumoniae* and *E. coli* recovered from a patient in Sweden that was transferred from a New Delhi hospital in 2008 (Yong et al., 2009). Since then, NDM-type carbapenemases have triggered global attention due to their rapid epidemiologic expansion among Enterobacterales and *Acinetobacter* spp., and more rarely, in *Pseudomonas aeruginosa* (Dortet et al., 2014). Of note, recent reports have documented the spreading of NDM producers beyond the boundary of human healthcare settings where they were originally related (Ranjan and Thatikonda, 2021). The environmental spread of NDM-producing bacteria has been associated to several human activities that result in chemical and microbial pollution mostly in aquatic environments (Ranjan and Thatikonda, 2021).

Particularly for marine environments, it has been demonstrated that anthropogenic pollution by improper discharge of effluents from hospitals, domestic sewage, and industrial, urban and/or agricultural wastewaters can runoff to ocean carrying MDR bacteria, antibiotic-resistant genes (ARGs), and heavy metals (Hatosy and Martiny, 2015; Li et al., 2020; Zhang et al., 2022). While it has been suggested that beaches and coastal waters from urbanized and densely populated coastlines are more prone to be contaminated by WHO critical priority bacteria, ocean currents and migratory animals can also favor the spread of these pathogens through long distances, sometimes reaching remote geographical areas with limited human footprints such Polar regions (Hernández and González-Acuña, 2016; Akhil Prakash et al., 2021) and inhospitable oceanic islands (Ewbank et al., 2022).

In this investigation, we report the occurrence of a carbapenem-resistant NDM-1- producing *E. coli* isolated from a pygmy sperm whale. In this regard, the pygmy sperm whale is a small cetacean from the *Kogiidae* family that is found in mesopelagic regions near the continental shelves (between 600 and 1,200 m depth) of the tropical and temperate Atlantic, Indian, and Pacific Oceans (Moura et al., 2016; Brentano and Petry, 2020; Kiszka and Braulik, 2020). Although cetacean research in oceanic waters has significantly progressed over the last decades, there is scarce information on the population, distribution, and behavior of pygmy sperm whales (Kiszka and Braulik, 2020). This could be explained by their short surfacing interval, cryptic surface behavior, and long deep dives, which make challenging to see these whales in the ocean (Kiszka and Braulik, 2020). Indeed, most data come from stranded animals, being generally affected by anthropogenic material, including accidental ingestion of plastic debris (Brentano and Petry, 2020). Alarmingly, increasing reports of WHO critical priority Gram-negative pathogens (MCR-type, carbapenemase- and/or ESBL-producing bacteria) on the Brazilian coast have been occurred in the last decade, which may indicate, in part, the adaptation of such pathogens in the sea. In this regard, the occurrence of such bacteria was documented in coastal waters from in densely coastal areas (Montezzi et al., 2015; Campana et al., 2017; Fernandes et al., 2017, 2020a; Paschoal et al., 2017; Sellera et al., 2017a; Corrêa et al., 2021; Furlan et al., 2021; Cordeiro-Moura et al., 2022), in marine fishes (Sellera et al., 2018a) and benthic invertebrates (Sellera et al., 2018b; Monte et al., 2019; Fernandes et al., 2020b), and also infecting penguins (Sellera et al., 2017b; Wink et al., 2021), a sea turtle (Goldberg et al., 2019), and a dolphin (Fuentes-Castillo et al., 2021b). More specifically, the presence of NDM-1-producing bacteria have been so far identified in *K. pneumoniae* and *Acinetobacter chengduensis* from coastal waters of Rio de Janeiro (Campana et al., 2017; Paschoal et al., 2017; Corrêa et al., 2021), whereas a single case of *E. coli* carrying $bla_{\mathrm{NDM-1}}$ infecting a penguin was also documented in the South coast of Brazil (Wink et al., 2021).

CONCLUSION

In summary, we report for the first time the occurrence of the NDM-1-producing *E. coli* ST162 clone in a marine cetacean. Our findings are worrisome because may indicate that NDM-producing *E. coli* can spill over from the human clinical context to the aquatic environment reaching marine animals with serious clinical implications in wildlife with a further threat to marine ecosystem maintenance. Indeed, recent studies have already demonstrated that WHO critical priority *E. coli* may display halotolerant behavior (Fernandes et al., 2020a), which could favor their spread and persistence in the marine environment. Considering that marine cetaceans are usually found in nearshore waters, exposure to critical priority carbapenemase-producing bacteria could emerge as a new challenge for the conservation of these threatened species. Last but not least, strengthening the epidemiological surveillance of antimicrobial resistance in the

ocean is crucial to understanding the ecological implications of these bacteria on marine populations.

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 animal study was reviewed and approved by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) of the Brazilian Ministry of Environment under ABIO N°755/2016; all animal handling procedures and protocols followed the required ethics and welfare practices.

AUTHOR CONTRIBUTIONS

FS, BC, DF-C, BF, ES, and FE performed data analysis. FS, BC, DF-C, BF, ES, FE, and HF conducted the experiments. FS, BC, DF-C, BF, ES, FE, DG, and NL wrote the manuscript. NL designed, coordinated the project, and supervised. MA, LS, SS, CK, and NL reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was supported by the Bill and Melinda Gates Foundation (Grand Challenges Explorations Brazil OPP1193112). Under the grant conditions of the Foundation, a CC BY or equivalent license is applied to the author accepted manuscript version arising from this submission. Additionally, this study was supported by the Fundação de Amparo ã Pesquisa do Estado de São Paulo (2020/08224-9 and 2019/15778-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico (AMR 443819/2018-1, 312249/2017-9, 422984/2021-3, and 314336/2021-4), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (88882.333054/2019-01). NL was a research fellow of CNPq (314336/2021-4). BC and HF were research fellows of CAPES (88882.333054/2019-01 and 88887.506496/2020-00). FE was a research fellow of FAPESP (2019/15578-4). BF was a research fellow of PNPD/CAPES (88887.358057/2019-00).

ACKNOWLEDGMENTS

We are grateful to FAPESP, CAPES, and CNPq. We also thank Cefar Diagnóstica Ltda. (São Paulo, Brazil) for kindly supplying the antibiotic disks for susceptibility testing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.915375/full#supplementary-material

REFERENCES

Akhil Prakash, E., Hromádková, T., Jabir, T., Vipindas, P. V., Krishnan, K. P., Mohamed Hatha, A. A., et al. (2021). Dissemination of multidrug resistant bacteria to the polar environment - Role of the longest migratory bird Arctic tern. *Sci. Total Environ.* 815:152727. doi: 10.1016/j.scitotenv.2021.152727

Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182. doi: 10.1016/j.tim.2006.02.006

Brentano, R., and Petry, M. V. (2020). Marine debris ingestion and human impacts on the Pygmy sperm whale (*Kogia breviceps*) in southern Brazil. *Mar. Pollut. Bull.* 150:110595. doi: 10.1016/j.marpolbul.2019.110595

Campana, E. H., Montezzi, L. F., Paschoal, R. P., and Picão, R. C. (2017). NDM-producing *Klebsiella pneumoniae* ST11 goes to the beach. *Int. J. Antimicrob. Agents*. 49, 119–121. doi: 10.1016/j.ijantimicag.2016.10.006

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. F. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods*. 148, 219–228. doi: 10.1016/j.mimet.2005.03.018

Cohen, R., Paikin, S., Rokney, A., Rubin-Blum, M., and Astrahan, P. (2020). Multidrug-resistant *Enterobacteriaceae* in coastal water: an emerging threat. *Antimicrob. Resist. Infect. Control.* 9, 169. doi: 10.1186/s13756-020-00826-2

CLSI (2020). *Performance Standards for Antimicrobial Susceptibility Testing — Twenty-Ninth. Edition: M100-S30*. European: CLSI.

Cordeiro-Moura, J. R., Kraychete, G. B., Longo, L., Corrêa, L. L., da Silva, N., Campana, E. H., et al. (2022). Description and comparative genomic analysis of a *Mcr-1*-carrying *Escherichia coli* ST683/CC155 recovered from touristic coastal water in Northeastern Brazil. *Infect. Genet. Evol.* 97:105196. doi: 10.1016/j.meegid.2021.105196

Corrêa, L. L., Kraychete, G. B., Rezende, A. M., Campana, E. H., Lima-Morales, D., Wink, P. L., et al. (2021). NDM-1-encoding plasmid in *Acinetobacter chengduensis* isolated from coastal water. *Infect. Genet. Evol.* 93:104926. doi: 10.1016/j.meegid.2021.104926

Dolejska, M., and Literak, I. (2019). Wildlife Is Overlooked in the Epidemiology of Medically Important Antibiotic-Resistant Bacteria. *Antimicrob. Agents Chemother.* 63, e1167–e1119. doi: 10.1128/AAC.01167-19

Dortet, L., Poirel, L., and Nordmann, P. (2014). Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. *Biomed. Res. Int.* 2014:249856. doi: 10.1155/2014/249856

Dubreuil, J. D. (2019). EAST1 toxin: an enigmatic molecule associated with sporadic episodes of diarrhea in humans and animals. *J. Microbiol.* 57, 541–549. doi: 10.1007/s12275-019-8651-4

Ewbank, A. C., Fuentes-Castillo, D., Sacristán, C., Cardoso, B., Esposito, F., Fuga, B., et al. (2022). Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* survey in wild seabirds at a pristine atoll in the southern Atlantic Ocean, Brazil: first report of the O25b-ST131 clone harboring $Bla_{CTX-M-8}$. *Sci. Total Environ.* 806:150539. doi: 10.1016/j.scitotenv.2021.150539

Fernandes, M. R., Sellera, F. P., Esposito, F., Sabino, C. P., Cerdeira, L., and Lincopan, N. (2017). Colistin-Resistant *Mcr-1*-Positive *Escherichia coli* on Public Beaches, an Infectious Threat Emerging in Recreational Waters. *Antimicrob. Agents Chemother.* 61, e234–e217. doi: 10.1128/AAC.00234-17

Fernandes, M. R., Sellera, F. P., Moura, Q., Esposito, F., Sabino, C. P., and Lincopan, N. (2020a). Identification and genomic features of halotolerant extended spectrum- β-lactamase (CTX-M)-producing Escherichia coli in urban-impacted coastal waters, Southeast Brazil. *Mar. Pollut. Bull.* 150:110689. doi: 10.1016/j.marpolbul.2019.110689

Fernandes, M. R., Sellera, F. P., Cunha, M. P. V., Lopes, R., Cerdeira, L., and Lincopan, N. (2020b). Emergence of CTX-M-27-producing Escherichia coli of ST131 and clade C1-M27 in an impacted ecosystem with international maritime traffic in South America. *J. Antimicrob. Chemother.* 75, 1647–1649. doi: 10.1093/jac/dkaa069

Fischer, J., Schmoger, S., Jahn, S., Helmuth, R., and Guerra, B. (2013). NDM-1 carbapenemase-producing *Salmonella enterica* subsp. *Enterica* serovar Corvallis isolated from a wild bird in Germany. *J. Antimicrob. Chemother.* 68, 2954–2956. doi: 10.1093/jac/dkt260

Fuentes-Castillo, D., Esposito, F., Cardoso, B., Dalazen, G., Moura, Q., Fuga, B., et al. (2020). Genomic data reveal international lineages of critical priority *Escherichia coli* harbouring wide resistome in Andean condors (*Vultur gryphus* Linnaeus, 1758). *Mol. Ecol.* 29, 1919–1935. doi: 10.1111/mec.15455

Fuentes-Castillo, D., Navas-Suárez, P. E., Gondim, M. F., Esposito, F., Sacristán, C., Fontana, H., et al. (2021a). Genomic characterization of multidrug-resistant ESBL-producing Escherichia coli ST58 causing fatal colibacillosis in critically endangered Brazilian merganser (Mergus octosetaceus). *Transbound. Emerg. Dis.* 68, 258–266. doi: 10.1111/tbed.13686

Fuentes-Castillo, D., Sellera, F. P., Goldberg, D. W., Fontana, H., Esposito, F., Cardoso, B., et al. (2021b). Colistin-resistant Enterobacter kobei carrying mcr-9.1 and bla CTX-M-15 infecting a critically endangered franciscana dolphin (Pontoporia blainvillei), Brazil. *Transbound. Emerg. Dis.* 68, 3048–3054. doi: 10.1111/tbed.13980

Furlan, J. P. R., Ramos, M. S., Dos Santos, L. D. R., Gallo, I. F. L., Lopes, R., and Stehling, E. G. (2021). Appearance of *Mcr-9*, Bla_{KPC}, *cfr* and other clinically relevsant antimicrobial resistance genes in recreation waters and sands from urban beaches. *Brazil. Mar. Pollut. Bull.* 167:112334. doi: 10.1016/j.marpolbul.2021.112334

Goldberg, D. W., Fernandes, M. R., Sellera, F. P., Costa, D. G. C., Loureiro Bracarense, A. P., and Lincopan, N. (2019). Genetic background of CTX-M-15-producing *Enterobacter hormaechei* ST114 and *Citrobacter freundii* ST265 co-infecting a free-living green turtle (*Chelonia mydas*). *Zoonoses Public Health* 66, 540–545. doi: 10.1111/zph.12572

Hatosy, S. M., and Martiny, A. C. (2015). The ocean as a global reservoir of antibiotic resistance genes. *Appl. Environ. Microbiol.* 81, 7593–7599. doi: 10.1128/AEM.00736-15

Hernández, J., and González-Acuña, D. (2016). Anthropogenic antibiotic resistance genes mobilization to the polar regions. *Infect. Ecol. Epidemiol.* 6:32112. doi: 10.3402/iee.v6.32112

Kim, M., Weigand, M. R., Oh, S., Hatt, J. K., Krishnan, R., Tezel, U., et al. (2018). Widely Used Benzalkonium Chloride Disinfectants Can Promote Antibiotic Resistance. *Appl. Environ. Microbiol.* 84, e1201–e1218. doi: 10.1128/AEM.01201-18

Kiszka, J., and Braulik, G. (2020). *Kogia breviceps. The IUCN Red List of Threatened Species 2020. e.T11047A50358334*. Available online at: https://www.iucnredlist.org/species/11047/50358334 [accessed on Feb 3, 2022].

Lee, Y. L., Chen, H. M., Hii, I. M., and Hsueh, P. R. (2022). Carbapenemase-producing Enterobacterales infections: recent advances in diagnosis and treatment. *Int. J. Antimicrob. Agents.* 59:106528. doi: 10.1016/j.ijantimicag.2022.106528

Li, W., Su, H., Cao, Y., Wang, L., Hu, X., Xu, W., et al. (2020). Antibiotic resistance genes and bacterial community dynamics in the seawater environment of Dapeng Cove, South China. *Sci. Total Environ.* 723:138027. doi: 10.1016/j.scitotenv.2020.138027

Liao, X., Yang, R. S., Xia, J., Chen, L., Zhang, R., Fang, L. X., et al. (2019). High colonization rate of a novel carbapenem-resistant *Klebsiella* lineage among migratory birds at Qinghai Lake. *China. J. Antimicrob. Chemother.* 74, 2895–2903. doi: 10.1093/jac/dkz268

Magiorakos, A.-P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Mairi, A., Barraud, O., Muggeo, A., de Champs, C., and Touati, A. (2020). Genomic analysis of one Multidrug-resistant Klebsiella pneumoniae ST11 strain recovered from Barbary Deer (Cervus elaphus barbarus) in the Akfadou forest, Algeria. *Algeria. J. Glob. Antimicrob. Resist.* 22, 515–518. doi: 10.1016/j.jgar.2020.04.027

Maluta, R. P., Leite, J. L., Rojas, T., Scaletsky, I., Guastalli, E., Ramos, M. C., et al. (2016). Variants of *AstA* gene among extra-intestinal *Escherichia coli* of human and avian origin. *FEMS Microbiol. Lett.* 364:fnw285. doi: 10.1093/femsle/fnw285

Mills, M. C., and Lee, J. (2019). The threat of carbapenem-resistant bacteria in the environment: evidence of widespread contamination of reservoirs at a global scale. *Environ. Pollut.* 255:113143. doi: 10.1016/j.envpol.2019.113143

Monte, D. F., Sellera, F. P., Fernandes, M. R., Moura, Q., Landgraf, M., and Lincopan, N. (2019). Genome Sequencing of an *Escherichia coli* Sequence Type 617 Strain Isolated from Beach Ghost Shrimp (*Callichirus major*) from a Heavily Polluted Ecosystem Reveals a Wider Resistome against Heavy Metals and Antibiotics. *Microbiol. Resour. Announc.* 8:e1471–e1418. doi: 10.1128/MRA.01471-18

Montezzi, L. F., Campana, E. H., Corrêa, L. L., Justo, L. H., Paschoal, R. P., da Silva, I. L., et al. (2015). Occurrence of carbapenemase-producing bacteria in coastal recreational waters. *Int. J. Antimicrob. Agents*. 45, 174–177. doi: 10.1016/j.ijantimicag.2014.10.016

Moura, J. F., Acevedo-Trejos, E., Tavares, D. C., Meirelles, A. C., Silva, C. P., Oliveira, L. R., et al. (2016). Stranding Events of *Kogia* Whales along the Brazilian Coast. *PLoS One* 11:e0146108. doi: 10.1371/journal.pone.0146108

Moura, Q., Fernandes, M. R., Silva, K. C., Monte, D. F., Esposito, F., Dropa, M., et al. (2018). Virulent nontyphoidal Salmonella producing CTX-M and CMY-2 β-lactamases from livestock, food and human infection, Brazil. *Virulence* 9, 281–286. doi: 10.1080/21505594.2017.1279779

Nordmann, P., Naas, T., and Poirel, L. (2011a). Global spread of Carbapenemase-producing *Enterobacteriaceae*. *Emerg. Infect. Dis.* 17, 1791–1798. doi: 10.3201/eid1710.110655

Nordmann, P., Poirel, L., Walsh, T. R., and Livermore, D. M. (2011b). The emerging NDM carbapenemases. *Trends Microbiol.* 19, 588–595. doi: 10.1016/j.tim.2011.09.005

Ochi, S., Shah, M., Odoyo, E., Bundi, M., Miringu, G., Guyo, S., et al. (2017). An Outbreak of Diarrhea in Mandera, Kenya, Due to *Escherichia coli* Serogroup O-Nontypable Strain That Had a Coding Gene for Enteroaggregative *E. coli* Heat-Stable Enterotoxin 1. *Am. J. Trop. Med. Hyg.* 96, 457–464. doi: 10.4269/ajtmh.16-0310

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., and Bonomo, R. A. (2011). Carbapenems: past, present, and future. *Antimicrob. Agents Chemother.* 55, 4943–4960. doi: 10.1128/AAC.00296-11

Paschoal, R. P., Campana, E. H., Corrêa, L. L., Montezzi, L. F., Barrueto, L. R. L., da Silva, I. R., et al. (2017). Concentration and Variety of Carbapenemase Producers in Recreational Coastal Waters Showing Distinct Levels of Pollution. *Antimicrob. Agents Chemother.* 61, e1963–e1917. doi: 10.1128/AAC.01963-17

Prodhan, S. H., Hossain, A., Nagamiya, K., Komamine, A., and Morishima, H. (2008). Improved salt tolerance and morphological variation in indica rice (*Oryza sativa* L.) transformed with a catalase gene from *E. coli*. *Plant Tissue Cult. Biotechnol.* 18, 57–63. doi: 10.3329/ptcb.v18i1.3266

Queenan, A. M., and Bush, K. (2007). Carbapenemases: the versatile beta-lactamases. *Clin. Microbiol. Rev.* 20, 440–458. doi: 10.1128/CMR.00001-07

Ranjan, R., and Thatikonda, S. (2021). β-Lactam Resistance Gene NDM-1 in the Aquatic Environment: a Review. *Curr. Microbiol.* 78, 3634–3643. doi: 10.1007/s00284-021-02630-6

Rimon, A., Tzubery, T., and Padan, E. (2007). Monomers of the NhaA Na+/H+ antiporter of Escherichia coli are fully functional yet dimers are beneficial under extreme stress conditions at alkaline pH in the presence of Na+ or Li+. *J. Biol. Chem.* 14, 26810–26821. doi: 10.1074/jbc.M704469200

Rossi, I., Royer, S., Ferreira, M., Braga, I. A., Campos, P., Batistão, D., et al. (2021). Novel ST1465/CC216 Nosocomial Lineage of Carbapenem-Resistant *Acinetobacter baumannii* Harboring an Unusual Plasmid Carrying Bla_{NDM-1} Gene. *Microb. Drug. Resist.* 27, 471–475. doi: 10.1089/mdr.2020.0219

Sellera, F. P., Fernandes, M. R., Moura, Q., Carvalho, M. P. N., and Lincopan, N. (2018a). Extended-spectrum-β-lactamase (CTX-M)-producing *Escherichia coli* in wild fishes from a polluted area in the Atlantic Coast of South America. *Mar. Pollut. Bull.* 135, 183–186. doi: 10.1016/j.marpolbul.2018.07.012

Sellera, F. P., Fernandes, M. R., Moura, Q., Lopes, R. B., Souza, T. A., Cerdeira, L., et al. (2018b). Draft genome sequence of a Bla_{CMY-2}/IncI1-harbouring

Escherichia coli D:ST457 isolated from coastal benthic organisms. *J. Glob. Antimicrob. Resist*. 14, 83–84. doi: 10.1016/j.jgar.2018.06.010

Sellera, F. P., Fernandes, M. R., Moura, Q., Souza, T. A., Cerdeira, L., and Lincopan, N. (2017a). Draft genome sequence of *Enterobacter cloacae* ST520 harbouring Bla_{KPC-2}, $bla_{CTX-M-15}$ and Bla_{OXA-17} isolated from coastal waters of the South Atlantic Ocean. *J. Glob. Antimicrob. Resist.* 10, 279–280. doi: 10.1016/j.jgar.2017.07.017

Sellera, F. P., Fernandes, M. R., Sartori, L., Carvalho, M. P., Esposito, F., Nascimento, C. L., et al. (2017b). *Escherichia coli* carrying IncX4 plasmid-mediated *Mcr-1* and Bla_{CTX-M} genes in infected migratory Magellanic penguins (*Spheniscus magellanicus*). *J. Antimicrob. Chemother*. 72, 1255–1256. doi: 10.1093/jac/dkw543

Silva, L. E., Souza, T. B., Silva, N. P., and Scaletsky, I. C. (2014). Detection and genetic analysis of the enteroaggregative *Escherichia coli* heat-stable enterotoxin (EAST1) gene in clinical isolates of enteropathogenic *Escherichia coli* (EPEC) strains. *BMC Microbiol.* 14:135. doi: 10.1186/1471-2180-14-135

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., and Monnet, D. L. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis*. 18, 318–327. doi: 10.1016/S1473-3099(17)30753-3

Tsai, C. J., Loh, J. M., and Proft, T. (2016). *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7, 214–229. doi: 10.1080/21505594.2015.1135289

Wink, P. L., Lima-Morales, D., Meurer, R., and Barth, A. L. (2021). *Escherichia coli* carrying Bla_{NDM-1} obtained from a migratory penguin (*Spheniscus magellanicus*) in the Brazilian seacoast. *Braz. J. Microbiol.* 53, 499–502. doi: 10.1007/s42770-021-00652-7

Wu, W., Feng, Y., Tang, G., Qiao, F., McNally, A., and Zong, Z. (2019). NDM Metallo-β-Lactamases and Their Bacterial Producers in Health Care Settings. *Clin. Microbiol. Rev*. 32, e115–e118. doi: 10.1128/CMR.00115-18

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., et al. (2009). Characterization of a new metallo-beta-lactamase gene, $Bla_{(NDM-1)}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob. Agents Chemother*. 53, 5046–5054. doi: 10.1128/AAC.00774-09

Zajacova, Z. S., Konstantinova, L., and Alexa, P. (2012). Detection of virulence factors of *Escherichia coli* focused on prevalence of EAST1 toxin in stool of diarrheic and non-diarrheic piglets and presence of adhesion involving virulence factors in *AstA* positive strains. *Vet. Microbiol*. 154, 369–375. doi: 10.1016/J.VETMIC.2011.07.029

Zhang, H., Wang, Y., Liu, P., Sun, Y., Dong, X., and Hu, X. (2022). Unveiling the occurrence, hosts and mobility potential of antibiotic resistance genes in the deep ocean. *Sci. Total Environ*. 816:151539. doi: 10.1016/j.scitotenv.2021.151539

Zubris, D., Minbiole, K., and Wuest, W. (2017). Polymeric quaternary ammonium compounds: versatile antimicrobial materials. *Curr. Top. Med. Chem*. 17, 305–318. doi: 10.2174/1568026616666160829155805

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 Sellera, Cardoso, Fuentes-Castillo, Esposito, Sano, Fontana, Fuga, Goldberg, Seabra, Antonelli, Sandri, Kolesnikovas and Lincopan. 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 Microbiology

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fmicb.2022.914764

Prevalence of Livestock-Associated MRSA ST398 in a Swine Slaughterhouse in Guangzhou, China

Xiaoshen Li[1,2,3], Longfei Xie[1,2,3], Honghao Huang[1,2,3], Zhi Li[1,2,3], Guihua Li[1,2,3], Peng Liu[4], Danyu Xiao[1,2,3], Xucai Zhang[1,2,3], Wenguang Xiong[1,2,3] and Zhenling Zeng[1,2,3]**

[1] Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, College of Veterinary Medicine, South China Agricultural University, Guangzhou, China, [2] National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou, China, [3] Guangdong Laboratory for Lingnan Modern Agriculture, South China Agricultural University, Guangzhou, China, [4] College of Veterinary Medicine, China Agricultural University, Beijing, China

OPEN ACCESS

Edited by:
Ziad Daoud,
Central Michigan University,
United States

Reviewed by:
Chen Kaichao,
City University of Hong Kong,
Hong Kong SAR, China
Kayode Fashae,
University of Ibadan, Nigeria

***Correspondence:**
Wenguang Xiong
xiongwg@scau.edu.cn
Zhenling Zeng
zlzeng@scau.edu.cn

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 07 April 2022
Accepted: 17 May 2022
Published: 23 June 2022

Citation:
Li X, Xie L, Huang H, Li Z, Li G, Liu P, Xiao D, Zhang X, Xiong W and Zeng Z (2022) Prevalence of Livestock-Associated MRSA ST398 in a Swine Slaughterhouse in Guangzhou, China. Front. Microbiol. 13:914764. doi: 10.3389/fmicb.2022.914764

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is an important zoonotic microorganism that is increasingly causing public health concern worldwide. The objective of this study was to determine the transmission and occurrence of MRSA in a slaughterhouse environment and evaluate its antimicrobial resistance and genetic characterization. In this study, we conducted a comprehensive epidemiological survey of *S. aureus* by *spa* typing and whole-genome sequencing (WGS) of samples obtained from the pork production chain, the environment, and community residents. To clarify the evolutionary relationships of MRSA sequence type (ST) 398 in this study and global isolates, 197 published whole-genome sequences data of MRSA ST398 strains were downloaded from the GenBank database and included in the phylogenetic analysis. A total of 585 porcine samples (snout and carcass swabs), 78 human nasal samples, and 136 environmental samples were collected. The MRSA isolates were detected at higher frequencies in samples from swine (15.0%) than carcasses (10.0%), slaughterhouse workers (8.0%), community residents (0%), and environment samples (5.9%). The *spa* typing results showed that t571 accounted for a higher proportion than other *spa* types. Closely related isolates from the samples of swine, slaughterhouse workers, carcasses, carrier vehicle, and surrounding fishpond water indicate that MRSA ST398 strains may spread among swine, humans, and the environment. MRSA ST398-t571 isolates were genetically different from global strains, except for two Korean isolates, which showed genetic closeness with it. In addition, a MRSA ST398 isolate recovered from an infected patient in Europe differed by only 31 SNPs from the airborne dust-associated strain isolated in this study, thereby suggesting potential transmission among different countries. Antimicrobial susceptibility testing results demonstrated that 99.0% (96/97) of MRSA and 95.1% (231/243) of methicillin-sensitive *S. aureus* (MSSA) showed multidrug-resistant (MDR) phenotypes. According to WGS analysis, the *poxtA*-carrying segment (IS*431mec-optrA*-IS*1216-fexB*-IS*431mec*) was reported in MRSA ST398 isolates for the first time. The coexistence of *cfr* and *optrA*

in a plasmid was first detected in MRSA ST398. The potential transmission of MRSA among humans, animals, and the environment is a cause for concern. The emergence and transmission of LA-MRSA ST398 with high levels of resistance profiles highlight the urgent need for LA-MRSA surveillance.

Keywords: swine slaughterhouse, multi-drug resistance, MRSA ST398, *spa* typing, whole-genome sequencing, public health

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen that causes community- and hospital-acquired infections (Larsen et al., 2015; Nakaminami et al., 2020). Postoperative secondary infections caused by multidrug-resistant (MDR) MRSA make clinical treatment difficult (Van Duin and Paterson, 2016; Bassetti et al., 2020). Swine is an important host for the MRSA strain. Previous studies highlighted the potential zoonotic risks associated with MRSA carriage in swine farmers. Such carriage may be a consequence of frequent contact with infected animals, causing public health concern worldwide (Armand-Lefevre et al., 2005; Kinross et al., 2017).

In 2005, the Netherlands reported a case of livestock-associated MRSA (LA-MRSA) infection in a 6-month-old infant residing on a swine farm with sequence type (ST) 398 (Voss et al., 2005). According to the data from the GenBank database, we found that MRSA ST398 has increasingly spread worldwide and is widespread in Europe. It is currently found in more than 20 countries, including Germany, Denmark, Switzerland, Belgium, Netherlands, France, Italy, United Kingdom, Greece, Spain, Czech Republic, Poland, United States, and Canada. The sources are varied, but the predominant ones are swine and humans. In particular, the application of whole-genome sequencing (WGS) has revealed potential drivers for LA-MRSA ST398 dissemination, such as trading of colonized swine, contaminated transport vehicles, and colonized human (Grøntvedt et al., 2016; da Silva et al., 2017; Sieber et al., 2018). Notably, *S. aureus* protein A (*spa*) t571 is more strongly connected with human infections than animal colonization strains (Bonnet et al., 2018; Wardyn et al., 2018). Methicillin-sensitive *S. aureus* (MSSA) ST398-t571 was detected in nine families from the Dominican Republic living in New York; these families had no contact with livestock (Bhat et al., 2009). Furthermore, it was disseminated among detainees and patients in America (Davies et al., 2011; David et al., 2013). ST398-t571 MSSA isolates caused fatal necrotizing pneumonia and bloodstream infections in the United States and Europe (Rasigade et al., 2010; Bonnet et al., 2018; Wardyn et al., 2018).

The prevalence of MDR phenomenon in MRSA strains also attracted global attention. Oxazolidinones have never been approved for veterinary use in any country worldwide, but linezolid-resistant MRSA isolate has been reported in animal hosts. Linezolid is a last resort antimicrobial agent used to treat serious infections in humans caused by MDR Gram-positive bacteria, such as vancomycin-resistant enterococci, MRSA, and penicillin-resistant pneumococci (Bozdogan and Appelbaum, 2004). To date, three acquired genes, namely, *cfr* (Li S.-M. et al., 2018; Sun et al., 2018), *optrA* (Sun et al., 2018), and *poxtA* (Chen et al., 2021) conferring linezolid resistance have been mainly described in staphylococci of livestock origin. Aside from mediating linezolid resistance, these genes could confer reduced susceptibility or resistance to other antimicrobial agents (Shen et al., 2013; Wang Y. et al., 2015; Antonelli et al., 2018). Considering that they are frequently located in plasmids or other mobile genetic elements, they could be transferred between species and genera (Shen et al., 2013; Wang J. et al., 2015; Sun et al., 2018; Chen et al., 2021).

Unlike the widespread use of ST398 type of LA-MRSA strains in European and North American countries, LA-MRSA-ST9 predominates in China (Chuang and Huang, 2015; Wang J. et al., 2015; Li S.-M. et al., 2018). Previous studies showed that ST398 type of MSSA strains was frequently colonized in slaughter pigs in northeast China (Yan et al., 2014), while LA-MRSA ST398 strains were mainly isolated from humans in China (Song et al., 2017; Chen et al., 2020). In the last several years, MRSA ST398 has been identified sporadically in swine farms (Li W. et al., 2018; Zou et al., 2021), pork (Li et al., 2021), milk (Cui et al., 2020), and monkey feces (Tang et al., 2021) in China. Continued monitoring for MRSA in food-producing animals is urgently required. Comprehensive studies on the transmission of LA-MRSA among swine, humans, and the environment in swine slaughterhouses in China are few. To gain insight into the prevalence and transmission of LA-MRSA and MSSA in a typical swine slaughterhouse, *spa* typing, WGS, and bioinformatic analysis were conducted. Samples were collected from swine, carcass, environment, slaughterhouse workers, and neighboring community residents in Guangzhou, China.

MATERIALS AND METHODS

Sampling Information

A slaughterhouse with a capacity of 3,000 swine in Guangzhou, Guangdong, China, was selected for sampling. A total of 799 samples from swine, carcasses, and humans, as well as samples from their surrounding environmental samples were collected from November 2020 to April 2021. The environment samples included airborne dusk (800–130 cm off the ground; n = 60), effluent (∼30 ml; n = 5), sludge (∼30 g; n = 5), workstations (surface swabs; n = 5), vehicles carrying swine (surface swabs; n = 5), river water (∼30 ml; n = 5), fishpond water (∼30 ml; n = 5), farmland soil, and vegetables (∼30 g; n = 46). Air samples were collected using a stationary method by placing the plates with an open lid inside the slaughterhouse area. Swine snout (n = 535), human nasal (slaughterhouse workers, n = 50; community

residents, n = 28), and carcass swabs (n = 50) were collected (**Supplementary Table 1**). The swine samples were mainly from Guangdong, Hunan, Guangxi, and Jiangxi Provinces. Samples from individual pigs were processed separately. The slaughterhouse workers and neighboring residents signed an informed consent form and were asked to agree to nose swabbing. Information details of the volunteers are summarized in **Supplementary Table 2**.

Isolation and Identification of MSSA and MRSA

The samples were soaked in 7.5% sodium chloride broth (Land Bridge, Beijing, China) and cultured at 37°C for 12 h. Then, a loopful of the broth was streaked onto chromogenic *S. aureus* agar (Huankai Microbial, Guangdong, China). One colony with typical *S. aureus* morphology was selected from each sample and streaked onto Mannitol salt agar (Huankai Microbial, Guangdong, China) to obtain a purified isolate colony. Each isolate colony was identified by polymerase chain reaction (PCR) using the *nuc* gene to detect *S. aureus* and the *nuc* and *mecA* (or *mecC*) genes to detect the MRSA isolate (Zhang et al., 2004; Harrison et al., 2013).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was tested using the agar dilution method according to the guidelines of Clinical and Laboratory Standards Institute guidelines (CLSI, 2018). Tigecycline were assessed by broth microdilution as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST Version 6.0) (EUCAST, 2016). *S. aureus* ATCC 29213 was set as the quality control strain. The antimicrobials were amoxicillin (AMO), oxacillin (OXA), penicillin (PEN), ceftiofur (CEF), cefoxitin (FOX), gentamicin (GEN), amikacin (AMI), doxycycline (DOX), tigecycline (TIG), florfenicol (FFC), erythromycin (ERY), tilmicosin (TMI), rifampicin (RIF), vancomycin (VAN), clindamycin (CLI), tiamulin (TIA), ciprofloxacin (CIP), enrofloxacin (ENR), fosfomycin (FOS), linezolid (LZD), tedizolid (TZD), and sulfamethoxazole-trimethoprim (SXT).

Molecular Typing and Whole-Genome Sequencing

All identified MSSA and MRSA isolates were subjected to *spa* typing by PCR amplification and sequencing of the polymorphic region of the *spa* gene (Harmsen et al., 2003). *Spa* type was assigned using *spa*-plugin in BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Minimum spanning trees based on *spa* types were constructed in BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Based on *spa* type, source, and antimicrobial resistance (AMR), 50 MRSA isolates were selected for WGS (**Supplementary Tables 4, 6**). The bacterial DNA was extracted using the HiPure Bacterial DNA Kit (Magen, Guangzhou, China). Illumina NOVAseq 6000 sequencing platform (Novogene Company, Beijing, China) and PacBio RS II sequencing platform (Biochip Company, Tianjin, China) were used for WGS. SMRTbell library (10–20 kb in size) was prepared by the ligation of hairpin adaptors at both ends, and the resulting library was selected according to size using Blue Pippin with a 7–10 kb cutoff for Pacbio sequencing. Illumina Novaseq sequences were assembled using CLC Genomics Workbench 10 (CLC Bio, Aarhus, Denmark). Pacbio sequences were assembled using a hierarchical genome assembly process (Chin et al., 2013). The assembled Pacbio sequences were corrected by using Burrows-Wheeler Aligner's Smith-Waterman Alignment software to ensure that their integrity was in accordance with those of Novaseq sequences (Li and Durbin, 2010). The genome assemblies of MRSA ST398 isolates were deposited in the GenBank and were registered with BioProject number PRJNA793117. The plasmid pSA159 carrying *cfr* and *optrA* in GD4SA159 and *poxtA*-positive strain GD4SA108 were annotated by RAST annotation server[1] (Overbeek et al., 2014). Multi-locus sequence type (MLST), acquired resistance genes, and Staphylococcal cassette chromosome *mec* (SCC*mec*) were identified against MLST (Aanensen and Spratt, 2005), ResFinder (Zankari et al., 2012), and SCC*mec*Finder (Kaya et al., 2018) databases (accessed on 16 June 2020) using a mapping approach implemented in SRST2 (Inouye et al., 2014).

To clarify the genetic relatedness of MRSA ST398 in this study and global isolates, 197 published whole-genome sequences data of MRSA ST398 strains were downloaded from the GenBank database and included for analysis (**Supplementary Table 3**). Based on the draft genome sequences, a core-genome single nucleotide polymorphism (SNP)-based maximum-likelihood (ML) phylogenetic tree was constructed. The genome of MRSA ST398 isolate (GenBank accession number: JAASKV000000000) was used as a reference. The tree was constructed using Parsnp in the Harvest package (Treangen et al., 2014) with default parameter settings and was annotated on the EvolView website https://evolgenius.info. A comparison of the genetic context was generated using Easyfig 2.1 (Sullivan et al., 2011) and BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011).

Statistical Analysis

Descriptive and comparative analyses were performed in Graphpad Prism 8.0 (Graphpad Software, La Jolla, CA, United States), and statistical analyses were performed in SPSS 20.0 (IBM Corp., Armonk, NY, United States). Fisher's exact test was used to test whether differences in drug resistance phenotypes among human-, environment-, and swine-associated isolates were significant, and also evaluated between MRSA and MSSA isolates.

RESULTS

Prevalence and Antimicrobial Susceptibility of MRSA and MSSA

Ninety-seven MRSA and 243 MSSA isolates were collected from 799 samples (**Supplementary Table 1**). MRSA isolates

[1]https://rast.nmpdr.org/

were detected in swine (15.0%, 80/535), carcass (10.0%, 5/50), slaughterhouse workers (8.0%, 4/50), and environmental samples (5.9%, 8/136), whereas no MRSA-positive isolate was found in the samples from community (**Figure 1A**). MSSA-positive isolates were detected in swine (35.5%, 190/535), carcasses (18.0%, 9/50), slaughterhouse workers (26.0%, 13/50), community residents (7.1%, 2/28), and environmental samples (21.3%, 29/136) (**Figure 1A**).

Antimicrobial susceptibility tests of 340 isolates were performed using 22 antimicrobials (**Supplementary Table 4**). According to the results, 99.0% (96/97) of MRSA and 95.1% (231/243) of MSSA showed MDR phenotypes. Swine- and environment-associated isolates were resistant to a larger number of antimicrobial agents compared with human-associated isolates (median 10, 11 vs. 5; $p < 0.001$) (**Figure 1C**). The swine- and environment-associated isolates showed high resistance levels to florfenicol, erythromycin, tilmicosin, and clindamycin (> 80%). The trimethoprim/sulfonamide resistance rate of environment-associated isolates was significantly higher than that of isolates from swine or human samples ($p < 0.05$). All isolates from different sources showed high resistance to penicillin (> 90%) but low resistance to tigecycline, linezolid, fosfomycin, and rifampicin (< 11.47%). All isolates were susceptible to amikacin, vancomycin, and Acetazolamide (**Figure 1B** and **Supplementary Table 5**). Compared with MSSA isolates, MRSA isolates were resistant to most of the antimicrobial agents (median 12 vs. 10; $p < 0.001$) (**Figure 1D**). The majority of isolates recovered in the study showed a MDR phenotype.

Spa Typing of MRSA and MSSA

In total, 26 *spa* types were identified among the 340 *S. aureus* isolates, with MSSA isolates (25 *spa* types) showing greater diversity than MRSA (six *spa* types) and three isolates were not typed (**Figure 2A** and **Supplementary Table 4**). Besides t571 (64.9%, 63/97), t034, t899, t114, and t1793 were also detected in MRSA isolates. In addition to t571 (72.8%, 177/243), t034, t899, t114, t1793, and t011 were also detected in MSSA isolates (**Figure 2A**).

Swine-associated MRSA isolates were dominated by t571 (71.4%, 60/84); *spa* type t034 and t899 accounted for a small proportion (**Figure 2B**). The human- and environment-associated MRSA isolates primarily had t034 (50%, 2/4) and t899 (50%, 4/8), respectively (**Figures 2C,D**). MSSA t571 clonal lineage predominated in swine, human, and environment-associated isolates (**Figure 2**).

Whole-Genome Phylogenetic Analysis of MRSA ST398

Among 50 MRSA isolates sequenced, ST398 was the dominant clonal lineage (84.0%, 42/50). Most MRSA isolates belonged to ST398-t571 (42.0%, 21/50), followed by ST398-t034 (30%, 15/50), ST398-t011 (10%, 5/50), ST9-t899 (14.0%, 7/50), and one ST1-t114 isolate associated with human.

Phylogenetic analyses revealed that MRSA ST398 was mainly clustered into three clades, corresponding to clonal lineage identified as SCC*mec* V-t034, SCC*mec* V-t011, and SCC*mec* IV-t011 (**Figure 3**). Notably, SCC*mec* were diversified in MRSA ST398-t571 clonal lineage, such as SCC*mec* V, IX, and XII subtypes, which were predicted with a template coverage of less than 60%. These isolates differed in 6,893 core genome SNPs. Interestingly, the isolates (block 1) found in this study were genetically different from global strains, except for the Korean strains (block 2) that showed close genetic relationship with the abovementioned isolates. Swine-associated Korean isolate PJFA-521M (GenBank accession number: SRKD00000000) was closely related to human-associated Korean isolate PJFH-522M (GenBank accession number: RKRI00000000) (2 SNPs difference) and swine-associated Chinese isolate (HN2SA18) (35 SNPs difference) (**Figure 3**). Furthermore, a strain OP17 (GenBank accession number: VKBM00000000) isolated from an infected patient in Europe differed by only 31 SNPs from the airborne dust-associated isolate (GDK3SA10) isolated in this study. The potential transmission of MRSA among swine, human, carcass, carrier vehicle, and fishpond water was observed around the slaughterhouse. Swine-associated isolate GDA3SA01 was closely related to GD4SA98 recovered from carrier vehicle (swine) (17 SNPs difference). Meanwhile, GD4SA98 was closely related to the GD4SA24 recovered from a slaughterhouse worker (1 SNP difference). GD4SA24 was closely related to GD4SA93 recovered from the surrounding fishpond water (with a difference of 8 SNPs). GD4SA24 was closely related to swine-associated isolate GDA3SA01 (with a difference of 16 SNPs). Swine-associated isolate GD4SA116 was closely related to GD4SA159 recovered from carcass (with a difference of 1 SNP). This was also observed among slaughterhouse workers, as GD3SAM4 was closely related to GD3SAT1 (with a difference of 1 SNP).

Distribution of Antibiotic Resistance Genes in MRSA ST398

Antibiotic resistance genes (ARGs) in MRSA ST398 isolates (42 sequenced isolates in this study and 197 records retrieved in the GenBank database) are displayed in **Supplementary Figure 1** (**Supplementary Table 7**). A total of thirty-three ARGs grouped into nine types were found in samples from swine, human, and environment. **Figure 3** shows that the abundance of resistance genes of sequenced MRSA ST398 isolates in this study was higher than that of global isolates. Notably, the prevalence rates of penicillin/methicillin resistance gene *blaZ* and tetracycline resistance gene *tetM* in MRSA ST398 were 95.82% and 88.28%, respectively. In addition, the detection rates of tetracycline resistance gene *tet*(K), trimethoprim resistance gene *dfrG*, lincomycin resistance gene *lnu*(B), macrolides-lincosamides-streptogramin B (MLS_B) resistance gene *erm*(C), lincosamides-pleuromutilins-streptogramins A resistance gene *lsa*(E), aminoglycoside resistance genes *aac(6′)-aph(2″)*, *ant(9)-Ia*, and *aadD*, and florfenicol resistance gene *fexA* ranged from 20% to 50%. The multiresistance genes *cfr*, *optrA*, and *poxtA* were detected in MRSA ST398. The coexistence of the *cfr* and *optrA* genes was detected in a plasmid of GD4SA159 with oxazolidinone sensitivity that was isolated from carcass. In addition, the swine-associated isolate GD4SA108 carried *poxtA*

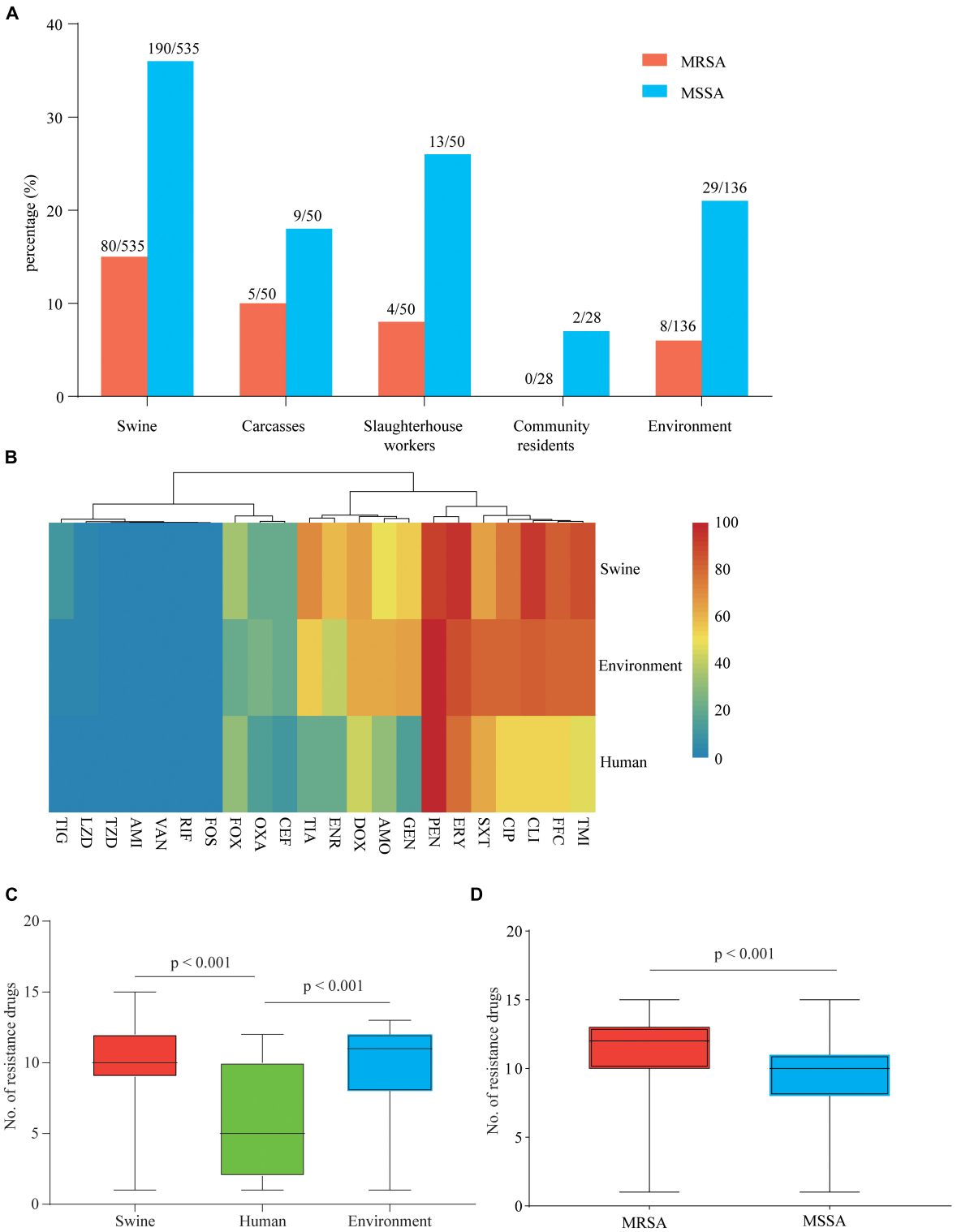


FIGURE 1 | Prevalence and drug resistance of isolates recovered from samples from various origins. **(A)** Prevalence of methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) in samples from various origins along and around a slaughterhouse in Guangzhou, China. **(B)** Each cell in the heat map indicates the percentage of strains with resistance to a particular drug. **(C)** The bar graph indicates the total number of drugs with resistance within each swine, human, and environment-associated isolate. **(D)** The bar graph indicates the total number of drugs with resistance within each MSSA and MRSA isolate. The numbers of swine, human, and environment-associated strains were 284, 19, and 37, respectively. The numbers of MSSA and MRSA isolates were 243 and 97, respectively.

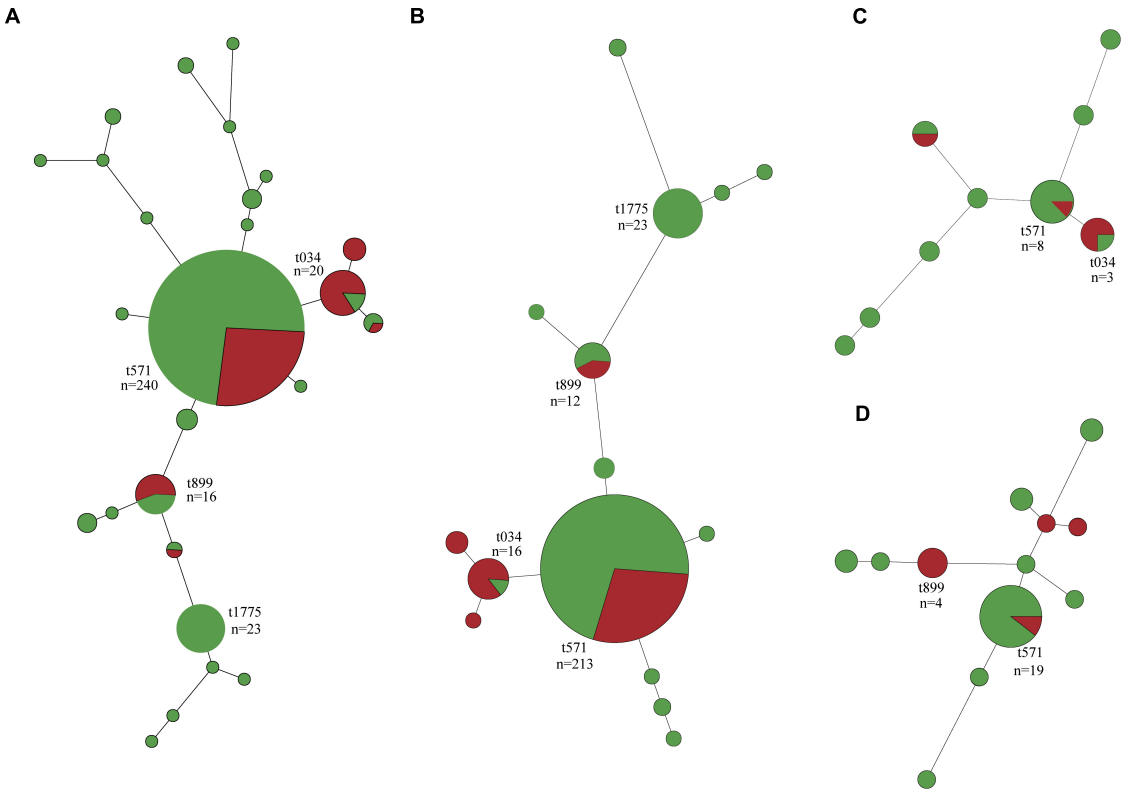


FIGURE 2 | Minimum spanning tree of methicillin-susceptible *Staphylococcus aureus* (MSSA, *n* = 243) and methicillin-resistant *S. aureus* (MRSA, *n* = 97) isolates by *spa* type. **(A)** All isolates. **(B)** Isolates of swine origin. **(C)** Isolates of human origin. **(D)** Isolates of environmental origin. Each node represents a single *spa* type. The size of the node is proportional to the number of isolates represented by a said node. Branch lengths between nodes are proportional to the number of alleles that differ between the two linked nodes. Selected nodes are labeled with corresponding *spa* type and number of isolates represented. Red nodes, MRSA; green nodes, MSSA.

along with the resistance genes *aac(6′)-aph(2″)*, *aadD*, *ant(6)-Ia*, *blaZ*, *dfrG*, *erm*(C), *erm*(T), *fexA*, *fexB*, *lnu*(B), *lsa*(E), *mecA*, *tet*(L), *tet*(M), and *qacG*. Alarmingly, two slaughterhouse worker-associated isolates (GD3SAM4 and GD3SAT1) carried the *cfr* gene, as well as the *ant*(9)-*Ia*, *blaZ*, *dfrG*, *erm*(A), *erm*(C), *fexA*, *mecA*, *tet*(K), *tet*(M), and *vga*(E). The two isolates showed an MDR phenotype, which indicated resistance to beta-lactams, tetracyclines, florfenicol, erythromycin, tilmicosin, clindamycin, tiamulin, and ciprofloxacin.

The Coexistence of *cfr* and *optrA* in a Plasmid

To characterize the genetic environment of the *cfr* and *optrA* genes, plasmid pSA159 (GenBank accession no. CP090408) from MRSA ST398 isolate GD4SA159 was completely sequenced. It was 52,881 bp in length and had an average GC content of 38.0%. A total of 53 open reading frames (ORFs) coding for proteins of > 50 amino acids were identified. Except for the 20 ORFs encoding hypothetical proteins with no defined function, the products of the remaining 36 ORFs exhibited identities ranging from 33.0% to 100.0% to proteins with known functions, such as AMR, plasmid replication, and conjugative transfer or transposition. The plasmid pSA159 with the *optrA-fexA-aacA-aphD-aadD-ble-cfr* resistance gene cluster was subjected to a comparative analysis with other plasmids (**Figure 4**). The 16,956 bp segment between *optrA* and *tnpA* showed 99.68% identity with the *cfr*- and *optrA*-carrying region of plasmid pwo28-3 from pig-associated *S. sciuri* (KT601170). The segment truncated *optrA* displayed 99.8% identity with the corresponding region of plasmid pk8D6P-*cfr* from duck-associated *S. sciuri* (CP065793). The segment between *tnpA* and IS*257* showed 99.96% identity with the corresponding region of plasmid pWo27-9 from *S. sciuri* (KX982169).

The Genetic Context of *poxtA*

The genome of MRSA-ST398 GD4SA108 (GenBank accession no. CP090375) containing the *poxtA* gene was completely sequenced and was 2,864,441 bp in length. It had an average GC content of 32.0%. As shown in **Figure 5**, the genetic context of *poxtA* from plasmids pY80, pC10, pfas4-1, and pDY32-*poxtA* was commonly flanked by two copies of IS*1216* elements in the same orientation. By contrast, the *poxtA* gene from chromosomal genome of GD4SA108 was flanked by two copies of IS*431mec* elements in the same orientation. It could form a circular intermediate (7,270 bp, region A) that contained IS*1216* and

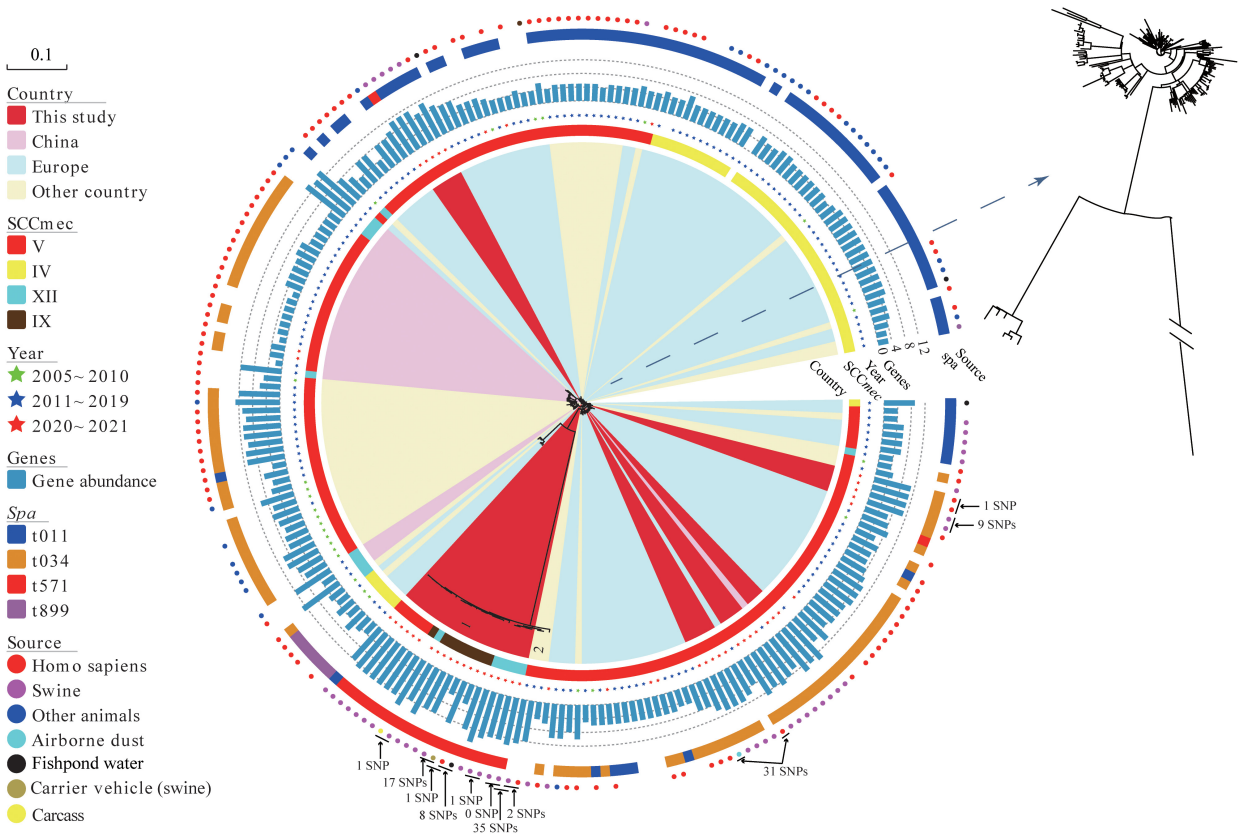


FIGURE 3 | The maximum likelihood (ML) phylogenetic tree of 42 methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 isolates from China based on single nucleotide polymorphisms in the core genomes. A total of 197 global MRSA ST398 isolates were included for comparison. Regions were indicated by leaf color. Sources and years of the isolates were indicated by circles and stars in different colors, respectively. SCC*mec* and *spa* types were indicated by strips in different colors. The branch of Block 2 represents Korean isolates. The total number of antibiotic resistance genes in each isolate was represented by the length of a blue bar chart. Details of the genes of each isolate are given in **Supplementary Table 7**. The core of phylogenetic tree was amplified for a clearer display.

fexB genes (**Figure 5**). Plasmids pC10, pfas4-1, and pDY32-*poxtA* from *Enterococcus faecalis*, *E. hirae*, and *E. faecium* carried the *poxtA* gene and tetracycline resistance genes *tetM* and *tetL*. Plasmid pY80 from *S. haemolyticus* contained *tetL* and *aadD1* aside from the *poxtA* gene. The results suggested that *poxtA* gene may be transmitted with other drug resistance genes, especially tetracycline-resistant genes.

DISCUSSION

MRSA ST9 is a common clonal lineage among swine-associated MRSA in China (Zhou et al., 2018). In this study, fewer MRSA ST9 isolates were found in swine. In contrast, the prevalence of MRSA ST398, which is typically associated with swine and farmers in Europe, was detected in the slaughterhouse. Phylogenetic analyses revealed that MRSA ST398 was predominantly associated with the t571 circulating in the slaughterhouse. This finding differed from those of previous studies, in which t011 and t034 were commonly associated with MRSA ST398 globally (Hansen et al., 2019; Pirolo et al., 2019; Schnitt et al., 2020). The swine contaminated with MRSA ST398 was probably raised in a farm setting before being transported to the slaughterhouse, and this assumption was supported by the occurrence of MRSA ST398-t034/t571 in a pig farm in southern China (Li W. et al., 2018). Additionally, the potential spread was predicted among swine, workers, carcass, carrier vehicle, and surrounding fishpond water, as supported by the analysis of SNP difference (Bartels et al., 2015; Schürch et al., 2018; Bouchami et al., 2020). A previous study on the drivers of LA-MRSA CC398 demonstrated that swine movements were crucial for the spread of MRSA CC398 (Sieber et al., 2018). As a means of transporting for swine, vehicles that test positive for MRSA ST398 were highly likely to contaminate the transport route, leading to widespread infection. In addition, the carcasses transported to the pork market were positive for MRSA ST398, thereby indicating a potential food safety risk. Slaughterhouse workers had a higher detection rate of MRSA ST398 compared with community residents, perhaps due to the prolonged exposure to slaughterhouse conditions. Although it was not detected in the samples from residents, MRSA ST398 was detected in the nearby fishpond and airborne dust, suggesting that residents exposed to the environment were still at risk of infection. The suggestion was corroborated by a previous report (Bos et al., 2016). The infected

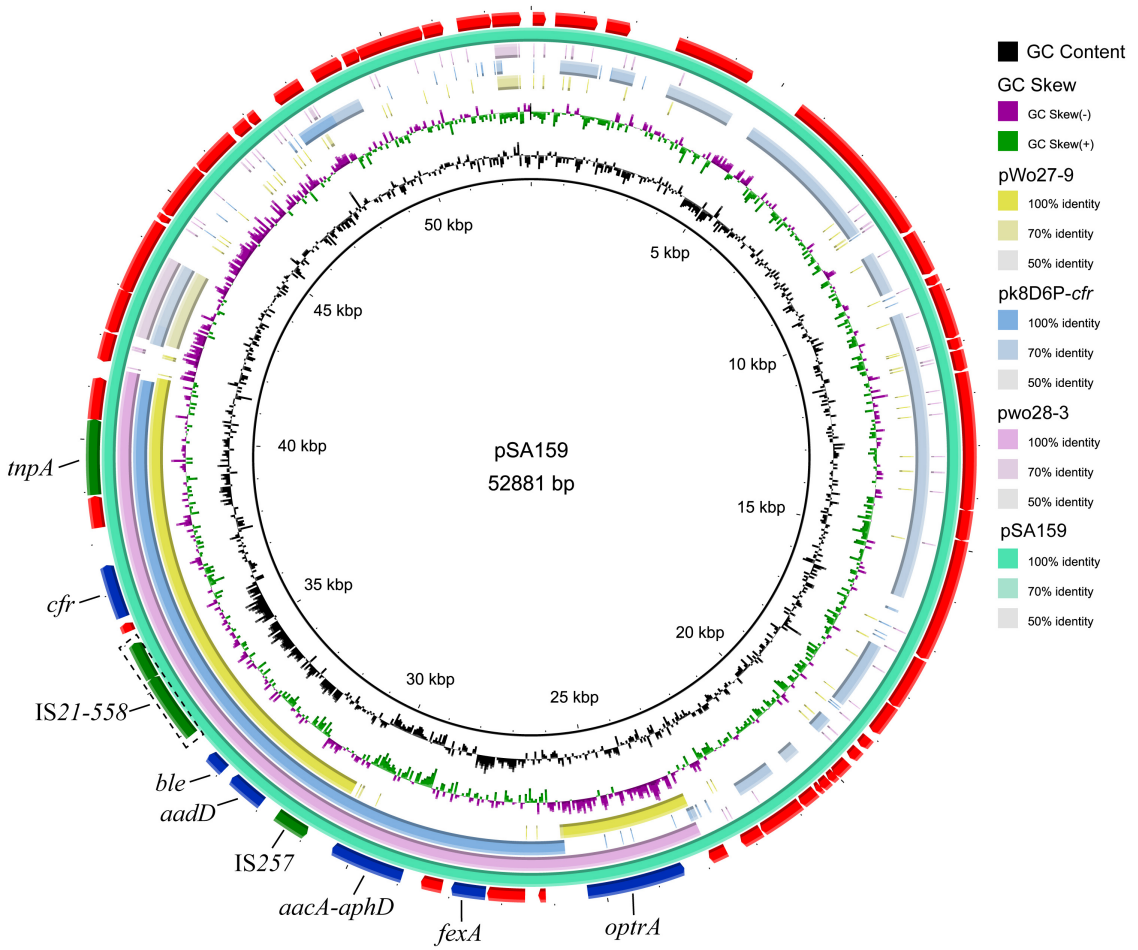


FIGURE 4 | Ring comparison of plasmid pSA159 (GD4SA159) using BRIG. Arrows indicate the positions and directions of gene transcription. Blue and green arrows indicate resistance genes and mobile genetic elements (MGEs), respectively. Genes encoding hypothetical proteins and proteins for plasmid stability are depicted in red.

slaughterhouse workers, carrier vehicles, fishponds, and carcasses could serve as a source for the transmission of MRSA ST398 in the community. However, large-scale sampling and further studies should be conducted to confirm this.

Phylogenetic analysis of MRSA ST398 suggested that infected human or swine may drive the spread of MRSA ST398 among different countries. The diversity of colonizing sources suggested their potential role as reservoirs for transmission. Half of the sequenced MRSA ST398 isolates showed difference in evolutionary relationship (block 1, *spa* type t571), indicating the development of diversity in the population of MRSA ST398 isolates. MSSA ST398-t571 was relatively rare among livestock isolates (Lienen et al., 2020) and was commonly associated with human infection. Amazingly, *spa* type t571 was the dominant clonal lineage in MRSA and MSSA isolates in the slaughterhouse (Bonnet et al., 2018; Wardyn et al., 2018). The virulence levels of MRSA- and MSSA-t571 isolates in this study were unclear. The frequent reports of invasive infections caused by *S. aureus* ST398-t571 isolates suggested that persistent genotypic surveillance of *S. aureus* strains in humans and animals should be practiced (Song et al., 2017; Chen et al., 2020). The prevalence of MRSA ST398 in the slaughterhouse may be closely related to foreign pig trading and traveling. The details of pig trading and history of human travel abroad could not be identified exactly. Thus, further studies are needed to clarify the reason for the prevalence of MRSA ST398 lineage.

The MRSA carriage among swine, carcass, slaughterhouse workers, and environmental samples in this study was higher than that in samples from slaughterhouses from Shandong (fatteners, 8.2%, 7/85; carcasses, 1.0%, 1/91; slaughterhouse workers, 0%, 0/16; environmental samples, 1.9%, 1/52) (Sun et al., 2019). The difference in MRSA carriage could be due to differences in the location and size of the slaughterhouse. The high prevalence of MRSA carriage in this slaughterhouse might result from the beta-lactam antibiotic usage pressure in animal production (Qiao et al., 2018). Antibiotic resistance is a serious threat to global public health. The resistance phenotypes of swine-associated isolates were generally more severe than those in human-associated isolates (e.g., resistance

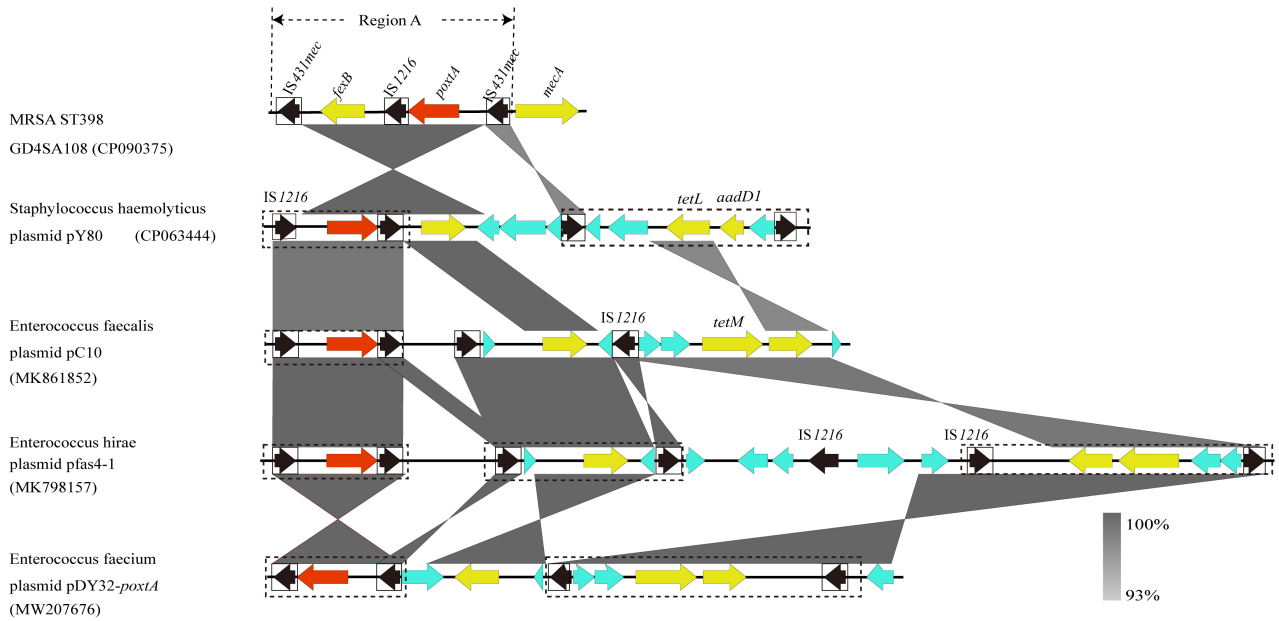


FIGURE 5 | Schematic presentation of the genetic context of *poxtA*-flanking regions in the chromosomal DNAs of the isolate GD4SA108 compared with the corresponding regions of other strains in the NCBI database. Regions of >93% homologies are shaded gray. Differently colored arrows indicate the direction of transcription of the different genes. The *poxtA* gene is shown as a red arrow, and yellow arrows indicate other resistance genes. Genes with unknown or other functions are depicted in light blue. MGEs are indicated as black dotted boxes. Insertion sequences are indicated as black boxes, with the black arrow inside the box showing the transposase gene. Region A could form a circular intermediate.

to florfenicol, gentamicin, doxycycline, clindamycin, tilmicosin, tiamulin, enrofloxacin, and ciprofloxacin) (**Figures 1B,C**). The differences in AMR phenotype between swine- and human-associated isolates could be due to differences in the type and amount of antimicrobial used. The analyses of AMR genotypes of MRSA ST398 showed that the extremely high detection of *tetM* (88.28%) was consistent with the characteristic of tetracycline resistance of CC398 clonal lineage (Ward et al., 2014), suggesting that heavy tetracycline use in the swine industry may have promoted the dissemination of this clonal lineage (Moodley et al., 2011).

Oxazolidinones are essential drugs used to address the MDR of Gram-positive bacteria. The linezolid resistance genes *cfr*, *optrA*, and *poxtA* are of concern, because they are often located in mobile genetic elements (MGEs), which could transmit among bacteria by horizontal gene transfer. The coexistence of *cfr* and *optrA* in a plasmid was first detected in MRSA ST398, although it has been reported in *S. sciuri* and *E. gallinarum* (Li et al., 2016; Fan et al., 2017; Coccitto et al., 2022). The *poxtA* gene is commonly flanked by IS*1216* element (Antonelli et al., 2018; Chen et al., 2021). However, the novel genetic context of *poxtA* gene flanked by two copies of IS*431mec* elements was identified, thereby increasing the awareness of the spread of *poxtA* gene. These *cfr*, *optrA*, and *poxtA*-carrying plasmids or fragments may be disseminated among MRSA in the slaughterhouse and possibly spread to MRSA of human origin. The *cfr*, *optrA*, and *poxtA* genes lead to resistance to chloramphenicol and florfenicol aside from linezolid (Long et al., 2006; Wang Y. et al., 2015; Antonelli et al., 2018). As the use of chloramphenicol in food animals was banned in China in 2002, florfenicol appears to be the only antimicrobial agent to select *cfr*, *optrA*, and *poxtA* from isolates of food animal origin. Thus, rational use of florfenicol in food-producing animals is urgently needed. In addition, SCC*mec* elements play a crucial role in the spread of methicillin resistance and evolution of MRSA. SCC*mec* V and SCC*mec* IV types among CC398-MRSA were prevalent worldwide (Wulf et al., 2008; Kadlec et al., 2009; Lienen et al., 2020). Compared with global isolates, SCC*mec* IV lineage isolate was absent, and the SCC*mec* V lineage dominated in MRSA ST398 isolates sequenced in this study. Interestingly, the types of SCC*mec* XII and IX were predicted in MRSA ST398-t571 isolates. These results indicated a rapid evolution of SCC*mec* elements in MRSA ST398-t571, which was similar to the findings from a previous study (Ji et al., 2020).

CONCLUSION

This study provided a longitudinal investigation of the presence of MRSA in a swine slaughterhouse in China. MRSA ST398 may spread among swine, humans, and the environment in the slaughterhouse, and it showed high levels of resistance profiles. This is the first report of the coexistence of *cfr* and *optrA* genes in a plasmid in MRSA ST398 isolates. The *poxtA*-carrying segment (IS*431mec-optrA*-IS*1216-fexB*-IS*431mec*) was reported in MRSA ST398 isolates for the first time. The *spa* typing results confirmed

that t571 clonal linage dominated in MRSA or MSSA strains. This study underscores the importance of surveillance for MRSA ST398 in swine and indicates a high likelihood for the spread of MRSA ST398 from the swine production chain to human.

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 study was approved by the South China Agriculture University (SCAU) Animal Ethics Committee. All animals were sampled under authorization from the Institutional Animal Care and Use Committees (IACUCs) of SCAU. 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

XL wrote the first draft of the manuscript. XL, WX, and ZZ contributed to the conception and design of the study. LX, HH, ZL, GL, PL, DX, and XZ performed the sampling and statistical analysis. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This study was supported by the Local Innovative and Research Teams Project of Guangdong Pearl River Talents Program [2019BT02N054] and Guangdong Basic and Applied Basic Research Foundation [2020A1515010917].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.914764/full#supplementary-material

REFERENCES

Aanensen, D. M., and Spratt, B. G. (2005). The multilocus sequence typing network: mlst. net. *Nucleic Acids Res.* 33(Suppl._2), W728–W733. doi: 10.1093/nar/gki415

Alikhan, N.-F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Antonelli, A., D'Andrea, M. M., Brenciani, A., Galeotti, C. L., Morroni, G., Pollini, S., et al. (2018). Characterization of *poxtA*, a novel phenicol–oxazolidinone–tetracycline resistance gene from an MRSA of clinical origin. *J. Antimicrob. Chemother.* 73, 1763–1769. doi: 10.1093/jac/dky088

Armand-Lefevre, L., Ruimy, R., and Andremont, A. (2005). Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. *Emerg. Infect. Dis.* 11, 711–714.

Bartels, M., Larner-Svensson, H., Meiniche, H., Kristoffersen, K., Schønning, K., Nielsen, J., et al. (2015). Monitoring meticillin resistant *Staphylococcus aureus* and its spread in Copenhagen, Denmark, 2013, through routine whole genome sequencing. *Euro Surveill.* 20:21112. doi: 10.2807/1560-7917.es2015.20.17.21112

Bassetti, M., Eckmann, C., Giacobbe, D. R., Sartelli, M., and Montravers, P. (2020). Post-operative abdominal infections: epidemiology, operational definitions, and outcomes. *Intensive Care Med.* 46, 163–172. doi: 10.1007/s00134-019-05841-5

Bhat, M., Dumortier, C., Taylor, B. S., Miller, M., Vasquez, G., Yunen, J., et al. (2009). *Staphylococcus aureus* ST398, New York City and Dominican Republic. *Emerg. Infect. Dis.* 15, 285–287.

Bonnet, I., Millon, B., Meugnier, H., Vandenesch, F., Maurin, M., Pavese, P., et al. (2018). High prevalence of *spa* type t571 among methicillin-susceptible *Staphylococcus aureus* from bacteremic patients in a French University Hospital. *PLoS One* 13:e0204977. doi: 10.1371/journal.pone.0204977

Bos, M. E., Verstappen, K. M., Van Cleef, B. A., Dohmen, W., Dorado-García, A., Graveland, H., et al. (2016). Transmission through air as a possible route of exposure for MRSA. *J. Expo. Sci. Environ. Epidemiol.* 26, 263–269. doi: 10.1038/jes.2014.85

Bouchami, O., Fraqueza, M. J., Faria, N. A., Alves, V., Lawal, O. U., de Lencastre, H., et al. (2020). Evidence for the dissemination to human of methicillin-resistant *Staphylococcus aureus* ST398 through the pork production chain: a study in a Portuguese slaughterhouse. *Microorganisms* 8:1892. doi: 10.3390/microorganisms8121892

Bozdogan, B., and Appelbaum, P. C. (2004). Oxazolidinones: activity, mode of action, and mechanism of resistance. *Int. J. Antimicrob. Agents* 23, 113–119. doi: 10.1016/j.ijantimicag.2003.11.003

Chen, H., Yin, Y., Li, X., Li, S., Gao, H., Wang, X., et al. (2020). Whole-genome analysis of livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 strains isolated from patients with bacteremia in China. *J. Infect. Dis.* 221(Suppl. 2), S220–S228. doi: 10.1093/infdis/jiz575

Chen, L., Hu, J.-X., Liu, C., Liu, J., Ma, Z.-B., Tang, Z.-Y., et al. (2021). Identification of the Multiresistance Gene *poxtA* in Oxazolidinone-Susceptible *Staphylococcus haemolyticus* and *Staphylococcus saprophyticus* of Pig and Feed Origins. *Pathogens* 10:601. doi: 10.3390/pathogens10050601

Chin, C.-S., Alexander, D. H., Marks, P., Klammer, A. A., Drake, J., Heiner, C., et al. (2013). Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nat. Methods* 10, 563–569. doi: 10.1038/nmeth.2474

Chuang, Y.-Y., and Huang, Y.-C. (2015). Livestock-associated meticillin-resistant *Staphylococcus aureus* in Asia: an emerging issue? *Int. J. Antimicrob. Agents* 45, 334–340. doi: 10.1016/j.ijantimicag.2014.12.007

CLSI (2018). *Performance Standards for Antimicrobial Susceptibility Testing*, Twenty-Eighth Edn. Wayne, PA: CLSI.

Coccitto, S. N., Cinthi, M., Fioriti, S., Morroni, G., Simoni, S., Vignaroli, C., et al. (2022). Linezolid-resistant *Enterococcus gallinarum* isolate of swine origin carrying *cfr*, *optrA* and *poxtA* genes. *J. Antimicrob. Chemother.* 77, 331–337. doi: 10.1093/jac/dkab408

Cui, M., Li, J., Ali, T., Kalim, K., Wang, H., Song, L., et al. (2020). Emergence of livestock-associated MRSA ST398 from bulk tank milk, China. *J. Antimicrob. Chemother.* 75, 3471–3474. doi: 10.1093/jac/dkaa367

da Silva, A. G., Baines, S. L., Carter, G. P., Heffernan, H., French, N. P., Ren, X., et al. (2017). A phylogenomic framework for assessing the global emergence and evolution of clonal complex 398 methicillin-resistant *Staphylococcus aureus*. *Microb. Genomics* 3:e000105. doi: 10.1099/mgen.0.000105

David, M. Z., Siegel, J., Lowy, F. D., Zychowski, D., Taylor, A., Lee, C. J., et al. (2013). Asymptomatic Carriage of Sequence Type 398, *spa* Type t571 Methicillin-Susceptible *Staphylococcus aureus* in an Urban Jail: a Newly Emerging, Transmissible Pathogenic Strain. *J. Clin. Microbiol.* 51, 2443–2447. doi: 10.1128/jcm.01057-13

Davies, P. R., Wagstrom, E. A., and Bender, J. B. (2011). Lethal necrotizing pneumonia caused by an ST398 *Staphylococcus aureus* strain. *Emerg. Infect. Dis.* 17, 1152–1153. doi: 10.3201/eid/1706.101394

EUCAST (2016). Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 6.0. Available online at: https://www.eucast.org/ (accessed November 20, 2016).

Fan, R., Li, D., Feßler, A. T., Wu, C., Schwarz, S., and Wang, Y. (2017). Distribution of *optrA* and *cfr* in florfenicol-resistant *Staphylococcus sciuri* of pig origin. *Vet. Microbiol.* 210, 43–48. doi: 10.1016/j.vetmic.2017.07.030

Grøntvedt, C. A., Elstrøm, P., Stegger, M., Skov, R. L., Skytt Andersen, P., Larssen, K. W., et al. (2016). Methicillin-resistant *Staphylococcus aureus* CC398 in humans and pigs in Norway: a "One Health" perspective on introduction and transmission. *Clin. Infect. Dis.* 63, 1431–1438. doi: 10.1093/cid/ciw552

Hansen, J. E., Ronco, T., Stegger, M., Sieber, R. N., Fertner, M. E., Martin, H. L., et al. (2019). LA-MRSA CC398 in dairy cattle and veal calf farms indicates spillover from pig production. *Front. Microbiol.* 10:2733. doi: 10.3389/fmicb.2019.02733

Harmsen, D., Claus, H., Witte, W., Rothganger, J., Claus, H., Turnwald, D., et al. (2003). Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *J. Clin. Microbiol.* 41, 5442–5448. doi: 10.1128/jcm.41.12.5442-5448.2003

Harrison, E. M., Paterson, G. K., Holden, M. T., Morgan, F. J., Larsen, A. R., Petersen, A., et al. (2013). A *Staphylococcus xylosus* isolate with a new *mecC* allotype. *Antimicrob. Agents Chemother.* 57, 1524–1528. doi: 10.1128/AAC.01882-12

Inouye, M., Dashnow, H., Raven, L.-A., Schultz, M. B., Pope, B. J., Tomita, T., et al. (2014). SRST2: rapid genomic surveillance for public health and hospital microbiology labs. *Genome Med.* 6:90. doi: 10.1186/s13073-014-0090-6

Ji, X., Krüger, H., Feßler, A. T., Liu, J., Zeng, Z., Wang, Y., et al. (2020). A novel SCC*mec* type V variant in porcine MRSA ST398 from China. *J. Antimicrob. Chemother.* 75, 484–486. doi: 10.1093/jac/dkz445

Kadlec, K., Ehricht, R., Monecke, S., Steinacker, U., Kaspar, H., Mankertz, J., et al. (2009). Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant *Staphylococcus aureus* ST398 from diseased swine. *J. Antimicrob. Chemother.* 64, 1156–1164. doi: 10.1093/jac/dkp350

Kaya, H., Hasman, H., Larsen, J., Stegger, M., Johannesen, T. B., Allesøe, R. L., et al. (2018). SCC *mec* Finder, a web-based tool for typing of staphylococcal cassette chromosome *mec* in *Staphylococcus aureus* using whole-genome sequence data. *mSphere* 3:e00612-17. doi: 10.1128/msphere.00612-17

Kinross, P., Petersen, A., Skov, R., Van Hauwermeiren, E., Pantosti, A., Laurent, F., et al. (2017). Livestock-associated meticillin-resistant *Staphylococcus aureus* (MRSA) among human MRSA isolates, European Union/European Economic Area countries, 2013. *Euro Surveill.* 22, 16–00696. doi: 10.2807/1560-7917.es.2017.22.44.16-00696

Larsen, J., Petersen, A., Sørum, M., Stegger, M., van Alphen, L., Valentiner-Branth, P., et al. (2015). Meticillin-resistant *Staphylococcus aureus* CC398 is an increasing cause of disease in people with no livestock contact in Denmark, 1999 to 2011. *Euro Surveill.* 20:30021. doi: 10.2807/1560-7917.es.2015.20.37.30021

Li, D., Wang, Y., Schwarz, S., Cai, J., Fan, R., Li, J., et al. (2016). Co-location of the oxazolidinone resistance genes *optrA* and *cfr* on a multiresistance plasmid from *Staphylococcus sciuri*. *J. Antimicrob. Chemother.*71, 1474–1478. doi: 10.1093/jac/dkw040

Li, H., and Durbin, R. (2010). Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* 26, 589–595. doi: 10.1093/bioinformatics/btp698

Li, H., Tang, T., Stegger, M., Dalsgaard, A., Liu, T., and Leisner, J. J. (2021). Characterization of antimicrobial-resistant *Staphylococcus aureus* from retail foods in Beijing, China. *Food Microbiol.* 93:103603. doi: 10.1016/j.fm.2020.103603

Li, S.-M., Zhou, Y.-F., Li, L., Fang, L.-X., Duan, J.-H., Liu, F.-R., et al. (2018). Characterization of the multi-drug resistance gene *cfr* in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from animals and humans in China. *Front. Microbiol.* 9:2925. doi: 10.3389/fmicb.2018.02925

Li, W., Liu, J.-H., Zhang, X.-F., Wang, J., Ma, Z.-B., Chen, L., et al. (2018). Emergence of methicillin-resistant *Staphylococcus aureus* ST398 in pigs in China. *Int. J. Antimicrob. Agents* 51, 275–276. doi: 10.1016/j.ijantimicag.2017.10.013

Lienen, T., Schnitt, A., Hammerl, J. A., Maurischat, S., and Tenhagen, B.-A. (2020). Genomic distinctions of LA-MRSA ST398 on dairy farms from different German federal states with a low risk of severe human infections. *Front. Microbiol.* 11:575321. doi: 10.3389/fmicb.2020.575321

Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S., and Vester, B. (2006). The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. *Antimicrob. Agents Chemother.* 50, 2500–2505. doi: 10.1128/AAC.00{\break}131-06

Moodley, A., Nielsen, S. S., and Guardabassi, L. (2011). Effects of tetracycline and zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 in pigs. *Vet. Microbiol.* 152, 420–423. doi: 10.1016/j.vetmic.2011.05.025

Nakaminami, H., Hirai, Y., Nishimura, H., Takadama, S., and Noguchi, N. (2020). Arthritis caused by MRSA CC398 in a patient without animal contact, Japan. *Emerg. Infect. Dis.* 26, 795–797. doi: 10.3201/eid2612.203738

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* 42, D206–D214.

Pirolo, M., Gioffrè, A., Visaggio, D., Gherardi, M., Pavia, G., Samele, P., et al. (2019). Prevalence, molecular epidemiology, and antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* from swine in southern Italy. *BMC Microbiol.* 19:51. doi: 10.1186/s12866-019-1422-x

Qiao, M., Ying, G.-G., Singer, A. C., and Zhu, Y.-G. (2018). Review of antibiotic resistance in China and its environment. *Environ. Int.* 110, 160–172. doi: 10.1016/j.envint.2017.10.016

Rasigade, J.-P., Laurent, F., Hubert, P., Vandenesch, F., and Etienne, J. (2010). Lethal necrotizing pneumonia caused by an ST398 *Staphylococcus aureus* strain. *Emerg. Infect. Dis.* 16:1330. doi: 10.3201/eid1608.100317

Schnitt, A., Lienen, T., Wichmann-Schauer, H., Cuny, C., and Tenhagen, B.-A. (2020). The occurrence and distribution of livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 on German dairy farms. *J. Dairy Sci.* 103, 11806–11819. doi: 10.3168/jds.2020-18958

Schürch, A., Arredondo-Alonso, S., Willems, R., and Goering, R. (2018). Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. *Clin. Microbiol. Infect.* 24, 350–354. doi: 10.1016/j.cmi.2017.12.016

Shen, J., Wang, Y., and Schwarz, S. (2013). Presence and dissemination of the multiresistance gene *cfr* in Gram-positive and Gram-negative bacteria. *J. Antimicrob. Chemother.* 68, 1697–1706. doi: 10.1093/jac/dkt092

Sieber, R. N., Skov, R. L., Nielsen, J., Schulz, J., Price, L. B., Aarestrup, F. M., et al. (2018). Drivers and dynamics of methicillin-resistant livestock-associated *Staphylococcus aureus* CC398 in pigs and human in Denmark. *mBio* 9:e2142-18. doi: 10.1128/mbio.02142-18

Song, Z., Gu, F.-F., Guo, X.-K., Ni, Y.-X., He, P., and Han, L.-Z. (2017). Antimicrobial resistance and molecular characterization of *Staphylococcus aureus* causing childhood pneumonia in Shanghai. *Front. Microbiol.* 8:455. doi: 10.3389/fmicb.2017.00455

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039

Sun, C., Chen, B., Hulth, A., Schwarz, S., Ji, X., Nilsson, L. E., et al. (2019). Genomic analysis of *Staphylococcus aureus* along a pork production chain and in the community, Shandong Province, China. *Int. J. Antimicrob. Agents* 54, 8–15. doi: 10.1016/j.ijantimicag.2019.03.022

Sun, C., Zhang, P., Ji, X., Fan, R., Chen, B., Wang, Y., et al. (2018). Presence and molecular characteristics of oxazolidinone resistance in staphylococci from household animals in rural China. *J. Antimicrob. Chemother.*73, 1194–1200. doi: 10.1093/jac/dky009

Tang, Y., Qiao, Z., Wang, Z., Li, Y., Ren, J., Wen, L., et al. (2021). The Prevalence of *Staphylococcus aureus* and the Occurrence of MRSA CC398 in Monkey Feces in a Zoo Park in Eastern China. *Animals* 11:732. doi: 10.3390/ani11030732

Treangen, T. J., Ondov, B. D., Koren, S., and Phillippy, A. M. (2014). The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* 15:524. doi: 10.1186/s13059-014-0524-x

Van Duin, D., and Paterson, D. L. (2016). Multidrug-resistant bacteria in the community: trends and lessons learned. *Infect. Dis. Clin.* 30, 377–390. doi: 10.1016/j.idc.2016.02.004

Voss, A., Loeffen, F., Bakker, J., Klaassen, C., and Wulf, M. (2005). Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerg. Infect. Dis.* 11, 1965–1966. doi: 10.3201/eid1112.050428

Wang, Y., Lv, Y., Cai, J., Schwarz, S., Cui, L., Hu, Z., et al. (2015). A novel gene, *optrA*, that confers transferable resistance to oxazolidinones and phenicols and its presence in *Enterococcus faecalis* and *Enterococcus faecium* of human and animal origin. *J. Antimicrob. Chemother.* 70, 2182–2190. doi: 10.1093/jac/dkv116

Wang, J., Lin, D.-C., Guo, X.-M., Wei, H.-K., Liu, X.-Q., Chen, X.-J., et al. (2015). Distribution of the multidrug resistance gene *cfr* in *Staphylococcus* isolates from pigs, workers, and the environment of a hog market and a slaughterhouse in Guangzhou, China. *Foodborne Pathog. Dis.* 12, 598–605. doi: 10.1089/fpd.2014.1891

Ward, M. J., Gibbons, C. L., McAdam, P. R., Bunnik, B. A. D. V., Girvan, E. K., Edwards, G. F., et al. (2014). Time-scaled evolutionary analysis of the transmission and antibiotic resistance dynamics of *Staphylococcus aureus* clonal complex 398. *Appl. Environ. Microbiol.* 80, 7275–7282.

Wardyn, S. E., Stegger, M., Price, L. B., and Smith, T. C. (2018). Whole-genome analysis of recurrent *Staphylococcus aureus* t571/ST398 infection in farmer, Iowa, USA. *Emerg. Infect. Dis.* 24, 153–154. doi: 10.3201/eid2401.161184

Wulf, M., Sørum, M., Van Nes, A., Skov, R., Melchers, W., Klaassen, C., et al. (2008). Prevalence of methicillin-resistant *Staphylococcus aureus* among veterinarians: an international study. *Clin. Microbiol. Infect.* 14, 29–34. doi: 10.1111/j.1469-0691.2007.01873.x

Yan, X., Yu, X., Tao, X., Zhang, J., Zhang, B., Dong, R., et al. (2014). *Staphylococcus aureus* ST398 from slaughter pigs in northeast China. *Int. J. Med. Microbiol.* 304, 379–383. doi: 10.1016/j.ijmm.2013.12.003

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, K., Sparling, J., Chow, B. L., Elsayed, S., Hussain, Z., Church, D. L., et al. (2004). New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. *J. Clin. Microbiol.* 42, 4947–4955. doi: 10.1128/jcm.42.11.4947-4955.2004

Zhou, W., Li, X., Osmundson, T., Shi, L., Ren, J., and Yan, H. (2018). WGS analysis of ST9-MRSA-XII isolates from live pigs in China provides insights into transmission among porcine, human and bovine hosts. *J. Antimicrob. Chemother.* 73, 2652–2661. doi: 10.1093/jac/dky245

Zou, G., Matuszewska, M., Jia, M., Zhou, J., Ba, X., Duan, J., et al. (2021). A survey of Chinese pig farms and human healthcare isolates reveals separate human and animal methicillin-resistant *Staphylococcus aureus* Populations. *Adv. Sci.* 9:e2103388. doi: 10.1002/advs.202103388

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 Li, Xie, Huang, Li, Li, Liu, Xiao, Zhang, Xiong and Zeng. 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 Microbiology

ORIGINAL RESEARCH
published: 27 June 2022
doi: 10.3389/fmicb.2022.859990

Genomic Characterization of *Enterococcus hirae* From Beef Cattle Feedlots and Associated Environmental Continuum

*Sani-e-Zehra Zaidi[1,2], Rahat Zaheer[1], Ruth Barbieri[1], Shaun R. Cook[1], Sherry J. Hannon[3], Calvin W. Booker[3], Deirdre Church[4,5], Gary Van Domselaar[6], Athanasios Zovoilis[2] and Tim A. McAllister[1]**

[1]Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada, [2]University of Lethbridge, Lethbridge, AB, Canada, [3]Feedlot Health Management Services, Okotoks, AB, Canada, [4]Cumming School of Medicine, University of Calgary, Calgary, AB, Canada, [5]Calgary Laboratory Services, Calgary, AB, Canada, [6]National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada

OPEN ACCESS

Edited by:
Ziad Daoud,
Central Michigan University, United States

Reviewed by:
Jørgen J Leisner,
University of Copenhagen, Denmark
Valentine Usongo,
Health Canada, Canada

***Correspondence:**
Tim A. McAllister
tim.mcallister@agr.gc.ca

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *22 January 2022*
Accepted: *08 April 2022*
Published: *27 June 2022*

Citation:
Zaidi S-e-Z, Zaheer R, Barbieri R, Cook SR, Hannon SJ, Booker CW, Church D, Van Domselaar G, Zovoilis A and McAllister TA (2022) Genomic Characterization of Enterococcus hirae From Beef Cattle Feedlots and Associated Environmental Continuum. Front. Microbiol. 13:859990. doi: 10.3389/fmicb.2022.859990

Enterococci are commensal bacteria of the gastrointestinal tract of humans, animals, and insects. They are also found in soil, water, and plant ecosystems. The presence of enterococci in human, animal, and environmental settings makes these bacteria ideal candidates to study antimicrobial resistance in the One-Health continuum. This study focused on *Enterococcus hirae* isolates (n = 4,601) predominantly isolated from beef production systems including bovine feces (n = 4,117, 89.5%), catch-basin water (n = 306, 66.5%), stockpiled bovine manure (n = 24, 0.5%), and natural water sources near feedlots (n = 145, 32%), and a few isolates from urban wastewater (n = 9, 0.2%) denoted as human-associated environmental samples. Antimicrobial susceptibility profiling of a subset (n = 1,319) of *E. hirae* isolates originating from beef production systems (n = 1,308) showed high resistance to tetracycline (65%) and erythromycin (57%) with 50.4% isolates harboring multi-drug resistance, whereas urban wastewater isolates (n = 9) were resistant to nitrofurantoin (44.5%) and tigecycline (44.5%) followed by linezolid (33.3%). Genes for tetracycline (*tetL, M, S/M,* and *O/32/O*) and macrolide resistance *erm(B)* were frequently found in beef production isolates. Antimicrobial resistance profiles of *E. hirae* isolates recovered from different environmental settings appeared to reflect the kind of antimicrobial usage in beef and human sectors. Comparative genomic analysis of *E. hirae* isolates showed an open pan-genome that consisted of 1,427 core genes, 358 soft core genes, 1701 shell genes, and 7,969 cloud genes. Across species comparative genomic analysis conducted on *E. hirae*, *Enterococcus faecalis* and *Enterococcus faecium* genomes revealed that *E. hirae* had unique genes associated with vitamin production, cellulose, and pectin degradation, traits which may support its adaptation to the bovine digestive tract. *E. faecium* and *E. faecalis* more frequently harbored virulence genes associated with biofilm formation, iron transport, and cell adhesion, suggesting niche specificity within these species.

Keywords: cattle production, antimicrobial resistance, enterococci, genomic signatures, pan-genome

INTRODUCTION

Antimicrobial resistance (AMR) is recognized as one of the major global health challenges of the 21st century. The interconnected microbiomes between humans, animals, and the environment contribute to the emergence, acquisition, and spread of AMR (Hiltunen et al., 2017). A One-health approach provides an in-depth knowledge of the evolution of AMR by focusing on those biological elements that influence the emergence of antimicrobial resistance genes (ARGs) within the microorganism and their dissemination among hosts (human and animals) and the environment (Hernando-Amado et al., 2019). Gram-positive enterococci are core members of the gastrointestinal microbiota of humans and animals and are frequently isolated from soil and water (Byappanahalli et al., 2012; Gilmore et al., 2014). Enterococci often carry ARGs as they compete within complex microbial communities and are exposed to antimicrobials in clinical settings and during livestock production (Murray, 1990; Moreno et al., 2006). Furthermore, depending on the species, enterococci exhibit intrinsic resistance to several antibiotics including cephalosporins, anti-staphylococcal penicillins, aztreonam, aminoglycosides, lincosamides, and streptogramins (Miller et al., 2014). Enterococci are typically commensals, but they can cause nosocomial infections in humans including septicemia, endocarditis, and urinary tract infections (Barnes et al., 2021). There are over 50 species of enterococci with *E. faecalis* and *E. faecium* most frequently linked to human infections. Occasionally, other species including *E. hirae, E. avium, E. durans, E. gallinarum, E. casseliflavus, and E. raffinosus* may also be associated with infections in people (Brayer et al., 2019; Pinkes et al., 2019; Winther et al., 2020). Due to their widespread occurrence and persistence in the environment, enterococci are considered indicators of fecal contamination (Byappanahalli and Fujioka, 2004; Yan et al., 2011) and also serve as key indicator bacteria for AMR surveillance systems in humans and animals (Harwood et al., 2000; Layton et al., 2010).

Studies have indicated that *E. faecium* and *E. faecalis* are more prevalent in human-associated environments, whereas *E. hirae* are prevalent in beef cattle production systems (Zaheer et al., 2020). *E. hirae* only accounts for 1% of enterococcal infections in humans (Heval Can et al., 2020) and is mainly linked to pyelonephritis (Chan et al., 2012; Pãosinho et al., 2016; Nakamura et al., 2021), endocarditis (Talarmin et al., 2011; Pinkes et al., 2019), and biliary tract infections (Tan et al., 2010; Bourafa et al., 2015). As with *E. faecalis* and *E. faecium, E. hirae* infections are typically treated with ampicillin, gentamicin, or vancomycin (Nakamura et al., 2021).

The focus of this study was to investigate the genomic relatedness of *E. hirae* across the environmental continuum and to identify the genetic nature of AMR in *E. hirae*. Furthermore, we applied a pan-genome analysis to identify genes that may account for the predominance of *E. hirae* within beef cattle production systems.

METHODOLOGY

Bacterial Isolates

A total of 8,430 *Enterococcus* strains were isolated in a One-Health surveillance study from different segments of the environmental continuum using samples collected from beef production systems (i.e., feedlot cattle feces, catch-basin water, and manure), natural water sources, urban wastewater, and human clinical samples (Zaheer et al., 2020). Bovine fecal samples came from four feedlots in southern Alberta over two years (March 2014–April 2016). Wastewater samples were collected from catch basins that accumulate runoff from the feedlots. Natural surface water samples came from up-stream and down-stream of the feedlots. Urban wastewater samples came from two wastewater plants located in southern Alberta. *Enterococcus* spp. recovered from patients with clinical infections were obtained through the Division of Medical Microbiology, Calgary Laboratory Services (now Alberta Precision Laboratories, Alberta Health Services) (Zaheer et al., 2020). This study focuses on *Enterococcus hirae*, collected as the most prevalent species from beef production system (n = 4,601 isolate) (Zaheer et al., 2020). **Figure 1** represents the prevalence of *E. hirae* isolates in the sampled sources.

Enterococci were recovered in parallel from two different media types including Bile Esculin Azide (BEA) agar without antibiotic and BEA supplemented with 8 μg/ml erythromycin, followed by species identification. *E. hirae* were identified *via* multiplex PCR targeting groES-EL and muramidase genes (Zaheer et al., 2020). As *E. hirae* was absent among clinical *Enterococcus* isolates (n = 1892; **Figure 1**), complete genomes

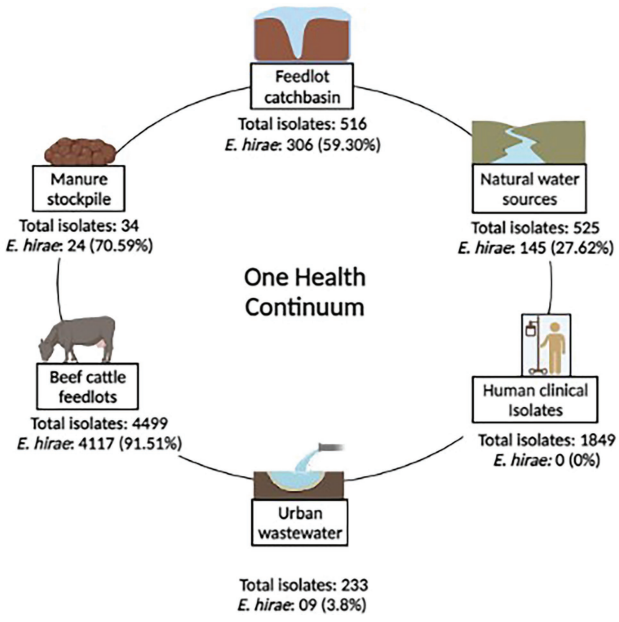


FIGURE 1 | Prevalence of *Enterococcus hirae* isolates (n = 4,601) identified across a One-health continuum.

($n = 3$) of clinical *E. hirae* were retrieved from NCBI database for comparative genomic analysis (**Supplementary Table S1**).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on a randomly selected subset ($n = 1319$, 29%) of *E. hirae* isolates using the disk diffusion method, as per the Clinical and Laboratory Standards Institute (CLSI) documents M02-A12 and M100-S24. A panel of twelve antibiotics was used for testing based on their common usage for treatment of human enterococcal infections that included those drugs of critical importance (levofloxacin, linezolid, quinupristin/dalfopristin, teicoplanin, vancomycin, and tigecycline), high importance (erythromycin, ampicillin, gentamicin, and streptomycin), and medium importance (nitrofurantoin and tetracycline). *Staphylococcus aureus* ATCC 25923 and *E. faecalis* ATCC 29212 were used as reference quality controls (Zaheer et al., 2020). The BioMic V3 imaging system (Giles Scientific, Inc., Santa Barbara, CA, USA) was used to read zones of inhibition. Isolates were categorized based on CLSI interpretive criteria, except for tigecycline for which EUCAST interpretive criteria (The European Committee on Antimicrobial Susceptibility Testing, 2014) were used.

Whole-Genome Sequencing

Whole-genomic sequencing of a subset of *E. hirae* isolates ($n = 286$), including isolates originating from bovine feces ($n = 168$), feedlot catch basin ($n = 62$), bovine manure stockpiles ($n = 8$), natural water sources ($n = 42$), and urban wastewater ($n = 7$), was performed using next-generation sequencing technology. Briefly, genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Montreal, QC, Canada) with modifications (Zaheer et al., 2020), followed by DNA quality assessment and quantification using a Nanodrop 2000 spectrophotometer and a Qubit Fluorometer with PicoGreen (Thermo Fisher Scientific, Mississauga, ON, Canada). Isolates were sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit V3 to generate 2×300 bp paired-end reads. Raw read FASTQ files were assessed for the quality of sequence data using FastQC (Galaxy Version 0.72 + galaxy1) (Wingett and Andrews, 2018) and *de novo* assemblies were performed using Shovill (Bankevich et al., 2012). Assembled contigs were then annotated by Prokka to identify all gene-coding sequences (Seemann, 2014).

AMR Determinants, Virulence, and Plasmid Detection

Assembled genomes were screened for the presence of AMR determinants, virulence genes, and plasmids using ABRicate (https://github.com/tseemann/abricate/) against the NCBI Bacterial Antimicrobial Resistance Reference Gene database (NCBI BioProject ID: PRJNA313047), VirulenceFinder [pmid 15,608,208], and PlasmidFinder databases (Zankari et al., 2012), respectively. Intact prophage were identified using PHASTER tool (Arndt et al., 2016).

Comparative Genomic Analysis

A total of 289 genomes including 286 assembled genomes from this study and 3 complete *E. hirae* genomes of clinical isolates retrieved from NCBI database (strain: 708, accession: NZ_CP055232.1; strain: FDAARGOS_234, accession: NZ_CP023011.2; and strain: 13344, accession: NZ_CP055229.1) were subjected to phylogenomic analysis. A core-genome phylogenomic tree was constructed using the (SNVPhyl v 1.0) pipeline (Petkau et al., 2017). Briefly, all paired-end reads were mapped against the *E. hirae* reference genome (strain R17; GenBank accession: CP015516.1) to produce read pileups (SMALT v.0.7.5; https://www.sanger.ac.uk/tool/smalt-0/). The read pileups were evaluated for mapping quality (minimum mean mapping quality score of 30), coverage cut offs (15X minimum depth of coverage), and a single-nucleotide variant (SNV) abundance ratio of 0.75 to generate a multiple sequence alignment of SNV containing sites. The final maximum likelihood-based phylogeny was generated by PhyML using unfiltered SNV alignment. Phylogenomic trees and associated metadata were visualized using Interactive Tree Of Life (iTOL) v5 tool (Letunic and Bork, 2021).

Comparative genome analysis was done using the Roary v3.12.0 pipeline with default parameters (Page et al., 2015). Genes identified by Prokka were used to construct pan-genomes. A pan-genome of 289 *E. hirae* isolates was reconstructed to identify core and accessory genes present in *E. hirae*. Furthermore, comparative analysis was performed between *E. faecium* and *E. faecalis,* which are predominantly associated with humans infections, and *E. hirae.* For this purpose, a small subset of *E. hirae* isolates ($n = 16$) representative of the various sources and phylogenetic clades were randomly selected. Similarly, a subset of *E. faecium* ($n = 26$) and *E. faecalis* ($n = 24$) isolates were randomly selected on the same bases from our previous study (Zaheer et al., 2020; BioProject PRJNA604849). The phandango interactive viewer tool (Hadfield et al., 2017) was used to interpret pan-genome data obtained from Roary analysis. This tool utilizes two of the Roary output files: one is a gene absence and presence matrix file that creates a heat map based on the number of genes present or absent in each isolate and a Newick-formatted tree file of accessory genomes used to plot a relatedness dendrogram of the accessory genes present in all isolates.

A pan-genome plot was generated using ggplot2 package of R Studio Version 1.4.1103 (R Studio Inc., Boston, MA, USA) based on two Roary output files (the number of conserved genes and the number of total genes). The number of conserved genes represented the size of the core genome. The number of total genes represented both the core and accessory genomes, creating a curve based on the pan-genome completeness. The pan-genome of an organism is considered "closed" if the curve is predicted to plateau or "open" if the curve is predicted to continue to rise. In contrast to a closed genome, the number of new gene families in an open genome increases with the inclusion of new genomes in the analysis.

Discriminatory genomic signatures between *E. hirae*, *E. faecium,* and *E. faecalis* were identified using Neptune v1.2.5 with default parameter (Marinier et al., 2017). The signature discovery process using Neptune identifies sequences that are

sufficiently common to a group of target sequences (inclusion group) and sufficiently absent from non-targets (exclusion group) using probabilistic models. Analyses was done using *E. hirae* genomes as the inclusion group and *E. faecium* and *E. faecalis* as independent exclusion groups, respectively. The genomic signature found in ≥90% of isolates in the inclusion group were selected and annotated using Prokka (Seemann, 2014).

RESULTS

Antimicrobial Susceptibility Testing

Phenotypic susceptibility testing was conducted on 1,319 *E. hirae* isolates originating from bovine feces, feedlot catch-basin water, stockpiled bovine manure, and natural and urban wastewater sources. (**Figure 2**). Fifty-one different resistance profiles were identified with the most frequent being resistant to doxycycline and erythromycin (364/1319, 27.6%) followed by resistance to doxycycline alone (242/1319, 18.3%) (**Supplementary Table S2**). Across all tested isolates, 14.1% (186/1319) were multi-drug resistant (resistant to ≥3 tested antimicrobials). Antimicrobial susceptibility profiles of all tested isolates are presented in **Supplementary Figure S2**.

Isolates Recovered From BEA Plates Without Erythromycin

Out of 1,319 total isolates tested for antimicrobial susceptibility, 666 isolates were recovered from BEA plates without erythromycin. From these, isolates originating from beef production systems (i.e., bovine feces, catch basin, and stockpiled bovine manure; $n = 632$) exhibited a high prevalence of resistance to tetracycline (376/632, 59.4%), followed by macrolides (200/632, 31.6%), nitrofurantoin (102/632, 16.1%), tigecycline (76/632, 12.0%), linezolid (40/632, 6.32%), ampicillin (9/632, 1.42%), quinupristin/dalfopristin (8/632, 1.26%), vancomycin (1/632,

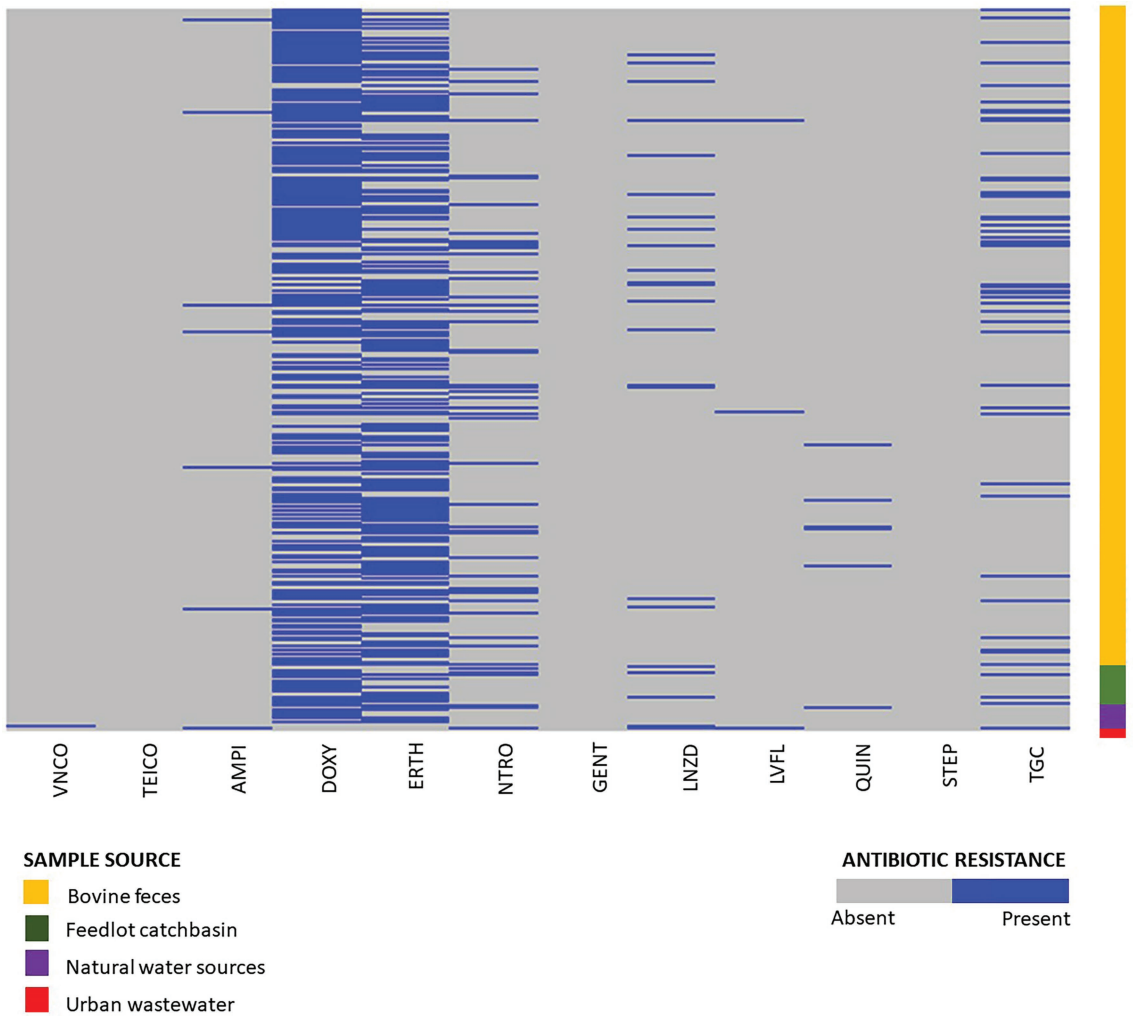


FIGURE 2 | Phenotypic resistance profiles of *Enterococcus hirae* isolated from beef production system ($n = 1,264$) including bovine feces and feedlot catch basin, natural water source ($n = 45$), and urban wastewater sample ($n = 9$).

0.15%), and teicoplanin (1/632, 0.15%) (**Supplementary Figure S1**).

The natural water source isolates recovered from BEA plates without antibiotics ($n = 28$) also showed a high prevalence of tetracycline resistance (22/28, 78.5%), followed by macrolides (8/28, 28.5%), nitrofurantoin (5/28, 17.8%), and tigecycline (1/28, 3.57%). Resistance against quinupristin/dalfopristin, linezolid, ampicillin, and fluoroquinolones was not detected (**Supplementary Figure S1**).

Of the 233 *Enterococcus* spp. isolates recovered from urban wastewater (Zaheer et al., 2020), only nine were identified as *E. hirae*. Six of those were recovered from media without erythromycin. Four of the six isolates exhibited resistance to tigecycline (4/6, 66.6%) followed by nitrofurantoin (3/6, 50%), linezolid (3/7, 48.85%), fluoroquinolones (2/6, 33.3%), vancomycin (1/6, 16.6%), and ampicillin (1/7, 14.2%) (**Supplementary Figure S1**).

Isolates Recovered From BEA Plates With Erythromycin

A total of 652 isolates from erythromycin plates were selected for phenotypic antimicrobial testing. Of these isolates, 632 originated from beef production (i.e., bovine feces, catch basin, and stockpiled bovine manure). Tetracycline resistance (437/632, 69%) was the most prevalent resistance in the beef isolates from production systems, followed by macrolides (525/632, 83%), tigecycline (67/632, 10.6%), nitrofurantoin (60/632, 9.5%), linezolid (33/632, 5.2%), quinupristin/dalfoprstin (13/632, 2.0%), ampicillin (9/632, 1.42%), fluoroquinolones (5/632, 0.79%), and gentamicin (1/632, 0.15%) (**Supplementary Figure S1**).

The isolates recovered from natural water sources ($n = 17$) showed a high prevalence of resistance to macrolides (15/17, 88.2%), followed by tetracycline (13/17, 76.4%), tigecycline (2/17, 11.76%), quinupristin/dalfoprstin (1/17, 5.88%), and nitrofurantoin (1/17, 5.88%). None of the isolates were resistant to linezolid, ampicillin, or fluoroquinolones (**Supplementary Figure S1**).

A total of three *E. hirae* isolates were recovered from urban waste sources on erythromycin plates. Two of those isolates showed macrolide resistance (2/3, 66.6%), followed by tetracycline (1/3, 33.3%), nitrofurantoin (1/3, 33.3%), quinupristin/dalfoprstin (1/3, 33.3%), and streptomycin (1/3, 33.3%). These isolates were sensitive to teicoplanin, ampicillin, vancomycin, gentamicin, tigecycline, fluoroquinolones, and linezolid (**Supplementary Figure S2**). Overall, 16.8% of isolates (110/652) recovered from erythromycin plates showed intermediate resistance to erythromycin.

Whole-Genome Sequencing

Of the *E. hirae* isolates tested for antimicrobial susceptibility, 286 randomly selected isolates were used for whole-genome sequencing. The size of *E. hirae* genomes as interpreted from the assembled sequence read data ranged from 2,307,753 bp to 3,200,875 bp, with a GC content of 36.7%. Detailed assembly statistics are provided in **Supplementary Table S3**.

AMR Determinants

Assembled genomes ($n = 286$) were screened for the presence of AMR determinants using the Abricate tool along with the NCBI AMR gene database. Ten different ARGs, including aminoglycosides ARGs *aac(6′)-Iid, ant(6)-Ia* and *aph(3)-III*, streptothricin *sat4,* tetracycline ARGs *tet (L, M, O, S/M, (O/32/O)),* and macrolide ARG *erm(B)* were identified across the examined genomes (**Figure 3**). Overall, nineteen different resistance genotypes were identified with the most frequent being *aac(6′)-lid-tet(L)-erm(B)* (87/286, 30.41%) followed by *aac(6′)-lid-tet(L)-tet(M)-erm(B)* (64/286, 22.37%) (**Supplementary Table S4**). The aminoglycoside resistance gene *aac(6′)-lid* was identified in all but two of the *E. hirae* genomes (284/286, 99.30%).

Of the 286 sequenced isolates, 238 were recovered from beef production systems (i.e., bovine feces, feedlot catch basin, and stockpiled bovine manure). *tet(L)* (199/238, 83.61%) was the most prevalent ARG identified in these isolates, followed by *erm(B)* (179/238, 75.21%) and the tetracycline resistance genes, *tet (M)* (73/238, 30.67%), *tetO* (36/238, 15.12%), *tet(O/32/O)* (13/238, 5.46%), and *tet(S/M)* (03/238, 1.26%).

Similar to beef production system isolates, *E. hirae* isolates recovered from natural water sources located near feedlots showed a high prevalence of *tet(L)* (38/41, 92.68%) followed by *erm(B)* (27/41, 65.85%). Occasionally, *tet(M)* (4/41, 9.75%), *tet(O)* (4/41, 9.75%), and tet(O/32/O) (2/41, 4.87%) were also present in these isolates.

Among seven *E. hirae* isolates recovered from urban wastewater, the streptomycin resistance gene *ant(6)-la* was present in two isolates (2/7, 28.57%). The kanamycin/neomycin *aph(3′)-III* and streptothricin *sat4* resistance genes were both found in a single urban wastewater isolate (1/7, 14.28%). Tetracycline resistance gene(s) were not found in any of these isolates, whereas *erm(B)* was only detected in one isolate (1/7, 14.28%).

tet(L) and *erm(B)* were found together in 63.63% of total isolates (182/286) indicating a strong correlation. Similarly, *tet (L)* and *tet (M)* coexisted in 24.12% of isolates (69/286) and in most cases were found on the same contig (60/69, 87%) in assembled genomes.

E. hirae genotypes generally associated with quinolone resistance (i.e., presence of quinolones resistant gene (qnr) or DNA gyrase and DNA topoisomerase IV genes mutations) and linezolid resistance (i.e., mutations in the 23S ribosomal RNA gene or presence of resistance genes including *cfr*, *cfrB*, *optrA,* and *poxtA*) were not identified.

Virulence Factors

Within the 286 *E. hirae* isolates, we identified nine different virulence genes associated with biofilm formation *(bopD)*, capsular polysaccharides biosynthesis *(cpsA, cpsB, and cap8E)*, hyaluronic acid production (*hasC*), proteolytic activity/chaperones *(clpP),* fibrinogen adhesions protein *(fss3)*, bile salt hydrolase *(bsh)*, and listeria adhesion protein *(lap).* All isolates carried *cap8E*, *clpP*, *cpsA*, *cpsB*, *bopD*, and *lap* genes, while *hasC*, *bsh,* and *fss3* were found in 98.95% (283/286), 89.86% (257/286),

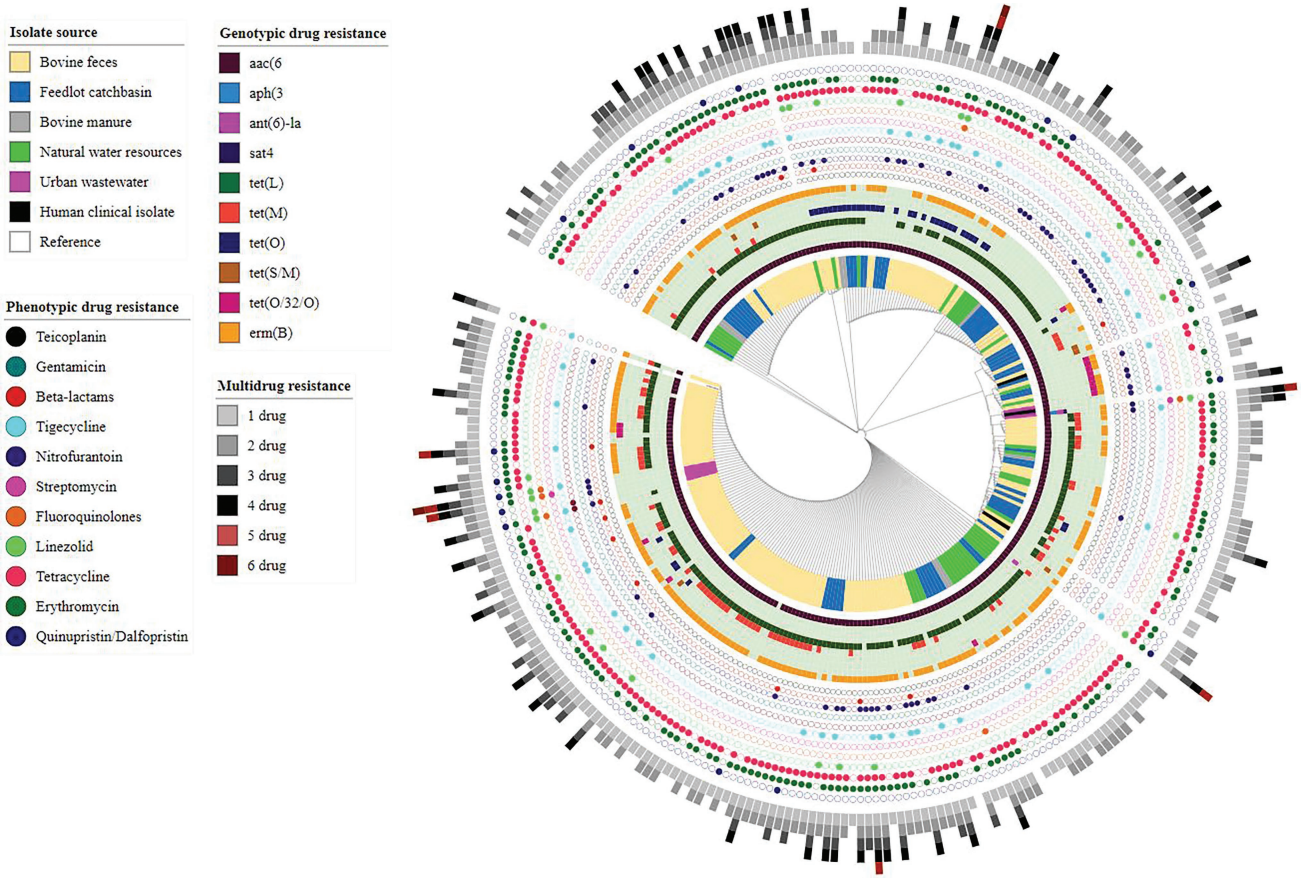


FIGURE 3 | Core-genome phylogenetic tree based on analysis of single-nucleotide polymorphisms (SNPs) of *Enterococcus hirae* genomes ($n = 291$) isolated from different environmental settings including beef production systems and human-related isolates. The genomes were compared using *E. hirae* OG1RF genome (GenBank accession # NZ_CP015516.1/CP015516.1) as a reference.

and 2.44% (7/286) of total isolates, respectively. These genes were also identified in publicly available clinical *E. hirae* genomes from humans. Pili protein-encoding gene *ebpC* was only detected in one of the human clinical isolates retrieved from NCBI. Detailed information is provided in **Supplementary Table S5**.

Plasmid Identification

Among all *E. hirae* isolates ($n = 286$), 16% carried plasmids. Seven different plasmids (rep1, rep2, rep11, rep17, rep18, repUS7, and repUS12) were identified. Among these, rep2 and rep17 were recovered from all sample types except urban wastewater. In contrast, rep1, rep18, and repUS7 were recovered from urban wastewater samples. Two out of fifteen rep17 plasmids carried *erm(B)*, whereas one out of twenty rep2 plasmids carried *tet(L)*. Of six repUS12 plasmids, five carried *tet (L)* and were recovered from bovine feces.

Prophage Identification

A total of 30 genomes were randomly selected from all sample sources to identify bacteriophage using PHASTER. All isolates contain at least one prophage ranging from 7 to 48 kb in size. Twenty-four intact prophage sequences were identified, with 95.5% identified as members of the family Siphoviridae. None of the identified prophages harbored ARGs.

Comparative Genomic Analysis

Core-genome phylogenomic analysis was conducted on the 286 *E. hirae* isolated in this study and the three *E. hirae* genomes retrieved from NCBI. The *E. hirae* isolates clustered into six different clades, with no obvious segregation by source (**Figure 3**).

Pan-genome analysis of *E. hirae* isolates identified 1,427 core genes (99 to 100% of strains), 358 soft core genes (95 to 99% of strains), 1701 shell genes (15 to 95% of strains), and 7,969 cloud genes (0 to 15% of strains) (**Figure 4A**). The pan-genome of *E. hirae* is open as the number of accessory genes progressively increased with increasing genomes (**Figure 4B**). In addition, the gene presence and absence heat map showed that the accessory genome constituted a large part of the pan-genome, indicative of a high level of genomic diversity within this species (**Figure 4C**). Cross-species comparative analysis of *E. hirae*, *E. faecium,* and *E. faecalis* genomes, highlighted the genomic diversity within

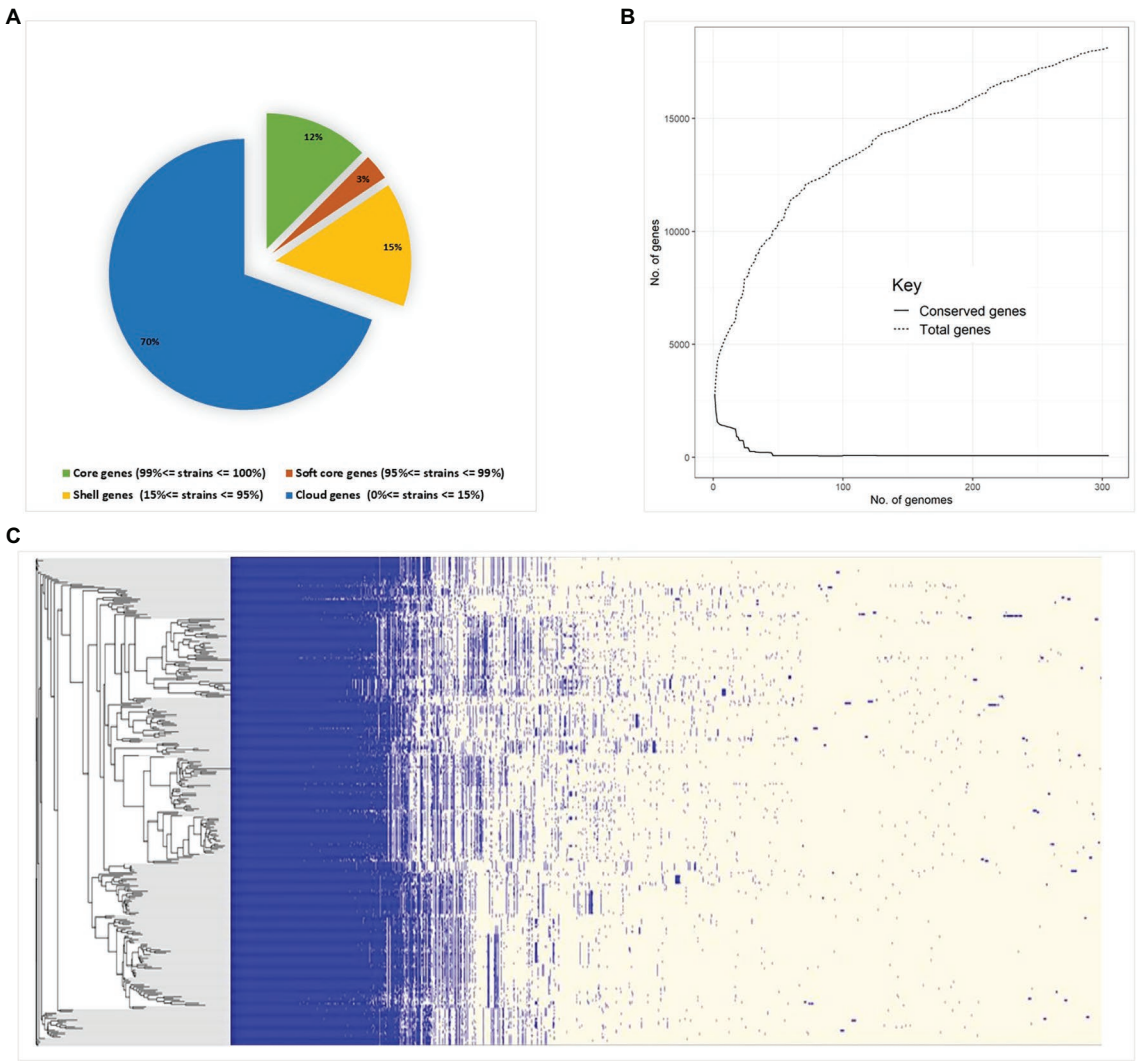


FIGURE 4 | Pan-genome estimation of *Enterococcus hirae* **(A)** genomic statistics and pan-genome estimation of 291 isolates. **(B)** Development of pan- and core genomes, illustrating the open nature of the pan-genome. **(C)** Heat map representing absence or presence of genes in isolates and the phylogenetic genetic tree generated from accessory genes.

Enterococcus spp. as the total core genome shared between three species was small (**Figure 5**). Furthermore, it also demonstrated the distinct genomic traits of each species as illustrated by the gene absence and presence heat map (**Figure 5**).

A total of 1,069 discriminatory genomic signatures were recognized in *E. hirae* compared to *E. faecalis* ($n = 808$) and *E. faecium* ($n = 261$). Most of these genomic signatures encoded for unknown hypothetical proteins (454/1069, 42.46%).

Neptune analysis is capable of identifying inter-species genomic variation, as discriminatory loci were identified in all three species. These loci coded for genes required for the synthesis of aromatic amino-acids including chorismate synthase (*aroC*), cyclohexadienyl dehydrogenase (*tyrC*), genes for sugar transport including permease (*yteP*), transcriptional regulator (*mtlR*), lichenan permease IIC component (*licC*), and lipoprotein (*lipO*).

Signatures found exclusively in *E. hirae* genomes included genes associated with the phosphotransferase system for galactitol (*gatA, B,* and *C*) and fructose (*fruA, frwA,* and *frwD*), peptidoglycan synthesis (*rodA ftsW, mur E, F, J,* and *Y*), teichoic acid synthesis (*tag H, G,* and *U*), coenzyme A biosynthesis (*coaD*), vitamin B12 synthesis (*nrdZ*), chitin degradation (*chiA*), capsule synthesis (*epsE, pglF, ywqD,* and *ywqC*), riboflavin synthesis (*ribBA*, *D, E,* and *H*), vitamin B6 synthesis (*yvgN*), vitamin uptake transporter (*queT*), gamma-aminobutyric acid (GABA) production (*glsA2, gadC,* and *amt*), cardiolipin biosynthesis (*clsA*), bacitracin export (*bceA* and *B*), xenobiotic degradation (*nylA*), and iron transport (*yqgN, feuC, feuB, fepC,* and *yfiY*).

Furthermore, genes that may be associated with antimicrobial resistance were also identified in *E. hirae* genomes such as those encoding for multi-drug transporters (*marA, mepE msrR,*

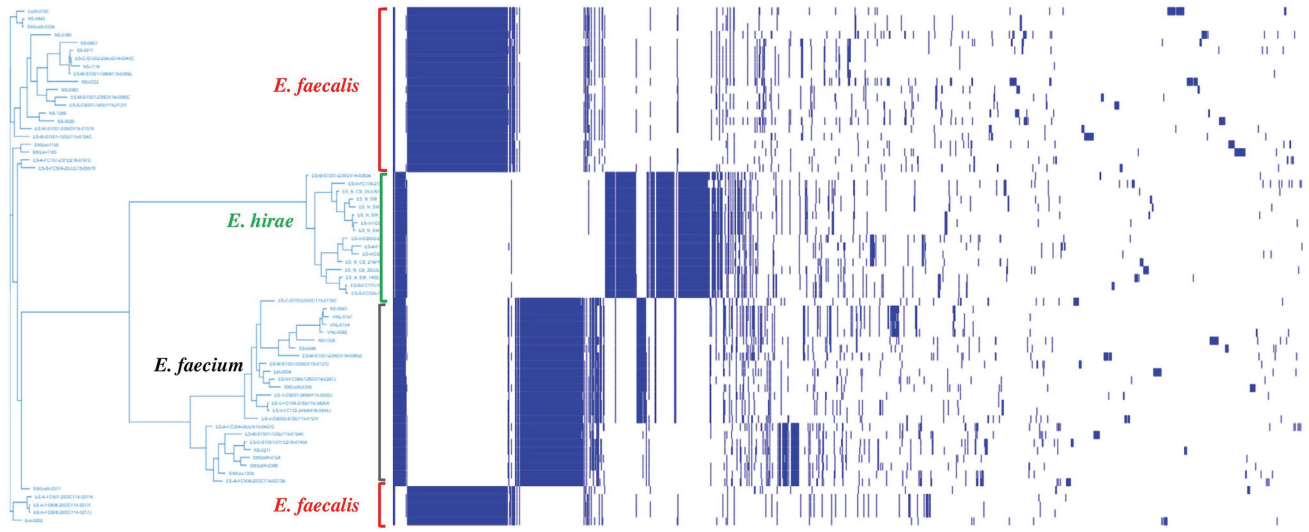


FIGURE 5 | Heat map representing absence or presence of genes and phylogenetic genetic tree generated from accessory genes of *Enterococcus hirae* (*n* = 16), *Enterococcus faecium* (*n* = 26), and *Enterococcus faecalis* (*n* = 24).

and *yxlF*), doxorubicin resistance (*drrA*), sulfonamide resistance (*bcr*), and penicillin-binding protein (*pbp*). Early secreted antigenic target (ESAT) system genes (*eccC, essB*, and *esxA*) were also found in *E. hirae* genomes.

Compared to *E. hirae*, 160 and 944 discriminatory genomic signatures were identified in *E. faecium* and *E. faecalis* genomes, respectively, with most of these genes associated with various aspects of cellular metabolism, such as molybdopterin biosynthesis (*mog*, *modB,* and *modB*); cadmium, zinc, and cobalt transport (*cadA*); copper export (*copY* and *copA*); phosphotransferase system for glucitol/sorbitol (*srlA*, *srlE,* and *srlB*), sorbose (*sorB*, *sorF,* and *sorA*), mannose (*manX* and manZ), cellobiose (*celA*), mannitol (*mtlA*, *mtlF,* and *mtlD*), maltose (*malX*), ascorbate (*ulaC* and *ulaA*), and beta-glucosides (*bglF*). In addition, genes encoding *E. faecalis* and *E. faecium* pathogenesis were also identified, such as biofilm formation genes (*brpA*, *icaA,* and *lytR*); virulence genes, including unsaturated chondroitin disaccharide hydrolase (*ugl*); anthrax toxin regulator positive (*atxA*); hemin transport system (*hmuU* and *hmuT*); sialic acid TRAP transporter small permease (*siaQ*); carnitine transport system (*opuCB* and *opuCA*); arginine/ornithine system (*argR* and *arcD1*); genes encoding the adhesions, including gelatinase (*gelE*) and collagen (*cna*); and genes encoding for antimicrobial resistance, including penicillin-binding protein (PbpE, PbpX, and PbpF), multi-drug resistance protein (YkkC, YkkD, and Stp), tetracycline repressor protein (TetR), fluoroquinolones export protein (Rv2688c), and macrolide export protein (MacB) (**Supplementary Table S7**).

DISCUSSION

Enterococci are ubiquitous Gram-positive bacteria. They colonize gastrointestinal tracts of most multicellular eukaryotic organisms including humans, animals, and insects and aide in digestion and gut metabolic pathways (De Graef et al., 2003; Farrow and Collins, 1985; Devriese et al., 1990; Andrew and Mitchell, 1997; de Vaux et al., 1998; Muniesa et al., 1999; Fogarty et al., 2003; Law-Brown and Meyers, 2003; Maria da Gloria et al., 2006; Layton et al., 2010; Giraffa, 2014). In addition, they are also found in food, plant, and water ecosystems (Müller et al., 2001; Svec et al., 2001; Klein, 2003; Švec et al., 2006; Byappanahalli et al., 2012). Enterococci are remarkably resilient to broad pH ranges, temperature variation, and osmotic pressure, traits that contribute to their broad distribution in nature (Heim et al., 2002; Caretti and Lubello, 2003; Anderson et al., 2005). Resistant bacterial populations are selected by the exposure of commensal gut microorganisms such as enterococci to antimicrobials that are used for disease treatment and prevention (Francino, 2016). The ubiquitous nature of enterococci may facilitate the dissemination of antimicrobial resistance genes between different environments. For this reason, it is imperative to identify antimicrobial resistance determinants and their role in the spread of antimicrobial resistance (Cameron and McAllister, 2016). Here, we focused on *E. hirae* isolates recovered from a One-Health surveillance study (Zaheer et al., 2020). The genomic relatedness of *E. hirae* was examined across various sampling matrices of the continuum and AMR determinants that contribute to antimicrobial resistance were identified. Furthermore, we examined the genomic traits of *E. hirae* that may facilitate their growth in the cattle gut as compared with other human-associated *Enterococcus* species.

As described previously, *E. hirae* is highly prevalent in cattle and thus can be readily isolated from bovine feces, bovine manure, and feedlot catch-basin water samples (Jackson et al., 2011; Zaheer et al., 2013, 2020; Beukers et al., 2015). The number of *E. hirae* isolates recovered from urban wastewater was low (3%) and most of the *Enterococcus* spp. from this source were identified as either *E. faecalis* or *E. faecium*. Similarly, only *E. faecalis* or *E. faecium* was identified among

human clinical isolates, confirming that *E. hirae* is generally not associated with human infections. However, *E. hirae* have occasionally been isolated from human cases of septicemia (Gilad et al., 1998), endocarditis (Poyart et al., 2002; Talarmin et al., 2011), urinary tract infections (Chan et al., 2012; Bourafa et al., 2015), spondylodiscitis (Canalejo et al., 2008), and acute pancreatitis (Dicpinigaitis et al., 2015). The rarity of this species among clinical enterococci isolates suggests that this species may not be as virulent as *E. faecalis* and *E. faecium*. This finding is also evident from our comparative genomic analysis where virulence genes were frequently identified in *E. faecalis* and *E. faecium,* but not in *E. hirae*.

The phenotypic resistance profiles of 1,319 *E. hirae* isolates showed that antimicrobial use and resistance phenotype were linked within a particular environment. For example, macrolides and tetracyclines are commonly used in beef cattle production systems for disease treatment and prevention including prophylaxis/metaphylaxis (Hurd and Malladi, 2008; Cameron and McAllister, 2016; Vikram et al., 2017). Isolates recovered from bovine feces, feedlot catch basin, stockpiled bovine manure, and natural surface water in the vicinity of the feedlots showed high occurrence of resistance to these antibiotics. Although only nine *E. hirae* isolates were recovered from urban wastewater, their resistance profiles indicated linkage with drugs commonly used to treat human infections including nitrofurantoin, tigecycline, and linezolid.

Genotypic resistance profiles of *E. hirae* corroborated to their phenotypic profiles, where tetracycline and macrolide resistance genes were predominantly present in isolates recovered from the beef cattle production system and natural water sources. This is consistent with previous studies where tetracycline and macrolide resistance genotypes were prevalent in beef production systems (Zaheer et al., 2013, 2019; Rovira et al., 2019). Tetracycline resistance was associated with the presence of *tetL, M,* and *O*. Two mosaic tetracycline genes *tetS/M* and *tetO/32/O* were also identified. *tetL* confers resistance *via* an efflux mechanism, while *tetM*, *tetS/M*, O, and *O/32/O* encode for ribosomal protection proteins (Safferling et al., 2003; Kazimierczak et al., 2008; Barile et al., 2012; Crespo et al., 2012; Dönhöfer et al., 2012). These genes are mostly found on transposable elements that are often linked with chloramphenicol and macrolide resistance determinants (Opal and Pop-Vicas, 2015). Macrolide resistance was associated with the presence of *erm(B),* which confers resistance against macrolide-lincosamide-streptogramin antibiotics (Okitsu et al., 2005). Others have also found *erm(B)* in *E. hirae* (Portillo et al., 2000; Chajęcka-Wierzchowska et al., 2016), as well as in *E. faecalis* and *E. faecium* isolated from chickens (Kim et al., 2019, 2021), turkies (Tremblay et al., 2011; Demirgül and Tuncer, 2017), pigs (Aarestrup, 2000), fermented food (Sanchez Valenzuela., 2013), and clinical settings (Schmitz et al., 2000; Chen et al., 2010; Wang et al., 2021). The macrolide resistance gene and tetracycline resistance genes in *E. hirae* appear to be identical to those in *E. faecalis* and *E. faecium* (Beukers et al., 2017; Zaheer et al., 2020). Considering that all of these species carry similar AMR determinants, the possibility of horizontal gene transfer across species seems probable (Palmer et al., 2010). Studies have identified the presence of pheromone responsive plasmids in *E. faecium* and *E. faecium* that either encode vancomycin resistance or facilitate the transfer of plasmids carrying vancomycin ARGs into recipient cells (Flannagan and Clewell, 2002; Johnson et al., 2021). These plasmids can also transfer between *Enterococcus* species, as the pMG1 plasmid has been shown to transfer between *E. faecium* and *E. faecalis*, and from *E. faecium* to *E. hirae* (Costa et al., 1993).

Aminoglycoside gene *acc(6′)-lid* is known to be intrinsic in *E. hirae* (Costa et al., 1993) and was detected in all but two genomes, likely as a result of gene coverage and assemblage issues. It is not surprising that vancomycin resistance genes were not identified in *E. hirae*, as this drug is not approved for veterinary use in North American cattle. Our result is consistent with a previous study where vancomycin resistance genes were not identified in *Enterococcus* spp. isolated from bovine feces (Beukers et al., 2017). The virulence genes identified in *E. hirae* were mostly associated with biofilm formation and polysaccharide biosynthesis, as described by others (Hashem et al., 2017). The 10 virulence genes that were identified in *E. hirae* were similar to those in *E. faecalis* and *E. faecium*, but many more (i.e., 49) virulence genes were found in *E. faecium* and *E. faecalis* (Zaheer et al., 2020).

Pan-genome analysis is an important comparative analysis tool that allows linkages between genetic changes and specific phenotypes as it describes core- and accessory genomes as well as species-specific genes (Vernikos, 2020). The core genome constituted only 64% of the total genome in *E. hirae*. Both horizontal and vertical transfer of genes, including those that confer antimicrobial resistance, play a significant role in shaping the pan-genome of a bacterial species (Ding et al., 2018). The pan-genome of *E. hirae* was considered "open" as there was no sign of saturation and it would be expected to expand with the addition of new genomes as illustrated by the pan-genome curve (**Figure 4B**). The high presence of cloud genes reflects the heterogeneity of the pan-genome of *E. hirae*. The existence of *E. hirae* in diverse environments may increase the chance of gene acquisition, in contrast to other *Enterococcus* species that may live in more specific environments that require less genomic variation for survival (Costa et al., 2020).

Gram-positive bacteria have sophisticated cell wall structures that ensure bacterial structural integrity and cellular viability and are also a major component of the host defense system (Koch, 2003; Silhavy et al., 2010). For this reason, numerous studies have been conducted to explore components of cell wall synthesis pathways as potential targets for drug therapy. Genes involved in cell wall synthesis were identified as discriminatory genomic signatures between *E. faecalis* and *E. hirae*. Identification of these different signatures [peptidoglycan synthesis genes (*Mur E, F,* and *Y*), penicillin-binding protein (*pbpE* and *pbpX*), teichoic acid synthesis genes (*tag H, G,* and *U*), and enterococcal polysaccharide antigen (*eps E, D, M,* and *N*)] may identify targets that offer more specific drug development against *E. faecalis* and *E. faecium* (Parisien et al., 2008).

Members of gut microflora compete with each other for nutrient availability. Therefore, the ability of one bacterial species to utilize multiple nutrients for energy generation provides an advantage

over other species. We found genes involved in the synthesis of cobalamin (vitamin B12), pyridoxine (vitamin B6), riboflavin (vitamin B2), biotin (vitamin B7), and folic acid exclusive to the *E. hirae* species compared with other enterococci analyzed in this study. With vitamins being undeniably important for both bacteria and the mammalian host, gut bacteria associated with their production directly contribute to the development and welfare of the host and thus may have a specific function within the microbiome of the digestive tract of cattle. In *E. hirae* genomes, multiple phosphotransferase systems (PTS) for fructose, galactitol, mannose, sorbose, glucose, N-acetyl glucosamine, and cellobiose were identified. The presence of these PTS promotes colonization of these bacterial populations (Jeckelmann and Erni, 2020). In addition, compared to *E. faecalis* and *E. faecium*, *E. hirae* harbored genes that were predicted to contribute to the synthesis of bacterial cellulose. These findings indicate that cellular metabolism genes identified in *E. hirae* may contribute to fitness within the cattle gut, accounting for its high prevalence in beef cattle.

Analysis of the annotated genomes indicated that *E. hirae* possessed genes coding for the production of antimicrobial agents like bacilysin, subtilosin, and narbonolide. Bacilysin is a dipeptide antimicrobial with antifungal and antibacterial activity (Özcengiz and Öğülür, 2015). Subtilosin belongs to the lantibiotics class of bacteriocins and has anti-biofilm activity (Babasaki et al., 1985; Algburi et al., 2017). These bacteria are also capable of producing gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter. GABA may increase feed intake in cattle and reduce anxiety and pain (Sarasa et al., 2020; Mamuad and Lee, 2021). These findings suggest that *E. hirae* may have probiotic properties that could benefit the gastrointestinal environment of cattle (Ben Braïek and Smaoui, 2019). Previously, *E. hirae* has been employed as a probiotic bacteria in freshwater fish (Adnan et al., 2017). Recent studies have also demonstrated that *E. hirae* may confer probiotic properties within the intestinal tract of cattle (Arokiyaraj et al., 2014; Daillère et al., 2016).

One of the goals of this study was to identify the niche-specific genes in *E. faecalis* and *E. faecium* that may contribute to virulence and infection. Several virulence factors that contribute to the pathogenesis of *E. faecalis* and *E. faecium* have been reported (Ali et al., 2017; Zhou et al., 2020). Several virulence genes were unique to *E. faecium* and/or *E. faecalis* and were not found in *E. hirae*. Members of the SlyA/ MarA family of proteins are associated with virulence gene regulation, promote biofilm formation, and act as cell adhesions. The presence of genomic signatures corresponding to these genes may in part account for the higher prevalence of *E. faecalis* and *E. faecium* infections than *E. hirae* infections in humans (Mäkinen et al., 1989; Michaux et al., 2011; Yang et al., 2015). Lipoproteins facilitate intake of nutrients and are often associated with ABC transporters that are linked to pathogenesis. This supports our findings as genes encoding lipoproteins mapped with ABC transport systems for manganese, arabinose, and methionine (Zhang et al., 1998; Hutchings et al., 2009). Furthermore, in *E. faecalis* and *E. faecium,* we also identified an arginine-ornithine antiporter which could contribute to cell fitness by facilitating arginine uptake. A study conducted to investigate the role of arginine-ornithine antiporter in *Streptococcus suis* reported that intercellular survival of this pathogen within epithelial cells was compromised in the absence of the antiporter (Fulde et al., 2014).

The potential of *E. hirae* as an opportunistic pathogen cannot be ignored, as it is occasionally recovered from both human and animal clinical samples (Nicklas et al., 2010; Dicpinigaitis et al., 2015; Ebeling and Romito, 2019; Pinkes et al., 2019; Bilek et al., 2020). Despite a higher prevalence of virulence genes in *E. faecium* and *E. faecalis,* some virulence genes were also identified in *E. hirae,* like genes associated with the ESX (or Type VII) secretion system, bicyclomycin resistance, capsule biogenesis, quorum sensing system, and an ABC transporter for iron import (Stauff et al., 2008; Rutherford and Bassler, 2012; Borst et al., 2015; Hatosy and Martiny, 2015; Poweleit et al., 2019). In addition, a lipoprotein gene associated with the iron transport system has been identified and is thought to play a role in *E. hirae* establishing opportunistic infections (Hutchings et al., 2009).

In conclusion, *E. hirae* has a tremendous ability for survival and adaptation. It has acquired resistance to the most common antimicrobials used in beef production systems. In addition, cellular metabolism genes involved in vitamin biosynthesis, multiple ABC and PTS transport systems, chitin degradation, and cellulose synthesis provide selective advantage and facilitate intestinal colonization of the cattle gut. As *E. hirae* appears to be uniquely adapted to cattle hosts, this likely limits the extent to which it transfers genes to bacteria that are important in human health. Regardless, the absence of resistance to critical antimicrobials in *E. hirae* gives credibility to limiting use of these drugs in feedlots and suggests that prudent management of antimicrobials in feedlot settings is an important practice.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in BioProject PRJNA604849 and in online repositories. The names of the repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Lethbridge Research Centre Animal Care and Use Committee and was conducted according to the Canadian Council of Animal Care Guidelines. Sampling procedures were conducted according to the protocol approved by the Animal Care Committee, University of Calgary (Protocol ID: AC14 -0029). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

RZ and TM designed the study. SH and CB arranged for collection of feedlot samples, metadata, and antimicrobial use

data. RB, SC, and RZ isolated and characterized enterococci. RB performed AST. S-e-ZZ, generated figures, analyzed overall data/results, and wrote first draft of the manuscript. GD provided and managed the bioinformatics cluster facility and bioinformatics tools. S-e-ZZ and RZ analyzed sequence data. TM and AZ provided funding and supervision. All authors participated in editing and reviewing the manuscript and approved the final manuscript.

FUNDING

Authors are grateful to the Major Innovation Fund of the Government of Alberta in conjunction with the University of Calgary AMR One Health Consortium, the Beef Cattle Research Council (BCRC) Project FOS 10.13, and Genomics Research and Development Initiative of the Government of Canada for financial support.

ACKNOWLEDGMENTS

The authors acknowledge Noriko Goji for sequencing, and Wendi Smart, Krysty Munns, Reuben Ha, and Taylor Davedow for technical assistance. Cooperation of the feedlot and wastewater treatment plant operators is also gratefully appreciated.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.859990/full#supplementary-material

REFERENCES

Aarestrup, F. M. (2000). Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. *J. Clin. Microbiol.* 38, 2774–2777. doi: 10.1128/JCM.38.7.2774-2777.2000

Adnan, M., Patel, M., and Hadi, S. (2017). Functional and health promoting inherent attributes of *Enterococcus hirae* F2 as a novel probiotic isolated from the digestive tract of the freshwater fish Catla catla. *PeerJ* 5:e3085. doi: 10.7717/peerj.3085

Algburi, A., Zehm, S., Netrebov, V., Bren, A. B., Chistyakov, V., and Chikindas, M. L. (2017). Subtilosin prevents biofilm formation by inhibiting bacterial quorum sensing. *Probiotics Antimicrob. Proteins* 9, 81–90. doi: 10.1007/s12602-016-9242-x

Ali, L., Goraya, M. U., Arafat, Y., Ajmal, M., Chen, J. L., and Yu, D. (2017). Molecular mechanism of quorum-sensing in *Enterococcus faecalis*: its role in virulence and therapeutic approaches. *Int. J. Mol. Sci.* 18:960. doi: 10.3390/ijms18050960

Anderson, K. L., Whitlock, J. E., and Harwood, V. J. (2005). Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Appl. Environ. Microbiol.* 71, 3041–3048. doi: 10.1128/aem.71.6.3041-3048.2005

Andrew, P. W., and Mitchell, T. J. (1997). *The biology of Streptococci and Enterococci. In: Symposium Series-Society for Applied Bacteriology (United Kingdom)*. Journals Customer Services Blackwell Scientific Publications Ltd, Hoboken.

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387

Arokiyaraj, S., Islam, V. I. H., Bharanidharan, R., Raveendar, S., Lee, J., Kim, D. H., et al. (2014). Antibacterial, anti-inflammatory and probiotic potential of *Enterococcus hirae* isolated from the rumen of Bos primigenius. *World J. Microbiol. Biotechnol.* 30, 2111–2118. doi: 10.1007/s11274-014-1625-0

Babasaki, K., Takao, T., Shimonishi, Y., and Kurahashi, K. (1985). Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. *J. Biochem.* 98, 585–603. doi: 10.1093/oxfordjournals.jbchem.a135315

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Barile, S., Devirgiliis, C., and Perozzi, G. (2012). Molecular characterization of a novel mosaic tet(S/M) gene encoding tetracycline resistance in foodborne strains of Streptococcus bovis. *Microbiology (Reading)* 158, 2353–2362. doi: 10.1099/mic.0.058206-0

Barnes, A. M. T., Frank, K. L., and Dunny, G. M. (2021). Enterococcal endocarditis: hiding in plain sight. *Front. Cell. Infect. Microbiol.* 11:722482. doi: 10.3389/fcimb.2021.722482

Ben Braïek, O., and Smaoui, S. (2019). Enterococci: between emerging pathogens and potential probiotics. *Biomed. Res. Int.* 5938210–5938213. doi: 10.1155/2019/5938210

Beukers, A. G., Zaheer, R., Cook, S. R., Stanford, K., Chaves, A. V., Ward, M. P., et al. (2015). Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers. *Front. Microbiol.* 6:483. doi: 10.3389/fmicb.2015.00483

Beukers, A. G., Zaheer, R., Goji, N., Amoako, K. K., Chaves, A. V., Ward, M. P., et al. (2017). Comparative genomics of *Enterococcus* spp. isolated from bovine feces. *BMC Microbiol.* 17:52. doi: 10.1186/s12866-017-0962-1

Bilek, H. C., Deveci, A., Ünal, S., Tanrıverdi Çaycı, Y., and Tanyel, E. (2020). *Enterococcus hirae* as a cause of bacteremic urinary tract infection: first case report from Turkey. *J. Infect. Dev. Ctries.* 14, 1780–1482. doi: 10.3855/jidc.12522

Borst, L. B., Suyemoto, M. M., Scholl, E. H., Fuller, F. J., and Barnes, H. J. (2015). Comparative genomic analysis identifies divergent genomic features of pathogenic *Enterococcus cecorum* including a type IC CRISPR-Cas system, a capsule locus, an epa-like locus, and putative host tissue binding proteins. *PLoS One* 10:e0121294. doi: 10.1371/journal.pone.0121294

Bourafa, N., Loucif, L., Boutefnouchet, N., and Rolain, J. M. (2015). *Enterococcus hirae*, an unusual pathogen in humans causing urinary tract infection in a patient with benign prostatic hyperplasia: first case report in Algeria. *New Microbes New Infect.* 8, 7–9. doi: 10.1016/j.nmni.2015.08.003

Brayer, S., Linn, A., Holt, S., Ellery, K., Mitchell, S., and Williams, J. (2019). *Enterococcus hirae* bacteremia in an infant: case report and review of the literature. *J. Pediatric. Infect. Dis. Soc.* 8, 571–573. doi: 10.1093/jpids/piz028

Byappanahalli, M., and Fujioka, R. (2004). Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils. *Water Sci. Technol.* 50, 27–32. doi: 10.2166/wst.2004.0009

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., and Harwood, V. J. (2012). Enterococci in the environment. *Microbiol. Mol. Biol. Rev.* 76, 685–706. doi: 10.1128/mmbr.00023-12

Cameron, A., and McAllister, T. A. (2016). Antimicrobial usage and resistance in beef production. *J. Animal Sci. Biotechnol.* 7, 1–22. doi: 10.1186/s40104-016-0127-3

Canalejo, E., Ballesteros, R., Cabezudo, J., García-Arata, M., and Moreno, J. (2008). Bacteraemic spondylodiscitis caused by *Enterococcus hirae*. *Eur. J. Clin. Microbiol. Infect. Dis.* 27, 613–615. doi: 10.1007/s10096-008-0476-8

Caretti, C., and Lubello, C. (2003). Wastewater disinfection with PAA and UV combined treatment: a pilot plant study. *Water Res.* 37, 2365–2371. doi: 10.1016/S0043-1354(03)00025-3

Chajęcka-Wierzchowska, W., Zadernowska, A., and Łaniewska-Trokenheim, Ł. (2016). Diversity of antibiotic resistance genes in *Enterococcus* strains isolated

from ready-to-eat meat products. *J. Food Sci.* 81, M2799–M2807. doi: 10.1111/1750-3841.13523

Chan, T.-S., Wu, M.-S., Suk, F.-M., Chen, C.-N., Chen, Y.-F., Hou, Y.-H., et al. (2012). *Enterococcus hirae*-related acute pyelonephritis and cholangitis with bacteremia: an unusual infection in humans. *Kaohsiung J. Med. Sci.* 28, 111–114. doi: 10.1016/j.kjms.2011.06.027

Chen, L., Ruan, Z., Wu, Q., Zhou, T., and Li, C. (2010). Relationship between ermB gene and transposons Tn1545 and Tn917 in *Enterococcus faecalis*. *Chin. J. Nosocomiol.* 20, 763–766.

Costa, Y., Galimand, M., Leclercq, R., Duval, J., and Courvalin, P. (1993). Characterization of the chromosomal aac(6′)-ii gene specific for *Enterococcus faecium*. *Antimicrob. Agents Chemother.* 37, 1896–1903. doi: 10.1128/aac.37.9.1896

Costa, S. S., Guimarães, L. C., Silva, A., Soares, S. C., and Baraúna, R. A. (2020). First steps in the analysis of prokaryotic pan-genomes. *Bioinf. Biol. Insights* 14:1177932220938064. doi: 10.1177/1177932220938064

Crespo, M. D., Olson, J. W., Altermann, E., Siletzky, R. M., and Kathariou, S. (2012). Chromosomal tet(O)-harboring regions in *Campylobacter coli* isolates from turkeys and swine. *Appl. Environ. Microbiol.* 78, 8488–8491. doi: 10.1128/aem.02258-12

Daillère, R., Vétizou, M., Waldschmitt, N., Yamazaki, T., Isnard, C., Poirier-Colame, V., et al. (2016). *Enterococcus hirae* and *Barnesiella intestinihominis* facilitate cyclophosphamide-induced therapeutic immunomodulatory effects. *Immunity* 45, 931–943. doi: 10.1016/j.immuni.2016.09.009

De Graef, E. M., Devriese, L. A., Vancanneyt, M., Baele, M., Collins, M. D., Lefebvre, K., et al. (2003). Description of *Enterococcus canis* sp. nov. from dogs and reclassification of *Enterococcus porcinus* Teixeira et al. 2001 as a junior synonym of *Enterococcus villorum* Vancanneyt et al 2001. *Int. J. Syst. Evol. Microbiol.* 53, 1069–1074. doi: 10.1099/ijs.0.02549-0

de Vaux, A., Laguerre, G., Diviès, C., and Prévost, H. (1998). *Enterococcus asini* sp. nov. isolated from the caecum of donkeys (Equus asinus). *Int. J. Syst. Bacteriol.* 48, 383–387. doi: 10.1099/00207713-48-2-383

Demirgül, F., and Tuncer, Y. (2017). Detection of antibiotic resistance and resistance genes in enterococci isolated from Sucuk, a traditional Turkish dry-fermented sausage. *Korean J. Food Sci. Anim. Resour.* 37, 670–681. doi: 10.5851/kosfa.2017.37.5.670

Devriese, L. A., Ceyssens, K., Rodrigues, U. M., and Collins, M. D. (1990). *Enterococcus columbae*, a species from pigeon intestines. *FEMS Microbiol. Lett.* 71, 247–251. doi: 10.1016/0378-1097(90)90228-i

Dicpinigaitis, P. V., De Aguirre, M., and Divito, J. (2015). *Enterococcus hirae* bacteremia associated with acute pancreatitis and septic shock. *Case Rep. Infect. Dis.* 2015, 1–3. doi: 10.1155/2015/123852

Ding, W., Baumdicker, F., and Neher, R. A. (2018). panX: pan-genome analysis and exploration. *Nucleic Acids Res.* 46:e5. doi: 10.1093/nar/gkx977

Dönhöfer, A., Franckenberg, S., Wickles, S., Berninghausen, O., Beckmann, R., and Wilson, D. N. (2012). Structural basis for TetM-mediated tetracycline resistance. *Proc. Natl. Acad. Sci. U. S. A.* 109, 16900–16905. doi: 10.1073/pnas.1208037109

Ebeling, C. G., and Romito, B. T. (2019). Aortic valve endocarditis from *Enterococcus hirae* infection. *Proc. (Bayl. Univ. Med. Cent.)* 32, 249–250. doi: 10.1080/08998280.2018.1551698

Farrow, J. A., and Collins, M. D. (1985). *Enterococcus hirae*, a new species that includes amino acid assay strain NCDO 1258 and strains causing growth depression in young chickens. *Int. J. Syst. Evol. Microbiol.* 35, 73–75.

Flannagan, S. E., and Clewell, D. B. (2002). Identification and characterization of genes encoding sex pheromone cAM373 activity in *Enterococcus faecalis* and *Staphylococcus aureus*. *Mol. Microbiol.* 44, 803–817. doi: 10.1046/j.1365-2958.2002.02922.x

Fogarty, L. R., Haack, S. K., Wolcott, M. J., and Whitman, R. L. (2003). Abundance and characteristics of the recreational water quality indicator bacteria *Escherichia coli* and enterococci in gull faeces. *J. Appl. Microbiol.* 94, 865–878. doi: 10.1046/j.1365-2672.2003.01910.x

Francino, M. P. (2016). Antibiotics and the human gut microbiome: dysbioses and accumulation of resistances. *Front. Microbiol.* 6:1543. doi: 10.3389/fmicb.2015.01543

Fulde, M., Willenborg, J., Huber, C., Hitzmann, A., Willms, D., Seitz, M., et al. (2014). The arginine-ornithine antiporter ArcD contributes to biological fitness of *Streptococcus suis*. *Front. Cell. Infect. Microbiol.* 4:107. doi: 10.3389/fcimb.2014.00107

Gilad, J., Borer, A., Riesenberg, K., Peled, N., Shnaider, A., and Schlaeffer, F. (1998). *Enterococcus hirae* septicemia in a patient with end-stage renal disease undergoing hemodialysis. *Eur. J. Clin. Microbiol. Infect. Dis.* 17, 576–577. doi: 10.1007/BF01708623

Gilmore, M. S., Clewell, D. B., Ike, Y., and Shankar, N. (Ed.) (2014): *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary, Boston.

Giraffa, G. (2014). "*Enterococcus*," in *Encyclopedia of Food Microbiology (2nd Edn.)*. eds. C. A. Batt and M. L. Tortorello (Oxford: Academic Press).

Hadfield, J., Croucher, N. J., Goater, R. J., Abudahab, K., Aanensen, D. M., and Harris, S. R. (2017). Phandango: an interactive viewer for bacterial population genomics. *Bioinformatics* 34, 292–293. doi: 10.1093/bioinformatics/btx610

Harwood, V. J., Whitlock, J., and Withington, V. (2000). Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. *Appl. Environ. Microbiol.* 66, 3698–3704. doi: 10.1128/AEM.66.9.3698-3704.2000

Hashem, Y. A., Amin, H. M., Essam, T. M., Yassin, A. S., and Aziz, R. K. (2017). Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. *Sci. Rep.* 7:5733. doi: 10.1038/s41598-017-05901-0

Hatosy, S. M., and Martiny, A. C. (2015). The ocean as a global reservoir of antibiotic resistance genes. *Appl. Environ. Microbiol.* 81, 7593–7599. doi: 10.1128/AEM.00736-15

Heim, S., Lleo, M., Bonato, B., Guzman, C. A., and Canepari, P. (2002). The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. *J. Bacteriol.* 184, 6739–6745. doi: 10.1128/jb.184.23.6739-6745.2002

Hernando-Amado, S., Coque, T. M., Baquero, F., and Martínez, J. L. (2019). Defining and combating antibiotic resistance from one health and Global Health perspectives. *Nat. Microbiol.* 4, 1432–1442. doi: 10.1038/s41564-019-0503-9

Heval Can, B., Aydın, D., Sema, Ü., Yeliz Tanrıverdi, Ç., and Esra, T. (2020). *Enterococcus hirae* as a cause of bacteremic urinary tract infection: first case report from Turkey. *J. Infect. Dev. Countries* 14, 1780–1482. doi: 10.3855/jidc.12522

Hiltunen, T., Virta, M., and Laine, A.-L. (2017). Antibiotic resistance in the wild: an eco-evolutionary perspective. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 372:20160039. doi: 10.1098/rstb.2016.0039

Hurd, H. S., and Malladi, S. (2008). A stochastic assessment of the public health risks of the use of macrolide antibiotics in food animals. *Risk Anal.: Int. J.* 28, 695–710. doi: 10.1111/j.1539-6924.2008.01054.x

Hutchings, M. I., Palmer, T., Harrington, D. J., and Sutcliffe, I. C. (2009). Lipoprotein biogenesis in gram-positive bacteria: knowing when to hold 'em, knowing when to fold 'em. *Trends Microbiol.* 17, 13–21. doi: 10.1016/j.tim.2008.10.001

Jackson, C. R., Lombard, J. E., Dargatz, D. A., and Fedorka-Cray, P. J. (2011). Prevalence, species distribution and antimicrobial resistance of enterococci isolated from US dairy cattle. *Lett. Appl. Microbiol.* 52, 41–48. doi: 10.1111/j.1472-765x.2010.02964.x

Jeckelmann, J.-M., and Erni, B. (2020). Transporters of glucose and other carbohydrates in bacteria. *Pflügers Archiv-Eur. J. Physiol.* 472, 1129–1153. doi: 10.1007/s00424-020-02379-0

Johnson, C. N., Sheriff, E. K., Duerkop, B. A., Chatterjee, A., and Margolin, W. (2021). Let me upgrade you: impact of mobile genetic elements on Enterococcal adaptation and evolution. *J. Bacteriol.* 203:e0017721. doi: 10.1128/JB.00177-21

Kazimierczak, K. A., Rincon, M. T., Patterson, A. J., Martin, J. C., Young, P., Flint, H. J., et al. (2008). A new tetracycline efflux gene, tet(40), is located in tandem with tet(O/32/O) in a human gut firmicute bacterium and in metagenomic library clones. *Antimicrob. Agents Chemother.* 52, 4001–4009. doi: 10.1128/aac.00308-08

Kim, Y. B., Seo, K. W., Jeon, H. Y., Lim, S.-K., Sung, H. W., and Lee, Y. J. (2019). Molecular characterization of erythromycin and tetracycline-resistant *Enterococcus faecalis* isolated from retail chicken meats. *Poult. Sci.* 98, 977–983. doi: 10.3382/ps/pey477

Kim, Y. B., Yoon, S., Seo, K. W., Shim, J. B., Noh, E. B., and Lee, Y. J. (2021). Detection of linezolid-resistant *Enterococcus faecalis* and *Enterococcus faecium*

isolates from the layer operation system in Korea. *Microb. Drug Resist.* 27, 1443–1449. doi: 10.1089/mdr.2020.0028

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int. J. Food Microbiol.* 88, 123–131. doi: 10.1016/s0168-1605(03)00175-2

Koch, A. L. (2003). Bacterial wall as target for attack: past, present, and future research. *Clin. Microbiol. Rev.* 16, 673–687. doi: 10.1128/CMR.16.4.673-687.2003

Law-Brown, J., and Meyers, P. R. (2003). *Enterococcus phoeniculicola* sp. nov., a novel member of the enterococci isolated from the uropygial gland of the red-billed Woodhoopoe, Phoeniculus purpureus. *Int. J. Syst. Evol. Microbiol.* 53, 683–685. doi: 10.1099/ijs.0.02334-0

Layton, B., Walters, S., Lam, L., and Boehm, A. (2010). *Enterococcus* species distribution among human and animal hosts using multiplex PCR. *J. Appl. Microbiol.* 109, 539–547. doi: 10.1111/j.1365-2672.2010.04675.x

Letunic, I., and Bork, P. (2021). Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. doi: 10.1093/nar/gkab301

Mäkinen, P. L., Clewell, D. B., An, F., and Mäkinen, K. K. (1989). Purification and substrate specificity of a strongly hydrophobic extracellular Metalloendopeptidase ("Gelatinase") from *Streptococcus faecalis* (strain 0G1-10). *J. Biol. Chem.* 264, 3325–3334. doi: 10.1016/S0021-9258(18)94069-X

Mamuad, L. L., and Lee, S.-S. (2021). The role of glutamic acid-producing microorganisms in rumen microbial ecosystems. *J. Life Sci.* 31, 520–526.

Maria da Gloria, S. C., Shewmaker, P. L., Steigerwalt, A. G., Morey, R. E., Sampson, A., Joyce, K., et al. (2006). *Enterococcus caccae* sp. nov., isolated from human stools. *Int. J. Syst. Evol. Microbiol.* 56, 1505–1508.

Marinier, E., Zaheer, R., Berry, C., Weedmark, K. A., Domaratzki, M., Mabon, P., et al. (2017). Neptune: a bioinformatics tool for rapid discovery of genomic variation in bacterial populations. *Nucleic Acids Res.* 45:e159. doi: 10.1093/nar/gkx702

Michaux, C., Sanguinetti, M., Reffuveille, F., Auffray, Y., Posteraro, B., Gilmore, M. S., et al. (2011). SlyA is a transcriptional regulator involved in the virulence of *Enterococcus faecalis*. *Infect. Immun.* 79, 2638–2645. doi: 10.1128/IAI.01132-10

Miller, W. R., Munita, J. M., and Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. *Expert Rev. Anti-Infect. Ther.* 12, 1221–1236. doi: 10.1586/14787210.2014.956092

Moreno, M. F., Sarantinopoulos, P., Tsakalidou, E., and De Vuyst, L. (2006). The role and application of enterococci in food and health. *Int. J. Food Microbiol.* 106, 1–24. doi: 10.1016/j.ijfoodmicro.2005.06.026

Müller, T., Ulrich, A., Ott, E. M., and Müller, M. (2001). Identification of plant-associated enterococci. *J. Appl. Microbiol.* 91, 268–278. doi: 10.1046/j.1365-2672.2001.01373.x

Muniesa, M., Jofre, J., and Lucena, F. (1999). Occurrence and numbers of bacteriophages and bacterial indicators in faeces of yellow-legged seagull (Larus cachinnans). *Lett. Appl. Microbiol.* 29, 421–423. doi: 10.1046/j.1365-2672.1999.00666.x

Murray, B. E. (1990). The life and times of the *Enterococcus*. *Clin. Microbiol. Rev.* 3, 46–65. doi: 10.1128/cmr.3.1.46

Nakamura, T., Ishikawa, K., Matsuo, T., Kawai, F., Uehara, Y., and Mori, N. (2021). *Enterococcus hirae* bacteremia associated with acute pyelonephritis in a patient with alcoholic cirrhosis: a case report and literature review. *BMC Infect. Dis.* 21:999. doi: 10.1186/s12879-021-06707-2

Nicklas, J. L., Moisan, P., Stone, M. R., and Gookin, J. L. (2010). In situ molecular diagnosis and histopathological characterization of enteroadherent *Enterococcus hirae* infection in pre-weaning-age kittens. *J. Clin. Microbiol.* 48, 2814–2820. doi: 10.1128/jcm.00916-09

Okitsu, N., Kaieda, S., Yano, H., Nakano, R., Hosaka, Y., Okamoto, R., et al. (2005). Characterization of ermB gene transposition by Tn1545 and Tn917 in macrolide-resistant *Streptococcus pneumoniae* isolates. *J. Clin. Microbiol.* 43, 168–173. doi: 10.1128/JCM.43.1.168-173.2005

Opal, S. M., and Pop-Vicas, A. (2015). "18 - molecular mechanisms of antibiotic resistance in bacteria". in *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, eds. J. E. Bennett, R. Dolin, M. J. Blaser, and W. B. Saunders. *(8th Edn.)*, Elsevier Saunders Philadelphia 235–251.

Özcengiz, G., and Öğülür, i. (2015). Biochemistry, genetics and regulation of bacilysin biosynthesis and its significance more than an antibiotic. *New Biotechnol.* 32, 612–619. doi: 10.1016/j.nbt.2015.01.006

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics (Oxford, England)* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Palmer, K. L., Kos, V. N., and Gilmore, M. S. (2010). Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr. Opin. Microbiol.* 13, 632–639. doi: 10.1016/j.mib.2010.08.004

Pãosinho, A., Azevedo, T., Alves, J. V., Costa, I. A., Carvalho, G., Peres, S. R., et al. (2016). Acute pyelonephritis with bacteremia caused by *Enterococcus hirae*: a rare infection in humans. *Case reports in infectious diseases* 2016:4698462. doi: 10.1155/2016/4698462

Parisien, A., Allain, B., Zhang, J., Mandeville, R., and Lan, C. Q. (2008). Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *J. Appl. Microbiol.* 104, 1–13. doi: 10.1111/j.1365-2672.2007.03498.x

Petkau, A., Mabon, P., Sieffert, C., Knox, N. C., Cabral, J., Iskander, M., et al. (2017). SNVPhyl: a single nucleotide variant phylogenomics pipeline for microbial genomic epidemiology. *Microb. Genom.* 3:e000116. doi: 10.1099/mgen.0.000116

Pinkes, M. E., White, C., and Wong, C. S. (2019). Native-valve *Enterococcus hirae* endocarditis: a case report and review of the literature. *BMC Infect. Dis.* 19:891. doi: 10.1186/s12879-019-4532-z

Portillo, A., Ruiz-Larrea, F., Zarazaga, M., Alonso, A., Martinez, J. L., and Torres, C. (2000). Macrolide Resistance Genes in *Enterococcus* spp. *Antimicrob. Agents Chemother.* 44, 967–971. doi: 10.1128/AAC.44.4.967-971.2000

Poweleit, N., Czudnochowski, N., Nakagawa, R., Trinidad, D. D., Murphy, K. C., Sassetti, C. M., et al. (2019). The structure of the endogenous ESX-3 secretion system. *eLife* 8:e52983. doi: 10.7554/eLife.52983

Poyart, C., Lambert, T., Morand, P., Abassade, P., Quesne, G., Baudouy, Y., et al. (2002). Native valve endocarditis due to *Enterococcus hirae*. *J. Clin. Microbiol.* 40, 2689–2690. doi: 10.1128/JCM.40.7.2689-2690.2002

Rovira, P., McAllister, T., Lakin, S. M., Cook, S. R., Doster, E., Noyes, N. R., et al. (2019). Characterization of the microbial resistome in conventional and "raised without antibiotics" beef and dairy production systems. *Front. Microbiol.* 10:1980. doi: 10.3389/fmicb.2019.01980

Rutherford, S. T., and Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* 2:a012427. doi: 10.1101/cshperspect.a012427

Safferling, M., Griffith, H., Jin, J., Sharp, J., De Jesus, M., Ng, C., et al. (2003). TetL tetracycline efflux protein from *Bacillus subtilis* is a dimer in the membrane and in detergent solution. *Biochemistry* 42, 13969–13976. doi: 10.1021/bi035173q

Sanchez Valenzuela, A., Lavilla Lerma, L., Benomar, N., Gálvez, A., Perez Pulido, R., and Abriouel, H. (2013). Phenotypic and molecular antibiotic resistance profile of *Enterococcus faecalis* and *Enterococcus faecium* isolated from different traditional fermented foods. *Foodborne Pathog. Dis.* 10, 143–149. doi: 10.1089/fpd.2012.1279

Sarasa, S. B., Mahendran, R., Muthusamy, G., Thankappan, B., Selta, D. R. F., and Angayarkanni, J. (2020). A brief review on the non-protein amino acid, gamma-amino butyric acid (GABA): its production and role in microbes. *Curr. Microbiol.* 77, 534–544. doi: 10.1007/s00284-019-01839-w

Schmitz, F.-J., Sadurski, R., Kray, A., Boos, M., Geisel, R., Köhrer, K., et al. (2000). Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. *J. Antimicrob. Chemother.* 45, 891–894. doi: 10.1093/jac/45.6.891

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Silhavy, T. J., Kahne, D., and Walker, S. (2010). The bacterial cell envelope. *Cold Spring Harb. Perspect. Biol.* 2:a000414. doi: 10.1101/cshperspect.a000414

Stauff, D. L., Bagaley, D., Torres, V. J., Joyce, R., Anderson, K. L., Kuechenmeister, L., et al. (2008). *Staphylococcus aureus* HrtA is an ATPase required for protection against heme toxicity and prevention of a transcriptional heme stress response. *J. Bacteriol.* 190, 3588–3596. doi: 10.1128/jb.01921-07

Svec, P., Devriese, L. A., Sedlácek, I., Baele, M., Vancanneyt, M., Haesebrouck, F., et al. (2001). *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis*

sp. nov., isolated from water. *Int. J. Syst. Evol. Microbiol.* 51, 1567–1574. doi: 10.1099/00207713-51-4-1567

Švec, P., Vancanneyt, M., Sedláček, I., Naser, S. M., Snauwaert, C., Lefebvre, K., et al. (2006). *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. *Int. J. Syst. Evol. Microbiol.* 56, 577–581. doi: 10.1099/ijs.0.63937-0

Talarmin, J.-P., Pineau, S., Guillouzouic, A., Boutoille, D., Giraudeau, C., Reynaud, A., et al. (2011). Relapse of *Enterococcus hirae* prosthetic valve endocarditis. *J. Clin. Microbiol.* 49, 1182–1184. doi: 10.1128/JCM.02049-10

Tan, C.-K., Lai, C.-C., Wang, J.-Y., Lin, S.-H., Liao, C.-H., Huang, Y.-T., et al. (2010). Bacteremia caused by non-faecalis and non-faecium *Enterococcus* species at a medical center in Taiwan, 2000 to 2008. *J. Infect.* 61, 34–43. doi: 10.1016/j.jinf.2010.04.007

Tremblay, C.-L., Letellier, A., Quessy, S., Boulianne, M., Daignault, D., and Archambault, M. (2011). Multiple-antibiotic resistance of *Enterococcus faecalis* and *Enterococcus faecium* from Cecal contents in broiler chicken and Turkey flocks slaughtered in Canada and plasmid Colocalization of tetO and ermB genes. *J. Food Prot.* 74, 1639–1648. doi: 10.4315/0362-028x.Jfp-10-451

Vernikos, G. S. (2020) "A review of Pangenome tools and recent studies," in *The Pangenome: Diversity, Dynamics and Evolution of Genomes*. eds. H. Tettelin and D. Medini (Springer, Berlin).

Vikram, A., Rovira, P., Agga, G. E., Arthur, T. M., Bosilevac, J. M., Wheeler, T. L., et al. (2017). Impact of "raised without antibiotics" beef cattle production practices on occurrences of antimicrobial resistance. *Appl. Environ. Microbiol.* 83, e01682–e01617. doi: 10.1128/AEM.01682-17

Wang, Y., Xiong, Y., Wang, Z., Zheng, J., Xu, G., Deng, Q., et al. (2021). Comparison of solithromycin with erythromycin in *Enterococcus faecalis* and *Enterococcus faecium* from China: antibacterial activity, clonality, resistance mechanism, and inhibition of biofilm formation. *J. Antibiot.* 74, 143–151. doi: 10.1038/s41429-020-00374-2

Wingett, S. W., and Andrews, S. (2018). FastQ screen: a tool for multi-genome mapping and quality control. *F1000Research* 7:7. doi: 10.12688/f1000research.15931.2

Winther, M., Dalager-Pedersen, M., Tarpgaard, I. H., and Nielsen, H. L. (2020). First Danish case of infective endocarditis caused by *Enterococcus hirae*. *BMJ Case Rep.* 13:e237950. doi: 10.1136/bcr-2020-237950

Yan, T., Goto, D. K., and Feng, F.: *Concentration Dynamics of Fecal Indicators in Hawaiian Coastal and Inland Sand, Soil, and Water during Rainfall Events*. Water Environment Research Foundation Alexandria, VA, (2011).

Yang, J., Tan, G., Zhang, T., White, R. H., Lu, J., and Ding, H. (2015). Deletion of the proposed iron chaperones IscA/SufA results in accumulation of a red intermediate cysteine Desulfurase IscS in *Escherichia coli*. *J. Biol. Chem.* 290, 14226–14234. doi: 10.1074/jbc.M115.654269

Zaheer, R., Cook, S. R., Barbieri, R., Goji, N., Cameron, A., Petkau, A., et al. (2020). Surveillance of *Enterococcus* spp. reveals distinct species and antimicrobial resistance diversity across a one-health continuum. *Sci. Rep.* 10:3937. doi: 10.1038/s41598-020-61002-5

Zaheer, R., Cook, S., Klima, C., Stanford, K., Alexander, T., Topp, E., et al. (2013). Effect of subtherapeutic vs. therapeutic administration of macrolides on antimicrobial resistance in *Mannheimia haemolytica* and enterococci isolated from beef cattle. *Front. Microbiol.* 4:133. doi: 10.3389/fmicb.2013.00133

Zaheer, R., Lakin, S. M., Polo, R. O., Cook, S. R., Larney, F. J., Morley, P. S., et al. (2019). Comparative diversity of microbiomes and Resistomes in beef feedlots, downstream environments and urban sewage influent. *BMC Microbiol.* 19, 1–17. doi: 10.1186/s12866-019-1548-x

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, H., Niesel, D. W., Peterson, J. W., and Klimpel, G. R. (1998). Lipoprotein release by bacteria: potential factor in bacterial pathogenesis. *Infect. Immun.* 66, 5196–5201. doi: 10.1128/iai.66.11.5196-5201.1998

Zhou, X., Willems, R. J. L., Friedrich, A. W., Rossen, J. W. A., and Bathoorn, E. (2020). *Enterococcus faecium*: from microbiological insights to practical recommendations for infection control and diagnostics. *Antimicrob. Resist. Infect. Control* 9:130. doi: 10.1186/s13756-020-00770-1

Conflict of Interest: CB is part owner and managing partner of Feedlot Health Management Services.

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 Sherry J. Hannon, Calvin W. Booker, Deirdre Church, Gary Van Domselaar, Athanasios Zovoilis and Her Majesty the Queen in Right of Canada, as represented by the Minister of Agriculture and Agri-Food Canada for the contribution of Tim A. McAllister, Sani-e-Zehra Zaidi , Rahat Zaheer, Ruth Barbieri and Shaun R. Cook. 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 Microbiology

ORIGINAL RESEARCH
published: 07 July 2022
doi: 10.3389/fmicb.2022.897905

Resistome Analysis of Global Livestock and Soil Microbiomes

*Katie Lawther[1], Fernanda Godoy Santos[1], Linda Boniface Oyama[1], Francesco Rubino[1], Steven Morrison[2], Chris J. Creevey[1], John W. McGrath[1] and Sharon Ann Huws[1]**

[1]School of Biological Sciences, Institute for Global Food Security, Queen's University Belfast, Belfast, United Kingdom, [2]Agri-Food and Biosciences Institute, Belfast, United Kingdom

Antimicrobial resistance (AMR) is a serious threat to public health globally; it is estimated that AMR bacteria caused 1.27 million deaths in 2019, and this is set to rise to 10 million deaths annually. Agricultural and soil environments act as antimicrobial resistance gene (ARG) reservoirs, operating as a link between different ecosystems and enabling the mixing and dissemination of resistance genes. Due to the close interactions between humans and agricultural environments, these AMR gene reservoirs are a major risk to both human and animal health. In this study, we aimed to identify the resistance gene reservoirs present in four microbiomes: poultry, ruminant, swine gastrointestinal (GI) tracts coupled with those from soil. This large study brings together every poultry, swine, ruminant, and soil shotgun metagenomic sequence available on the NCBI sequence read archive for the first time. We use the ResFinder database to identify acquired antimicrobial resistance genes in over 5,800 metagenomes. ARGs were diverse and widespread within the metagenomes, with 235, 101, 167, and 182 different resistance genes identified in the poultry, ruminant, swine, and soil microbiomes, respectively. The tetracycline resistance genes were the most widespread in the livestock GI microbiomes, including *tet*(W)_1, *tet*(Q)_1, *tet*(O)_1, and *tet*(44)_1. The *tet*(W)_1 resistance gene was found in 99% of livestock GI tract microbiomes, while *tet*(Q)_1 was identified in 93%, *tet*(O)_1 in 82%, and finally *tet*(44)_1 in 69%. Metatranscriptomic analysis confirmed these genes were "real" and expressed in one or more of the livestock GI tract microbiomes, with *tet*(40)_1 and *tet*(O)_1 expressed in all three livestock microbiomes. In soil, the most abundant ARG was the oleandomycin resistance gene, *ole*(B)_1. A total of 55 resistance genes were shared by the four microbiomes, with 11 ARGs actively expressed in two or more microbiomes. By using all available metagenomes we were able to mine a large number of samples and describe resistomes in 37 countries. This study provides a global insight into the diverse and abundant antimicrobial resistance gene reservoirs present in both livestock and soil microbiomes.

Keywords: ruminant, poultry, swine, soil, metagenome, resistome, antimicrobial resistance gene, antimicrobial resistance

OPEN ACCESS

Edited by:
Ziad Daoud, Central Michigan University, United States

Reviewed by:
Jinxin Liu, Nanjing Agricultural University, China
Pimlapas Leekitcharoenphon, Technical University of Denmark, Denmark

***Correspondence:**
Sharon Ann Huws
s.huws@qub.ac.uk

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *16 March 2022*
Accepted: *24 May 2022*
Published: *07 July 2022*

Citation:
Lawther K, Santos FG, Oyama LB, Rubino F, Morrison S, Creevey CJ, McGrath JW and Huws SA (2022) Resistome Analysis of Global Livestock and Soil Microbiomes. Front. Microbiol. 13:897905. doi: 10.3389/fmicb.2022.897905

INTRODUCTION

It has been 20 years since, the WHO published their first "Global Strategy for Containment of Antimicrobial Resistance (AMR)," AMR remains a serious threat to both animal and human health as well as the world's economy (WHO, 2001, 2021). The Centre for Disease Control and Prevention (CDC) estimates an annual cost of $55 billion dollars associated with AMR within the United States, including additional healthcare costs of $20 billion (CDC, 2013). Over 2.8 million antibiotic-resistance infections occur annually in the United States, and treatments can be lengthy and expensive. For example, each case of extensively drug-resistant tuberculosis costs $526,000 to treat, and extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* cost $1.2 billion in healthcare costs per year (CDC, 2019). Within the United Kingdom, there were an estimated 65,162 resistant infections in 2019, an increase of over 3,000 compared to the previous year [English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) Report, 2020], with 21% of all key pathogen bloodstream infections reported in England showing resistance to at least one antibiotic [English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) Report, 2020]. By 2050, it is estimated that AMR will have cost 100 trillion USD from the world's economy (O'Neill, 2016). Globally, 1.27 million deaths were caused by bacterial AMR in 2019 (Antimicrobial Resistance Collaborators et al., 2022) with up to 10 million deaths predicted to occur annually by 2050 (O'Neill, 2016).

Surveillance using a "One Health" approach, covering the use of antibiotics and resistance rates within humans, animals, and the environment in which they coexist, is essential for effectively assessing the spread of AMR (WHO, 2001, 2020; Zinsstag et al., 2011). This approach considers the close interactions between humans, animals, and the environment, with the World Organisation for Animal Health, the Food and Agriculture Organization of the United Nations, and the WHO recognizing AMR as a critical issue requiring such a One Health surveillance strategy (Zinsstag et al., 2011). As an example, humans can be exposed to AMR pathogens through the food chain, including through consumption of contaminated meat leading to food borne illnesses (Zhao et al., 2008). Additionally, a high exchange frequency of mobile antimicrobial resistance genes (ARGs) can occur between bacteria that infect animals and humans (Hu et al., 2016). Human–animal-shared mobile ARGs have been identified in human, chicken, pig, and cattle guts, conferring resistance to six major antibiotic classes: tetrayclcines, aminoglycosides, macrolide-lincosamide-streptogramin B (MLSB), chloramphenicols, β-lactams, and sulphonamides. These ARGs that transfer between human and animal guts are also harbored by human pathogens (Hu et al., 2016). Additionally, there is a shared resistome between soil bacteria and human pathogens, and it has been suggested that the soil microbiota act as a reservoir of resistance genes (Forsberg et al., 2012). Such exchange can happen *via* horizontal gene transfer; these mechanisms allow bacteria to exchange genes including ARGs, making both inter- and intraspecies exchange possible. This allows for the development of mobile genetic elements such as plasmids which include multiple ARGs, leading to multidrug resistant or extensively drug-resistant bacteria; these plasmids can transfer to different bacteria in various environments (Sun et al., 2019).

Antibiotics are commonly administered to livestock, particularly in countries where prophylactic use is allowed. For example over 17 million kg of antimicrobials sold in the United States in 2019 was for use in food-producing animals, with over one-third of this amount being antimicrobials important to human health (FDA, 2020). The BRICS countries (Brazil, Russia, India, China, and South Africa) are estimated to double their antimicrobial use in animals by 2030, and the use of antimicrobials applied to chickens is set to triple in India by the same year (Van Boeckel et al., 2015). Intensive farming conditions, including high animal densities in indoor production systems and poor biosecurity (as typically seen in monogastric farming), can lead to livestock being more vulnerable to disease thus leading to even higher antimicrobial usage (FAO, 2016). Antimicrobials can also be widely abused due to their low cost and over the counter availability in some countries (FAO, 2016). Such practices can lead to excess antibiotic use, which places a selection pressure on the microbiota within the animal gut flora, leading to an alteration of the diversity of the host microbiota and the promotion of exchange of genetic material (FAO, 2016).

Antimicrobial resistance within livestock farming will also impact AMR in the environment as well as human and animal health. A significant quantity of those antimicrobials applied to animals are thought to be largely unmetabolized, with up to 90% of antibiotics used in food animals being excreted (Marshall and Levy, 2011). Tetracycline applied to pigs has been shown to be rapidly excreted, with up to 72% of the active ingredient recovered in the animals' feces and urine (Winckler and Grafe, 2001). While soil microbiota do naturally harbor ARGs, known as the intrinsic resistome (Zhu et al., 2019), feces and urine of grazing animals and the application of animal manure as fertilizer can lead to the dissemination of resistance in soil bacteria (Ghosh and LaPara, 2007; Iwu et al., 2020). For example, Zhu et al. (2013) have shown that manure sourced from Chinese pig farms was enriched by up to 28,000 fold in ARGs with a concomitant 90,000-fold increase in transposons, when compared to antibiotic free manure and soil. Soil can also be exposed to antimicrobials, ARGs and resistant organisms through the application of sewage sludge for crop production and wastewater from industrial, agricultural, pharmaceutical, and municipal treatment plants for irrigation (Fouz et al., 2020). These exposures to antimicrobials can lead to changes in the diversity of the soil resistome; it has been suggested that microorganisms that have acquired resistance now dominate over the intrinsic resistant organisms (Zhu et al., 2019). Resistance genes identical in sequence to those found in a range of human pathogens have also been identified in environmental soil bacteria, including multidrug resistance cassettes, providing resistance against five different antibiotic classes (Forsberg et al., 2012).

Given the intrinsic link between animal manures, soil as a reservoir of ARGs (Forsberg et al., 2012), and the associated potential impact on human health, we now present a global surveillance study of acquired ARGs in the gastrointestinal tracts of ruminants, swine, and poultry and the soil environment.

Additionally, we confirm that these ARGs were functional and expressed within metatranscriptomic datasets of the microbiomes from these environments. This study provides a global outlook of antimicrobial resistance across different microbiomes in livestock farming landscapes, and soil environments.

MATERIALS AND METHODS

Selection of Metagenomic Data for Identification of ARGs

The National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (Leinonen et al., 2010) was searched for metagenomic sequencing datasets for the following microbiomes: ruminant (species *Bos Taurus*), poultry, swine, and soil. All available metagenomic data on the NCBI, as of July 31, 2019, for each microbiome were considered for this study. The search terms used are outlined in **Table 1**, with individual searches completed for each search term.

Bioprojects matching at least one of these search terms were filtered to only include whole shotgun metagenomic sequence data and sequencing performed on Illumina sequencing platforms to ensure consistency between samples, both single and paired end reads were included. Bioprojects were only included if they were of known geographical location, i.e., the country of sample origin. Bioprojects were excluded if they were genomic sequences from pure culture and 16S rDNA sequencing data, involved bench top or post sampling enrichment or involved animals participating in an antibiotic trial (as the purpose of this study was to investigate baseline resistance). The study design and abstract for each Bioproject on the NCBI SRA were checked and, where available, the associated papers were read. All biosamples, within the bioprojects that met these criteria, were included in the final dataset for analysis (**Table 2**).

Identification of Antimicrobial Resistance Genes

All bioinformatic analyses described below were performed on the QUB High Performance Cluster system, Kelvin. All metagenomic sequences meeting selection criteria, as described above, were downloaded from the NCBI databases and local blast searches performed. Each sequence run was downloaded in compressed sra format (.sra) using the *prefetch* tool. The files were converted from sra to fasta format using the SRA-Toolkit version 2.9.6 (Leinonen et al., 2010). An AMR database file was created by downloading ARGs (excluding any ARGs due to point mutations) from the ResFinder database resulting in a fasta file containing 2,470 acquired ARG sequences (downloaded on July 31, 2019) (Zankari et al., 2012). To overcome the potential challenge that nucleotide redundancy in ARGs (with the same function) may lead to their separation using Resfinder, clustering was performed via CD-HIT-EST analysis using Galaxy version 1.2 (Fu et al., 2012; Afgan et al., 2018). This clustering avoided multiple ARGs of similar sequence being overrepresented by matching to the same stretch of metagenomic sequence clustering resulted in 302 ARGs. The clustering cut offs were 90% similarity and sequence length difference cutoff of 99%, i.e., the shorter sequences need to be at least 99% length of the representative of the cluster.

Using NCBI BLAST version 2.2.30, nucleotides searches were performed to identify the clustered ResFinder ARGs within the metagenomic sequences. Default BLASTn parameters and the output format six were used, outputting a tab-separated table with no preset column headers. A specific text file output was requested including the query accession, sequence accession, e-value, query start, query end, sequence start, sequence end, bitscore, percentage of identical matches, and alignment length (Madden, 2008).

Summary files were then created from the BLAST result files to filter the BLAST hits such that only those with a percentage identity ≥75%, and a bitscore of ≥60 were included. This was completed using a shell script for example, cat soil1.txt | awk '{if ($8 >= 60 && $9 >= 75) print $0}' | sort -k2,2 | cut -f2 | uniq -c | sort -rn>summarysoil1.txt. The output included the incidence of the AMR gene in that sequence that met the criteria. Any metagenome fasta file (biosample) that had no AMR genes identified after applying the cut off filters were removed from this point and did not proceed for downstream analysis, this included 23 soil, five ruminant, two poultry, and two swine biosamples. Results were further filtered to remove low frequency hits, with ARG hits included if they appeared >25 times within the biosample. This filter was selected to balance the retention of data with the removal of background noise and low frequency hits (**Supplementary Figure 1**). Essentially, any gene that appeared >25 times within a sample was considered as present and denoted as 1, any gene present ≤25 times was considered absent, 0. All countries that contained AMR genes following application of these cutoffs are highlighted in **Figure 1**. Datasets from five countries including Argentina, Colombia, Madagascar, Papua New Guinea, and Sri Lanka did not contain ARGs meeting these cutoffs and were consequently excluded from further analysis.

TABLE 1 | NCBI SRA search terms.

Microbiome	Search term 1	Search term 2	Search term 3	Search term 4
Ruminant	Rumen gut	Rumen feces*	Bovine feces*	Bovine gut
Poultry	Poultry feces*	Poultry gut	Chicken gut	Chicken feces*
Swine	Swine gut	Swine feces*	Pig feces*	Pig gut
Soil	Soil			

Each search term was followed by the word metagenome.
**Feces/fecal/feces/fecal.*

Metatranscriptomics: Gene Expression Analysis

In order to evaluate the potential functionality and active expression of identified ARGs, the ARG sequences were aligned to metatranscriptome datasets. Metatranscriptome datasets were selected for each of the four microbiomes: ruminant, poultry, swine, and soil. The metatranscriptomic studies for both gut microbiomes of swine and poultry were selected from the

FAANG database,[1] and the largest available metatranscriptomic datasets from published studies and free from antibiotic treatment were selected. These included the poultry metranscriptomic dataset: PRJEB23255, Germany (Reyer et al., 2018) and the swine datasets: PRJNA529662, PRJNA529214, United States of America (Keel et al., 2020). Similarly, the largest antibiotic free metatranscriptomic soil dataset was selected from the ENA database,[2] PRJNA366008, United States of America (unpublished). These metatranscriptomic datasets were downloaded using *wget* version 1.14. Finally, to represent the ruminant category, the metatranscriptome data from the study by Shi et al. (2014) (PRJNA202380, New Zealand) were investigated.

Analysis was performed using Bowtie2 (version 2.3.5.1) and the methods described in Sabino et al. (2019) and Langmead and Salzberg (2012). In brief, the DNA sequences of ARGs contained on the ResFinder database were indexed using the Bowtie2-build tool. The downloaded metatranscriptomic datasets were then aligned to these indexes, default end-to-end alignment cut offs were used, and minimum score threshold was −0.6 + −0.6 * L, where L is the read length. The presence and absence of alignment to an ARG were then summarized from the SAM files produced from bowtie2.

Statistics and Graphical Representation

All statistical analyses were performed using GraphPad Prism version 9.1.0.221 (GraphPad Software, Inc., San Diego, CA), including Spearman r correlations, normality testing, chi-square, and ANOVA Kruskal-Wallis tests (significance at $p < 0.05$). **Figure 1** was prepared using the My Maps function on Google

TABLE 2 | Sequencing datasets selected for analysis.

Microbiome	Total number of sequencing datasets	Total size of sequencing data (Gb)
Ruminant	592	4,794.3
Poultry	306	1,092.8
Swine	784	1,772.4
Soil	4,161	19,513.2

The total number of sequencing datasets and sizes of data used in this study collected from the NCBI SRA database.

[1]https://data.faang.org/home
[2]https://www.ebi.ac.uk/ena/browser/

FIGURE 1 | Geographical locations of the selected metagenomic samples containing ARGs. Metagenomes selected from NCBI SRA including 509 ruminant metagenomes from eight different countries, 277 poultry metagenomes from six countries, 710 swine metagenomes sourced from seven countries, and soil metagenomes from 23 countries.

maps (Google Maps, 2022). MyDraw version 5.0.0 software was used to produce **Figure 2** (Nevron Software, 2020). **Figures 3–5** were prepared using GraphPad Prism version 9.1.0.221 (GraphPad Software, Inc., GraphPad Software Inc., 10855 Sorrento Valley Rd.# 203., San Diego, CA 92121). **Figure 6** was prepared using mapchart webpage (MapChart, 2022) and **Figure 7** was prepared using RStudio (Wickham, 2016; RStudio Team, 2020).

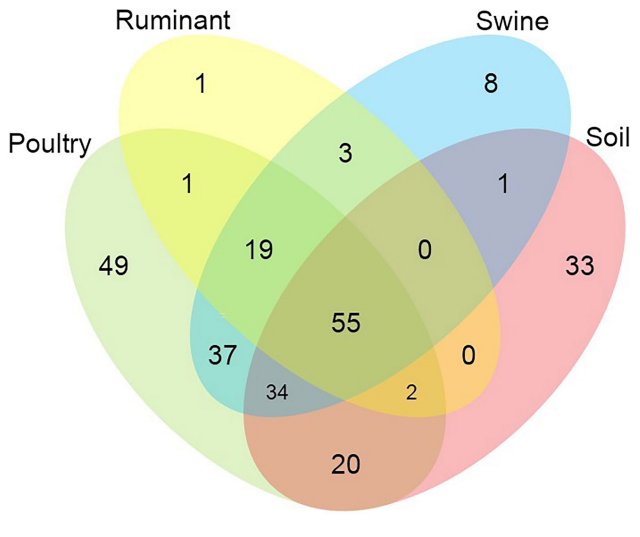


FIGURE 2 | Venn diagram showing the number of shared and unique antimicrobial resistance genes among the analysed microbiomes.

RESULTS

Summary of Resistance Within Soil and Livestock Microbiomes

Metagenomes from the soil environment and three livestock microbiomes [ruminant (species *Bos Taurus*), swine, and poultry] were selected from the NCBI SRA for resistome characterization. The soil microbiome was the largest dataset included, covering 28 countries, 4,161 samples, and 19,513 Gb of sequencing data. The livestock microbiomes covered 18 different countries, with ruminant samples from 8 countries, poultry samples from 6 countries, and swine samples from 7 countries. In this study, 1,682 livestock metagenomes and 7,659.5 Gb of metagenomic sequencing data were included (592 ruminant samples, 4,794.3 Gb, 306 poultry samples, 1,092.8 Gb, and 784 swine samples, 1,772.4 Gb). Acquired resistance genes contained within the ResFinder database were used to characterize the resistomes present within these four microbiomes.

A large range of ARGs were identified within each environment, the poultry microbiome datasets being the most diverse, with an average of 47 resistant genes identified per sample, significantly higher than the other microbiomes ($p < 0.001$). The soil dataset had the lowest average number of resistance genes per sample (4) and was significantly lower than all other microbiomes (adjusted $p < 0.0001$) (**Table 3**). Soil of agriculture origin and contaminated soil (antimony, arsenic, metal, mines, oil, pharmaceutical waste, polychlorinated biphenyl, and uranium) had significantly more different ARGs per sample than forest soil ($p < 0.0001$). On average forest soil samples contained three resistance genes per sample, and in comparison, agricultural soil had 8 ARGs per sample and contaminated soil had 5 ARGs. The highest number of resistance genes per

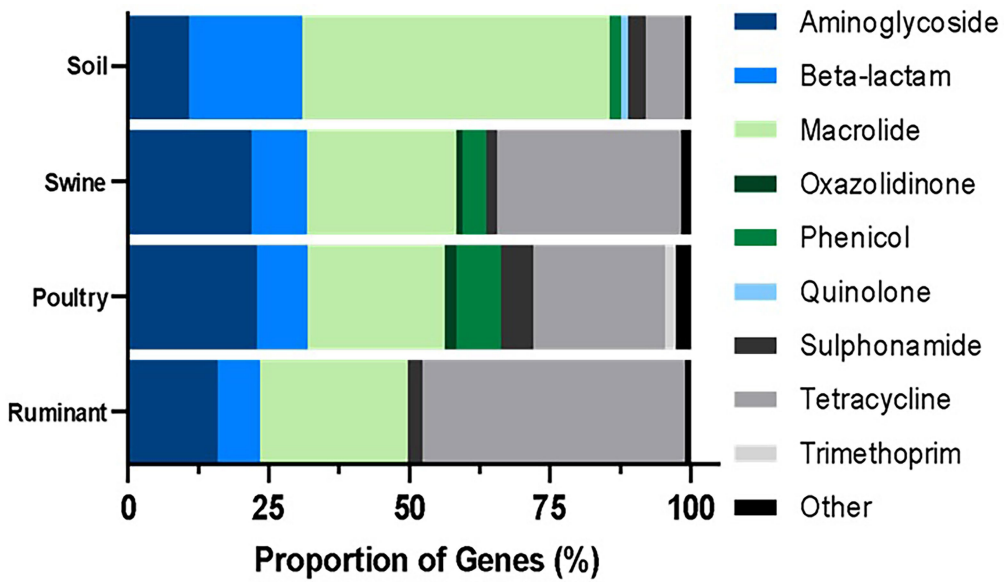


FIGURE 3 | Percentage resistance genes grouped by antibiotic class per microbiome type. The ARG were identified by BLASTn and the proportion was estimated by the dividing the number of distinct resistance genes per antibiotic class by the total number of resistance genes for all classes.

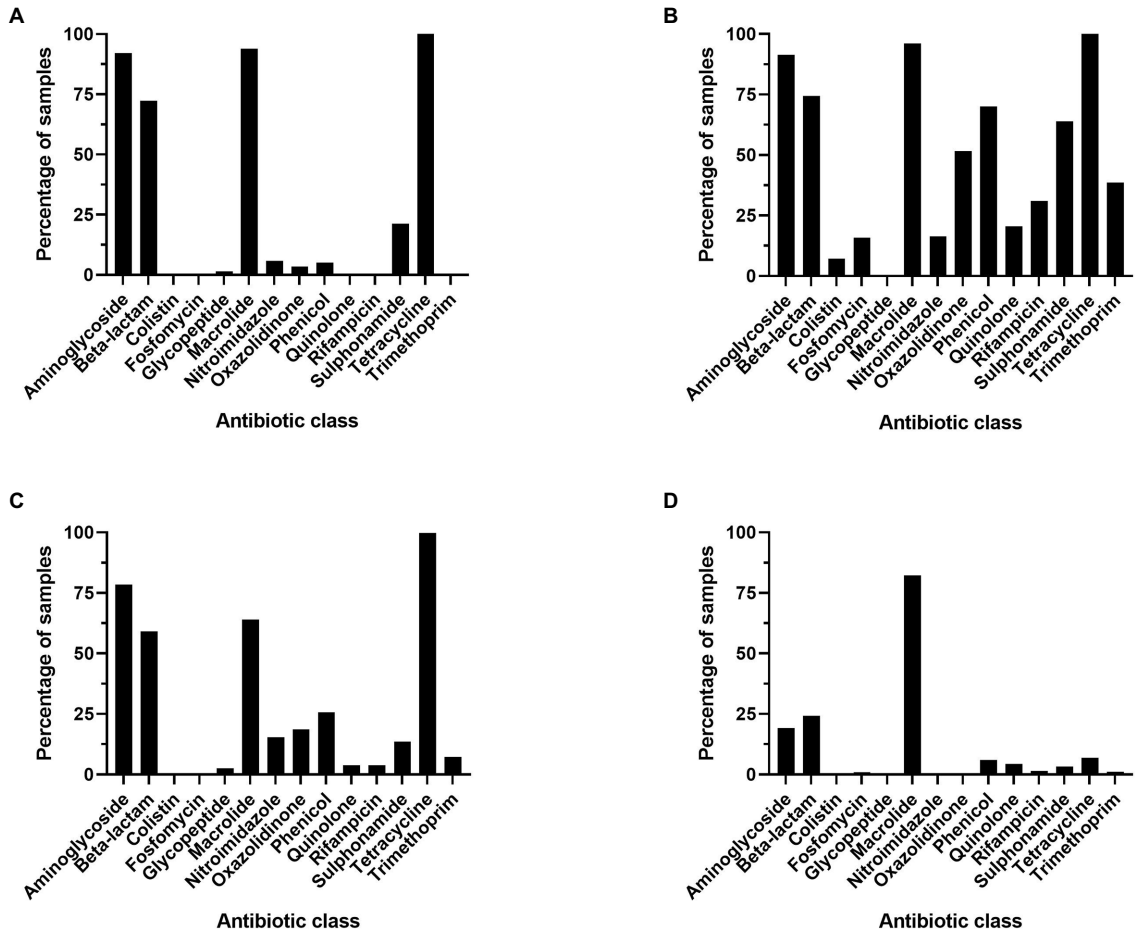


FIGURE 4 | Percentage of metagenomes that contain at least one resistance gene per antibiotic class, genes were considered present if occurring >25 times in a sample. **(A)** Ruminant, **(B)** poultry, **(C)** swine, and **(D)** soil.

sample, 73 ARGs, was observed in American soils that had been treated with manure. The ruminant microbiome contained the least number of distinct resistance genes (101), in comparison to the poultry, swine, and soil microbiomes that harbored 235, 167, and 182 different ARGs, respectively.

In total, 55 AMR genes were shared between all environments, conferring resistance to six antibiotic classes (tetracycline, macrolide, aminoglycoside, beta-lactam, sulphonamide, and phenicols) (**Figure 2**). Almost one-third of these genes (18 of 55 genes) conferred resistance to tetracycline antibiotics, followed by macrolide resistance (13 of 55 genes) and aminoglycoside resistance genes (11 of 55). Poultry and soil microbiomes contained the largest number of distinct genes, which were not present in the other microbiomes (49 and 33 genes, respectively). The soil microbiome harbored 13 β-lactam resistance genes, which were not present in any other microbiome, while 11 aminoglycoside and 11 β-lactam resistance genes were present only in the poultry microbiome. The soil microbiome was also the only microbiome to contain the colistin-resistance genes, *mcr-7.1* and *mcr-5.1*. The ruminant and swine microbiomes contained the lowest numbers of distinct genes, ruminants contained one distinct resistance gene, *aph(2″)-If* conferring aminoglycoside resistance, while the swine microbiome contained 8 ARGs, which were not present in other microbiomes; aminoglycoside (*aph(2″)-Ih*, *rmtf*), beta-lactam (*bla*OXA-2, *bla*OXA-34, *bla*OXA-36) macrolide [*vga*(A)], and nitroimidazole (*nimB*, *nimH*). In total, 19 resistance genes were distinct to the livestock microbiomes and conferred resistance to the following antibiotic classes: β-lactam (5 genes), macrolide (5 genes), aminoglycoside (3 genes), phenicols (2 genes), tetracycline (2 genes), nitroimidazole (1 gene), and trimethoprim (1 gene). Poultry and swine microbiomes shared 37 resistance genes that were present in no other microbiome; this was the largest number of shared genes between microbiomes. These 37 genes conferred resistance to 11 different antibiotic classes (aminoglycoside, beta-lactam, phenicols, trimethoprim, quinolone, macrolide, colistin, nitroimidazole, oxazolidinone, rifampicin, and sulphonamide) and included the colistin resistance gene *mcr-1.11*.

ARG Abundance and Diversity in Microbiomes by Antibiotic Class

The majority (54.70%) of genes identified in the soil environment encoded resistance against macrolide antibiotics (**Figure 3**).

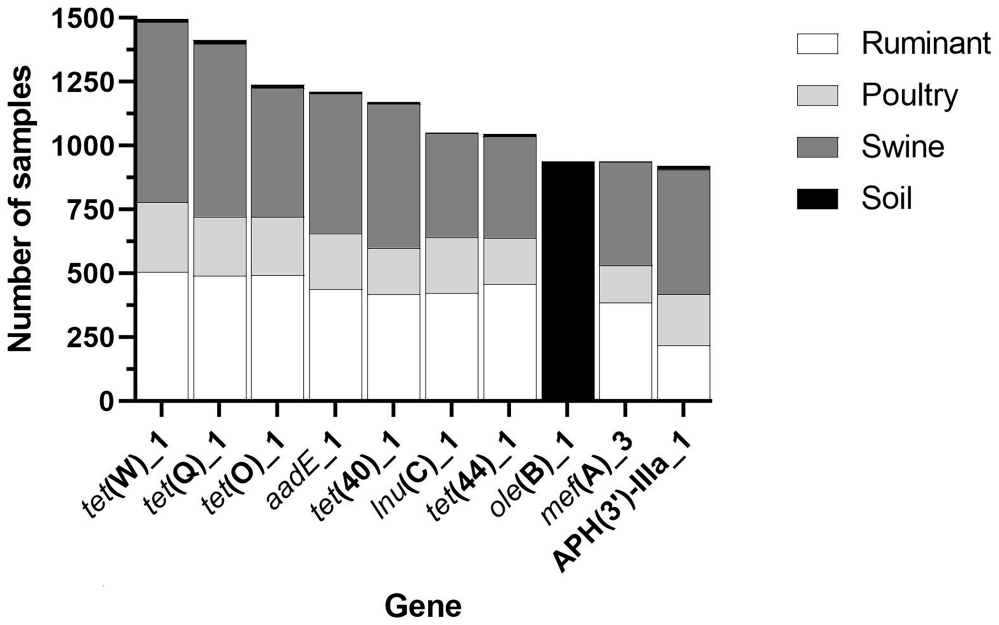


FIGURE 5 | The most widespread resistance genes across all the four microbiome types.

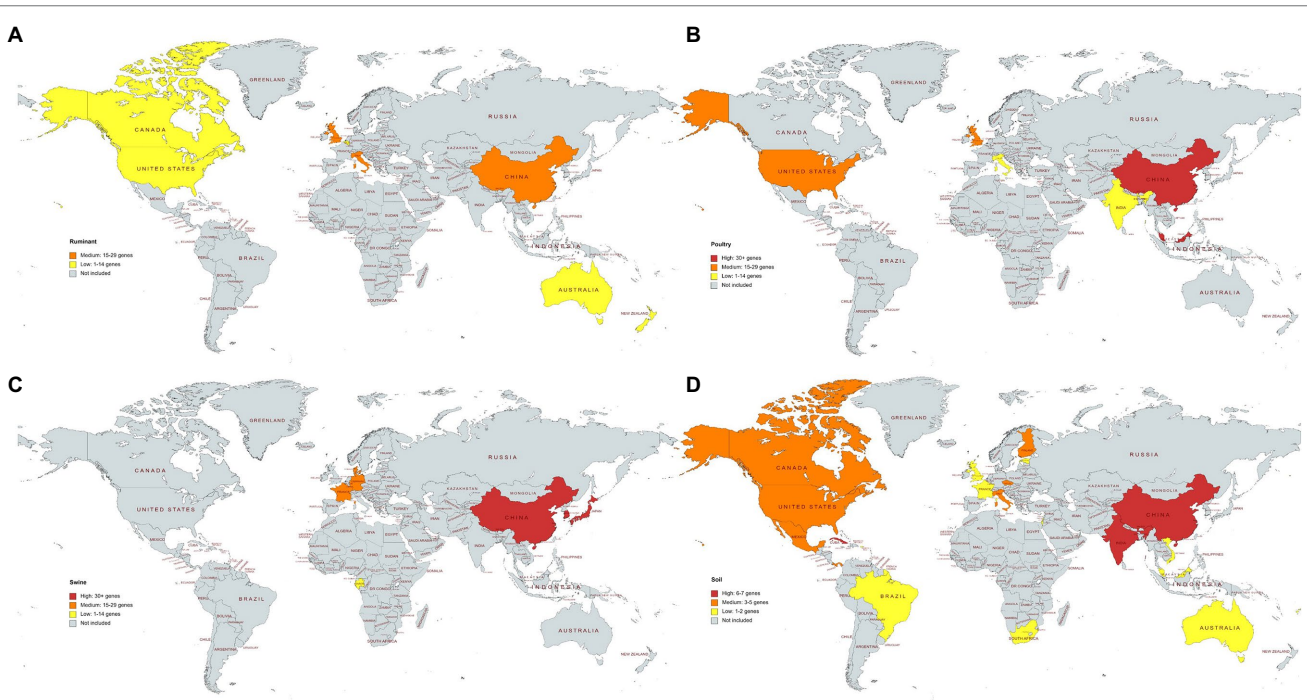


FIGURE 6 | (A–D) The average number of different antimicrobial resistance genes per country and microbiome. Data for countries represented by one sample are not shown. **(A)** Ruminant, **(B)** poultry, **(C)** swine, **(D)** soil. **(A–C)** High: 30+ genes, medium: 15–29 genes, low: 1–14 genes. **(D)** Soil samples. High: 6–7 genes, medium: 3–5 genes, low: 1–2 genes. Soils samples from Antarctica are not included, and they showed medium average resistance (average of three distinct resistance genes per sample).

The most widespread macrolide-resistant gene within this environment was *ole*(B)_1, which was identified in 930 soil samples (60.08% of analyzed soil samples). This gene was spread across 73 different soil types including agriculture, desert, forest, grasslands, garden, permafrost, and soils, both with and without crop. Macrolide resistance genes were also the most prevalent

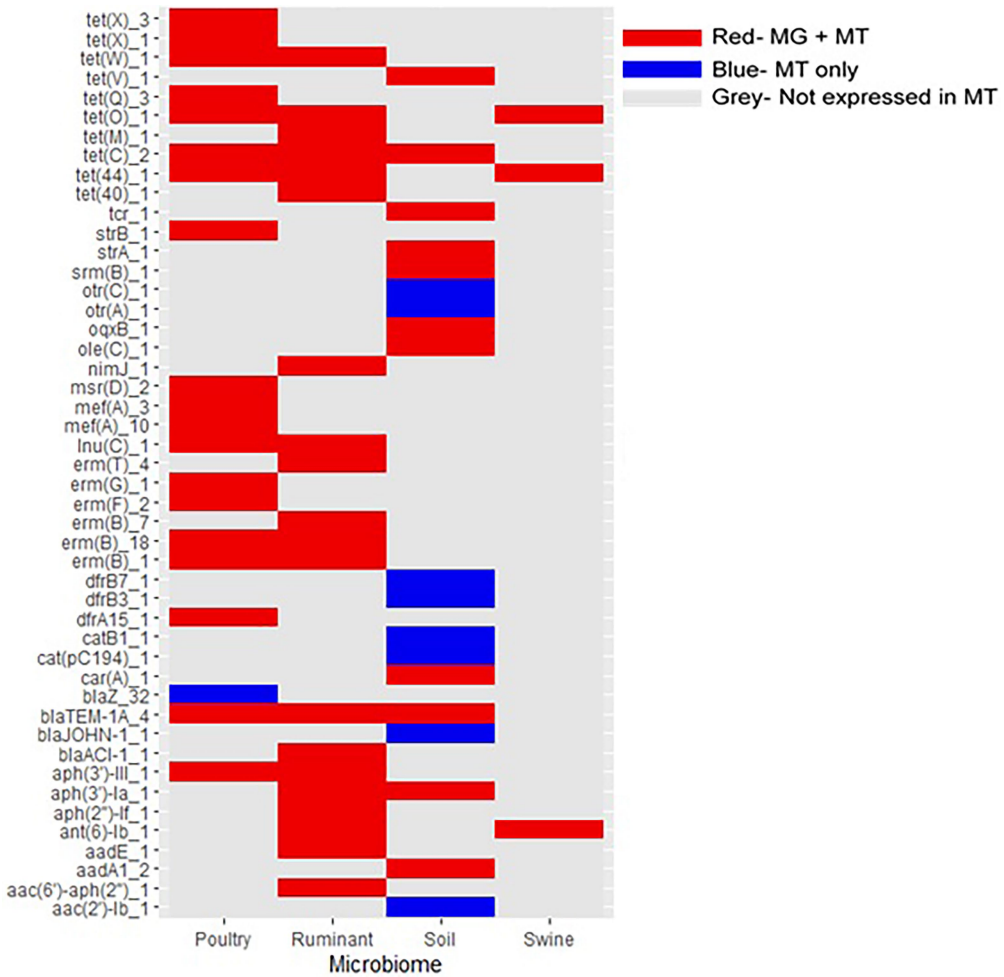


FIGURE 7 | Expression of antimicrobial resistance genes in livestock and soil microbiomes. Only ARGs expressed in at least one metatranscriptome are included. MT, metatranscriptomes and MG, metagenomes. Red gene was present in both the metagenome and metatranscriptome datasets. Blue gene was present in the metatranscriptome dataset only. Gray gene was not present in metatranscriptomic datasets.

in poultry samples with over 24.38% of ARGs identified in this microbiome conferring resistance against macrolides. The most widespread gene within poultry metagenomes was the *erm*(B)_7 gene, identified in 256 samples (92.41%). In contrast, the most commonly identified class in both swine and ruminant environments were tetracycline resistance genes, which represented 46.64% and 32.71% of resistance genes identified in ruminant and swine microbiomes, respectively (**Figure 3**). The most commonly identified tetracycline resistance gene in both environments was *tet*(W)_1, identified in 99.41% of ruminant samples and 99.44% of swine samples.

The resistome profiles identified in each of the four microbiomes were significantly different ($p < 0.0001$). The macrolide resistance genes were widespread across all microbiomes, identified in >80% of all samples. Tetracycline and aminoglycoside resistance genes were widespread across all livestock microbiomes but were present in small proportion of soil samples (6.85% of samples contained at least one tetracycline resistance gene). Also prevalent in livestock samples were β-lactam antibiotic resistance genes, which were identified in more than half of livestock microbiome samples. Resistance genes against colistin, fosfomycin, oxazolidinone, phenicol, quinolone, rifampicin, sulphonamide, and trimethoprim were present in more poultry samples than in any other microbiome (**Figure 4**).

Three antibiotic classes were represented in the 10 most common genes, including tetracycline [*tet*(W)_1, *tet*(Q)_1, *tet*(O)_1, *tet*(40)_1 and *tet*(44)_1], macrolide (lincosamide-streptogramin B) [*lnu*(C)_1, *ole*(B)_1, *mef*(A)_3], and aminoglycoside [*aadE*_1, APH(3′)-IIIA_1] (**Figure 5**). Fifty percent of the most widespread genes conferred tetracycline resistance. All genes, except for *ole*(B)_1, were identified in a large number of livestock microbiome samples but were not widespread in soil samples (**Figure 5**). The macrolide resistance gene, *ole*(B)_1, was identified in 939 (60.66%) of soil samples but was only identified in 3 poultry and 6 ruminant samples and was not identified in any swine samples. Also, widespread across soil samples was the macrolide resistance gene, *tlr(C)_1*, identified in 589 soil samples.

TABLE 3 | Total antimicrobial resistance gene (ARGs) per microbiome.

	Number of samples	Resistance genes identified	Total occurrences of resistance genes	Avg. number of genes per sample (SEM)
Ruminant	509	101	8,248	16.20 (±0.365)
Poultry	277	235	13,210	47.69 (±1.875)
Swine	710	167	16,342	23.02 (±0.725)
Soil	1,548	182	5,930	3.83 (±0.146)

Summary showing the number of antimicrobial resistance genes (ARGs) identified per microbiome and the average number of ARGs identified in each group. Resistance genes identified refers to the number of distinctly different resistance genes identified across the microbiomes. Total occurrences of resistance genes was calculated as the number of incidences all resistance genes were identified in the microbiome. SEM, standard error of the mean.

ARG Abundance in Microbiomes by Country of Origin

Metagenomes for all four microbiomes sourced from China were analyzed. Chinese soil, poultry and swine microbiomes were classified as containing high number of average resistance genes per sample, while the Chinese ruminant microbiomes showed medium resistance levels. The United States showed medium resistance levels in both the soil and poultry microbiomes and low resistance in the ruminant microbiome (**Figure 6**). All European swine microbiomes showed medium resistance. The ruminant and poultry microbiomes selected from the United Kingdom also showed medium levels of resistance genes. Cuba, India, and China soil microbiomes showed high resistance in comparison to other countries, but only one metagenome sequence was available for Cuba (**Figure 6**). In general, soil samples across all the countries harbored less AMR genes than the livestock microbiomes.

Expression of ARGs in Microbiomes

Metatranscriptomic data for each microbiome was used to ascertain whether the ARGs were expressed and thus functionally active (**Figure 7**). The expression of ARGs, previously identified in the metagenome datasets, were confirmed in metatransciptomes for all four of the microbiomes. Of the 76 poultry metatranscriptomic sequences, 72 contained ARGs (94.7% of sequences). Of the 32 ruminant metatranscriptomes, 19 contained ARGs (59.4%), similarly 46.8% of soil metatranscriptomes contained ARGs (22 of 47 metatranscriptomes), while ARGs were identified in only 3 of the 60 swine metatranscriptomes analysed (5.0%).

All of the ARGs expressed in the ruminant and swine metatranscriptomes were identified in the associated metagenomes, similarly 19 of the 20 ARGs identified in the poultry metatranscriptomes were present in the poultry metagenomic sequences. Of the 18 ARGs expressed in the soil metatranscriptomes, 11 were also identified in the soil metagenomic dataset. The percentages of ARGs identified in metagenomes that were also identified in metatranscriptomic data are as follows: 8.1% poultry, 19.8% ruminant, 1.8% swine, and 6.0% soil. The expression of ARGs in the microbiomes did report similarities, with aminoglycoside and tetracycline genes being expressed in all of the microbiomes. Additionally, ruminant, poultry, and soil microbiomes all expressed β-lactam, macrolide, and quinolone ARGs.

DISCUSSION

In this study, we identified the acquired ARGs in the soil environment and the gastrointestinal tracts of livestock animals including ruminants, swine, and poultry. We aimed to take a global approach to assess the resistomes in these four microbiomes by using publicly available metagenomic datasets on the NCBI SRA database and metatranscriptomic sequencing data from 37 different countries. The four microbiomes investigated in this study contained a large diversity of ARGs, with each environment containing over 100 different resistance genes, conferring resistance to at least 11 different antibiotic classes.

The livestock microbiomes, namely, poultry, ruminant, and swine, showed similarities in their resistomes, with ARGs against tetracyclines, aminoglycosides, and β-lactam antibiotics being widespread among livestock microbiomes but less abundant in the soil microbiomes. The soil microbiome also showed very low average number of ARGs per sample (4 genes) in comparison to the livestock microbiomes (25 genes). This study provides a global and more expansive insight and is in agreement with previous findings that soil in general had lower ARG abundance than livestock microbial communities (Pal et al., 2016).

Tetracycline genes were spread across all livestock microbiomes, particularly, the tetracycline resistance genes *tet*(Q)_1 and *tet*(W)_1. Both of these tetracycline genes have been identified in important human pathogens including *Prevotella* spp. and *Clostridium difficile* (Arzese et al., 2000; Spigaglia et al., 2008). The *tet*(W)_1 gene, identified in the rumen anaerobe *Butyrivibrio fibrisolvens* has also been identified in human feces (Scott et al., 2000). The potential widespread distribution of this gene could be due to it's ability to use horizontal gene transfer through association with integrative and conjugative elements (ICE) including the newly identified ICE_RbtetW_07 (Sabino et al., 2019). Tetracycline genes were also confirmed to be expressed in each of the four microbiomes, with *tet*(44) and *tet*(O)_1 expressed in all livestock microbiomes. The extensive use of tetracycline antibiotics, extensive host range seen for the *tet* resistance gene and the genes associations with mobile genetic elements such as ICEs may all contribute to the widespread identification of these genes in this study (Spigaglia et al., 2008).

The poultry microbiome was the most diverse, harboring the highest number of different ARGs and contained the highest number of unique ARGs. So far, research has been relatively limited on the poultry resistome and how it compares to other livestock microbiomes (Ma et al., 2021), therefore this study sheds new light on the poultry resistome. Such high resistance in poultry farming could be due to the high antibiotic usage associated with the widespread intensive farming used in this livestock sector (Kirchhelle, 2018).

Additionally, to the livestock microbiomes, agriculture soil also showed high numbers of ARGs. Specifically agricultural soils that had been fertilized using manure showed particularly high numbers of ARGs. The spread of ARGs in soil due to manure application has been considered a serious public concern (Checcucci et al., 2020). The most widespread resistance gene within the soil samples was *ole*(B)_1, this gene was not widespread across the livestock microbiomes. The *ole*(B)_1 gene confers resistance against oleandomycin through the production of a ribosome protection protein (Kerr et al., 2005). Oleandomycin is an active ingredient of a last resort drug used to treat mastitis in cattle where other treatments have failed (Zoetis, 2016). This study highlights that the *ole*(B)_1 resistance gene is widespread in different soil types including agriculture, desert, forest, grasslands, garden, permafrost, and soils both with and without crop, and builds upon previous identifications of this resistance gene in arable farmland soil, forest soil, and compost from Poland (Popowska et al., 2012). The *ole*(B)_1 gene has been identified in zoonotic pathogens within soil and is also widely distributed in the human gut (Yang et al., 2016). The gene product of *ole*(B)_1 is a member of the ABC-transporter superfamily, and in addition to conferring antibiotic resistance, members of this gene family are also involved in transport, DNA repair, enzyme regulation, and translational control (Davidson et al., 2008; Sharkey et al., 2016). Therefore, although the *ole*(B)_1 genes found in the soil samples in this study may confer resistance in these environments, their gene products may also serve other functions in that environment, highlighting that ARG hits within samples does not always equal resistance.

The majority of the metagenomic studies included in this analysis did not have associated metatranscriptomic data. Therefore, we employed the most suitable alternative methods and analyzed the largest publicly available metatranscriptomic dataset for each microbiome to explore if the ARGs identified within the metagenomes could be expressed in their associated microbiomes. Low numbers of the ARGs identified in the metagenomes were identified in metatranscriptomes, we suggest this is due to the much smaller amount of metatranscriptomes analyzed in comparison to the large number of metagenomes. Resistance genes expressed in the four microbiomes were diverse covering eight different antibiotics classes. In total, 28 ARGs were expressed in the livestock microbiomes that were not expressed in the soil microbiome, suggesting these genes are more adapted to expression in the gut microbiome.

Resistance to one of the last resort drugs, colistin, was detected in poultry, swine, and soil microbiomes. In total, 20 poultry metagenomes (7.22% of poultry samples) contained colistin resistance genes, with the *mcr-1.11* gene being the most widespread. Of the 20 colistin ARG containing poultry samples, 19 contained the *mcr-1.11* gene and one sample contained the *mcr-3.1* gene. The *mcr-1.11* was also the only colistin-resistant gene identified in swine microbiome. The *mcr-1.11* gene is a variant of the *mcr-1* gene, which likely emerged due to spontaneous mutation within a plasmid structure (Deshpande et al., 2019). The plasmid-borne *mcr-1* gene and its variants pose a significant challenge to human healthcare, as colistin and the polymyxins are currently last resort treatments against serious multidrug resistant Gram-negative human pathogens (Gao et al., 2016). In this study, livestock microbiome datasets from China and soil microbiome datasets from the United States harbored the *mcr-1.11* gene and the *mcr-5.1 and mcr-7.1* colistin-resistant genes, respectively. These soils included both agriculture and uncultivated soils. Both the *mcr-5.1 and mcr-7.1* genes are associated with mobile genetic elements including transposons and plasmids and both were identified in human pathogens (Han et al., 2006; Borowiak et al., 2017). The *mcr-5.1* gene has been identified in *Salmonella. enterica* subsp. *enterica* serovar Paratyphi B dT+ (*S. Paratyphi* B dT+; formerly called *Salmonella Java*), which causes gastroenteritis and is currently an emerging problem worldwide (Han et al., 2006; Borowiak et al., 2017). While the *mcr-7.1* gene has been identified from serious human pathogen *Klebsiella pneumoniae* (Yang et al., 2018). Previous studies have identified the *mcr-5.1* and *mcr-7.1* genes in Chinese soil (Zheng et al., 2017; Shen et al., 2018; Anyanwu et al., 2020). However, this study also shows for the first time that they are present in soil from the USA.

Vancomycin-resistant genes were identified across all four microbiomes; vancomycin is an important treatment option in endocarditis and other serious infections including the treatment of methicillin-resistant *Staphylococci* infections (Bennett et al., 2019). The swine microbiome harbored the largest diversity, containing four different vancomycin resistance genes: *vanG*, *vanA-B*, *vanH-B*, and *vanXY-G*. Vancomycin resistance was more widely disseminated than colistin resistance, with vancomycin resistance genes identified in 10 counties including Canada, China, Denmark, England, France, Germany, Japan, New Zealand, South Korea, and United States.

A large number of metagenomic sequences were available from China, and it was the only country to have samples included in each of the four microbiomes. In this study, medium levels of ARGs were observed within the ruminant microbiome and high resistance levels in the poultry, swine, and soil microbiomes. High ARGs occurrence in different livestock environments have been previously reported in China (Wang et al., 2016; Qiao et al., 2018). The Chinese Ministry of Agriculture have aimed to reduce the use of veterinary antibiotics through their "National Action Plan for Restraining Bacteria of Animal Origin" (Ministry of Agriculture of the People's Republic of China, 2017). One objective of the action plan is to improve the monitoring system of veterinary antibiotics including the implementation of regulatory actions to strengthen the supervision and management of veterinary antimicrobials (Ministry of Agriculture of the People's Republic of China, 2017).

The microbiomes included from the United Kingdom and United States all showed low to medium average number of resistance genes per sample. The poultry and ruminant microbiomes from the United Kingdom showed medium resistance levels, as did poultry and soil microbiomes from the United States. Both the United Kingdom and United States have launched AMR action plans aimed at reducing antimicrobial consumption and AMR (HM Government, 2019; Federal Task Force on Combating Antibiotic-Resistant Bacteria, 2020). The United Kingdom has seen large reductions in antibiotic usage in animals, with a 52% reduction of antibiotics usage for

food-producing animals observed from 2014 to 2019 (UK-VARSS, 2020). Although low to medium levels of ARGs in livestock microbiomes were observed in United States, the United States does not have such reductions in antibiotic consumptions as those reported in the United Kingdom. However, the United States are introducing guidance in the prescription of antibiotics in the livestock sector including the implementation of the FDA's Guidance for Industry #213 (CDC, 2021). Differences in countries ARG levels could be due to varying regulations on antibiotic use.

All four microbiomes shared 55 AMR genes in common, with a number of genes being widespread across the sample types, stressing the need for a One Health approach to AMR surveillance encompassing both livestock and environmental microbiomes in addition to human AMR surveillance. This study successfully used available metagenomic and metatranscriptomic data to identify the ARG reservoirs present in four microbiomes across 37 countries (no ARGs were present in the analysed datasets from five of these countries). AMR and ARGs have been identified as a critical human health challenge (WHO, 2019). By expanding our knowledge on the resistomes present in different systems such as those included in this study, policies supporting issues such as antimicrobial usage and applications to soil, e.g., manure can be better informed. As highlighted in this study, ARGs are both persisting and being expressed in the microbiomes with microbiomes from many countries harboring high number of ARGs, and this is a global problem. Therefore, in addition to a One Health approach, a global approach must be employed to aid our understanding of ARG abundance and diversity globally, and so improve our ability to tackle the AMR problem in a holistic manner.

DATA AVAILABILITY STATEMENT

All metagenomic sequences are freely available on the NCBI SRA, while metatranscriptomics were sourced from the FAANG database and ENA database. Raw data is provided in the **Supplementary Table 1**. The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KL performed the analysis with guidance from CC, FS, and SH. FR assisted with metatranscriptomic work. FS and LO contributed significantly to manuscript preparation. SH is PI of KL and oversaw each stage of this study. JM and SM are also supervisors to KL. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Northern Irish Department of Agriculture, Environment and Rural Affairs.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.897905/full#supplementary-material

REFERENCES

Afgan, E., Baker, D., Batut, B., Van Den Beek, M., Bouvier, D., Čech, M., et al. (2018). The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* 46, W537–W544. doi: 10.1093/nar/gky379

Antimicrobial Resistance Collaborators, Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*, 399.

Anyanwu, M. U., Jaja, I. F., and Nwobi, O. C. (2020). Occurrence and characteristics of mobile colistin resistance (mcr) gene-containing isolates from the environment: a review. *Int. J. Environ. Res. Public Health* 17:1028. doi: 10.3390/ijerph17031028

Arzese, A. R., Tomasetig, L., and Botta, G. A. (2000). Detection of tetQ and ermF antibiotic resistance genes in Prevotella and Porphyromonas isolates from clinical specimens and resident microbiota of humans. *J. Antimicrob. Chemother.* 45, 577–582. doi: 10.1093/jac/45.5.577

Bennett, J. E., Dolin, R., and Blaser, M. J., (2019). *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases E-Book*. New York: Elsevier Health Sciences.

Borowiak, M., Fischer, J., Hammerl, J. A., Hendriksen, R. S., Szabo, I., and Malorny, B. (2017). Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. enterica serovar Paratyphi B. *J. Antimicrob. Chemother.* 72, 3317–3324. doi: 10.1093/jac/dkx327

CDC (2013). *Antibiotic Resistance Threats in the United States, 2013*. Atlanta, GA: U.S. Department of Health and Human Services.

CDC (2019). *Antibiotic Resistance Threats in the United States, 2019*. Atlanta, GA: U.S. Department of Health and Human Services.

CDC (2021). Where Resistance Spreads: Food Supply. https://www.cdc.gov/drugresistance/food.html (Accessed February 2, 2022).

Checcucci, A., Trevisi, P., Luise, D., Modesto, M., Blasioli, S., Braschi, I., et al. (2020). Exploring the animal waste resistome: the spread of antimicrobial resistance genes through the use of livestock manure. *Front. Microbiol.* 11:1416. doi: 10.3389/fmicb.2020.01416

Davidson, A. L., Dassa, E., Orelle, C., and Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol. Mol. Biol. Rev.* 72, 317–364. doi: 10.1128/MMBR.00031-07

Deshpande, L. M., Hubler, C., Davis, A. P., and Castanheira, M. (2019). Updated prevalence of mcr-like genes among Escherichia coli and *Klebsiella pneumoniae* in the SENTRY program and characterization of mcr-1.11 variant. *Antimicrob. Agents Chemother.* 63, e02450–e02418. doi: 10.1128/AAC.02450-18

English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) Report (2020). Public Health England. Available at: https://www.gov.uk/government/publications/english-surveillance-programme-antimicrobial-utilisation-and-resistance-espaur-report (Accessed December 20, 2021).

FAO (2016). Drivers, dynamics and epidemiology of antimicrobial resistance in animal production. Available at: http://www.fao.org/3/a-i6209e.pdf

FDA (2020). *Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals*. United States: FDA.

Federal Task Force on Combating Antibiotic-Resistant Bacteria, (2020). National Action Plan for Combating Antibiotic-Resistant Bacteria, 2020–2025. Available at: https://www.hhs.gov/sites/default/files/carb-national-action-plan-2020-2025.pdf (Accessed January 4, 2022).

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O., and Dantas, G. (2012). The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337, 1107–1111. doi: 10.1126/science.1220761

Fouz, N., Pangesti, K. N., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., et al. (2020). The contribution of wastewater to the transmission of antimicrobial resistance in the environment: implications of mass gathering settings. *Trop. Med. Infect. Dis.* 5:33. doi: 10.3390/tropicalmed5010033

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Gao, R., Hu, Y., Li, Z., Sun, J., Wang, Q., Lin, J., et al. (2016). Dissemination and mechanism for the MCR-1 colistin resistance. *PLoS Pathog.* 12:e1005957. doi: 10.1371/journal.ppat.1005957

Ghosh, S., and LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J.* 1, 191–203. doi: 10.1038/ismej.2007.31

Google Maps (2022). Metagenomic Sample Locations. Available at: https://www.google.com/maps (Accessed February 23, 2022).

Han, K. H., Choi, S. Y., Lee, J. H., Lee, H., Shin, E. H., Agtini, M. D., et al. (2006). Isolation of *Salmonella enterica* subspecies enterica serovar Paratyphi B dT+, or Salmonella Java, from Indonesia and alteration of the d-tartrate fermentation phenotype by disrupting the ORF STM 3356. *J. Med. Microbiol.* 55, 1661–1665. doi: 10.1099/jmm.0.46792-0

HM Government (2019). Tackling Antimicrobial Resistance 2019–2024. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/784894/UK_AMR_5_year_national_action_plan.pdf (Accessed January 12, 2022).

Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., et al. (2016). The bacterial mobile resistome transfer network connecting the animal and human microbiomes. *Appl. Environ. Microbiol.* 82, 6672–6681. doi: 10.1128/AEM.01802-16

Iwu, C. D., Korsten, L., and Okoh, A. I. (2020). The incidence of antibiotic resistance within and beyond the agricultural ecosystem: a concern for public health. *Microbiology* 9:e1035. doi: 10.1002/mbo3.1035

Keel, B. N., Snelling, W. M., Lindholm-Perry, A. K., Oliver, W. T., Kuehn, L. A., and Rohrer, G. A. (2020). Using SNP weights derived from gene expression modules to improve gwas power for feed efficiency in pigs. *Front. Genet.* 10:1339. doi: 10.3389/fgene.2019.01339

Kerr, I. D., Reynolds, E. D., and Cove, J. H. (2005). ABC proteins and antibiotic drug resistance: is it all about transport? *Biochem. Soc. Trans.* 33:1000. doi: 10.1042/BST20051000

Kirchhelle, C. (2018). Pharming animals: a global history of antibiotics in food production (1935–2017). *Palgrave Communications* 4, 1–13. doi: 10.1057/s41599-018-0152-2

Langmead, B., and Salzberg, S. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Leinonen, R., Sugawara, H., and Shumway, M. (2010). And international nucleotide sequence database collaboration, 2010. the sequence read archive. *Nucleic Acids Res.* 39(suppl_1), D19–D21. doi: 10.1093/nar/gkq1019

Ma, T., McAllister, T. A., and Guan, L. L. (2021). A review of the resistome within the digestive tract of livestock. *J. Anim. Sci. Biotechnol.* 12, 1–20. doi: 10.1186/s40104-021-00643-6

Madden, T. (2008). Appendices. [Updated 2021 Jun 24]. In: BLAST® Command Line Applications User Manual [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2008–. Available at: https://www.ncbi.nlm.nih.gov/books/NBK279684 (Accessed August 4, 2021).

MapChart (2022). Choropleth Map. Available at: https://mapchart.net/ (Accessed February 21, 2022)

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24, 718–733. doi: 10.1128/CMR.00002-11

Ministry of Agriculture of the People's Republic of China (2017). National Action Plan for Restraining Bacteria of Animal Origin (2017–2020). Available at: http://www.moa.gov.cn/govpublic/SYJ/201706/t20170623_5726086.htm (Accessed January 10, 2022).

Nevron Software (2020). MyDraw (5.0.0) [Software]. Available at: https://www.mydraw.com/ (Accessed January 20, 2022).

O'Neill, J. (2016). *Tackling Drug-Resistant Infections Globally: Final Report and Recommendation*. University College, London: The Review on Antimicrobial Resistance.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., and Larsson, D. J. (2016). The structure and diversity of human, animal and environmental resistomes. *Microbiome* 4, 1–15. doi: 10.1186/s40168-016-0199-5

Popowska, M., Rzeczycka, M., Miernik, A., Krawczyk-Balska, A., Walsh, F., and Duffy, B. (2012). Influence of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated resistance genes. *Antimicrob. Agents Chemother.* 56, 1434–1443. doi: 10.1128/AAC.05766-11

Qiao, M., Ying, G. G., Singer, A. C., and Zhu, Y. G. (2018). Review of antibiotic resistance in China and its environment. *Environ. Int.* 110, 160–172. doi: 10.1016/j.envint.2017.10.016

Reyer, H., Metzler-Zebeli, B. U., Trakooljul, N., Oster, M., Muráni, E., Ponsuksili, S., et al. (2018). Transcriptional shifts account for divergent resource allocation in feed efficient broiler chickens. *Sci. Rep.* 8, 1–9. doi: 10.1038/s41598-018-31072-7

RStudio Team (2020). RStudio: Integrated Development for R RStudio. PBC, Boston, MA. Available at: http://www.rstudio.com/ (Accessed April 25, 2022).

Sabino, Y. N. V., Santana, M. F., Oyama, L. B., Santos, F. G., Moreira, A. J. S., Huws, S. A., et al. (2019). Characterization of antibiotic resistance genes in the species of the rumen microbiota. *Nat. Commun.* 10, 1–11. doi: 10.1038/s41467-019-13118-0

Scott, K. P., Melville, C. M., Barbosa, T. M., and Flint, H. J. (2000). Occurrence of the new tetracycline resistance gene tet (W) in bacteria from the human gut. *Antimicrob. Agents Chemother.* 44, 775–777. doi: 10.1128/AAC.44.3.775-777.2000

Sharkey, L. K., Edwards, T. A., and O'Neill, A. J. (2016). ABC-F proteins mediate antibiotic resistance through ribosomal protection. *MBio* 7, e01975–e01915. doi: 10.1128/mBio.01975-15

Shen, Y., Xu, C., Sun, Q., Schwarz, S., Ou, Y., Yang, L., et al. (2018). Prevalence and genetic analysis of mcr-3-positive Aeromonas species from humans, retail meat, and environmental water samples. *Antimicrob. Agents Chemother.* 62, e00404–e00418. doi: 10.1128/AAC.00404-18

Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., et al. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Res.* 24, 1517–1525. doi: 10.1101/gr.168245.113

Spigaglia, P., Barbanti, F., and Mastrantonio, P. (2008). Tetracycline resistance gene tet (W) in the pathogenic bacterium *Clostridium difficile*. *Antimicrob. Agents Chemother.* 52, 770–773. doi: 10.1128/AAC.00957-07

Sun, D., Jeannot, K., Xiao, Y., and Knapp, C. W. (2019). Horizontal gene transfer mediated bacterial antibiotic resistance. *Front. Microbiol.* 10:1933. doi: 10.3389/fmicb.2019.01933

UK-VARSS (2020). *Veterinary Antibiotic Resistance and Sales Surveillance Report (UK-VARSS 2019)*. New Haw, Addlestone: Veterinary Medicines Directorate.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2015). Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci.* 112, 5649–5654. doi: 10.1073/pnas.1503141112

Wang, N., Guo, X., Yan, Z., Wang, W., Chen, B., Ge, F., et al. (2016). A comprehensive analysis on spread and distribution characteristic of antibiotic resistance genes in livestock farms of Southeastern China. *PLoS One* 11.

WHO (2001). *WHO Global Strategy for Containment of Antimicrobial Resistance*. Geneva: WHO.

WHO (2019). Ten Threats to Global Health in 2019. Available at: https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019 (Accessed February 6, 2022).

WHO (2020). Antimicrobial Resistance, Available at: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance (Accessed July 2, 2021).

WHO (2021). *Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2021*. Geneva: WHO.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Winckler, C., and Grafe, A. (2001). Use of veterinary drugs in intensive animal production. *J. Soil. Sediment.* 1, 66–70. doi: 10.1007/BF02987711

Yang, Z., Guo, Z., Qiu, C., Li, Y., Feng, X., Liu, Y., et al. (2016). Preliminary analysis showed country-specific gut resistome based on 1267 feces samples. *Gene* 581, 178–182. doi: 10.1016/j.gene.2016.01.043

Yang, Y. Q., Li, Y. X., Lei, C. W., Zhang, A. Y., and Wang, H. N. (2018). Novel plasmid-mediated colistin resistance gene mcr-7.1 in *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* 73, 1791–1795. doi: 10.1093/jac/dky111

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhao, S., White, D. G., Friedman, S. L., Glenn, A., Blickenstaff, K., Ayers, S. L., et al. (2008). Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006. *Appl. Environ. Microbiol.* 74, 6656–6662. doi: 10.1128/AEM.01249-08

Zheng, B., Huang, C., Xu, H., Guo, L., Zhang, J., Wang, X., et al. (2017). Occurrence and genomic characterization of ESBL-producing, MCR-1-harboring *Escherichia coli* in farming soil. *Front. Microbiol.* 8:2510. doi: 10.3389/fmicb.2017.02510

Zhu, Y. G., Johnson, T. A., Su, J. Q., Qiao, M., Guo, G. X., Stedtfeld, R. D., et al. (2013). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci.* 110, 3435–3440. doi: 10.1073/pnas.1222743110

Zhu, Y. G., Zhao, Y., Zhu, D., Gillings, M., Penuelas, J., Ok, Y. S., et al. (2019). Soil biota, antimicrobial resistance and planetary health. *Environ. Int.* 131:105059. doi: 10.1016/j.envint.2019.105059

Zinsstag, J., Schelling, E., Waltner-Toews, D., and Tanner, M. (2011). From "one medicine" to "one health" and systemic approaches to health and well-being. *Prev. Vet. Med.* 101, 148–156. doi: 10.1016/j.prevetmed.2010.07.003

Zoetis (2016). Treatment-Brochure. https://www.zoetis.co.nz/livestocksolutions/dairywellness/assets/pdfs/15384-lims-iphone-treatment-brochure-135x424_hj.pdf (Accessed February 9, 2022)

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 Lawther, Santos, Oyama, Rubino, Morrison, Creevey, McGrath and Huws. 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 Microbiology

TYPE Original Research
PUBLISHED 22 July 2022
DOI 10.3389/fmicb.2022.885132

Check for updates

OPEN ACCESS

EDITED BY
Ziad Daoud,
Central Michigan University,
United States

REVIEWED BY
Ximin Zeng,
The University of Tennessee, Knoxville,
United States
Evelyn Madoroba,
University of Zululand,
South Africa

*CORRESPONDENCE
Biao Tang
tangbiao@zaas.ac.cn
Baoan Ding
dingbaoan1967@163.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

RECEIVED 30 March 2022
ACCEPTED 04 July 2022
PUBLISHED 22 July 2022

CITATION
Tang B, Wang J, Zheng X, Chang J, Ma J, Wang J, Ji X, Yang H and Ding B (2022) Antimicrobial resistance surveillance of *Escherichia coli* from chickens in the Qinghai Plateau of China. *Front. Microbiol.* 13:885132. doi: 10.3389/fmicb.2022.885132

COPYRIGHT
© 2022 Tang, Wang, Zheng, Chang, Ma, Wang, Ji, Yang and Ding. 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.

Antimicrobial resistance surveillance of *Escherichia coli* from chickens in the Qinghai Plateau of China

Biao Tang[1*†], Jingge Wang[1,2†], Xue Zheng[1], Jiang Chang[3], Jiangang Ma[1], Juan Wang[4], Xiaofeng Ji[1], Hua Yang[1] and Baoan Ding[2*]

[1]State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products, Institute of Agro-product Safety and Nutrition, Zhejiang Academy of Agricultural Sciences, Hangzhou, China, [2]College of Agriculture and Animal Husbandry, Qinghai University, Xining, China, [3]State Key Laboratory of Microbial Metabolism, School of Agriculture and Biology, MOST-USDA Joint Research Center for Food Safety, Shanghai Jiao Tong University, Shanghai, China, [4]College of Veterinary Medicine, Northwest A&F University, Yangling, China

Antimicrobial resistance (AMR) may lead to worldwide epidemics through human activities and natural transmission, posing a global public safety threat. Colistin resistance mediated by the *mcr*-1 gene is the most prevalent among animal-derived *Escherichia coli*, and *mcr*-1-carrying *E. coli* have been frequently detected in central-eastern China. However, animal-derived *E. coli* with AMR and the prevalence of *mcr*-1 in the Qinghai Plateau have been rarely investigated. Herein, 375 stool samples were collected from 13 poultry farms in Qinghai Province and 346 *E. coli* strains were isolated, of which eight carried *mcr*-1. The AMR rates of the *E. coli* strains to ampicillin, amoxicillin/clavulanic acid, and tetracycline were all above 90%, and the resistance rates to ciprofloxacin, cefotaxime, ceftiofur, and florfenicol were above 70%. Multidrug-resistant strains accounted for 95.66% of the total isolates. Twelve *E. coli* strains showed colistin resistance, from which a total of 46 AMR genes and 36 virulence factors were identified through whole-genome sequencing. The *mcr*-1 gene resided on the IncHI2, IncI2-type and IncY-type plasmids, and *mcr*-1 was located in the *nikA-nikB-mcr*-1-*pap2* gene cassette (three strains) or the *pap2-mcr*-1-IS*Apl1* structure (one strain). Completed IncI2-type plasmid pMCR4D31–3 sequence (62,259bp) revealed that it may cause the horizontal transmission of *mcr*-1 and may increase the risk of its spread through the food chain. Taken together, the AMR of chicken-derived *E. coli* in the plateau is of concern, suggesting that it is very necessary for us to strengthen the surveillance in various regions under the background of one health.

KEYWORDS

***Escherichia coli*, antimicrobial resistance, Qinghai Plateau, genome sequences, *mcr*-1**

Introduction

With rising antimicrobial resistance (AMR) to less toxic antibiotics, colistin is used as a backup drug to treat infections caused by carbapenem-resistant Enterobacteriaceae pathogens (Tonny and Anti, 2015; Sarica and Yurttutan, 2018). Due to the unregulated use of colistin in livestock before the antibiotics ban, colistin-resistant Enterobacteriaceae have already become prevalent. The gene *mcr*-1(1,626 bp), which mediates colistin resistance, was first reported on the IncI2-type plasmid of *Escherichia coli* (SHP45; Liu et al., 2016). Subsequently, the *mcr* gene series (*mcr*-1 to *mcr*-10) that mediates colistin resistance in plasmids of enterobacteria has been successively reported in more than 50 countries (Ling et al., 2020; Yang et al., 2020; Pan et al., 2021; Xu et al., 2021). The *mcr*-1 gene is present in various types of *E. coli* plasmids, including IncI, IncX, IncHI, IncP, and IncF (Wang et al., 2017). Of these, IncI2, IncX4, and IncHI2 are common and were the earliest reported plasmid types carrying the *mcr*-1 gene. The IncHI2-type plasmids from different origins are similar to each other and have become the most common effective carrier for the spread of *mcr*-1 in humans, animals, and food (Chang et al., 2020).

Escherichia coli is a common pathogenic bacteria in human infections and has caused various medical and community public safety issues. Manges et al. retrospectively analyzed relevant studies from 1995 to 2018 and found that the epidemiology and population dynamics of *E. coli* are complex and dynamic (Manges et al., 2019). It is worth noting that *E. coli*, as an indicator of the prevalence of Gram-negative bacteria, acts as a rich antibiotic resistance gene pool and a mobile center of resistance gene exchange (Poirel et al., 2018). The high prevalence of *mcr*-1, a colistin resistance gene in *E. coli*, poses a clinical threat, and its diverse genetic mechanisms have jointly promoted its spread and long-term presence (Wang et al., 2018; Zhong et al., 2018). China and Vietnam are the two countries with the most *mcr*-1-positive isolates verified through genome sequencing (Wang et al., 2018). However, studies on *mcr*-1 in China have been mostly concentrated in the central, eastern, and southern regions, while data in the northwestern region, especially in Qinghai Province, are still limited. This disparity may be related to the high altitude, low population density, and low avicultural intensity of Qinghai Province, allowing for poultry farming to be kept away from other human activities in most areas in this region. In the past, studies related to animal-derived *mcr*-1-carrying *E. coli* in Qinghai included four *E. coli* strains isolated from sheep diarrhea samples (Tang et al., 2019b), 14 *E. coli* strains isolated from pig feces specimens, and six extended-spectrum beta-lactamases (ESBLs)-carrying *E. coli* strains found in pigs and yaks in Xining and Haidong, respectively (Tong et al., 2018; Wang et al., 2020). Of the above-described resistance gene-carrying *E. coli* strains, only the sheep-derived *E. coli* strain has been fully genome sequenced. Moreover, animal-derived drug-resistant *E. coli* strains in this region have rarely been reported, and the scattered published results are insufficient for comprehensively understanding animal-derived AMR in Qinghai Province and its risks.

Surveillance of AMR in China is mainly in developed provinces and rarely involved in remote areas. Qinghai Province represents a unique geographical unit and is located in the hinterland of the Qinghai-Tibet Plateau, which is known as the third pole of the earth and the roof of the world. As one of the areas in China least disturbed by human activities, Qinghai is the origin of three rivers, i.e., the Yangtze River, the Yellow River, and the Lancang River, and has rich biodiversity (Wu et al., 2016). From 2012 to 2013, an *E. coli* strain obtained from wild *Marmota himalayana* in Yushu, Qinghai, was found to be sensitive to 23 antibiotics including penicillins, cephalosporins, and carbapenems (Bai et al., 2016; Lu et al., 2016). However, all *E. coli* strains obtained from wild plateau pika (*Ochotona curzoniae*) in Qinghai Province in 2016 were penicillin-resistant and some strains were cephalosporin- and streptomycin-resistant (Bai et al., 2016). Therefore, the evaluation of the AMR of zoonotic *E. coli* in Qinghai Province can help us understand the status of antibiotic pollution in animal husbandry and apicultural environments and provide references for formulating animal husbandry and natural environmental protection policies in this unique area.

In this study, we collected fresh stool samples from chickens of different varieties and different ages under different farming environments in Qinghai Province (at altitudes of ~2,500–4,000 m) from June 2020 to July 2021 and analyzed drug-resistant *E. coli* strains in the samples to address the inadequacy of previous studies on drug-resistant bacteria of animal origin in Qinghai Province. We performed whole-genome sequencing on the colistin-resistant *E. coli* isolates to reveal the prevalence of the animal-derived AMR gene *mcr*-1 and its transmission patterns in the environment.

Materials and methods

Isolate information

From June 2020 to July 2021, 375 cloaca stool samples were collected from 13 chicken farms in Qinghai Province (Figure 1A; Supplementary Table 1). Then, they were cultured in 5 ml of sterile buffered peptone water (BPW) at 37°C for enrichment, and then sequentially cultured in MacConkey agar (MacConkey, MAC) and eosin methylene blue (EMB) agar medium for identification. Putative *E. coli* colonies were selected and cultured on Luria broth (LB) plates and purified through three rounds of streaking, from which single *E. coli* colonies were determined through Bruker time-of-flight mass spectrometry (MALDI-TOF MS, Germany; Kostrzewa, 2018; Tang et al., 2021). The identified *E. coli* strains were stored at −80°C in a freezer in a 25% glycerol solution.

Antimicrobial susceptibility testing

All isolated strains of *E. coli* were tested for antibiotic resistance using the standard broth dilution method according to the guidelines

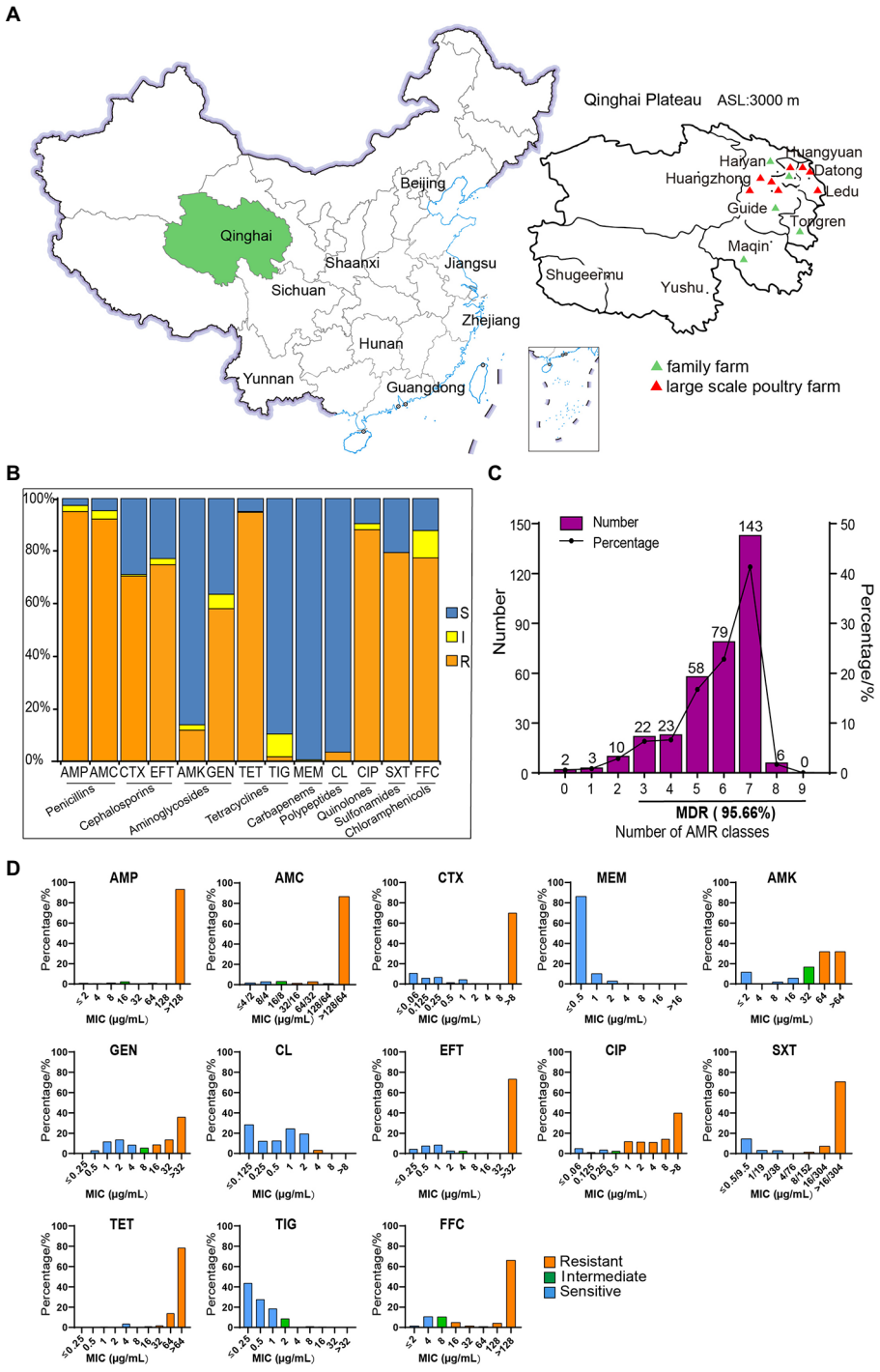


FIGURE 1
The sampling locations and susceptibility test results of 346 strains of *Escherichia coli* to 13 antibiotics. **(A)** Sampling areas in Qinghai Province, China. **(B)** AMR rates of *E. coli*. **(C)** The distribution of multidrug-resistant strains. **(D)** MIC distributions of 13 antibiotics in isolated *E. coli* strains.

of the Clinical and Laboratory Standards Institute (CLSI). The minimal inhibitory concentration (MIC) of 13 antibiotics was detected using a drug susceptibility panel for Gram-negative aerobic bacteria. The panel of antimicrobial compounds tested included ampicillin (AMP; 2–128 μg/ml), amoxicillin/clavulanate potassium (AMC; 4/2–128/64 μg/ml), cefotaxime (CTX; 0.06–8 μg/ml), meropenem (MEM; 0.5–16 μg/ml), amikacin (AMK; 2–64 μg/ml), gentamicin (GEN; 0.25–32 μg/ml), colistin (CL; 0.125–8 μg/ml), ceftiofur (EFT; 0.25–32 μg/ml), ciprofloxacin (CIP; 0.06–8 μg/ml), sulfamethoxazole (SXT; 9.5–304 μg/ml), tetracycline (TET; 0.25–64 μg/ml), tigecycline (TIG; 0.25–32 μg/ml), and florfenicol (FFC; 2–128 μg/ml; Tang et al., 2020).

To determine the bacterial viability of *E. coli*, serially diluted mid-log cultures were spotted on LB plates supplemented with different concentrations of colistin (0, 0.25, 0.5, 1, 2, 4, 8, and 16 μg/ml) and incubated at 37°C for 12 h. The *E. coli* strain ATCC 25922 was used as a negative control (Tang et al., 2019a).

A MIC test strip (Liofilchem®, Italy) was used to detect the MIC value of colistin-resistant *E. coli* and strain ATCC 25922 in this experiment. A sterile cotton swab was immersed in 0.5 McFarland standard suspension, which was then spread evenly on the MH (OXOID, England) agar medium (Tang et al., 2019a; Chang et al., 2020).

Whole-genome sequencing, identification of antimicrobial resistance genes, multilocus sequence typing analysis, virulence factors, and plasmid replicon types

DNA was extracted from strains of *E. coli* isolates showing colistin resistance using a commercially available bacterial genomic DNA isolation kit (Generay, China), according to the manufacturer's instructions. The concentration of DNA in the extracted samples was detected with a NanoDrop 2000 spectrophotometer (Thermo Fisher, United States). The presence of the *mcr*-1 gene in *E. coli* isolates was detected by amplification using *mcr*-1-specific primers (Chang et al., 2020).

Indexed Illumina sequencing libraries were prepared using a TruSeq DNA PCR-free sample preparation kit (Illumina Inc., San Diego, CA) following the standard protocol, and samples were sequenced on the Illumina NovaSeq 6,000 platform according to the manufacturer's protocols, thus producing 150-bp paired-end reads. The sequence was assembled with a CLC Genomics Workbench 12. Libraries of genomic DNA of representative strain were prepared by SQK-LSK109 kit (Oxford Nanopore Technologies, United Kingdom) and sequenced using R9.4 flow cell technology on a GridION sequencer. The reads were assembled using Unicycler 0.4.8. The NCBI Prokaryotic Genome Annotation Pipeline was used for gene prediction and annotation of the genome.

Antimicrobial resistance genes were predicted using ResFinder 4.1 software (Zankari et al., 2012).[1] Virulence factors were predicted using Virulence Finder 2.0 software[2] and the virulence factor database.[3] Plasmid replicon types were predicted using PlasmidFinder 2.1 software.[4] Multilocus sequence typing (MLST) was carried out as previously described using MLST 2.0 software.[5] The BLAST Ring Image Generator (BRIG) and Easyfig software were used in the comparative analysis of plasmids (Alikhan et al., 2011; Sullivan et al., 2011).

1 https://cge.cbs.dtu.dk/services/ResFinder/
2 https://cge.cbs.dtu.dk/services/VirulenceFinder/
3 http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi
4 https://cge.cbs.dtu.dk/services/PlasmidFinder/
5 https://cge.cbs.dtu.dk/services/MLST/

S1-pulsed-field gel electrophoresis, Southern blotting, and conjugation transfer

The *Salmonella enterica* serovar Braenderup H9812 (as size marker) and *E. coli* cells harboring the *mcr-1* gene were fixed by SeaKem Gold Agarose (Lonza Group AG, United States) and subsequently lysed. The embedded DNAs were digested using *Xba* I or S1 nuclease (Takara Bio, China) in a 37°C water bath for 3 h. The restricted DNA fragments were separated in 0.5 × Tris-Borate-EDTA buffer (Sangon Biotech, Shanghai, China) at 14°C for 18 h using a pulsed-field electrophoresis system (CHEF Mapper, Bio-Rad, United States) with pulse times of 2.2 to 63.8 s. The gel block was stained and observed with a gel imager (Tang et al., 2019a; Liang et al., 2021b). The *mcr-1*-specific probe was labeled using a DIG High Prime DNA Labeling and Detection Starter Kit (Roche, Sant Cugat del Vallés, Spain) following the manufacturer's instructions. *E. coli* J53 was used as the recipient strain, and *E. coli* strains harboring *mcr-1* were used as the donor strain in the conjugation transfer assay. Donor and recipients bacteria were grown together overnight and then inoculated onto LB plates with colistin and sodium azide.

Statistical analysis

The error values of conjugation transfer efficiency were calculated by GraphPad Prism software. The heatmap of clustering of strains and AMR phenotypes were performed by TBtools. In this analysis, the presence of the above resistance phenotype received a score of 2, intermediate received a score of 1, and the susceptibility received a score of 0.

Results

Isolation of *Escherichia coli* and antimicrobial susceptibility

A total of 346 strains of *E. coli* were isolated and identified from 375 stool samples (Figure 1A) collected in Qinghai Province, with an isolation rate of 92.27%. The sensitivity test results of 346 of the *E. coli* strains to 13 antibiotics are shown in Figures 1B,C. In terms of the MIC distribution (Figure 1D), the MIC values of the antibiotics AMP, AMC, CIP, CTX, EFT, TET, and FFC were significantly higher, indicating that the isolated *E. coli* strains are highly resistant to these antibiotics. Among these, AMP, AMC, and TET showed the highest resistance rates of over 90% and were followed by that of CIP, at 88.15%, while CTX, EFT, and FFC exhibited resistance rates greater than 70%. Among the isolates, strains with multidrug resistance (MDR) (resistance to more than three kinds of antibiotics) accounted for 95.66% of the total isolates. The tested strains showed a high resistance rate to GEN (58.09%) and a low resistance rate to AMK (11.85%). In addition,

their resistance rates to colistin and TIG were 3.47% and 1.73%, respectively. All of the tested *E. coli* strains were sensitive to MEM. These results indicate that the AMR of chicken-derived *E. coli* in Qinghai Province is serious.

In this study, *E. coli* isolates showed a broad spectrum of resistance to most antibiotics, as shown in Table 1; Figure 2, among which the AMP-AMC-CTX-GEN-EFT-CIP-SXT-TET-FFC-resistant phenotype was the highest proportion (32.08%) out of a total of 111 strains. Among the 346 *E. coli* isolates, only two (0.58%) were sensitive to all of the antibiotics assayed, and the rest exhibited 81 types of resistance across a spectrum. Six of these strains (1.73%) showed resistance to eight of the tested antibiotic categories, with two types of resistance, AMP-AMC-CTX-AMK-GEN-CL-EFT-CIP-SXT-TET-FFC-resistant (four strains) and AMP-AMC-CTX-GEN-CL-EFT-CIP-SXT-TET-FFC-resistant (two strains). One hundred forty-three strains (41.33%) showed resistance to seven of the tested antibiotic categories, with seven types of resistance. Seventy-nine strains (22.83%) showed resistance to six of the tested antibiotic categories, with 18 types of resistance. In the multidrug resistance assay, 95.66% of the isolates showed resistance to the 13 tested antibiotics, indicating that in Qinghai Province, multidrug-resistant chicken-derived *E. coli* strains are common and have diverse and wide AMR spectra.

Through the agar dilution method (Figure 3A), we found that the control strain ATCC 25922, could grow normally on plates without colistin but could not grow on plates with colistin ($\geq$0.25 μg·ml^{-1}). With 8 μg·ml^{-1} of colistin, the growth of QH20-4D3-1, QH20-5 T-2, QH21-3-15, QH21-5-14, QH21-2-13, QH21-3-13, and QH21-7-8 was completely inhibited.

The colistin resistance of eight *mcr*-1-carrying *E. coli* strains was verified with the E-test strip: (0.016–256 μg·mL^{-1} of colistin; Figure 3B). The MIC of the negative control strain ATCC 25922 was 0.75 μg·ml^{-1}, while those of the *mcr*-1-carrying *E. coli* strains were all $\geq$4 μg·ml^{-1}, demonstrating colistin resistance.

Distribution of antimicrobial resistance genes, STs, virulence factors, and plasmid replicon types of *Escherichia coli* isolates

We sequenced the genomes of all 12 colistin-resistant *E. coli* isolates, from which 46 AMR genes were predicted, as shown in Figure 4A. The complete genome sequence of strain QH20-4D3-1 was performed by third-generation sequencing. Among them, 15 aminoglycoside resistance genes [*aac*(*3*)-*IIa*, *aac*(*3*)-*IV*, *aadA1*, *aadA2*, *aadA5*, *aadA16*, *aadA22*, *aadA24*, *aadA2b*, *aph*(*3′*)-*Ia*, *aph*(*3′*)-*IIa*, *aph*(*3″*)-*Ib*, *aph*(*4*)-*IA*, *aph*(*6*)-*Id*, and *rmtB*], eight beta-lactam resistance genes ($bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-64}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{OXA-10}}$, $bla_{\text{TEM-141}}$, $bla_{\text{TEM-206}}$, $bla_{\text{TEM-214}}$, $bla_{\text{TEM-1B}}$, and $bla_{\text{TEM-1C}}$), seven folate antagonist pathway resistance genes (*dfrA12*, *dfrA14*, *dfrA17*, *dfrA27*, *sul1*, *sul2*, and *sul3*), four fluoroquinolone resistance genes (*oqxA*, *oqxB*, *qnrS1*, and *qnrS2*), one lincosamide resistance gene [*lnu*(F)], two macrolide resistance genes [*mdf*(A) and *mph*(A)], one peptide resistance gene (*mcr*-1), two phenol resistance genes (*cmlA1* and *floR*), one fosfomycin resistance gene (*fosA3*), two rifampicin resistance genes (*arr*-2 and *arr*-3), and two TET resistance genes [*tet*(A) and *tet*(M)] were detected. These strains were predicted to have nine different ST types (ST29735, ST43, ST10, ST93, ST162, ST2329, ST189, ST4689, and ST259; Supplementary Table 2), indicating that the *E. coli* isolates in this study are highly diverse. 8 of these 12 strains harbored the *mcr*-1 gene.

A total of 36 virulence factors (Figure 4B) and 19 plasmid replicons (Figure 4C) were predicted from the 12 colistin-resistant *E. coli* isolates. Among them, the tellurium ion resistance virulence gene *terC* was found in all 12 strains, and the virulence gene *astA*, which is related to the EAST-1 heat-stable toxin and is a typical marker for entero-aggregating *E. coli* (EAEC), was found in QH20-4B1-2, QH20-2B4-2, QH20-4D2-1, QH20-2 T-1, and QH21-7-8. The plasmid replicators were as follows: Col156, Col8282, Col (BS512), IncB/O/K/Z, IncFIB (AP001918), IncFII (29), IncFII (pCoo), IncFII (pSE11), IncFII (pHN7A8), IncHI2, IncHI2A, INCI (Gamma), IncI2-type (Delta), IncN, INCR, IncX1, IncY, IncQ1, and po111. In the eight *mcr*-1-containing colistin-resistant isolates, the *mcr*-1 gene was located on the IncHI2-, IncI2-, or IncY-type plasmid (Figure 4C). Plasmids carrying the *mcr-1* gene were verified by S1-PFGE and Southern blot (Supplementary Figure 1). The conjugation transfer frequency was ~10^{-2}–10^{-6} for four strains QH20-4D2-1, QH21-5-14, QH20-4D3-1, and QH20-5 T-2, with QH20-4D2-1 exhibiting the highest frequency $(2.97 \pm 0.15) \times 10^{-2}$. Whereas, the *mcr*-1-harboring plasmid in strains QH21-3-15, QH20-4B1-2, QH20-2B4-2, and QH20-5D4-1 were not found to be conjugatively transferred (Supplementary Figure 2).

In QH20-2 T-1, the *etsC, hlyF,* and *ompT* virulence genes all resided on the replicon of the IncFIB-type plasmid. The virulence gene *etpD* was also predicted to be on the IncFIB-type plasmid replicon of QH21-2-13 and QH21-3-13, while the virulence gene *traT* was predicted to be on the IncFII replicon of Plasmid 4 of QH21-3-13 and QH20-4D3-1. The virulence genes *traT* and *ompT* are related to the activity of biofilm outer membrane proteins, *etsC* confers resistance to the serum complement, and the *ets* operon and *ompT* and *hlyF* are related to the conserved virulence region of the FIB replicon (Moran and Hall, 2018). Therefore, different types of plasmid replicons facilitate the horizontal spread of virulence genes.

Sequence alignment analysis of homologous IncI2-type and IncHI2-type plasmids carrying the *mcr*-1 gene

In different isolates, the contig on which *mcr*-1 resides ranged from 11,011–123,144 bp in size. In QH21-5-14, the contig that contains *mcr*-1 was too short, making it impossible to analyze the sequence. In the other seven *E. coli* isolates, the *mcr*-1 gene was located on the IncI2-type (4 strains) or

10.3389/fmicb.2022.885132

TABLE 1 Antimicrobial resistance profiles of 346 *Escherichia coli* strains.

ID	Antibiotic resistance pattern	Number	Percentage (%)
1	AMP-AMC-CTX-GEN--EFT-CIP-SXT-TET-FFC	111	32.08
2	AMP-AMC-CTX-EFT-CIP-SXT-TET-FFC	40	11.56
3	AMP-AMC-CIP-SXT-TET-FFC	21	6.07
4	AMP-AMC-CTX-AMK-GEN-EFT-CIP-SXT-TET-FFC	19	5.49
5	AMP-AMC-CTX-EFT-CIP-TET-FFC	7	2.02
6	AMP-AMC-EFT-CIP-SXT-TET-FFC	7	2.02
7	AMP-AMC-SXT-TET	7	2.02
8	AMP-AMC-TET	7	2.02
9	AMP-AMC-CTX-GEN-EFT-CIP-TET-FFC	5	1.45
10	AMP-AMC-GEN-CIP-SXT-TET-FFC	5	1.45
11	AMP-AMC-CTX-AMK-GEN-CL-EFT-CIP-SXT-TET-FFC	4	1.16
12	AMP-AMC-CTX-AMK-GEN-EFT-CIP-TET-FFC	4	1.16
13	AMP-AMC-CTX-GEN-EFT-CIP-SXT-TET	4	1.16
14	AMP-AMC-CTX-EFT-CIP-SXT-TET	4	1.16
15	AMP-AMC-GEN-EFT-CIP-FFC	4	1.16
16	AMP-AMC-GEN-CIP-SXT-TET	4	1.16
17	AMP-CTX-GEN-EFT-CIP-SXT-TET-FFC	4	1.16
18	GEN-CIP-TET	4	1.16
19	AMP-AMC-CTX-GEN-EFT-CIP-SXT-TET-TIG-FFC	3	0.87
20	AMP-AMC-CTX-AMK-GEN-EFT-CIP-SXT-TET	3	0.87
21	AMP-AMC-CTX-EFT-SXT-TET-FFC	3	0.87
22	AMP-AMC-EFT-CIP-SXT-TET	3	0.87
23	AMP-AMC-CIP-SXT-TET	3	0.87
24	AMP-AMC-CIP-TET-FFC	3	0.87
25	AMP-AMC-CIP-TET	3	0.87
26	AMP-AMC-CTX-GEN-CL-EFT-CIP-SXT-TET-FFC	2	0.58
27	AMP-AMC-GEN-EFT-CIP-SXT-TET-FFC	2	0.58
28	AMP-AMC-CTX-EFT-TET-FFC	2	0.58
29	AMP-AMC-CTX-EFT-CIP-TET	2	0.58
30	AMP-AMC-CTX-EFT-TET	2	0.58
31	AMP-AMC-EFT-CIP-FFC	2	0.58
32	--	2	0.58
33	AMP-AMC-CTX-GEN-CL-EFT-CIP-SXT-TET	1	0.29
34	AMP-AMC-CTX-CL-EFT-CIP-SXT-TET-FFC	1	0.29
35	AMP-AMC-CTX-EFT-CIP-SXT-TET-TIG-FFC	1	0.29
36	AMP-AMC-CTX-GEN-CIP-SXT-TET-FFC	1	0.29
37	AMP-AMC-CTX-GEN-EFT-SXT-TET-FFC	1	0.29
38	AMP-AMC-AMK-GEN-EFT-CIP-SXT-FFC	1	0.29
39	AMP-AMC-GEN-CL-CIP-SXT-TET-FFC	1	0.29
40	AMC-CTX-AMK-GEN-EFT-CIP-SXT-TET	1	0.29
41	AMP-AMC-CTX-CL-EFT-CIP-TET-FFC	1	0.29
42	AMP-AMC-CTX-EFT-CIP-TET-TIG	1	0.29
43	AMP-AMC-CTX-CIP-SXT-TET-FFC	1	0.29
44	AMP-AMC-GEN-EFT-CIP-SXT-FFC	1	0.29
45	AMC-CTX-AMK-GEN-CIP-SXT-TET	1	0.29
46	CTX-AMK-GEN-EFT-CIP-SXT-TET	1	0.29
47	AMP-AMC-CTX-GEN-CIP-TET-FFC	1	0.29
48	AMP-AMC-CTX-GEN-EFT-CIP-TET	1	0.29
49	AMP-CTX-AMK-GEN-EFT-CIP-TET	1	0.29
50	AMP-CTX-GEN-EFT-CIP-SXT-TET	1	0.29

(Continued)

TABLE 1 Continued

ID	Antibiotic resistance pattern	Number	Percentage (%)
51	AMK-GEN-EFT-CIP-SXT-TET	1	0.29
52	CTX-AMK-GEN-CIP-SXT-TET	1	0.29
53	AMP-GEN-EFT-CIP-SXT-FFC	1	0.29
54	AMP-CTX-EFT-SXT-TET-FFC	1	0.29
55	AMP-CTX-EFT-CIP-SXT-TET	1	0.29
56	AMP-AMC-GEN-CIP-TET-FFC	1	0.29
57	AMP-AMC-GEN-CIP-TET-TIG	1	0.29
58	AMP-AMC-GEN-EFT-CIP-TET	1	0.29
59	AMP-AMC-AMK-CIP-TET-FFC	1	0.29
60	AMP-AMC-AMK-CIP-SXT-TET	1	0.29
61	AMP-AMC-CTX-EFT-SXT-TET	1	0.29
62	AMP-AMC-CTX-EFT-CIP-SXT	1	0.29
63	AMP-AMC-GEN-CIP-FFC	1	0.29
64	AMP-AMC-EFT-CIP-TET	1	0.29
65	AMP-AMC-SXT-TET-FFC	1	0.29
66	AMP-CTX-AMK-GEN-TET	1	0.29
67	AMC-CTX-CIP-TET-FFC	1	0.29
68	CTX-AMK-GEN-SXT-TET	1	0.29
69	AMP-CTX-CIP-TET	1	0.29
70	AMP-AMC-TET-FFC	1	0.29
71	AMP-AMC-CL-TET	1	0.29
72	AMC-CTX-CIP-TET	1	0.29
73	AMP-AMC-EFT	1	0.29
74	AMP-SXT-TET	1	0.29
75	AMP-CL-TET	1	0.29
76	AMP-GEN-TET	1	0.29
77	AMP-AMC-FFC	1	0.29
78	AMC-SXT-TET	1	0.29
79	AMP-AMC	1	0.29
80	AMP-FFC	1	0.29
81	TET	1	0.29
82	SXT	1	0.29

IncHI2-type (1 strain) plasmid. Here, we selected the sequences of the *mcr*-1-carrying IncI2-type plasmid pHNSHP45 (Acc. No. KP347127) and the IncHI2-type plasmid pHNSHP45-2 (Acc. No. KU341381) that were the earliest to be reported for sequence alignment analysis.

As shown in Figure 5A, the alignment of the IncI2-type plasmid pHNSHP45 sequence with the sequences of plasmids pMCR4D31–3 in QH20-4D3-1, QH21-3-15, QH20-5 T-2, QH20-4D2-1, and QH20-4B1-2 (123.144 kp, GC content: 42.6%, CP085520), QH21-3-15 contig_75 (≈59.073 kp, GC content: 42.2%, JAJDKL010000075), QH20-5 T-2 contig_2 (≈56.045 kp, GC content: 42.2%, JAJDKK010000002), QH20-4D2-1 contig_81 (32.841 kp, GC content: 42.6%, JAJDKI010000081), and QH20-4B1-2 (≈11.011 kp, GC content: 42.3%, JAJDKH010000139) indicates that all sequences were highly homologous, contained the backbone of the typical IncI2-type plasmid, and included the replication-related gene (*repA*), conjugative transfer-related pilus gene cluster genes (*tra*, *pil*), and the plasmid stability-associated gene (*parA*). The backbone region of the IncI2-type plasmid is conserved, containing a shufflon region led by the site-specific recombinase gene (*rci*; Sekizuka et al., 2017). Moreover, upstream of *mcr*-1, all sequences lacked the insertion element IS*Apl1*. The *pap2* gene was always present downstream of *mcr*-1 except for QH20-4D2-1 contig_81 (JAJDKI010000081), which contained $bla_{\text{CTX-M-64}}$, an independent resistance region of the ESBL resistance gene. This region was located in IS*Ecp1*-$bla_{\text{CTX-M-64}}$-*Δorf477*, a typical transposon unit, of which the IS*Ecp1* insertion sequence can mediate the transposition of many types of the $bla_{\text{CTX-M}}$ gene (Zhao et al., 2020). Except in the case of QH20-4B1-2 contig_139 (JAJDKH010000139), the resistance gene *mcr*-1 was integrated at a location downstream to the *nikB* gene, which is identical to that of the majority of *mcr*-1-positive

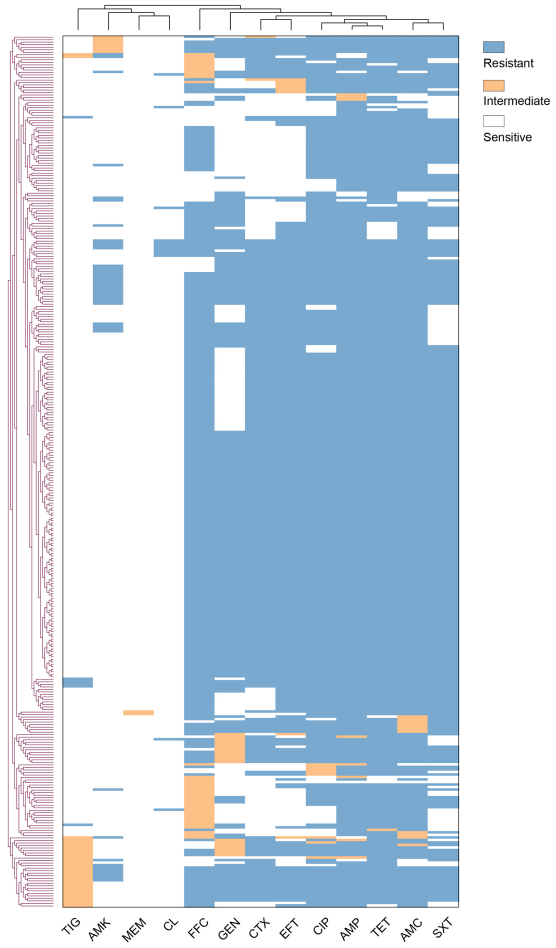


FIGURE 2
Antimicrobial resistance profiles of 346 *Escherichia coli* strains. Vertical and horizontal trees represent clustering relationships. The abscissa is the antibiotic, and the ordinate is the *E. coli* isolate.

IncI2-type plasmids reported previously. The other five sequences all lacked the IS*683* insertion sequence present in pHNSHP45, while two IS*2* insertion elements were present in the transposase genes *insD* and *insC* downstream of *mcr*-1 in pMCR4D31–3. In pMCR4D31–3, QH21-3-15 contig_75 (JAJDKL010000075) and QH20-5 T-2 contig_2 (JAJDKK010000002), a *virB*/*virD4* secretion system similar to that of pHNSHP45 was present.

As shown in Figure 5B, the sequence alignment of the IncHI2-type plasmid pHNSHP45-2 and QH20-5D4-1 contig_94 (≈107.905 kp, GC content: 44.0%, JAJDKJ010000094) and QH20-2B4-2 contig_135 (≈38.480 kp, GC content: 49.1%, JAJDKG010000135) indicates that the three sequences were highly homologous. In the two IncHI2-type *mcr*-1-containing plasmids obtained in this study, the pHNSHP45-2 toxin-antitoxin system (*higB* and *hipB-A*) and the region mediating resistance to heavy metal tellurium (*terDDCBAD*) were present. QH20-5D4-1 contig_94 (JAJDKJ010000094) carried four partition-associated genes (*parR*, *parM*, *parB*, and *parA*) and 16 plasmid-independent conjugation genes (*traN*, *traU*, *traC*, *traV*, *traB*, *traK*, *traE*, *traL*, *htdO*, *htdW*, *orf4*, *orf17*, *orf16*, *trhP*, *trhO*, and *trhZ*). Moreover, in all cases, the *pap2* gene was present downstream; however, in QH20-2B4-2 contig_135 (JAJDKG010000135), the insertion element IS*Apl1* present in QH20-5D4-1 contig_94 was absent. In the QH20-2B4-2 contig_135 sequence, the 3-terminus of *mcr*-1 was incomplete, and we were therefore unable to obtain information about the genetic elements of plasmid replicon. However, in the QH20-5D4-1 contig_94 sequence, the *pap2-mcr*-1-IS*Apl1* structure that mediates the horizontal transfer of *mcr*-1 and is present in pHNSHP45-2 was observed.

Analysis of IncI2-type *mcr*-1-carrying plasmids from different sources

For sequence alignment analysis, we selected nine plasmids from the NCBI database with the highest homology to the sequence of pMCR4D31–3 carried in strain QH20-4D3-1 (Figure 6) and found that the homology between pMCR4D31–3 and all the selected plasmids was over 99% (Supplementary Table 3). Among the selected plasmids, pEC13-1 was from a water sample in a Malaysian pond (Yu et al., 2017), p2018-10-2CC was from a fecal sample of healthy residents in a rural community in Vietnam (Yamaguchi et al., 2020), p1106-IncI2-type and pAH62-1 were from poultry meat samples of Anhui Province, China (Liu et al., 2019; Yin et al., 2021), pHLJ109-11 was from the cecal content of chickens that had died of illness in Heilongjiang Province, China (Li et al., 2020), and the other four plasmids, pColR644SK1, pL889-MCR1, p5CRE51-MCR-1, and p778, were from patients from Switzerland, Zhejiang, China, Taiwan, China, and Ecuador, respectively (Zurfluh et al., 2017; Lin et al., 2019; Loayza-Villa et al., 2020; Liang et al., 2021a). Unfortunately, the patient from which p5CRE51-MCR-1 was isolated died of illness despite treatment. Seven plasmids, including pMCR4D31–3, all contained the *nikA-nikB-mcr*-1-*pap2* structure. Among the other three plasmids, pColR644SK1 and pEC13-1 (from Switzerland and Malaysia, respectively) lacked the *nikA* gene in the *mcr*-1 cassette; p2018-10-2CC only contained the *pap2* gene in sequences flanking the *mcr*-1 gene. Overall, the *mcr*-1 gene in pMCR4D31–3 showed a genetic neighborhood similar to that of the IncI2-type plasmids from various origins, emphasizing the feasibility of horizontal transmission.

Discussion

In the 21st century, antimicrobial resistance is considered to be one of the most serious global threats to human health. The AMR of *E. coli* is closely associated with the abuse of antibiotics, with a high rate of resistance to antibiotics that have been used for a long time in poultry farming but a very low rate of resistance to antibiotics that are prohibited or restricted in poultry farming (Rhouma et al., 2016; Shen et al., 2016). Poultry farming varies

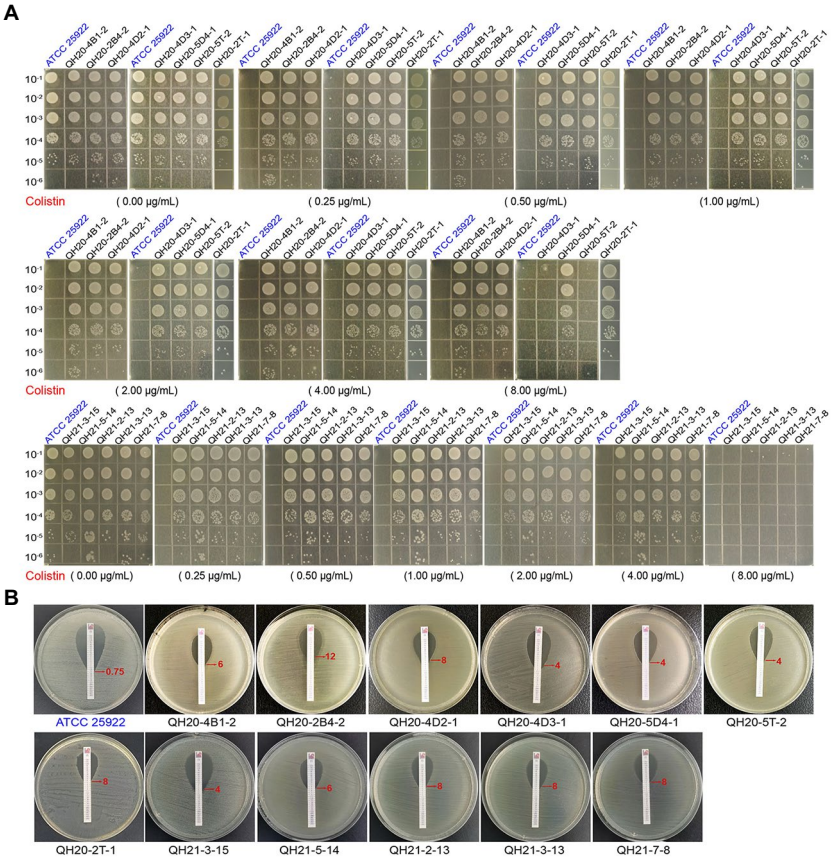


FIGURE 3
The insusceptibility of *mcr*-1-harboring *Escherichia coli* strains to colistin. **(A)** Bacterial viability of *E. coli* strains on LB plates containing different concentrations of colistin. The diluted suspension (10μl) was inoculated onto the prepared LB plates. *Escherichia coli* ATCC 25922 was used as a negative control. **(B)** E-test to compare the MIC of colistin in *E. coli* strains. *Escherichia coli* ATCC 25922 was used as a negative control.

profoundly in different regions of China in terms of scale and method, and the use of antibiotics also varies greatly among regions due to differences in the geographic environment and farming methods. In this study, we found that the antibiotic resistance rate of *E. coli* in poultry farms of Qinghai Province was high, with a resistance rate of 3.47% to colistin and a resistance rate of 1.73% to TIG, and all the *E. coli* isolates in this study were sensitive to MEM. Although poultry farming in Qinghai Province is smaller in scale, shorter in history, and has a more independent poultry farming environment, the drug-resistant *E. coli* in Qinghai is as serious as that in inland areas, suggesting antibiotic abuses in poultry farming in this region. Given that Qinghai Province has a small population and a small number of poultry farms in the western region, and the samples of this study were mostly collected from the eastern areas of the province, the data were limited, and more follow-up monitoring is needed on the drug-resistant *E. coli* strains from chickens in China's plateau region.

Colistin resistance derived from the horizontal transfer of the *mcr*-1 gene has led to an epidemic risk of colistin-resistant bacteria (Wang et al., 2017). In 8022 stool samples, Zhong et al. found a *mcr*-1 positivity rate of up to 6.2%. It is the diverse genetic mechanisms of the *mcr*-1-carrying IncI and IncHI2/HI2A plasmids that cause the high horizontal transfer rate of *mcr*-1, e.g., the *mcr*-1-related insertion element IS*Apl1* (1,070 bp; Zhong et al., 2018), which was originally discovered in *Actinobacillus pleuropneumoniae* (Tegetmeyer et al., 2008), is the most important mobile element of the IS*30* family mediating the spread of *mcr*-1. The Tn*6330* transposon (IS*Apl1*-*pap2*-*mcr*-1-IS*Apl1*) can mediate the horizontal transfer of *mcr*-1, in which IS*Apl1* forms a ring-shaped intermediate in a "cut-and-paste" manner to mediate the spread of *mcr*-1 among different types of plasmids (Girardello et al., 2021). In the QH20-5D4-1 isolate, the transposon element IS*Apl1* was present downstream of *mcr*-1, and the *pap2* gene present upstream of *mcr*-1 encodes the phospholipase closely related to the horizontal transmission of *mcr*-1 (Ma et al., 2021), both playing a very important role in *mcr*-1 mediated bacterial colistin resistance (Choi et al., 2020). Although we did not observe the Tn*6330* transposon unit that mediates the rapid spread of the *mcr*-1 gene in the poultry farms of Qinghai Province, the insertion element IS*Apl1* and the *pap2* gene neighboring *mcr*-1 similarly mobilized the plasmid-mediated horizontal spread of the *mcr*-1 gene.

With the exception of QH20-4D2-1 contig_81 (JAJDKI010000081), all the sequences of the *mcr*-1-carrying *E. coli*

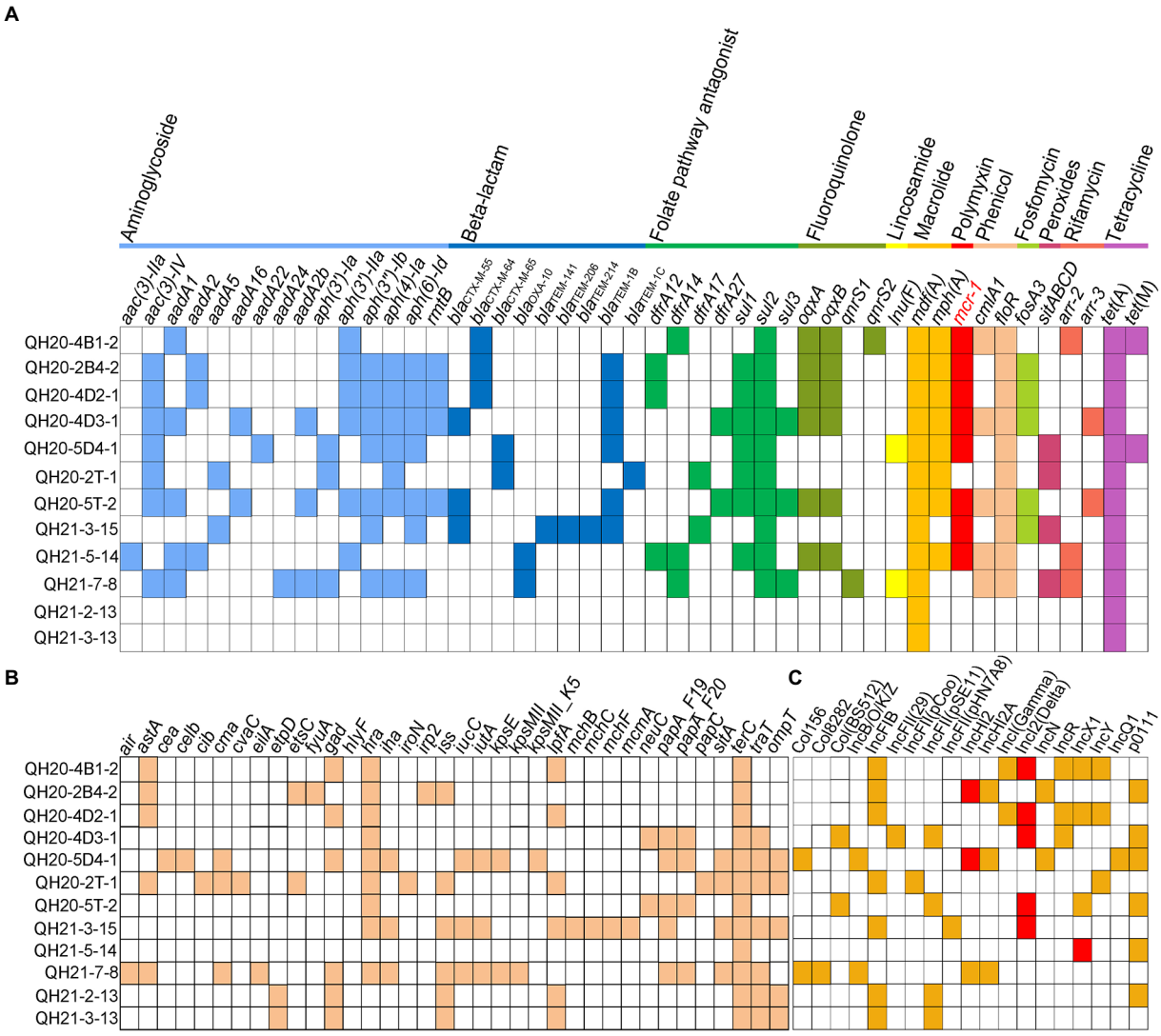


FIGURE 4
Acquired resistance genes, virulence genes, and plasmid replicon types of *Escherichia coli* strains with whole-genome sequences. **(A)** Antimicrobial resistance (AMR) gene profiles. Various types of AMR genes are labeled with different colors. White squares indicate no AMR genes. **(B)** Virulence gene profiles. Colored squares represent virulence genes, and white squares indicate no virulence genes. **(C)** Plasmid replicon profiles. Colored squares represent the presence of plasmids, and white squares indicate no plasmids.

isolates contained the *pap2* gene nearby; this gene is associated with colistin resistance and the horizontal transmission of *mcr*-1. However, only in QH20-5D4-1 contig_94 (JAJDKJ010000094), the *pap2-mcr*-1-IS*Apl1* structure was present near *mcr*-1, and Wang et al. noted that the missing IS*Apl1* insertion sequence is a remanent of the frequent shuffling of the *pap2-mcr*-1-IS*Apl1* structure (Wang et al., 2017). It is worth noting that in the QH20-4D2-1 contig_81 sequence downstream to *mcr*-1, the ESBL gene $bla_{\text{CTX-M-64}}$, which is capable of degrading the third and fourth generations of cephalosporins, was observed. This gene also mediates resistance to amoxicillin, AMP, aztreonam, cefepime, CTX, ceftazidime, ceftriaxone, piperacillin, ticarcillin, and other antibiotics. This gene is located in IS*Ecp1*-$bla_{\text{CTX-M-64}}$-*Δorf477*, a typical transposon unit, in which IS*Ecp1* and IS*Apl1* are two important insertion sequences for the horizontal transfer of *mcr*-1 (Wang et al., 2017). In 2016, the co-occurrence of $bla_{\text{CTX-M}}$ and *mcr*-1 in the same *E. coli* strain was first reported (Zhang et al., 2016), and the IncI2-type *E. coli* plasmids simultaneously carrying the two genes have since been continuously reported (Gao et al., 2016; Sun et al., 2016), possibly because the IncI2-type plasmids are also important carriers and transmission vectors of the $bla_{\text{CTX-M}}$ resistance gene (Wu et al., 2018). The co-presence of *mcr*-1 and $bla_{\text{CTX-M-64}}$ on the same plasmid may accelerate the spread of the two genes through co-selection (Sun et al., 2016), which provides a way for the bacterium to acquire multidrug resistance more quickly. Consequently, the risk and threat of the epidemic spread of multidrug-resistant bacteria is increased, further suggesting that *E. coli* with the *mcr*-1 gene may have pan-drug resistance (PDR) and cause infections that are

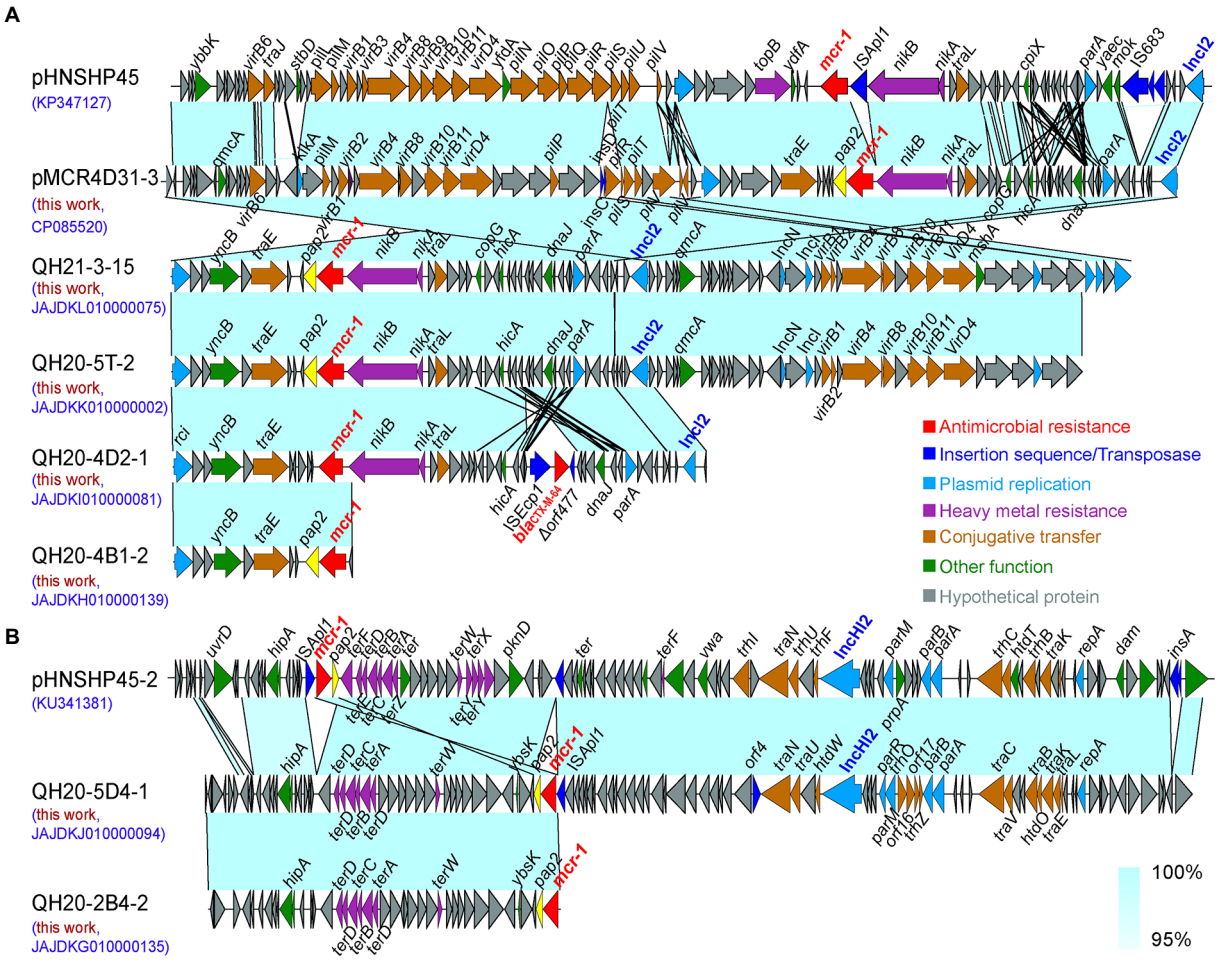


FIGURE 5
Sequence alignment analysis of IncI2 **(A)** and IncHI2-type **(B)** plasmids carrying the *mcr*-1 gene. The arrows indicate the direction of gene transcription. Functional genes are shown in different colors. Regions of >95% nucleotide homology are indicated by colored shading.

difficult to treat clinically. To our knowledge, this study represents the first report on the co-existence of the animal-derived ESBL resistance gene and *mcr*-1 resistance gene in the same *E. coli* plasmid in Qinghai Province.

pMCR4D31–3 shows high homology to the IncI2-type *mcr*-1-carrying plasmids that are geographically distant and different in species. Loayza-Villa et al. discovered the first IncI2-type *mcr*-1-carrying *E. coli* plasmid (p778) in Ecuador, which was isolated from a 14-year-old boy. Meanwhile, three non-identical *E. coli* clones isolated from the chickens and dogs of the boy's family contained similar IncI2-type plasmids, which supports the notion that *mcr*-1 can be horizontally transmitted between animals and humans living in the same environment (Loayza-Villa et al., 2020). When pHNSHP45, a plasmid carrying the *mcr*-1 gene, was first reported, Liu et al. proposed that given that the proportion of *mcr*-1-positive samples in animals is far greater than that of *mcr*-1-positive samples in humans, the *mcr*-1-mediated colistin resistance most likely originated in animals and was then transmitted to humans (Liu et al., 2016). Zurfluh et al. analyzed the *mcr*-1-carrying IncI2-type plasmids isolated from the patient's urethra, diarrhea patients, and those with travel history to Asian countries (pColR644SK1) as well as from retail poultry meat and turkey meat, and found that the horizontal transfer of the *mcr*-1 gene *via* plasmids is the primary method of spread along the food chain, indicating that the food chain may be an important transmission route of *mcr*-1-carrying plasmids (Zurfluh et al., 2017). It is worth noting that p778 and pColR644SK1 have high homology to pMCR4D31–3 isolated in this study, with similar *mcr*-1 gene cassettes; thus, it is necessary to prevent and control the pMCR4D31-3-mediated horizontal transmission of the *mcr*-1 gene between animals and humans in the same environment and the risk of its spread along the food chain.

Conclusion

In this study, we provide data on the AMR of chicken-derived *E. coli* in Qinghai Province and the genetic

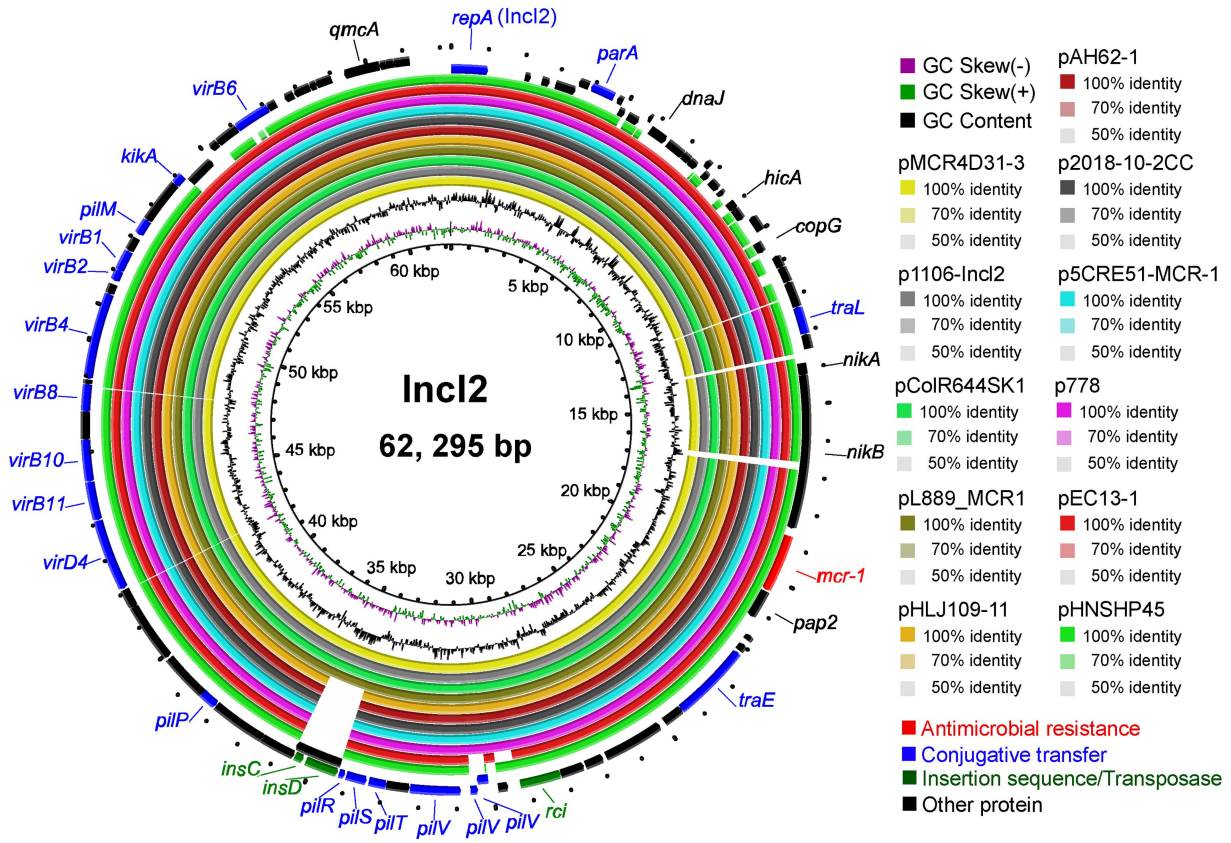


FIGURE 6
Circular diagram of the IncI2 plasmids. Circles, from inside to outside, indicate the GC skew, GC content, and ORFs in the positive and negative DNA strands. Genes are shown in different colors.

environment of the *mcr*-1 gene and for the first time, generated a complete plasmid profile of the IncI2-type *mcr*-1-carrying plasmid from Qinghai, which helps us understand the prevalence of *mcr*-1 in animal-derived *E. coli* in this area. In summary, our study indicates that it is necessary to continuously monitor the AMR of *E. coli* and the prevalence of *mcr*-1 in the plateau region, which is of great significance to understanding and controlling the spread of bacterial antibiotic resistance.

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 animal study was reviewed and approved by Zhejiang Academy of Agricultural Sciences. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BT: conceptualization. HY: funding acquisition. BT, JC, XZ, JM, JuW, XJ, and BD: investigation. JiW, XZ, and BD: methodology. BT and BD: supervision. JM, JiW, and BT: visualization. BT and JiW: writing—original draft. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Key Research and Development Program of Zhejiang Province (2020C02031), the State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products (2010DS700124-ZZ2008, ZZ2102), and the Collaborative Extension Plan of Major Agricultural Technologies in Zhejiang Province (2021XTTGXM03).

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.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.885132/full#supplementary-material

References

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST ring image generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Bai, X., Zhang, W., Tang, X., Xin, Y., Xu, Y., Sun, H., et al. (2016). Shiga toxin-producing *E. coli* in plateau Pika (*O. curzoniae*) on the Qinghai-Tibetan plateau, China. *Front. Microbiol.* 7:375. doi: 10.3389/fmicb.2016.00375

Chang, J., Tang, B., Chen, Y., Xia, X., Qian, M., and Yang, H. (2020). Two IncHI2 plasmid-mediated colistin-resistant *E. coli* strains from the broiler chicken supply chain in Zhejiang Province, China. *J. Food Prot.* 83, 1402–1410. doi: 10.4315/JFP-20-041

Choi, Y., Lee, J. Y., Lim, S. K., and Ko, K. S. (2020). Intact pap2 downstream of mcr-1 appears to be required for colistin resistance. *Diagn. Microbiol. Infect. Dis.* 97:114997. doi: 10.1016/j.diagmicrobio.2020.114997

Gao, R., Hu, Y., Li, Z., Sun, J., Wang, Q., Lin, J., et al. (2016). Dissemination and mechanism for the *mcr-1* Colistin resistance. *PLoS Pathog.* 12:e1005957. doi: 10.1371/journal.ppat.1005957

Girardello, R., Piroupo, C. M., Martins, J. Jr., Maffucci, M. H., Cury, A. P., Franco, M. R. G., et al. (2021). Genomic characterization of mcr-1.1-Producing *E. coli* recovered from human infections in São Paulo, Brazil. *Front. Microbiol.* 12:663414. doi: 10.3389/fmicb.2021.663414

Kostrzewa, M. (2018). Application of the MALDI Biotyper to clinical microbiology: progress and potential. *Expert Rev. Proteomics* 15, 193–202. doi: 10.1080/14789450.2018.1438193

Li, X., Sun, R., Song, J., Fang, L., Zhang, R., Lian, X., et al. (2020). Within-host heterogeneity and flexibility of mcr-1 transmission in chicken gut. *Int. J. Antimicrob. Agents* 55:105806. doi: 10.1016/j.ijantimicag.2019.09.010

Liang, Z., Pang, J., Hu, X., Nie, T., Lu, X., Li, X., et al. (2021b). Low prevalence of mcr-1 Among clinical *Enterobacteriaceae* isolates and co-transfer of mcr-1 and bla_{NDM-1} from separate donors. *Microb. Drug Resist.* 27, 476–484. doi: 10.1089/mdr.2020.0212

Liang, G., Rao, Y., Wang, S., Chi, X., Xu, H., and Shen, Y. (2021a). Co-occurrence of NDM-9 and MCR-1 in a human gut Colonized *E. coli* ST1011. *Infect. Drug Resist.* 14, 3011–3017. doi: 10.2147/IDR.S321732

Lin, Y. C., Kuroda, M., Suzuki, S., and Mu, J. J. (2019). Emergence of an *E. coli* strain co-harbouring mcr-1 and bla_{NDM-9} from a urinary tract infection in Taiwan. *J. Glob. Antimicrob. Resist.* 16, 286–290. doi: 10.1016/j.jgar.2018.10.003

Ling, Z., Yin, W., Shen, Z., Wang, Y., Shen, J., and Walsh, T. R. (2020). Epidemiology of mobile colistin resistance genes mcr-1 to mcr-9. *J. Antimicrob. Chemother.* 75, 3087–3095. doi: 10.1093/jac/dkaa205

Liu, X., Geng, S., Chan, E. W.-C., and Chen, S. (2019). Increased prevalence of *E. coli* strains from food carrying bla_{NDM} and mcr-1-bearing plasmids that structurally resemble those of clinical strains, China, 2015 to 2017. *Euro Surveill.* 24:1800113. doi: 10.2807/1560-7917.ES.2019.24.13.1800113

Liu, Y., Wang, Y., Walsh, T. R., Yi, L., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/s1473-3099(15)00424-7

Loayza-Villa, F., Salinas, L., Tijet, N., Villavicencio, F., Tamayo, R., Salas, S., et al. (2020). Diverse *E. coli* lineages from domestic animals carrying colistin resistance gene mcr-1 in an Ecuadorian household. *J. Glob. Antimicrob. Resist.* 22, 63–67. doi: 10.1016/j.jgar.2019.12.002

Lu, S., Jin, D., Wu, S., Yang, J., Lan, R., Bai, X., et al. (2016). Insights into the evolution of pathogenicity of *E. coli* from genomic analysis of intestinal *E. coli* of *M. himalayana* in Qinghai-Tibet plateau of China. *Emerg. Microbes Infect.* 5, 1–9. doi: 10.1038/emi.2016.122

Ma, Z., Liu, J., Chen, L., Liu, X., Xiong, W., Liu, J., et al. (2021). Rapid increase in the IS26-mediated cfr gene in *E. coli* isolates with IncP and IncX4 plasmids and co-existing cfr and mcr-1 genes in a swine farm. *Pathogens* 10:33. doi: 10.3390/pathogens10010033

Manges, A. R., Geum, H. M., Guo, A., Edens, T. J., Fibke, C. D., and Pitout, J. D. D. (2019). Global Extraintestinal Pathogenic *E. coli* (ExPEC) lineages. *Clin. Microbiol. Rev.* 32, e00135–e00118. doi: 10.1128/cmr.00135-18

Moran, R. A., and Hall, R. M. (2018). Evolution of regions containing antibiotic resistance genes in FII-2-FIB-1 ColV-Colla virulence plasmids. *Microb. Drug Resist.* 24, 411–421. doi: 10.1089/mdr.2017.0177

Pan, Y., Fang, Y., Song, X., Lyu, N., Chen, L., Feng, Y., et al. (2021). Co-occurrence of mcr-9, extended spectrum beta-lactamase (ESBL) and AmpC genes in a conjugative IncHI2A plasmid from a multidrug-resistant clinical isolate of Salmonella diarizonae. *J. Infect.* 82, 84–123. doi: 10.1016/j.jinf.2020.11.008

Poirel, L., Madec, J. Y., Lupo, A., Schink, A. K., Kieffer, N., Nordmann, P., et al. (2018). Antimicrobial Resistance in *E. coli*. *Microbiol. Spectr.* 6: ARBA-0026-2017. doi: 10.1128/microbiolspec.ARBA-0026-2017

Rhouma, M., Beaudry, F., Theriault, W., and Letellier, A. (2016). Colistin in pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and one health perspectives. *Front. Microbiol.* 7:1789. doi: 10.3389/fmicb.2016.01789

Sarica, S., and Yurttutan, S. (2018). An evaluation of hearing in infants administered with colistin in the premature neonatal intensive care unit. *J. Matern. Fetal Neonatal Med.* 31, 2918–2922. doi: 10.1080/14767058.2018.1479388

Sekizuka, T., Kawanishi, M., Ohnishi, M., Shima, A., Kato, K., Yamashita, A., et al. (2017). Elucidation of quantitative structural diversity of remarkable rearrangement regions, shufflons, in IncI2 plasmids. *Sci. Rep.* 7:928. doi: 10.1038/s41598-017-01082-y

Shen, Z., Wang, Y., Shen, Y., Shen, J., and Wu, C. (2016). Early emergence of mcr-1 in *E. coli* from food-producing animals. *Lancet Infect. Dis.* 16:293. doi: 10.1016/S1473-3099(16)00061-X

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039

Sun, J., Li, X., Yang, R., Fang, L., Huo, W., Li, S., et al. (2016). Complete nucleotide sequence of an IncI2 plasmid Coharboring $bla_{CTX-M-55}$ and mcr-1. *Antimicrob. Agents Chemother.* 60, 5014–5017. doi: 10.1128/aac.00774-16

Tang, B., Chang, J., Cao, L., Luo, Q., Xu, H., Lyu, W., et al. (2019a). Characterization of an NDM-5 carbapenemase-producing E. coli ST156 isolate from a poultry farm in Zhejiang, China. *BMC Microbiol.* 19:82. doi: 10.1186/s12866-019-1454-2

Tang, B., Chang, J., Zhang, L., Liu, L., Xia, X., Hassan, B. H., et al. (2020). Carriage of distinct mcr-1-harboring plasmids by unusual serotypes of Salmonella. *Adv. Biosyst.* 4:e1900219. doi: 10.1002/adbi.201900219

Tang, B., Ma, Y., He, X., Zhou, Q., Chang, J., Qian, M., et al. (2021). Similar antimicrobial resistance of *E. coli* strains isolated from retail chickens and poultry farms. *Foodborne Pathog. Dis.* 18, 489–496. doi: 10.1089/fpd.2021.0019

Tang, F., Wang, J., Li, D., Gao, S., Ren, J., Ma, L., et al. (2019b). Comparative genomic analysis of 127 *E. coli* strains isolated from domestic animals with diarrhea in China. *BMC Genomics* 20:212. doi: 10.1186/s12864-019-5588-2

Tegetmeyer, H. E., Jones, S. C., Langford, P. R., and Baltes, N. (2008). ISApl1, a novel insertion element of *A. pleuropneumoniae*, prevents ApxIV-based serological detection of serotype 7 strain AP76. *Vet. Microbiol.* 128, 342–353. doi: 10.1016/j.vetmic.2007.10.025

Tong, H., Liu, J., Yao, X., Jia, H., Wei, J., Shao, D., et al. (2018). High carriage rate of mcr-1 and antimicrobial resistance profiles of mcr-1-positive *E. coli* isolates in swine faecal samples collected from eighteen provinces in China. *Vet. Microbiol.* 225, 53–57. doi: 10.1016/j.vetmic.2018.09.018

Tonny, L., and Anti, D. (2015). Colistin: an antibiotic and its role in multiresistant gram-negative infections. *Acta Med. Indones.* 47, 157–168.

Wang, Q., Sun, J., Li, J., Ding, Y., Li, X. P., Lin, J., et al. (2017). Expanding landscapes of the diversified mcr-1-bearing plasmid reservoirs. *Microbiome* 5:70. doi: 10.1186/s40168-017-0288-0

Wang, R., van Dorp, L., Shaw, L. P., Bradley, P., Wang, Q., Wang, X., et al. (2018). The global distribution and spread of the mobilized colistin resistance gene mcr-1. *Nat. Commun.* 9:1179. doi: 10.1038/s41467-018-03205-z

Wang, Y., Zhou, J., Li, X., Ma, L., Cao, X., Hu, W., et al. (2020). Genetic diversity, antimicrobial resistance and extended-spectrum beta-lactamase type of *E. coli* isolates from chicken, dog, pig and yak in Gansu and Qinghai provinces, China. *J. Glob. Antimicrob. Resist.* 22, 726–732. doi: 10.1016/j.jgar.2020.06.028

Wu, J., Duan, D., Lu, J., Luo, Y., Wen, X., Guo, X., et al. (2016). Inorganic pollution around the Qinghai-Tibet plateau: An overview of the current observations. *Sci. Total Environ.* 550, 628–636. doi: 10.1016/j.scitotenv.2016.01.136

Wu, C., Wang, Y., Shi, X., Wang, S., Ren, H., Shen, Z., et al. (2018). Rapid rise of the ESBL and mcr-1 genes in *E. coli* of chicken origin in China, 2008-2014. *Emerg. Microbes Infect.* 7:30. doi: 10.1038/s41426-018-0033-1

Xu, T., Zhang, C., Ji, Y., Song, J., Liu, Y., Guo, Y., et al. (2021). Identification of mcr-10 carried by self-transmissible plasmids and chromosome in Enterobacter roggenkampii strains isolated from hospital sewage water. *Environ. Pollut.* 268:115706. doi: 10.1016/j.envpol.2020.115706

Yamaguchi, T., Kawahara, R., Hamamoto, K., Hirai, I., Khong, D. T., Nguyen, T. N., et al. (2020). High prevalence of Colistin-Resistant *E. coli* with chromosomally carried mcr-1 in healthy residents in Vietnam. *mSphere* 5, e00117–e00120. doi: 10.1128/mSphere.00117-20

Yang, X., Peng, K., Zhang, Y., Liu, L., and Li, R. (2020). Characterization of a novel mcr-8.2-bearing plasmid in ST395 *Klebsiella pneumoniae* of chicken origin. *Infect. Drug Resist.* 13, 1781–1784. doi: 10.2147/IDR.S256544

Yin, D., Cheng, B., Yang, K., Xue, M., Lin, Y., Li, Z., et al. (2021). Complete genetic analysis of plasmids carrying mcr-1 and other resistance genes in avian pathogenic E. coli isolates from diseased chickens in Anhui Province in China. *mSphere* 6, e01135–e01120. doi: 10.1128/mSphere.01135-20

Yu, C. Y., Ang, G. Y., Chong, T. M., Chin, P. S., Ngeow, Y. F., Yin, W. F., et al. (2017). Complete genome sequencing revealed novel genetic contexts of the mcr-1. *J. Antimicrob. Chemother.* 72, 1253–1255. doi: 10.1093/jac/dkw541

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, H., Seward, C. H., Wu, Z., Ye, H., and Feng, Y. (2016). Genomic insights into the ESBL and MCR-1-producing ST648 Escherichiacoli with multi-drug resistance. *Sci. Bull.* 61, 875–878. doi: 10.1007/s11434-016-1086-y

Zhao, Q., Chen, P., Yang, L., Cai, R., Zhu, J., Fang, L., et al. (2020). Transmission of plasmid-borne and chromosomal $bla_{CTX-M-64}$ among E. coli and Salmonella isolates from food-producing animals *via* ISEcp1-mediated transposition. *J. Antimicrob. Chemother.* 75, 1424–1427. doi: 10.1093/jac/dkaa044

Zhong, L., Phan, H. T. T., Shen, C., Vihta, K. D., Sheppard, A. E., Huang, X., et al. (2018). High rates of human fecal carriage of mcr-1-positive multidrug-resistant Enterobacteriaceae emerge in China in association With successful plasmid families. *Clin. Infect. Dis.* 66, 676–685. doi: 10.1093/cid/cix885

Zurfluh, K., Nüesch-Inderbinen, M., Klumpp, J., Poirel, L., Nordmann, P., and Nordmann, P. (2017). Key features of mcr-1-bearing plasmids from *E. coli* isolated from humans and food. *Antimicrob. Resist. Infect. Control* 6:91. doi: 10.1186/s13756-017-0250-8

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 28 July 2022
DOI 10.3389/fmicb.2022.936843

Check for updates

OPEN ACCESS

EDITED BY
Ziad Daoud,
Central Michigan University,
United States

REVIEWED BY
Michael Brouwer,
Wageningen University and Research,
Netherlands
Virginia Carfora,
Experimental Zooprophylactic Institute of the Lazio and Tuscany Regions (IZSLT), Italy

*CORRESPONDENCE
Ana Hurtado
ahurtado@neiker.eus

SPECIALTY SECTION
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

RECEIVED 05 May 2022
ACCEPTED 29 June 2022
PUBLISHED 28 July 2022

CITATION
Tello M, Ocejo M, Oporto B, Lavín JL and Hurtado A (2022) Within-farm dynamics of ESBL-producing *Escherichia coli* in dairy cattle: Resistance profiles and molecular characterization by long-read whole-genome sequencing.
Front. Microbiol. 13:936843.
doi: 10.3389/fmicb.2022.936843

COPYRIGHT
© 2022 Tello, Ocejo, Oporto, Lavín and Hurtado. 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.

Within-farm dynamics of ESBL-producing *Escherichia coli* in dairy cattle: Resistance profiles and molecular characterization by long-read whole-genome sequencing

Maitane Tello[1], Medelin Ocejo[1], Beatriz Oporto[1], José Luis Lavín[2] and Ana Hurtado[1]*

[1]Department of Animal Health, NEIKER – Basque Institute for Agricultural Research and Development, Basque Research and Technology Alliance (BRTA), Derio, Bizkaia, Spain, [2]Department of Applied Mathematics, NEIKER – Basque Institute for Agricultural Research and Development, Bioinformatics Unit, Basque Research and Technology Alliance (BRTA), Derio, Bizkaia, Spain

A longitudinal study was designed in five dairy cattle farms to assess the within-farm dynamics of ESBL-/AmpC-/carbapenemase-producing *E. coli* and their resistance profiles, along with the genes conferring the resistance phenotypes. Twelve samplings were performed over a period of 16 months, collecting rectal feces from apparently healthy animals in three age groups (calves, heifers, and lactating cows) that were subjected to selective isolation in cefotaxime-containing media. Minimum inhibitory concentrations were determined by broth microdilution for 197 cefotaxime-resistant *E. coli* (1–3 isolates per age group and sampling date), and 41 of them were selected for long-read whole-genome sequencing. Cefotaxime-resistant *E. coli* were detected in the five farms, but isolation frequency and resistance profiles varied among farms and age groups. The genetic profiling of a selection of isolates recovered in two of the farms was described in full detail, showing the predominance of a few genomic subtypes of *E. coli* in one farm (F1) and great variability of strains in another one (F4). Two predominant distinct strains carrying the $bla_{CTX\text{-}M\text{-}1}$ gene in IncX1 plasmids successively spread and persisted in F1 over a prolonged period. In F4, 13 different MLST types carrying a high diversity of ESBL-encoding genes in 6 different plasmid types were observed, probably as the result of multiple source contamination events. In both farms, the presence of certain plasmid types with the same repertoire of ARGs in different *E. coli* STs strongly suggested the occurrence of horizontal transfer of such plasmids among strains circulating within the farms. Considering the public health importance of ESBL-producing *E. coli* both as pathogens and as vectors for resistance mechanisms, the presence of β-lactamase- and other AMR-encoding genes in plasmids that can be readily transferred between bacteria is a concern that highlights the need for One Health surveillance.

KEYWORDS

antimicrobial resistance, dairy cattle, extended-spectrum β-lactamases, ESBL-producing *Escherichia coli*, minimum inhibitory concentration, whole-genome sequencing, long-read WGS

Introduction

Cephalosporins (third- and higher-generation) and carbapenems are critically important antimicrobials for human medicine since in some instances, they are either the sole or one of the limited therapies available to treat multidrug-resistant (MDR) bacteria in human infections (WHO, 2019). *E. coli* strains can become resistant to these antimicrobials by the acquisition of antimicrobial resistance genes (ARGs) coding for enzymes like extended-spectrum β-lactamases (ESBL), AmpC cephalosporinases, and carbapenemases (CP). ESBL and AmpC enzymes are capable of hydrolyzing various β-lactam antibiotics such as penicillins, third- and higher-generation cephalosporins, and monobactams, while AmpC enzymes are additionally active against cephamycins and resistant to inhibition by clavulanate. CPs confer resistance to a broad spectrum of β-lactams, including carbapenems, a last resort for treating MDR Gram-negative bacterial infections.

ESBL/AmpC-producing *E. coli* are widely distributed in livestock (Poirel et al., 2018; Dantas Palmeira and Ferreira, 2020), but their contribution as a source of human infection remains controversial (Collis et al., 2019). On the other hand, CP-producing *E. coli* are still scarcely detected in cattle (Madec et al., 2017; Kock et al., 2018; EFSA and ECDC, 2021). The spread of ESBL-/AmpC-/CP-producing *E. coli* can be the result of the selection of resistance (usually at the intestinal level) under the pressure of antibiotic usage, and the dissemination of such resistant bacteria by cross-contamination of fecal material among animals (Seiffert et al., 2013). In a previous cross-sectional survey conducted in the Basque Country in 2014–2016 to study the herd-level prevalence of ESBL-/AmpC- and carbapenemase-producing commensal *E. coli* in ruminants, a higher prevalence was detected in dairy cattle compared with beef cattle and sheep (Tello et al., 2020). However, the association of animal age with the likelihood of ESBL-/AmpC-producing *E. coli* shedding was not investigated. Besides, cross-sectional studies do not provide information on the long-term dynamics of bacterial shedding, which is relevant for understanding their potential for spread and persistence within the farm. Longitudinal data on fecal shedding of ESBL-/AmpC-producing *E. coli* within farm animals remains limited. Other longitudinal studies performed on dairy cattle either focused on a single farm, were short time-framed, or applied different approaches and methodologies (Hordijk et al., 2013, 2019; Horton et al., 2016; Gay et al., 2019; Plassard et al., 2021), but none combined long-term monitoring with a detailed genomic analysis.

To further explore the epidemiology of ESBL-/AmpC-producing *E. coli* on dairy cattle farms, we studied the dynamics of fecal shedding in animals from different age groups in five dairy cattle farms in the Basque Country. To increase detection efficiency, selective pre-enrichment was used. Phenotypic antimicrobial susceptibility of isolates recovered from the five farms was tested, and in-depth genome characterization of isolates from two of the farms was performed using long-read sequencing (Oxford Nanopore Technologies, ONT) to investigate ARG transmission dynamics. Bacterial chromosomes and plasmids were reconstructed and typed.

Materials and methods

Study design

A longitudinal study was carried out in dairy cattle farms in the Basque Country (northern Spain) to monitor the occurrence of ESBL-/AmpC-/CP-producing *E. coli* in apparently healthy animals. Five commercial farms (designated F1, F2, F3, F4, and F5), representative of the style of farming in the region, were selected to be enrolled in the study. Farms were located in the three counties of the Basque Country, and the distance between farms ranged from 15–25 km for those located within the same county (i.e., F3-F4 and F1-F2, respectively) and up to 160 km (F4-F5). Before the study started, our team paid a visit to each farm and, in the presence of the farm veterinary clinicians, farmers were interviewed face to face using a questionnaire that addressed general information about farm characteristics, management practices, vaccine programs, and antimicrobial drug use. Farm size based on the combined number of lactating and dry cows, heifers, and calves, ranged between 140 and 320 animals (mean = 240), with the number of lactating cows ranging from 75 (F5) to 200 cows (F1).

Monthly visits over a 1 year-period were planned for fecal sample collection. However, one of the farms (F5) dropped out after five samplings due to operational changes; samplings in the other four farms were interrupted midway through the study due to the COVID-19 pandemic and resumed at different times after the lockdown to complete the 12 samplings scheduled. Overall, the collection of fecal samples commenced in February 2019 and ended in October 2020, and extended over 16–17 months within individual farms. Samples were collected from apparently healthy animals from different age groups defined according to the different management practices, i.e., 1–5 month-old calves, 5–22 month-old heifers, and lactating cows. At each sampling time, rectal fecal samples (minimum of 5 g) were collected with a gloved hand from five animals randomly selected within each age group, and analyzed in a single 25 g pool per age group (5 g per animal). In seven time points, heifers could not be sampled in the two farms (five sampling times in F2 and two in F4) that raised heifer replacements at a breeding center. A total of 760 rectal fecal samples were collected and analyzed in 152 pools. Additionally, environmental slurry samples were also collected from F3 and F4 (two samplings each).

Selective isolation of ESBL-/AmpC- and carbapenemase (CP)-producing *Escherichia coli*

Upon arrival, samples were refrigerated at 4°C and sample processing was carried out within 3 days after collection, at the latest. Pooled fecal samples (25 g) were thoroughly mixed, diluted 1:10 in buffered peptone water (BPW, bioMérieux), and incubated at 37°C for 20 ± 2 h. For the isolation of ESBL-/AmpC-producing

E. coli, two loops (20 µl) of BPW were subcultured onto MacConkey agar supplemented with 1 mg/l of cefotaxime and incubated at 37°C for 20 ± 2 h. Two morphologically different colonies per plate were harvested and confirmed as *E. coli* by species-specific real-time PCR detection of the *uidA* gene (Frahm and Obst, 2003).

For the isolation of CP-producing *E. coli*, two loops (20 µl) of BPW were subcultured onto MacConkey agar without antibiotics. A loopful of grown colonies was then harvested for DNA extraction and subjected to a real-time PCR amplification screening targeting the CP-coding genes bla_{NDM}, bla_{VIM}, bla_{KPC}, and bla_{OXA-48} (Ellington et al., 2016). If any of these genes tested positive, a loopful of bacterial growth from the MacConkey agar was subcultured on ChromID® Carba Smart selective agar plates (bioMérieux), and isolated colonies were identified by *uidA* gene detection as above.

Antimicrobial susceptibility testing by broth microdilution

Between 1 and 3 isolates per plate were selected and tested to assess antimicrobial susceptibility. Minimum inhibitory concentrations (MICs) were determined by broth microdilution using two Sensititre® MIC susceptibility plates (EUVSEC1 and EUVSEC2, Thermo Fisher Scientific) following the recommendations in Commission Implementing Decision 2013/652/EU[1] concerning antimicrobials and dilution ranges, and the results were interpreted using epidemiological cutoff values (ECOFF). For antimicrobials with no ECOFFs assigned at the time, the results were interpreted as follows: for temocillin, ECOFF was fixed at 16 mg/l based on 2020/1729/EU; for azithromycin, 16 mg/l was used as a reference based on the bibliography (Sjölund-Karlsson et al., 2011; Clinical and Laboratory Standards Institute, 2015).

Whole-genome sequencing and bioinformatic analyses

Based on their phenotypic AMR profile, sampling time, and age group isolation source, 41 isolates (27 from F4, 11 from F1, and one each from F2, F3, and F5) were selected for WGS. For in-depth genome characterization, genomic DNA was extracted from pure cultures using NZY Microbial gDNA Isolation kit (NZYtech) and subjected to long-reads (Oxford Nanopore Technologies, ONT) WGS. For ONT sequencing, a library was prepared using the Ligation Sequencing Kit (SQK-LSK109). Native barcoding genomic DNA kits (EXP-NBD104 and EXP-NBD114) were used for sample multiplexing except for three isolates that were sequenced in singleplex. Libraries were run in FLO-MIN106 (R9.4.1) or FLO-MIN111 (R10.3) flow cells on a MinION Mk1C device (ONT). For validation purposes, five isolates also underwent short-reads (Illumina) WGS; genomic DNA was submitted to Eurofins Genomics, where libraries were prepared based on the NEBNext Ultra II FS DNA library prep kit (Illumina) and sequenced with Illumina NovaSeq 6,000 (150-bp paired-end reads). The output files generated by ONT sequencing were basecalled in high-accuracy mode (HAC) and quality-filtered using Guppy (Qscore >7 in v4.2 and v4.3, and Qscore >8 in v5.0). Then, reads were adapter-trimmed and filtered by length and quality, as described before (Tello et al., 2022) and the resulting fastq reads were *de novo* assembled using Unicycler (Wick et al., 2017b). For one particular sample, Flye assembler (Kolmogorov et al., 2019) was used after retrieving inconsistent results in the draft genome generated with Unicycler, and the resultant assembly was the one further used in this study. For isolates sequenced by both technologies, Illumina reads were pre-processed for assembly as described elsewhere (Tello et al., 2020) and the outputs were further used to generate hybrid Nanopore-Illumina assemblies with Unicycler (Wick et al., 2017b). As previously described (Tello et al., 2022), isolates were subjected to *in silico* typing to determine their serogroup and phylogroup. MLST profiles were determined from unassembled long-reads using Krocus (Page and Keane, 2018). New sequence type (ST) assignations were obtained after submitting WGS reads to the Enterobase database (Zhou et al., 2020). Draft genomes were processed to predict plasmid- and chromosome-derived contigs using PlasFlow (v.1.1; Krawczyk et al., 2018). Molecular characterization of the isolates, including screening of ARGs, chromosomal point mutations associated with AMR, virulence factors detection, and plasmid replicon identification were performed, as previously described (Tello et al., 2020). Databases used for molecular characterization (ResFinder, PointFinder, PlasmidFinder, and ecoli_vf) were all updated on 20 October 2021. ResFinder hits were filtered at 90% coverage and identity and those with values below 100% were individually revised for frameshifts and amino acid changes, removing those considered not potentially functional. Virulence genes were filtered at 75% identity and 95% coverage, and the pattern of presence/absence of these genes was used as a typing scheme for genetic diversity. Genome annotations were carried out with Prokka (Seemann, 2014) and RAST (Aziz et al., 2008), and were graphically represented using SnapGene v.5.2.4.[2] Genome alignments were performed using MAUVE (Darling et al., 2010) in Geneious Prime v. 2020.2.4 software.[3] Blast Ring Image Generator (BRIG) v.0.95 was used for plasmid structural comparison (Alikhan et al., 2011).

Phenotypic resistance profiles and the genetic determinants of resistance (GDR) in each sequenced sample (chromosome and

1 https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri_CELEX:32013D0652&from_EN

2 http://www.snapgene.com/

3 https://www.geneious.com

plasmids) were represented in heatmaps. The plasmid heatmap was graphed along with a dendrogram illustrating the similarity among plasmids based on their AMR pattern. The hierarchical clustering analysis for the dendrogram was performed with the unweighted pair-group method with arithmetic mean (UPGMA) based on the Jaccard distance matrix, using the function hclust (v.3.6.1) of the R statistical package v.3.6.3. To identify the shared and unique phenotypic antimicrobial resistance profiles among the different age groups within each farm, Venn diagrams were constructed with the online tool InteractiVenn (Heberle et al., 2015).

Statistical analyses

To evaluate differences between age groups and farms in the shedding prevalence of cefotaxime-resistant *E. coli* and in the occurrence of phenotypic antimicrobial resistance for each antimicrobial, multivariate logistic regressions were performed including age group and farms as the explanatory variables. Adjusted odds ratios (OR_{adj}) were used as the measure of association between positivity and the explanatory variables and were expressed with their confidence interval at 95% (95% CI). Differences were considered statistically significant if $p < 0.05$. Simpson indices were estimated to calculate the diversity of phenotypic antimicrobial resistance profiles for each farm.

Results

Farms' descriptive data derived from the questionnaire

Following common practice in dairy farms in the Basque Country, all farms were closed production systems where replacement heifers originated from the same farm. Two of the farms (F2 and F4) raised their heifer replacements off-site in two different breeding centers. In both cases, animals leave the farm at 3–4 months of age and return already pregnant a few months before calving. A blanket antimicrobial treatment program was routinely used at dry-off that included the intramammary application of antimicrobials and teat sealant. The antimicrobials used for intramammary dry-cow therapy (DCT) were benzylpenicillin-benethamine/framycetin sulfate (Mamyzin) in F1 and F2, and cephapirin benzathine (Cefa-safe) in F3, F4, and F5. Farms participating in the study also used antimicrobials belonging to 12 antimicrobial drug classes for the treatment of disease in calves and cows. The antimicrobials most commonly used were third- and fourth-generation cephalosporins, followed by fluoroquinolones, and tetracyclines. Other antimicrobials used included penicillins, aminoglycosides, macrolides, and sulfonamides. Parenteral administration of fluoroquinolones was the most common treatment for mastitis during lactation in all except farm F2 where mastitis was not treated with antimicrobials. Third- and fourth-generation cephalosporins were the most common drugs used to treat reproductive diseases, diarrhea, and lameness that warranted systemic antimicrobial treatment. Reproductive diseases for which the producer opted to use antimicrobials included metritis, retained placenta, or other diseases related to reproduction.

Vaccination programs were quite different among farms. For example, vaccination against mastitis was only performed in F1. The vaccination program in F1 included vaccines against Infectious Bovine Rhinotracheitis (IBR), clostridia, and mastitis; IBR, *Leptospira,* and respiratory pathogens (Parainfluenza, bovine respiratory syncytial virus - BRS, *Mannheimia*) in F2; no vaccines at all were used in F3; IBR, Bovine viral diarrhea virus, and clostridia in F4; and clostridia, diarrhea in calves, and respiratory pathogens (Parainfluenza, BRS, *Mannheimia*) in F5.

Cefotaxime-resistant *Escherichia coli* isolates were frequently recovered in the five dairy cattle farms, but differences were found among age groups and farms

Escherichia coli was isolated in cefotaxime-containing media in 92 of the 152 pooled fecal samples analyzed (60.5%) and in the 4 slurry samples collected from F3 and F4. These included samples collected from all farms and age groups, but differences in frequencies among age groups and farms were observed (Figure 1). Overall, isolation frequency of cefotaxime-resistant *E. coli* was higher in lactating cows [$OR_{adj} = 4.71$ (1.76–12.64), $p = 0.002$] and calves [$OR_{adj} = 4.21$ (1.59–11.18), $p = 0.004$] compared with heifers, and lower in F1 and F2 compared with the other three farms (LR $\chi^2 = 21.55$, $p < 0.001$).

The majority of cefotaxime-resistant *Escherichia coli* isolates were also resistant to several other antimicrobials

When available, between 1 and 3 cefotaxime-resistant *E. coli* isolates per age group and sampling date were selected in each farm for antimicrobial susceptibility testing. Thus, 187 isolates from fecal samples (73 isolated from lactating cows, 40 from heifers, and 74 from calves) and 10 isolates from slurry were analyzed. Since isolates had been obtained by selective isolation in a medium containing cefotaxime, they were all resistant to cefotaxime and ampicillin. Most isolates were also resistant to cefepime (99.0%) and ceftazidime (98.0%). Resistance to cefoxitin was detected in 36 isolates (18.3%), but 19 of them displayed a MIC value just one dilution step above the ECOFF. All 197 isolates were susceptible to tigecycline and colistin. Two isolates obtained from the same pool of feces

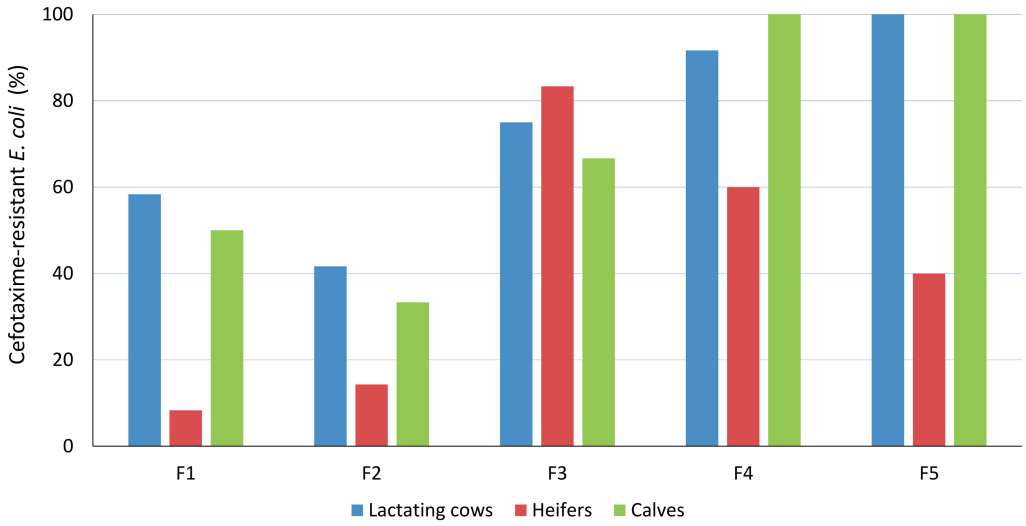


FIGURE 1
Isolation frequency of cefotaxime-resistant *E. coli* among age groups and farms. Results were based on 12 samplings per farm and age group, except for heifers in F2 and F4 where only 7 and 10 pool samples were collected, respectively, and F5, which dropped out from the study after 5 samplings due to operational changes.

collected from calves in F4 were resistant to all β-lactams tested, including temocillin and carbapenems (ertapenem, imipenem, and meropenem).

Co-resistance to other antimicrobial classes was also observed in most isolates (161/197, 81.7%) and 72.1% (142/197) showed multidrug resistance (MDR, resistance to 3 or more antimicrobial classes). Overall, resistance to tetracycline (53.8%), nalidixic acid (45.7%), ciprofloxacin (66.5%), sulfamethoxazole (69.0%), trimethoprim (48.7%), and chloramphenicol (47.7%) was very frequent, while resistance to gentamicin (29.9%) and azithromycin (14.2%) was lower and mainly associated to F5. The prevalence of resistance to each antimicrobial tested did not differ between age groups. However, statistically significant differences between farms were observed in the occurrence of resistance to several antimicrobials. Compared to other farms, F1 and F5 presented a significantly higher prevalence of tetracycline, chloramphenicol, and trimethoprim (all with $p < 0.001$). Resistance to gentamicin ($p < 0.001$), azithromycin ($p < 0.001$), ciprofloxacin ($p = 0.002$), and nalidixic acid ($p = 0.009$) was higher in F5 than in other farms, while resistance to cefoxitin was significantly higher in F1 and F2 ($p = 0.003$).

The diversity of phenotypic resistance profiles varied among farms

A total of 45 different profiles of microbiological resistance (Supplementary Table S1) resulting from the combination of antimicrobial agents that showed MICs above the ECOFF were observed in the study. Each phenotypic resistance profile was designated a letter of the Latin alphabet, and their distribution within each farm is represented in Figure 2. Within each farm, the number of different profiles ranged between 5 and 16 along the 12 samplings, the lowest diversity being found in F1 (Simpson index = 0.609) and the highest in F4 (Simpson index = 0.905). In F1, resistance to tetracycline, chloramphenicol, sulfamethoxazole, and trimethoprim remained stable during the entire study, whereas resistance to gentamicin, ciprofloxacin, and nalidixic acid was only observed in the second half of the study. This observation might reflect a shift in the circulating resistance profiles, where profile B, which dominated at the beginning of the study in all the age groups, was displaced by profile G in the second half of the study. On the contrary, the highest diversity in resistance profiles was observed in F4, where the three predominant profiles (A, C, and H) coexisted with 12 other profiles, with A and H dominating in the first half of the study, and profile C in the second half. Profile A only included resistance to ESBLs (penicillins and cephalosporins), whereas C and H included resistance to additional antimicrobials (Figure 2; Supplementary Table S1).

In F2, the prevalence of cefotaxime-resistant *E. coli* was the lowest, and fewer isolates were recovered and typed, particularly in heifers. Still, 11 different profiles were identified among 24 isolates, but profile D was the only one recovered in more than one sampling, in S7 in lactating cows and in S8 both in calves and lactating cows. In F3 high diversity in resistance profiles was observed, with a total of 16 different profiles, and a shift in the predominant resistance profiles occurred with time as happened in F4. Finally, the most outstanding feature of isolates recovered in F5 was the MDR pattern of all of them, with resistance to gentamicin and azithromycin being common in all age groups. On the other hand, isolates recovered from slurry samples shared their resistance profiles with isolates from fecal samples

10.3389/fmicb.2022.936843

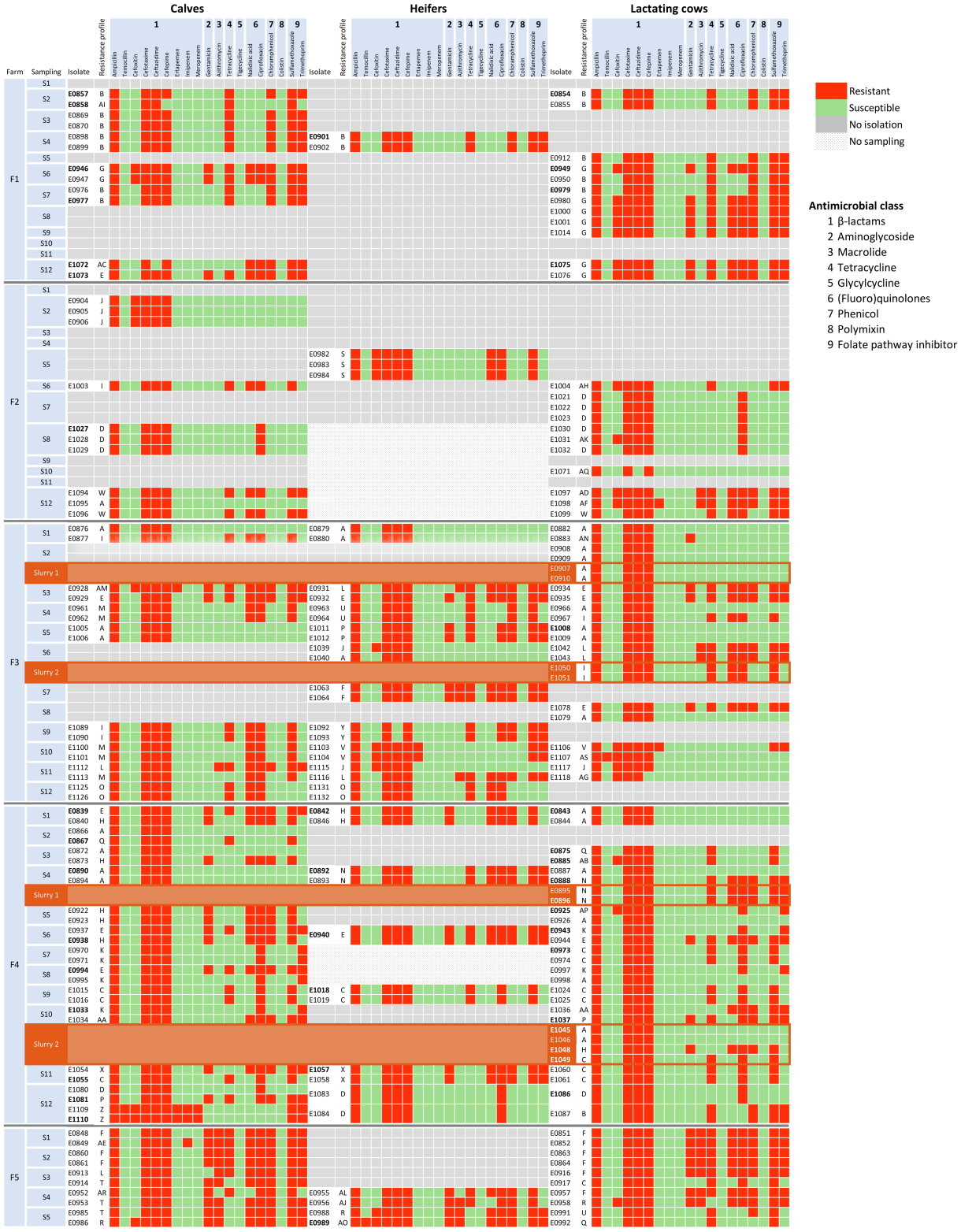


FIGURE 2
Distribution of AMR phenotypic profiles of the 197 *E. coli* isolates by farm, sampling, and age group. Each phenotypic profile is represented with a letter of the Latin alphabet as described in Supplementary Table S1. Antimicrobial susceptibility, determined by the broth microdilution method and interpreted using epidemiological cutoff values (see text), is shown in green for susceptible, and in red for resistant isolates. Slurry samples are indicated with a different background color (brown) and placed below lactating cows to save space. Antimicrobial classes are indicated with numbers: 1=β-lactam, 2=Aminoglycoside, 3=Macrolide, 4=Tetracycline, 5=Glycylcycline, 6=(Fluoro)quinolone, 7=Phenicol, 8=Polymyxin, 9=Folate pathway inhibitor.

collected within the corresponding farms (Figure 2; Supplementary Figure S1).

Sequences generated by ONT sequencing successfully assembled into complete and circular chromosomes and plasmids

ONT sequencing provided a median of 60,729 reads per sample (IQR = 22,781–405,021) in a median of 631 Mb per sample (IQR = 501–1,011 Mb) corresponding to a median coverage of 114X (IQR = 84X–182X; Supplementary Table S2). Upon assembly, the 5 isolates sequenced by both Illumina and ONT technologies, and 24 of the 36 ONT sequenced isolates resulted in circularized chromosomes. In all cases, the chromosome size of the assembled draft genome corresponded to the expected size of *E. coli* (median = 4,999,307 bp; IQR = 4,871,651 bp - 5,059,042 bp). Plasmid replicons were identified in a total of 125 contigs that in most cases (120/125, 96.0%) were assembled into complete circular plasmids. At least one plasmid replicon was identified in each isolate. IncF type plasmids were the most common (38/125, 30.4%), followed by IncB/O/K/Z (15/125, 12.0%), IncX1 (13/125, 10.4%), and IncY (13/125, 10.4%) along with 13 other replicon types. Screening for ARGs and SNPs associated with AMR identified 41 acquired ARGs and point mutations (9) in 4 other genes, coding for resistance to antimicrobials representing 9 different classes (Figure 3). The combination of GDRs detected in each isolate resulted in 22 different genotypic profiles of resistance (Supplementary Table S3). Sixty-two plasmids contained at least one ARG (Figure 4). None of the IncL, IncP, IncX4, or Col plasmids carried ARG genes.

WGS confirmed the predominance of certain genomic subtypes of *Escherichia coli* in F1 and great variability of strains in F4

A selection of isolates, mainly from farms F1 and F4, were analyzed by WGS to confirm whether the distribution of the different AMR profiles within the farms was due to different strains coexisting throughout the sampling period or to successive colonization by different strains. The 41 isolates were assigned to 18 MLST types, including two novel STs, i.e., ST-11626 in F4 and ST-12870 in F3. In F1, the 11 isolates tested belonged to 4 ST types, and 2 of them (ST-69 and ST-2930) included more than one isolate (Figure 3). Thus, ST-69 was represented by 5 isolates from the 3 age groups recovered at samplings S2, S4, and S7, which were identical in all other features, i.e., phylogroup (D), serotype (O15:H18), phenotypic resistance profile (B), and genotypic profile (a). ST-2930 included 4 isolates recovered in samplings S6 and S12 from calves and lactating cows that also shared all their genetic features, i.e., they were all assigned to phylogroup A, serotype O100:H25, and genotypic profile c. However, they split into two phenotypic resistance profiles differing only in susceptibility to FOX (profile E, 1 susceptible isolate, $MIC_{FOX} = 2$; and profile G, 3 resistant isolates, $MIC_{FOX} = 16$). The remaining two isolates sequenced (E0858 and E1072) were recovered during the second and last samplings, and had unique features.

In F4, 27 isolates were sequenced and assigned to 4 phylogroups (A, B1, C, and D) and 13 different ST types. Eight of the ST types were identified in more than one isolate ($n = 2$–4) and 5 were represented by a single isolate (Figure 3). As in F1, the most prevalent MLST type was ST-69. It included isolates recovered from lactating cows (S7), heifers (S9), calves (S11), and slurry. They all shared the same phylogroup (D), serotype (O15:H18), and genotypic (b) and phenotypic AMR profiles (C), suggesting that the same clone spread after sampling 7 in all animal groups and was also detected in slurry. As described in F1, differences associated with FOX among otherwise similar isolates were also observed in F4 within ST-23 (profiles AP and K) and ST-109 (profiles AB and Q). Other identical clones infecting several animals in F4 were those with MLST types ST-88 ($n = 2$), and ST-448 ($n = 3$).

On the other hand, several isolates with identical ST differed in other features (Figure 3). These included differences in resistance due to the carriage of ARG-harboring plasmids (i.e., ST-4981 and ST-69) and occasionally also in chromosomally encoded features (i.e., ST-58 isolates, which differed in ARG-harboring plasmids as well as serotype and chromosomally encoded ARGs). Finally, the isolates from F2, F3, and F5 were unique in all their features. When virulence genes were examined, patterns of presence/absence were highly conserved within ST types, with ST-58 and ST-69 being the only exceptions (Supplementary Table S4). Thus, for these epidemiologically related isolates, the typing scheme based on the presence/absence of virulence genes confirmed the diverse genetic profile inferred from the combination of all other features (phylogroup, serotype, and GDR profile).

Resistance to cephalosporins was mainly due to plasmid-encoded $bla_{CTX\text{-}M}$ genes. F1 differs from F4 regarding the diversity and location of cephalosporin resistance genes

ARG-harboring plasmids were present in all but two of the isolates (E1072 and E1027); 16 isolates carried a single plasmid and 23 carried 2 types of plasmids with ARGs. Overall, ARGs were present in 9 different types of plasmids, and 7 of them harbored ESBL-encoding genes, alone or in combination with several other ARGs (Figure 4). GDRs associated with ESBL production were only sporadically located in the chromosome. These included $bla_{CTX\text{-}M\text{-}14}$ ($n = 1$), $bla_{CTX\text{-}M\text{-}15}$ ($n = 5$), and the point mutation (nt 42 C → T) in the *ampC* promoter ($n = 1$). However,

10.3389/fmicb.2022.936843

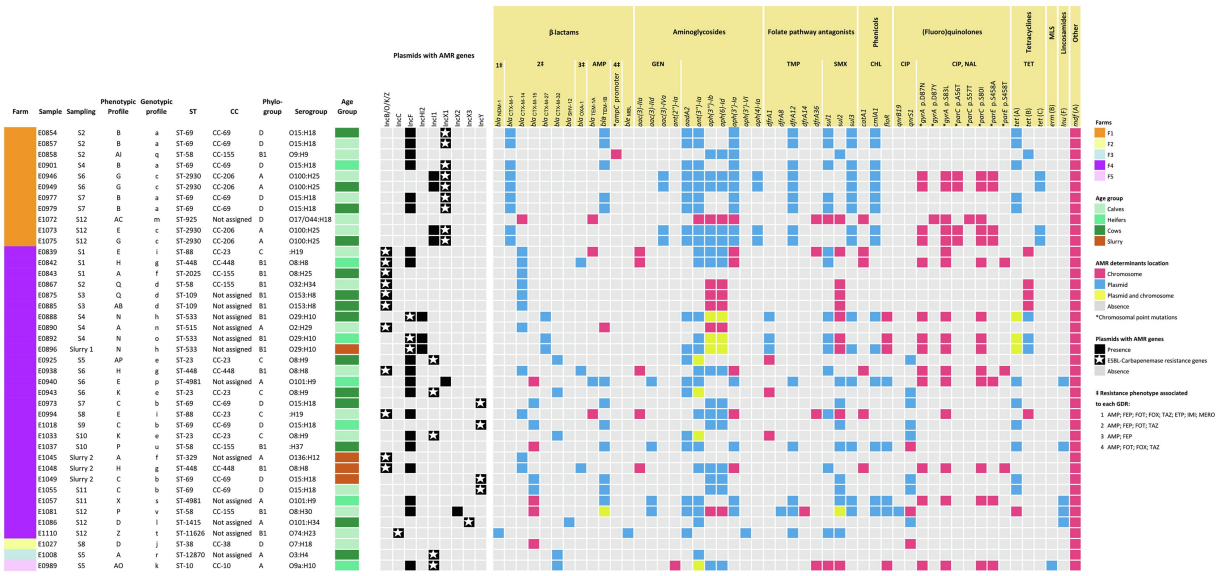


FIGURE 3
Heatmap showing the distribution of the AMR genes and plasmids detected by WGS (presence or absence and location are indicated as per the legend). Isolates are arranged per farm, sampling, and source (age group or slurry). Additional information including MLST type (ST and CC), phylogroup, and serogroup are included. AMR phenotypic resistance profiles are as indicated in Figure 2 and described in Supplementary Table S1. Each AMR genotypic profile resulting from an identical combination of GDR is represented with a letter of the Latin alphabet in lower case. The resistance phenotypes associated with each GDR are indicated for those antimicrobials tested, which were abbreviated as follows: ampicillin (AMP), cefepime (FEP), cefotaxime (FOT), cefoxitin (FOX), ceftazidime (TAZ), ertapenem (ETP), imipenem (IMI), meropenem (MERO), gentamicin (GEN), trimethoprim (TMP), sulfamethoxazole (SMX), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), tetracycline (TET). MLS, macrolide-lincosamide-streptogramin. Descriptions of the phenotypic and genotypic profiles of resistance can be found in Supplementary Tables S1 and S3, respectively.

differences between farms were found concerning the diversity and location of these genes.

In F1, the most prevalent ESBL-encoding gene was $bla_{\text{CTX-M-1}}$ (9/11 isolates), present in isolates recovered from all animal groups and at different sampling times along the study (Figure 3). This gene was located in IncX1 type plasmids which always carried the same repertoire of ARGs. These plasmids were structurally compared using MAUVE and showed a high degree of similarity demonstrating the presence of largely conserved collinear coding blocks (data not shown). Hence, in addition to $bla_{\text{CTX-M-1}}$, IncX1 plasmids harbored the aminoglycoside resistance genes *aadA2*, *ant(3″)-Ia*, and *aph(3′)-Ia*, a trimethoprim resistance gene (*dfrA12*), a sulfamethoxazole resistance gene (*sul3*), and a chloramphenicol resistance gene (*cmlA1*; Figure 4). This plasmid was present in all isolates assigned to ST-69 and ST-2930.

In F4, a higher diversity of ESBL-encoding genes was observed (Figure 3). These included $bla_{\text{CTX-M-14}}$ ($n = 11$), $bla_{\text{CTX-M-15}}$ ($n = 8$), $bla_{\text{CTX-M-27}}$ ($n = 3$), $bla_{\text{CTX-M-32}}$ ($n = 3$), and $bla_{\text{SHV-12}}$ ($n = 1$). In addition, $bla_{\text{NDM-1}}$ was detected in one isolate (E1110). All were located in plasmids except for 4 chromosomally encoded $bla_{\text{CTX-M-15}}$. The $bla_{\text{CTX-M-14}}$ gene was always located in IncB/O/K/Z type plasmids that did not carry any additional ARGs (Figure 4). This plasmid was found in *E. coli* of different ST, genotypic and phenotypic profiles, isolated from slurry and animals of all age groups throughout the study. The $bla_{\text{CTX-M-15}}$ gene, which was also detected in all animal groups and environmental samples, was the predominant ESBL-encoding gene in isolates recovered in the second half of the study. This gene was located in IncY plasmids (all 4 ST-69 isolates) or in the chromosome (ST-4981 and ST-58). Besides $bla_{\text{CTX-M-15}}$, IncY plasmids harbored 6 other ARGs (Figure 4). The $bla_{\text{CTX-M-27}}$ gene was detected in the IncF plasmid of 3 ST-533 isolates, along with 5 other identical ARGs. Three isolates (ST-23) carried the $bla_{\text{CTX-M-32}}$ gene in an IncI1 plasmid, and the bla_{SHV} gene was present in an IncX3 plasmid in one isolate recovered from lactating cows in the last sampling. The isolation in F4 of a carbapenem-resistant *E. coli* harboring the $bla_{\text{NDM-1}}$ gene in an IncC plasmid was a significant finding extensively reported elsewhere (Tello et al., 2022).

Cephalosporin-resistant *Escherichia coli* isolates carried additional plasmids with ARGs and exhibited other chromosomally encoded GDRs

In F1, ST-2930 isolates, besides IncX1, also carried an IncI1 plasmid that harbored another 5 ARGs [*aac(3)-IVa*, *aph(3″)-Ib*, *aph(6)-Id*, *aph(4)-Ia*, and *tet*(C)], while ST-69 isolates carried an IncF plasmid that harbored another four different ARGs [$bla_{\text{TEM-1B}}$, *ant(3″)-Ia*, *sul1* and *tet*(A)]. A different IncF plasmid with a

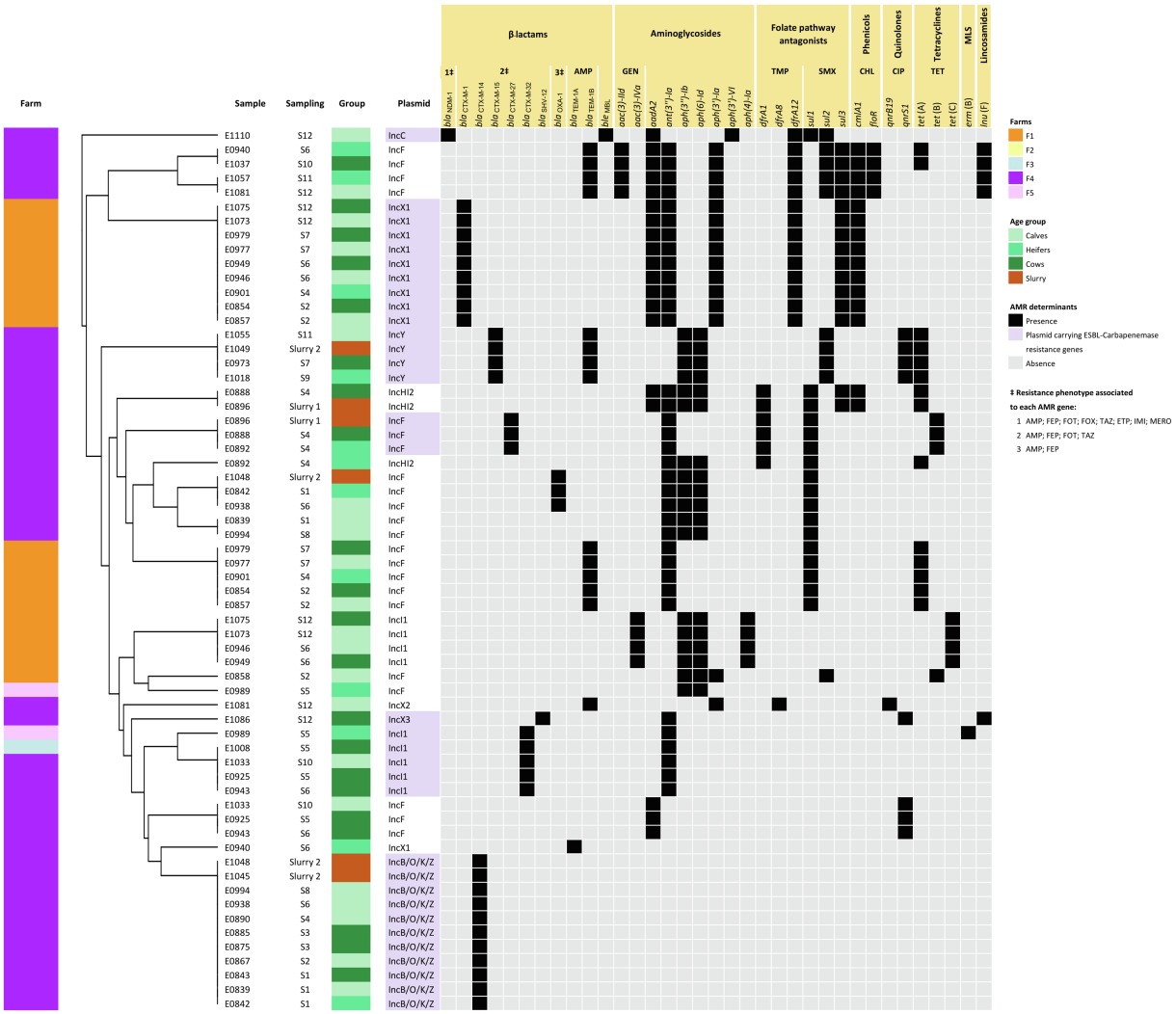


FIGURE 4
Heatmap showing ARG-harboring plasmids along with a dendrogram illustrating the similarity among plasmids based on their AMR pattern. Plasmids were grouped based on their antimicrobial resistance pattern (ARGs) according to the result of the hierarchical clustering using the average linkage method (UPGMA) on the Jaccard distance matrix. Further information including sampling and source (age group or slurry) is also included.

different collection of ARGs was present in one isolate (E0858). This isolate also carried a chromosomally encoded mutation in the *ampC* promoter. No plasmids were detected in the remaining isolate of F1 sequenced (ST-925), which harbored several GDRs in its chromosome, including $bla_{\text{CTX-M-14}}$ (Figure 3). Resistance to (fluoro)quinolones was always associated with point mutations in the gyrase and topoisomerase genes (*gyrA*, *parC*, and *parE*) and only observed in *E. coli* strains assigned to ST-2930 and ST-925 (Figure 3).

In F4, the 3 ST-23 isolates, which carried an Incl1 plasmid harboring the $bla_{\text{CTX-M-32}}$ gene, also carried an IncF plasmid, resulting in an identical genotypic profile. Instead, ST-533 isolates (E0888, E0892, and E0896) carried a second plasmid (IncHI2) with a different repertoire of ARGs (Figure 4). These IncHI2 plasmids were structurally compared (Supplementary Figure S2) and showed extensive sequence similarity, but E0896 lacked an 11.000 bp fragment that included *aadA2*, *cmlA1*, *ant(3″)-Ia*, and *sul3* genes which was present in E0888 and E0892. Other genes coding only for resistance to narrow-spectrum β-lactamases like $bla_{\text{OXA-1}}$ ($n = 3$), $bla_{\text{TEM-1A}}$ ($n = 4$), and $bla_{\text{TEM-1B}}$ ($n = 14$) were mostly located in IncF plasmids, and less frequently in the chromosome or other type of plasmids such as IncX2 and IncY (Figures 3, 4). Resistance to (fluoro)quinolones in F4 was associated with point mutations in the gyrase and topoisomerase genes (*gyrA*, *parC*, and *parE*) in 10 isolates, and with the *qnrS1* gene in another 10 (along with *qnrB19* in one of them). Interestingly, the gene that codes for resistance to lincosamides, *lnuF*, was present in 5 isolates recovered from all age groups in the second half of the study. *lnuF* was always located in IncF and IncX3 plasmids (Figure 4).

Discussion

This longitudinal study was designed to monitor the occurrence of ESBL-/AmpC-/CP-producing *E. coli* and their antimicrobial resistance profiles in apparently healthy animals in dairy cattle farms for over 16 months. Longitudinal surveillance allows the assessment of the bacterial population dynamics throughout time, enabling the detection of emerging genotypes and changes in the AMR profiles over time. The longitudinal survey presented here encompassed five farms that represented the style of farming in the Basque Country, and therefore, might provide a useful understanding of the regional situation regarding cephalosporin-resistant *E. coli* distributions and AMR transmission dynamics.

In a previous cross-sectional survey conducted in the Basque Country in 2014–2016, ESBL/AmpC producers were isolated in 32.9% of the 82 dairy cattle herds tested (Tello et al., 2020). Here, cephalosporin-resistant *E. coli* were detected in all the five investigated dairy cattle farms, surely due to the more intensive longitudinal sampling strategy used that comprised 12 samplings and three age groups. Isolation frequency varied along time, as well as among farms and age groups. Both calves and lactating cows had a higher prevalence of cephalosporin-resistant *E. coli* than heifers, but no difference was observed between them. This could be associated with age-related differences in management practices. Pregnant heifers and dry cows had access to the outside pastures, whereas lactating cows were permanently housed indoors, where increased infection pressure and a higher probability of recirculation of resistant isolates occur. In this sense, ruminants raised under less intensive management systems have been associated with a lower prevalence of infection with cefotaxime-resistant *E. coli*, e.g., beef cattle and sheep in the Basque Country (Tello et al., 2020) and elsewhere (Hille et al., 2017; Collis et al., 2019). The higher incidence found in lactating cows compared with heifers could also be explained by the continuous and prolonged exposure of older cows to antimicrobials used to treat intramammary and other infections during their lifespan. These treatments include the commonly used cephalosporins, which do not require a withdrawal period for milk. On the other hand, calf management practices differed from those in heifers and lactating cows. Calves are kept in different housing facilities and are administered a different diet. Moreover, calves are susceptible to different diseases such as neonatal diarrhea and pneumonia, which are the main reasons for antimicrobial treatment in this age group. Besides, young calves rapidly acquire antibiotic-resistant *E. coli*, which are often multiresistant (Hordijk et al., 2013; Gay et al., 2019), and their resistome has been reported to be more diverse than that of adult cattle (Noyes et al., 2016).

Antimicrobial use (AMU) in food animals has been linked to an increased prevalence of resistant bacteria, but this relation depends on the antimicrobial class, microorganism, and sector (ECDC, EFSA, and EMA, 2021). Here, in the absence of detailed records of AMU, differences extracted from the questionnaires were related to mastitis treatments and DCT. Fluoroquinolones were the antibiotics of choice for mastitis treatment in F1, F3, and F5, the combination of parenteral enrofloxacin with an intramammary ointment containing cefquinome was common practice in F4, and no antimicrobials were used to treat mastitis in F2. On the other hand, the antimicrobials used for blanket DCT to control mastitis were penicillins and aminoglycosides in F1 and F2, and a first-generation cephalosporin in F3, F4, and F5. This could somehow explain the higher prevalence of cephalosporin-resistant *E. coli* found in F3, F4, and F5 compared to F1 and F2. Differences in farm infrastructure and management practices (e.g., vaccine programs and hygiene) may impact animal disease incidence and, consequently, influence the use of antimicrobials and the subsequent increase in AMR prevalence.

Antimicrobial susceptibility testing of 197 cefotaxime-resistant *E. coli* isolates identified 72.1% of them as MDR. This is not unexpected since ESBL-producing *E. coli* are commonly co-resistant to other classes of antimicrobials (Seiffert et al., 2013). However, the diversity of phenotypic resistance profiles varied among farms. Therefore, to thoroughly compare the relationship of the circulating strains, a selection of isolates from the two farms that showed the lowest (F1) and largest (F4) AMR profile diversity were further whole-genome characterized. This analysis identified certain isolates with phenotypic AMR profiles that differed only in their susceptibility to FOX; the FOX-resistant isolates showed a MIC value of just a single two-fold dilution above the ECOFF ($MIC_{FOX} = 16$ mg/l), and, therefore, within the widely accepted margin of error of the microdilution method. These isolates did not carry any GDR associated with AmpC production, and based on WGS results (ST, phylogroup, serotype, GDR, and virulence genes) these isolates could be considered the same strains as their FOX-susceptible counterparts within the same ST type. The opposite situation, i.e., isolates with the same phenotypic profile but clearly different ARGs was also observed. This occurred in F4 and was due to changes in the chromosome and the carriage of different plasmids (ST-58) or the loss of a fragment within an otherwise similar plasmid (ST-533). Isolates with different ST and serotypes that shared the same ARGs were also found.

Even though the genomic data provided in this study represents only two farms, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}14}$, and $bla_{CTX\text{-}M\text{-}15}$ were the most common ESBL-encoding genes, as reported in a previous cross-sectional study carried out in the Basque Country (Tello et al., 2020). Noteworthy was the detection of a gene coding for CP production in F4. The identification, in the frame of this study, of a $bla_{NDM\text{-}1}$-carrying *E. coli* was described in more detail elsewhere (Tello et al., 2022). Previous to this study, CP-producing *E. coli* had not been detected in food-producing animals in the Basque Country, and $bla_{NDM\text{-}1}$-carrying *E. coli* had never been isolated from cattle neither in the Basque Country nor elsewhere.

ESBL-/CP-encoding genes were mostly located in plasmids, with an apparent association of each gene with certain types of plasmid. IncB/O/K/Z plasmids are frequently found in *E. coli* from animal sources and have been associated with the spread of $bla_{CTX\text{-}M\text{-}14}$ in Europe, especially in Spain and the UK (Rozwandowicz et al., 2018). IncF is the most frequently described plasmid type from human and animal sources (Rozwandowicz et al., 2018) and encodes different $bla_{CTX\text{-}M}$ variants. Here, IncF was the most

abundant plasmid but only sporadically carried ARG coding for resistance to ESBL, specifically $bla_{CTX\text{-}M\text{-}27}$ gene, an association already described in cattle (Tadesse et al., 2018). Other ESBL-encoding gene and plasmid associations found here, such as $bla_{CTX\text{-}M\text{-}1}$ in IncX1, $bla_{CTX\text{-}M\text{-}15}$ in IncY, $bla_{CTX\text{-}M\text{-}32}$ in IncI1, and bla_{SHV} in IncX3 plasmids, are not so frequently described (Rozwandowicz et al., 2018). In the cross-sectional study previously carried out in the region (Tello et al., 2020), most of the ESBL/AmpC gene-carrying plasmids were identified as IncI1, but since Illumina was the sequencing technology then used, the type of many of the plasmids could not be assigned. Here, using long-read ONT sequencing, most of the genomes (both the chromosomes and plasmids) were completely sequenced and circularized, allowing a better characterization of plasmids, which is one of the main advantages of this technique (Wick et al., 2017a).

Genome sequencing and characterization of this selection of isolates allowed elucidation of whether transmission of ESBL genes was the result of the persistence of certain strains or multiple source contamination. In F1, only four different strains were identified, two of them being recovered multiple times and from all age groups. One predominated during the first half of the study and was then replaced by a very different strain. Their chromosomally encoded features (all 7 ST alleles, CC, phylogroup, serotype, virulence genes profile, and point mutations associated with quinolone resistance) were completely different, but both carried the same ESBL-encoding gene ($bla_{CTX\text{-}M\text{-}1}$) harbored by an identical IncX1 plasmid. Further differences between both strains were due to genes present in different additional plasmids. These results may reflect an endemic situation where, due to clonal expansion, just a few strains persisted in the farm over a long time thus giving the opportunity for plasmid transfer. Conversely, the situation in F4 was completely different. Although a few genotypes persisted for some time, there was a large diversity of genotypes carrying multiple and diverse GDRs both in the chromosome and in different plasmids, likely due to multiple source contamination events. Yet, different *E. coli* isolates containing the same type of plasmids that carry the same repertoire of ARGs were also identified (e.g., IncB/O/K/Z in 7 different STs). This strongly suggested that horizontal transfer of ESBL-carrying plasmids occurred within the farm.

Conclusion

In conclusion, this study illustrates the within-farm diversity and dynamics of cefotaxime-resistant *E. coli* over time in dairy cattle, and shows the power of genomic surveillance in deciphering the complex epidemiology underlying multidrug resistance dissemination within a farm. Despite the differences observed between both farms, the presence of certain plasmid types with the same repertoire of ARGs in different *E. coli* STs might be indicative of the occurrence of horizontal transfer of such plasmids among strains circulating within the farms. AMU, environmental selection pressure, or co-selection with other advantageous genes might drive these events. Although we cannot rule out the existence of certain niche-specific clones that are better adapted to the calf intestinal environment, we found that the more widespread clones could readily infect animals of all age groups. Recommendations for the implementation of biosecurity measures to prevent the introduction of ESBL-producing *E. coli* and management protocols that limit contact between animals of different age groups were made to farmers to avoid cross-contamination and the spread of resistant bacteria. Considering the public health importance of ESBL-producing *E. coli* both as pathogens and as vectors for resistance mechanisms, the presence of β-lactamase- and other AMR-encoding genes in plasmids that can be readily transferred between bacteria is a concern that highlights the need for One Health surveillance.

Data availability statement

The raw sequencing data presented in this study can be found online at the NCBI Sequence Read Archive (SRA) database, associated with the BioProjects PRJNA833969 and PRJNA680938. The accession number(s) can be found in Table S5 in the Supplementary material.

Ethics statement

Ethical review and approval by the Ethics Committee for Animal Experimentation was not required for the animal study because sample collection was carried out by veterinary practitioners strictly following Spanish ethical guidelines and animal welfare regulations (Real Decreto 53/2013) as part of their routine veterinary practice. Informed consent was obtained from the farm owners at the time of sample collection. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AH conceived and coordinated the study. BO and MT performed laboratory analyses. JL, MO, and MT carried out bioinformatic data analyses. MO performed statistical analyses. AH, MO, and MT interpreted the data and wrote the manuscript. BO and JL contributed to the manuscript revision. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Basque Government: The Department of Economic Development, Sustainability, and Environment (URAGAN 17-00892). MT is the recipient of a predoctoral fellowship from the Basque Government (Department of Economic Development, Sustainability, and Environment).

Acknowledgments

We express our thanks to the veterinary clinicians who carried out the samplings and to the farmers for their collaboration in this study. The authors would like to thank Dr. Gorka Aduriz (NEIKER) for his technical advice and helpful discussions.

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.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.936843/full#supplementary-material

References

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST ring image generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Aziz, R. K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9:75. doi: 10.1186/1471-2164-9-75

Clinical and Laboratory Standards Institute (2015). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CSLI Document M100-S25. Wayne, PA.

Collis, R. M., Burgess, S. A., Biggs, P. J., Midwinter, A. C., French, N. P., Toombs-Ruane, L., et al. (2019). Extended-spectrum beta-lactamase-producing Enterobacteriaceae in dairy farm environments: a New Zealand perspective. *Foodborne Pathog. Dis.* 16, 5–22. doi: 10.1089/fpd.2018.2524

Dantas Palmeira, J., and Ferreira, H. M. N. (2020). Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in cattle production – a threat around the world. *Heliyon* 6:e3206. doi: 10.1016/j.heliyon.2020.e03206

Darling, A. E., Mau, B., and Perna, N. T. (2010). Progressivemauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5:e11147. doi: 10.1371/journal.pone.0011147

ECDC, EFSA, and EMA (2021). Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA. *EFSA J.* 19:6712. doi: 10.2903/j.efsa.2021.6712

EFSA and ECDC (2021). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. *EFSA J.* 19:e6490. doi: 10.2903/j.efsa.2021.6490

Ellington, M. J., Findlay, J., Hopkins, K. L., Meunier, D., Alvarez-Buylla, A., Horner, C., et al. (2016). Multicentre evaluation of a real-time PCR assay to detect genes encoding clinically relevant carbapenemases in cultured bacteria. *Int. J. Antimicrob. Agents* 47, 151–154. doi: 10.1016/j.ijantimicag.2015.11.013

Frahm, E., and Obst, U. (2003). Application of the fluorogenic probe technique (TaqMan PCR) to the detection of Enterococcus spp. and *Escherichia coli* in water samples. *J. Microbiol. Methods* 52, 123–131. doi: 10.1016/S0167-7012(02)00150-1

Gay, E., Bour, M., Cazeau, G., Jarrige, N., Martineau, C., Madec, J. Y., et al. (2019). Antimicrobial usages and antimicrobial resistance in commensal *Escherichia coli* from veal calves in France: evolution during the fattening process. *Front. Microbiol.* 10:792. doi: 10.3389/fmibc.2019.00792

Heberle, H., Meirelles, V. G., da Silva, F. R., Telles, G. P., and Minghim, R. (2015). InteractiVenn: A web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics* 16:169. doi: 10.1186/S12859-015-0611-3

Hille, K., Ruddat, I., Schmid, A., Hering, J., Hartmann, M., von Munchhausen, C., et al. (2017). Cefotaxime-resistant *E. coli* in dairy and beef cattle farms – joint analyses of two cross-sectional investigations in Germany. *Prev. Vet. Med.* 142, 39–45. doi: 10.1016/j.prevetmed.2017.05.003

Hordijk, J., Fischer, E. A. J., Van Werven, T., Sietsma, S., Van Gompel, L., Timmerman, A. J., et al. (2019). Dynamics of faecal shedding of ESBL- or AmpC-producing *Escherichia coli* on dairy farms. *J. Antimicrob. Chemother.* 74, 1531–1538. doi: 10.1093/JAC/DKZ035

Hordijk, J., Mevius, D. J., Kant, A., Bos, M. E. H., Graveland, H., Bosman, A. B., et al. (2013). Within-farm dynamics of ESBL/AmpC-producing *Escherichia coli* in veal calves: a longitudinal approach. *J. Antimicrob. Chemother.* 68, 2468–2476. doi: 10.1093/JAC/DKT219

Horton, R. A., Duncan, D., Randall, L. P., Chappell, S., Brunton, L. A., Warner, R., et al. (2016). Longitudinal study of CTX-M ESBL-producing *E. coli* strains on a UK dairy farm. *Res. Vet. Sci.* 109, 107–113. doi: 10.1016/J.RVSC.2016.09.018

Kock, R., Daniels-Haardt, I., Becker, K., Mellmann, A., Friedrich, A. W., Mevius, D., et al. (2018). Carbapenem-resistant Enterobacteriaceae in wildlife, food-producing, and companion animals: a systematic review. *Clin. Microbiol. Infect.* 24, 1241–1250. doi: 10.1016/j.cmi.2018.04.004

Kolmogorov, M., Yuan, J., Lin, Y., and Pevzner, P. A. (2019). Assembly of long, error-prone reads using repeat graphs. *Nat. Biotechnol.* 37, 540–546. doi: 10.1038/S41587-019-0072-8

Krawczyk, P. S., Lipinski, L., and Dziembowski, A. (2018). PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures. *Nucleic Acids Res.* 46:e35. doi: 10.1093/nar/gkx1321

Madec, J. Y., Haenni, M., Nordmann, P., and Poirel, L. (2017). Extended-spectrum β-lactamase/AmpC- and carbapenemase-producing Enterobacteriaceae in animals: a threat for humans? *Clin. Microbiol. Infect.* 23, 826–833. doi: 10.1016/j.cmi.2017.01.013

Noyes, N. R., Yang, X., Linke, L. M., Magnuson, R. J., Cook, S. R., Zaheer, R., et al. (2016). Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. *Sci. Rep.* 6, 1–12. doi: 10.1038/srep24645

Page, A. J., and Keane, J. A. (2018). Rapid multi-locus sequence typing direct from uncorrected long reads using Krocus. *PeerJ* 6:e5233. doi: 10.7717/peerj.5233

Plassard, V., Gisbert, P., Granier, S. A., and Millemann, Y. (2021). Surveillance of extended-spectrum β-lactamase-, cephalosporinase- and carbapenemase-producing gram-negative bacteria in raw milk filters and healthy dairy cattle in three farms in Île-de-France. *France. Front. Vet. Sci.* 8:633598. doi: 10.3389/fvets.2021.633598

Poirel, L., Madec, J.-Y., Lupo, A., Schink, A.-K., Kieffer, N., Nordmann, P., et al. (2018). "Antimicrobial resistance in *Escherichia coli*," in *Antimicrobial Resistance in Bacteria from Livestock and Companion Animals*. eds. S. Schwarz, L. M. Cavaco and J. Shen (USA: ASM Press), 289–316.

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., et al. (2018). Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *J. Antimicrob. Chemother.* 73, 1121–1137. doi: 10.1093/jac/dkx488

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Seiffert, S. N., Hilty, M., Perreten, V., and Endimiani, A. (2013). Extended-spectrum cephalosporin-resistant gram-negative organisms in livestock: an emerging problem for human health? Drug resist. *Updat.* 16, 22–45. doi: 10.1016/j.drup.2012.12.001

Sjölund-Karlsson, M., Joyce, K., Blickenstaff, K., Ball, T., Haro, J., Medalla, F. M., et al. (2011). Antimicrobial susceptibility to azithromycin among *Salmonella enterica* isolates from the United States. *Antimicrob. Agents Chemother.* 55, 3985–3989. doi: 10.1128/AAC.00590-11

Tadesse, D. A., Li, C., Mukherjee, S., Hsu, C. H., Bodeis Jones, S., Gaines, S. A., et al. (2018). Whole-genome sequence analysis of CTX-M containing *Escherichia coli* isolates from retail meats and cattle in the United States. *Microb. Drug Resist.* 24, 939–948. doi: 10.1089/MDR.2018.0206

Tello, M., Ocejo, M., Oporto, B., and Hurtado, A. (2020). Prevalence of cefotaxime-resistant *Escherichia coli* isolates from healthy cattle and sheep in northern Spain: phenotypic and genome-based characterization of antimicrobial susceptibility. *Appl. Environ. Microbiol.* 86:e00742-20. doi: 10.1128/AEM.00742-20

Tello, M., Oporto, B., Ocejo, M., and Hurtado, A. (2022). Characterization of a carbapenem-resistant *Escherichia coli* from dairy cattle harbouring bla_{NDM-1} in an IncC plasmid. *J. Antimicrob. Chemother.* 77, 843–845. doi: 10.1093/jac/dkab455

WHO (2019). Critically Important Antimicrobials for human medicine, 6th Revision. Geneva. Available at: https://www.who.int/publications/i/item/9789241515528

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017a). Completing bacterial genome assemblies with multiplex MinION sequencing. *Microb. Genomics* 3:e000132. doi: 10.1099/mgen.0.000132

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017b). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13:e1005595. doi: 10.1371/journal.pcbi.1005595

Zhou, Z., Alikhan, N. F., Mohamed, K., Fan, Y., and Achtman, M. (2020). The EnteroBase user's guide, with case studies on Salmonella transmissions, *Yersinia pestis* phylogeny, and Escherichia core genomic diversity. *Genome Res.* 30, 138–152. doi: 10.1101/gr.251678.119

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 29 July 2022
DOI 10.3389/fmicb.2022.929800

Check for updates

OPEN ACCESS

EDITED BY
Ziad Daoud,
Central Michigan University,
United States

REVIEWED BY
Po-Xing Zheng,
Academia Sinica, Taiwan
Jeanette Teo,
National University Hospital, Singapore

*CORRESPONDENCE
Ping Chen
1243377523@qq.com
Xuejun Guo
xuejung2021@163.com

SPECIALTY SECTION
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

RECEIVED 27 April 2022
ACCEPTED 28 June 2022
PUBLISHED 29 July 2022

CITATION
Zheng L, Zhang X, Zhu L, Lu G, Guan J, Liu M, Jing J, Sun S, Wang Y, Sun Y, Ji X, Jiang B, Chen J, Liu J, Chen P and Guo X (2022) A clinical *Pseudomonas juntendi* strain with bla_{IMP-1} carried by an integrative and conjugative element in China. *Front. Microbiol.* 13:929800. doi: 10.3389/fmicb.2022.929800

COPYRIGHT
© 2022 Zheng, Zhang, Zhu, Lu, Guan, Liu, Jing, Sun, Wang, Sun, Ji, Jiang, Chen, Liu, Chen and Guo. 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.

A clinical *Pseudomonas juntendi* strain with bla_{IMP-1} carried by an integrative and conjugative element in China

Lin Zheng[1,2], Xinfang Zhang[3], Lingwei Zhu[2], Gejin Lu[2], Jiayao Guan[2], Mingwei Liu[1,2], Jie Jing[2], Shiwen Sun[2], Ying Wang[2], Yang Sun[2], Xue Ji[2], Bowen Jiang[2], Jun Chen[2], Jun Liu[2], Ping Chen[1]* and Xuejun Guo[2]*

[1]School of Food and Engineering, Jilin Agricultural University, Changchun, China, [2]Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Changchun Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Changchun, China, [3]China-Japan Union Hospital, Jilin University, Changchun, China

Objective: To precisely determine the species of a carbapenem-resistant *Pseudomonas* strain 1809276 isolated from the urine of a Chinese patient and analyze its integrative and conjugative element (ICE) *1276* formation mechanism.

Methods: Single-molecule real-time (SMRT) sequencing was carried out on strain 18091276 to obtain the complete chromosome and plasmid (pCN1276) sequences, and average nucleotide identity (ANI) was used for precise species identification. The ICEs in GenBank with the same integrase structure as ICE *1276* were aligned. At the same time, the transfer ability of bla_{IMP-1} and the antibiotic sensitivity of *Pseudomonas juntendi* 18091276 were tested.

Results: This bacterium was *P. juntendi*, and its drug resistance mechanism is the capture of the *accA4'* gene cassette by the Tn*402*-like type 1 integron (*IntI1*-bla_{IMP-1}) to form In*1886* before its capture by the ΔTn*4662a*-carrying ICE *1276*. The acquisition of bla_{IMP-1} confers carbapenem resistance to *P. juntendi* 18091276.

Conclusion: The formation of bla_{IMP-1}-carrying ICE *1276*, its further integration into the chromosomes, and transposition and recombination of other elements promote bacterial gene accumulation and transmission.

KEYWORDS

***Pseudomonas juntendi*, integrative and conjugative, carbapenem-resistant, reorganization, bla_{IMP-1}**

Introduction

Pseudomonas sp. is a genus of varied and complex Gram-negative bacteria commonly isolated from soil and water, with a wide host diversity, including animals and plants. It consists of 254 species (List of Prokaryotic names with Standing in Nomenclature, LPSN; www.bacterio.net) divided into three lineages and 13 groups based on the sequences of the

16S rRNA, *gyrB*, *rpoB,* and *rpoD* genes (Peix et al., 2018). Among them, several species of *Pseudomonas sp.* were regarded as important opportunistic pathogens affecting humans, including *Pseudomonas aeruginosa*, *Pseudomonas asiatica*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas cepacia*, *Pseudomonas stutzeri*, *Pseudomonas maltophilia,* and *Pseudomonas putrefaciens* (Tohya et al., 2019). Their capabilities of acquirement of exogenous genes lead to the prone of antibiotics resistance in this genus, including carbapenem antibiotics resistance, which enhances the difficulties of treatment. *Pseudomonas sp.* can acquire carbapenem resistance genes by integrons, which further can be captured by other mobile elements (such as plasmids, transposons, etc.), and then, these genes begin to spread among other bacteria.

In this study, we analyzed a *P. juntendi* strain separated from a urine specimen of a patient in China in 2018, which was tentatively identified as *P. putida* by VITEK2 (bioMerieux), and the strain carried a carbapenem resistance gene bla_{IMP-1}. To our knowledge, this is the first report of clinical strain of *P. juntendi* carrying bla_{IMP-1} in a new gene context in China.

Materials and methods

Bacterial isolation and identification

Strain 18091276 was isolated from a urine specimen from a patient in a tertiary hospital in northeast China in 2018 and the species of the strain was determined by the part of the sequence of the 16S rRNA gene (Edwards et al., 1989). Then, a maximum likelihood (ML) evolutionary tree was constructed using MEGA 7.0 software to evaluate the sequence similarity of the 16S rRNA genes that were aligned and listed in the BLASTN program (top 100).

The minimum inhibitory concentration (MIC) of imipenem was determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines, and Escherichia coli ATCC 25922 was used as a control. MICs of amikacin, gentamicin, meropenem, cefazolin, ceftazidime, cefotaxime, cefepime, aztreonam, ampicillin, piperacillin, amoxicillin-clavulanate, ampicillin-sulbactam, piperacillin-tazobactam, colistin, trimethoprim-sulfamethoxazole, chloramphenicol, ciprofloxacin, levofloxacin, moxifloxacin, and tetracycline were tested by BD Phoenix-100.

Sequencing and sequence assembly

Bacterial genomic DNA was extracted from the strain 18091276 using the UltraClean Microbial Kit (Qiagen, NW, Germany) and sequenced by a PacBio RSII sequencer (Pacific Biosciences, CA, USA). The reads were assembled *de novo* by using *SMARTdenovo* (http://github.com/ruanjue/smartdenovo).

Sequence annotation and comparison

Rast 2.0 (Brettin et al., 2015) and *BLASTP*/*BLASTN* (Boratyn et al., 2013) searches were used to predict open reading frames (ORFs), online databases *CARD* (https://card.mcmaster.ca/; Alcock et al., 2020), and *ResFinder 4.1* (https://cge.cbs.dtu.dk/services/ResFinder/; Bortolaia et al., 2020) to find out resistance genes, and *ISfinder* (https://www-is.biotoul.fr/; Lastest Database Update 2021-9-21; Varani et al., 2011), *TnCentral* (https://tncentral.proteininformationresource.org/), and *ICEberg 2.0* (http://db-mml.sjtu.edu.cn/ICEberg/; Liu et al., 2019) were used to find out the mobile elements. Pairwise sequence comparisons were carried out by *BLASTN*. Gene organization diagrams were drawn by *Inkscape 1.0* (http://inkscape.org/en/).

Bacterial precise species identification and evolutionary tree construction

Bacterial precise species identification was performed using the pair-wise average nucleotide identity (ANI) analysis between strain 18091276 and the reference genome (http://www.ezbiocloud.net/tools/ani). A ≥95% ANI cutoff was used to define bacterial species (Yoon et al., 2017). *CSI Phylogeny* (https://cge.cbs.dtu.dk/services/CSIPhylogeny/; Kaas et al., 2014) calls and filters single nucleotide polymorphisms (SNPs) of strain 18091276, does site validation, and infers a phylogeny based on the concatenated alignment of the high-quality SNPs. In addition, the *MUSCLE* software program was used to align multiple single-copy core-encoded proteins identified by the core-/pan-genome analysis. The aligned sequences were then subjected to phylogenetic analysis using the *TreeBeST* (Version 1.9.2) program, a neighbor-joining tree reconstruction algorithm, and 1,000 bootstrap replicates (Nandi et al., 2010).

Conjugation experiments

Conjugation experiments were performed as described previously (Mizuno et al., 2020). Briefly, strain 18091276 was used as a donor and sodium azide-resistant *E.coli* J53 as a recipient. Donor and recipient strains were cultured overnight at 37°C separately. Then, 3 ml of 18091276 culture was mixed up with an equal volume of *E.coli* J53 culture. The mixed cells were harvested by centrifugation for 3 min at 12,000 × *g*, washed with 3 ml of lysogeny broth (LB), and resuspended in 150 μl of LB. The mixture was spotted on a 1 cm^2 hydrophilic nylon membrane filter (Millipore) with a 0.45-μm pore size, which was

then placed on an LB agar plate and then incubated for mating at 37°C for 6 h. The cells were recovered from the filter membrane and spotted on LB agar containing 100 μg/ml sodium azide and 4 μg/ml imipenem for selecting carbapenem-resistant *E. coli* transconjugant.

Nucleotide sequence accession numbers

The complete sequence of 18091276 has been submitted to GenBank under the accession number CP091311.

Results and discussion

After the 18091276 strain was cultured overnight at 37°C, 2 mm round protruding colonies with smooth and regular edges, non-fusion growth, non-pyocyanin production, and the absence of metallic sheen were found on brain heart infusion agar (imipenem concentration: 4 μg/ml).

Strain 18091276 was identified by the BD Phoenix-100 identification system and VITEK 2 as *P. putida*, and Table 1 shows its drug resistance spectrum. The similarity of the 16S rRNA sequence of this bacterium and *P. putida*, *Pseudomonas monteilii*, *Pseudomonas plecoglossicida*, and *P. juntendi* in the *P. putida* group was more than 99% and the strain was identified to be in the *P. putida* group (Supplementary Figure 1). After single-molecule real-time (SMRT) sequencing (basic information about SMRT sequence results was provided in Supplementary Table 1), it was found that the ANI value of strain 18091276 was more than 95% with the reference strain *P. juntendi* BML3 (GCA_009932375.1), and this strain was confirmed to be *P. juntendi* (ANI value of *P. juntendi* 18091276 were provided in Supplementary Table 2A). In 2019, Tohya M. was the first to carry out a systematic identification of *P. juntendi* and officially named this species (Tohya et al., 2019). The analysis of all 14 strains that were annotated as *P. juntendi* in GenBank (cutoff date was November 2021; Table 2) found that the clinical isolates before 2018 were from Brazil, and the isolates in China, the USA, Japan, and Russia only appeared after 2018. Although *P. juntendi* 18091276 and 14181154 were all isolated in China, they had a far phylogenetic relationship (Figure 1). According to the SNPs and core-genome phylogenetic tree, *P. juntendi* 18091276 was the closest relative to *P. juntendi* 12349 (Brazil, 2012) and *P. juntendi* PSB00036 (the USA, 2018; Figure 1). We speculate

TABLE 1 Antimicrobial susceptibility of 18091276.

Antimicrobial type	Antimicrobial	MIC (μg/mL)[a]	SIR[b]
Aminoglycosides	Amikacin	≤8	S
	Gentamicin	4	S
β-lactam	Imipenem	32	R
	Meropenem	>8	R
	Cefazolin	>16	R
	Ceftazidime	>16	R
	Cefotaxime	>32	R
	Cefepime	>16	R
	Aztreonam	16	I
	Ampicillin	>16	R
	Piperacillin	8	S
	Amoxicillin-Clavulanate	>16/8	R
	Ampicillin-Sulbactam	>16/8	R
	Piperacillin-Tazobactam	8/4	S
Colistin	Colistin	1	NA
Sulfonamide	Trimethoprim-Sulfamethoxazole	>2/38	R
Chloramphenicol	Chloramphenicol	>16	R
Quinolones	Ciprofloxacin	≤0.5	S
	Levofloxacin	≤1	S
	Moxifloxacin	4	NA
Tetracycline	Tetracycline	4	S

[a]MIC, minimum inhibitory concentration.
[b]SIR, Susceptible (S), Intermediate (I), Resistant (R).
NA, not applicable.

TABLE 2 The information of *P. juntendi*.

Strain	Source	Time	Country	Size (Mb)	GC content (%)	Resistance gene	Assembly ID
10918	Urine	2010	Brazil	6.09	62	*aadA1, aph(3')-VIa, aacA4', sul1,* bla_{IMP-16}, bla_{VIM-2}, *blaOXA-129*	GCA_014062235.1
10618	Blood	2010	Brazil	5.71	62.2	bla_{VIM-2}, *aacA4', blaOXA-129, sul1*	GCA_014062305.1
11213	Blood	2010	Brazil	5.70	62.2	*aacA4', sul1,* $bla_{OXA-129}$, bla_{VIM-2}	GCA_014062275.1
12273	Urine	2012	Brazil	5.59	62.5		GCA_014062265.1
12349	Blood	2012	Brazil	5.64	62.3	*aacA4', sul1,* bla_{IMP-16}, bla_{VIM-2}	GCA_014062185.1
12815	Urine	2013	Brazil	5.69	62.5	*sul1, aadA1, aacA4', aph(3')-VIa, aadA1,* bla_{IMP-1}	GCA_014062135.1
14181154	Catheter	2014	China	5.61	62.3	*aacA4', aadA1,* bla_{OXA-21}, bla_{IMP-1}	CP045554.1
18091276	Urine	2018	China	5.89	62.3	*aacA4',* bla_{IMP-1}	This study
BML3	Sputum	2018	Japan	5.73	62.7		GCA_009932375.1
PSB00022	Urine	2018	USA	5.77	62.1		GCA_016009075.1
PSB00036	Urine	2018	USA	5.42	62.4		GCA_016337345.1
PSB00020	Sputum	2018	USA	6.20	62.2		GCA_016009085.1
14535	Blood	2019	Brazil	6.07	62.1	*aacA4', sul1,* bla_{VIM-2}, bla_{IMP-16}	GCA_014062085.1
SCPM-O-B-9248	Sputum	2021	Russia	5.67	62.3		GCA_018138545.1

that *P. juntendi* 18091276 might be transferred from the USA or Brazil through international food (animal- and plant-based) trade and travel. The identification method at the time was not precise, many *P. juntendi* isolates were wrongly identified as *P. putida* or other species in the *P. putida* group (Morimoto et al., 2020), resulting in fewer epidemiological data and whole-genome data of this strain in other countries. ResFinder screening found that, out of the 14 *P. juntendi* strains, six Brazilian strains carried the carbapenem resistance genes bla_{IMP} and bla_{VIM} and two Chinese strains carried bla_{IMP} (see Table 2 for strain information), showing that *P. juntendi* could acquire bla_{IMP} and bla_{VIM}, like *P. aeruginosa* and *P. putida,* and develop carbapenem resistance, and it is potentially harmful.

The chromosome of *P. juntendi* 18091276 contained an 88.61 kb integrative and conjugative element (ICE) that was named ICE *1276*. ICE *1276* contained *attL* and *attR* produced from recombination, a complete integrase gene (*int)*, the F-type IV secretion system responsible for the ICE transfer, the conjugation gene *cpl*, and *rlx* responsible for autonomous rolling circle replication (Johnson and Grossman, 2015). ICE *1276* did not contain the cleavage enzyme *xis* or the origin of replication *oriT*. *crpP* (quinolone resistance gene) was also found in the backbone region of ICE *1276*. *crpP* was mainly present in the ICEs of *Pseudomonas aeruginosa* and could increase ciprofloxacin resistance (Zhu et al., 2021). However, *P. juntendi* 18091276 did not possess quinolone resistance. In addition, a 15.96-kb accessory module was discovered in ICE *1276*, and this structure had the closest phylogenetic relationship with Tn*4662a* (a Tn*3* family transposon) in plasmid pDK1 (NC_014124.1; coverage: 58%, identity: 98.65%). Moreover, it had the same 38 bp inverted repeat sequence (IRs) as Tn*4662a,* and a 5-bp (AGTAT) directed repeat sequence (DRs) was generated during insertion (detailed information is shown in Supplementary Table 3). Tn*4662a* was first found on a plasmid pDK1 carried by *P. putida* HS (Yano et al., 2010). In contrast to Tn*4662a* (pDK1), the insertion structure of *P. juntendi* 18091276 also contained a Tn*6811* remnant (Tn*3* family), a single copy insertion element IS*Psfu1* (IS*5* family), and a complete In*1886* (Tn*402*-like type 1 integron) carrying bla_{IMP-1} and *aacA4'*. However, the res_site_II, res_site_III, *tnpR*, and *tnpA* sequences in Tn*4662a* (ICE *1276*) were partially deleted, which should have a Tn*4662a*-derived structure (Figure 2).

Until November 2021, only 4 ICEs with the same integrase as ICE *1276* were indexed in GenBank (Table 3 shows the strain information). Interestingly, these ICE *1276*-carrying strains were *Pseudomonas* sp. from China. ANI calculator was used to precisely analyze the identified *Pseudomonas* sp. 14181154 and LTGT-11-2Z. The ANI value of *Pseudomonas sp.* 14181154 and the reference strain *P. juntendi* BML3 was more than 95% (ANI value in Supplementary Table 2B), so *Pseudomonas sp.* 14181154 was identified as *P. juntendi* (Figure 1 shows the phylogenetic relationship with other *P. juntendi* strains), but the ANI of LTGT-11-2Z and all *Pseudomonas* species were lower than 95%. Therefore, LTGT-11-2Z was only identified as *Pseudomonas sp.* All ICEs were obtained from *Pseudomonas* sp. chromosomes, and *E. coli* transconjugants were not obtained after repeated conjugation experiments. Except for the exogenous insertion sites, the remaining structure of the ICEs was nearly identical. Another exception is that a 14.29 kb accessory module was

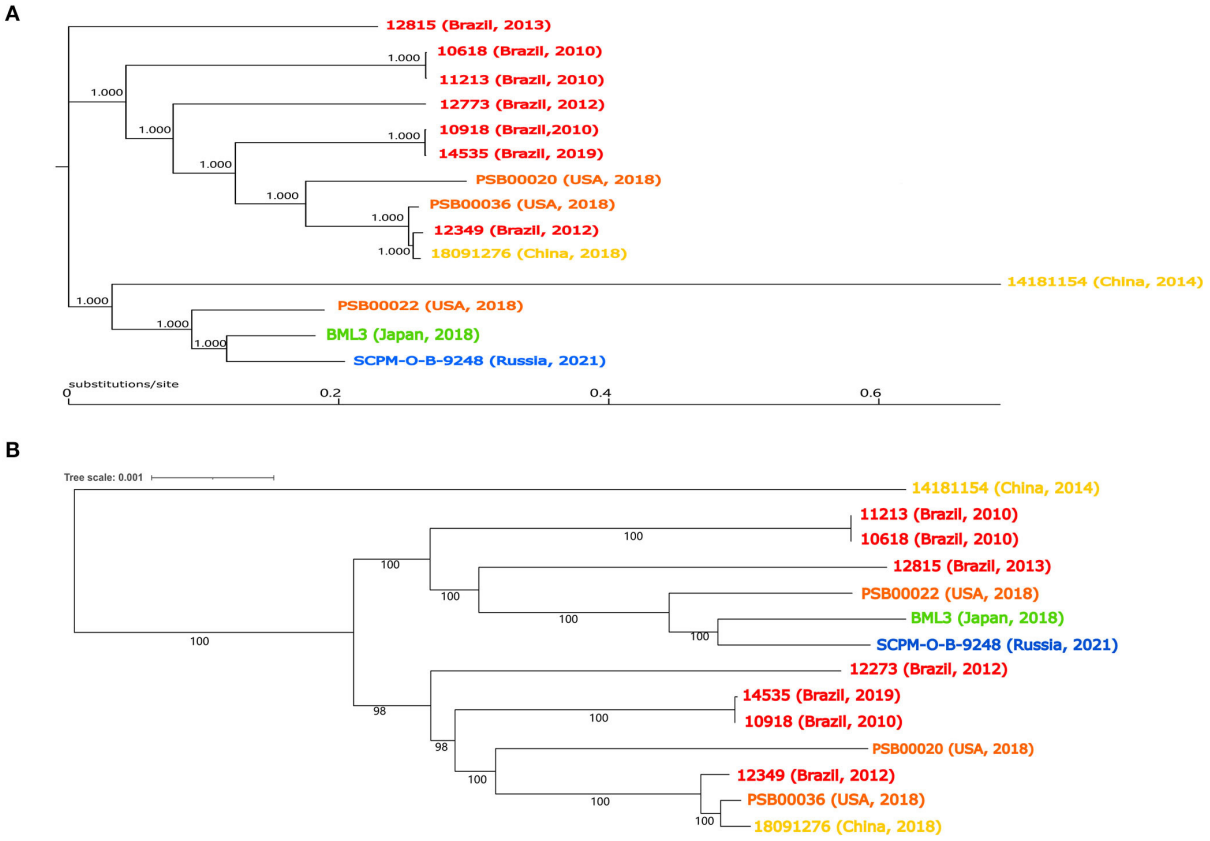


FIGURE 1
Pseudomonas juntendi genome phylogenetic tree. The top half is the phylogenetic tree constructed from SNPs of genomes **(A)**. The bottom half of the figure is the phylogenetic tree constructed from core genomes **(B)**. The Brazilian isolates are labeled in red, Chinese isolates are labeled in khaki, American isolates are labeled orange, Japanese isolates are labeled green, and Russian isolates are labeled blue.

inserted at 74 bp upstream of *ssb* (single-strand DNA binding protein) in the backbone region of ICE *LTGT-11-2Z* (plant-derived *Pseudomonas sp.* LTGT-11-2Z). This ICE seemed to have caused the transfer of large fragments between *Pseudomonas* species under specific conditions.

As the downstream region of *Tn4662a* is truncated by *Tn6811*, it is unable to carry out normal replicative transposition (Grindley and Reed, 1985), but it still contains an intact res_site_I (Supplementary Table 4A) and can recombine with In*1886* and IS*Psfu1* (Grindley and Reed, 1985; Brovedan et al., 2021) (Figure 2). IRs and DRs were absent at both ends of IS*Psfu1*-In*1886* and did not constitute a composite transposon (Zong, 2014). Alignment with 10 Tn*402*-like integrons containing bla_{IMP-1} and *aacA4'* gene cassettes that were indexed before November 2021 (Table 4 shows the strain information) found that except for In*1960*, In*1771*, and In*1886* located in ICEs, the IS*Psfu1* element was not inserted upstream or downstream of the remaining integrons (regardless of whether it is on the plasmid or chromosome) and there were two independent insertion processes. The Tn*4662a*-drived structure in ICE *LTGT-11-2Z* (Figure 2) indicated that, after IS*Psfu1* had undergone non-replicative transposition, In*1886* was inserted upstream of the res_site_I of ΔTn*4662a* (Figure 3 shows the formation process of ICE *1276*).

IMP-1 was first discovered in a *P. aeruginosa* strain from Japan (Walsh et al., 2005) and is now the most widely circulating IMP enzyme in Southeast Asia (Walsh et al., 2005). Up until November 2021, 86 IMP family members were indexed in GenBank, and most bla_{IMP-1} were carried and transferred by type 1 integrons (Diene and Rolain, 2014). After the Tn*402* integron captures bla_{IMP-1}, Tn*402* possesses the self-transfer capability and can be integrated into transposons or res sites on plasmids to expand its transmission range (Gillings, 2017). In 2014, pNXM63 (MW150990) from *Morganella morganii* nx_m63 that was isolated in a hospital in China contained a Tn402-like type 1 integron (intI1-bla_{IMP-1}; Xiang et al., 2021). Based on the repeat sequence (AACG) on both sides

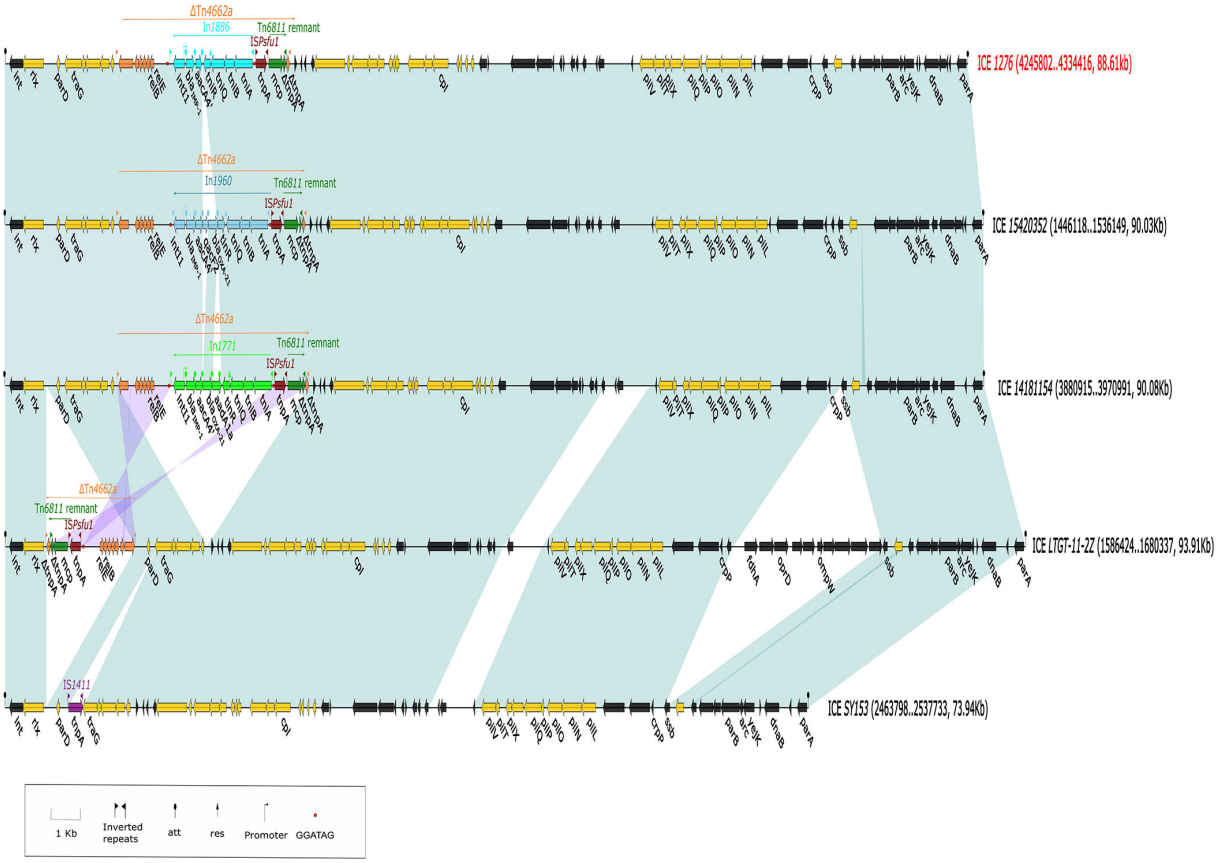


FIGURE 2
Linear alignment map of integrative and conjugative elements (ICEs). The integration region is yellow, the backbone region is black, In*1886* is light blue, In*1960* is blue, In*1771* is green, Tn*4662a* is orange, IS*Psfu1* is dark red, Tn*6811* is dark green, and IS*1411* is purple. The solid red dot is the integron inverted repeat (IR) recognition sequence GGATAG, and the shaded region represents regions with >90% nucleotide identity. Except for *Pseudomonas putida* SY153 ICE *SY153*, the other ICEs were all Tn*4662a*-derived structures. IS*1411* (ISL3 family) recognizes the 8 bp binding site (ATCAAAGG) on *traG* through IR, thereby inserting it into ICE *SY153* and causing the binding site to form directed repeat (DR). Except for the Tn*4662a*-derived structure in the ICE of *Pseudomonas* sp. LTGT-11-2Z that was inserted in the 257 bp site downstream of *rlx* after inversion, Tn*4662a*-derived structures from other strains were inserted in the positive direction 296 bp upstream of the integrating conjugative element protein of the TIGR03757 family in these ICEs. The integron IR recognition site (GATAGG) was similarly present at 1,153 bp upstream of the res site on Tn*4662a* (ICE *LTGT-11-2Z*), but no integron was inserted. A 14.29-kb accessory module (dark pink) was also inserted 74 bp upstream of the *ssb* (single-strand DNA binding protein) in the backbone region of ICE *LTGT-11-2Z* and contains *fdhA* (formate dehydrogenase), *oprD* (outer membrane porin protein), and *ompW* (outer membrane protein). Alignment of ICE *1276* with ICE *14181154* from *P. juntendi* 14181154 and ICE *15420352* from *P. putida* 15420352 found that only the number of gene cassettes captured by the Tn*402*-like integron was different, and other structures were identical.

of the gene cassette, it can be deduced that In*1886* is a structure formed after the integron on pNXM63 captured the *aacA4*' gene cassette (Figure 4). The Tn*402*-like transposon module at the 3' CS end of In*1886* could capture other drug resistance genes. An example is In*1886* in the PA15W plasmid in *P. aeruginosa* (GeneBank accession no.: MN961673). However, as this In*1886* IS*Cfr1* fragment mediated the insertion of *strA-strB-aac(3)-IId* to the Tn*402* recombination site on PA15W-In*1886*, it resulted in the truncation of the res_site_r1 site (Figure 4, Supplementary Table 4B), leading to irreversible recombination (Rowland et al., 2020). In*1886* can also capture *aadA6*, bla_{OXA-21}, *aadA1a*, *qacF2*, and *guc162* gene cassettes through recombination at specific sites to obtain a series of derived structures (Labbate et al., 2009; Table 4). This integron and its derived structures were mostly present on *Pseudomonas sp.* chromosomes and were also discovered in *K. pneumoniae* and *K. oxytoca* plasmids (Table 4).

Therefore, bla_{IMP-1} can be recombined upstream of res_site_I in Tn*4662a* using a Tn*402*-like integron as a medium to achieve transfer from other species to *P. juntendi* 18091276. After that, large fragments are transferred between *Pseudomonas* sp. through ICEs. The Tn*4662a*-derived structures in these ICEs contain the intact res_site_I sequence. It can also continue recombination with other elements. In addition, it was also found that, in addition to capturing gene cassettes, the res_site_r1 on *tniABQR* at 3' CS can also undergo

TABLE 3 The information of strains carrying ICEs.

Strain	Source	Species identification		Year	Country	Size (Mb)	GC content (%)	Assembly ID
		GenBank	This study					
LTGT-11-2Z	Alhagi sparsifolia Shap.	*Pseudomonas sp.*	*Pseudomonas sp.*	2014	Xinjiang, China	6.07	61.7	CP033104.1
14181154	Homo sapiens, Catheter	*Pseudomonas sp.*	*P.juntendi*	2014	Hunan, China	5.61	62.3	CP045554.1
SY153	Homo sapiens, urine	*P. putida*	*P. putida*	2012	Sanya, China	5.60	62.0	CP062218.1
15420352	Homo sapiens, urine	*P. putida*	*P. putida*	2015	Hunan, China	6.17	61.6	CP045551.1
18091276	Homo sapiens, urine	*P.juntendi*	*P.juntendi*	2018	Changchun, China	5.89	62.3	This study

TABLE 4 Genetic contexts of In*1886*.

Strain	Country	Year	Source	Location	Structure	Genbank AN
P. aeruginosa PA15W	China	NA	Human	P	*Int1-bla*$_{IMP-1}$*-aacA4'-Tni402*	MN961673
P. juntendi 18091276	China	2018	Urine	C	*Int1-bla*$_{IMP-1}$*-aacA4'-Tni402*-IS*Psful*	In this study
Pseudomonas sp. 14181154	China	2014	Catheter	C	*Int1-bla*$_{IMP-1}$*-aacA4'-bla*$_{OXA-21}$*-aadA1a-Tni402*-IS*Psful*	CP045554.1
P. putida 15420352	China	2015	Urine	C	*Int1-bla*$_{IMP-1}$*-aacA4'-qacF2-bla*$_{OXA-21}$*-Tni402*-IS*Psful*	CP045551.1
K. pneumoniae 2013050801	China	2013	Blood	P	*Intl1-bla*$_{IMP-1}$*-guc162-aacA4'-aadA6-Tni402*	KT345947
K. oxytoca 7121	China	2014	Sputum	P	*Intl1-bla*$_{IMP-1}$*-guc162-aacA4'-aadA6-Tni402*	KX784502
P. aeruginosa 97	Ghana, Western Africa	2015	Urine	C	*Intl1-bla*$_{OXA-10}$*/aacA4-bla*$_{IMP-1}$*-Tni402*	CP031449
P. aeruginosa JUNP133	Japan	NA	NA	C	*Intl1-bla*$_{OXA-10}$*/aacA4-bla*$_{IMP-1}$*-Tni402*	LC636409
E. coli MBL1-07[a]	India	2008	NA	NA	*Intl1-bla*$_{OXA-10}$*/aacA4-bla*$_{IMP-1}$	LC169568
P. alcaligenes KAM426	Japan	2020	wastewater	C	*Int1-fosE-aacA4'-3-aacA4'-3-blaIMP-1-qacG8-qacEΔ1-sul1-Tni402*	AP024354

[a]Since the uploaded of this strain was only an integrator sequence, unable to determine the mobile genetic elements upstream and downstream.
P, Plasmid.
C, Chromosome.
NA, not applicable.

further recombination, resulting in the production of multidrug resistance. Regarding different hosts, ICE *1276*-like integration elements can also undergo recombination with exogenous genes through *ssb* to carry out adaptive evolution.

Conclusion

This study was the first to report a clinical isolate of *P. juntendi* in China. At the same time, it was found that this bacterium can capture Tn*402*-like type 1 integron containing *bla*$_{IMP-1}$ through the ICE of Tn*4662a*. This provides a new vector and host for the horizontal transfer of *bla*$_{IMP-1}$. Hence, there is a need to improve bacterial identification methods and drug resistance monitoring in opportunistic pathogens in hospitals. At the same time, the ICE *1276*-Tn*4662a*-In*1886* structure and its mutant-derived structures should be closely monitored, particularly its epidemiology in China.

10.3389/fmicb.2022.929800

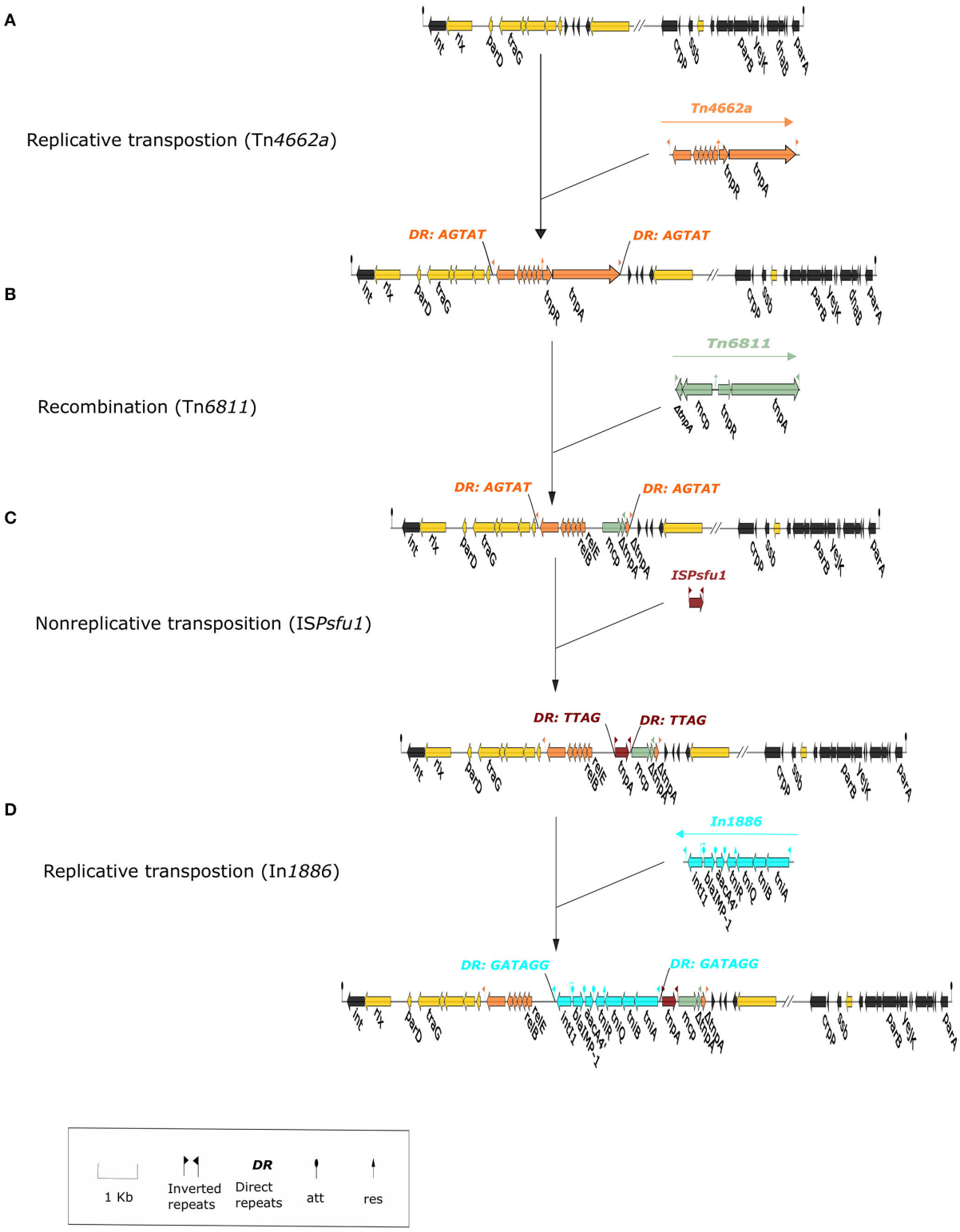


FIGURE 3
The formation process of integrative and conjugative element (ICE) *1276*. The figure shows the ICE *1276* structure as a local structure. The integration region of ICE *1276* is yellow, the backbone region is black, In*1886* is light blue; **(A)** Tn*4662a* wasrecombined in ICE *1276* through replicative transposition and produces bilateral directed repeat sequence (DRs) (AGTAT); **(B)** Tn*6811* and Tn*4662a* underwent recombination to form the ΔTn*4662a*-ΔTn*6811* structure; the truncated part of Tn*4662a* includes *tnpA*, complete deletion of *tnpR*, and the recombination sites res_site_II and res_site_III; **(C)** IS*Psfu1* was inserted into ΔTn*4662a*-ΔTn*6811* through non-replicative transposition and produced bilateral DRs (TTAG); **(D)** In*1886* was inserted upstream of the res_site_I in ΔTn*4662a* through replicative transposition and produced bilateral DRs (GATAGG).

10.3389/fmicb.2022.929800

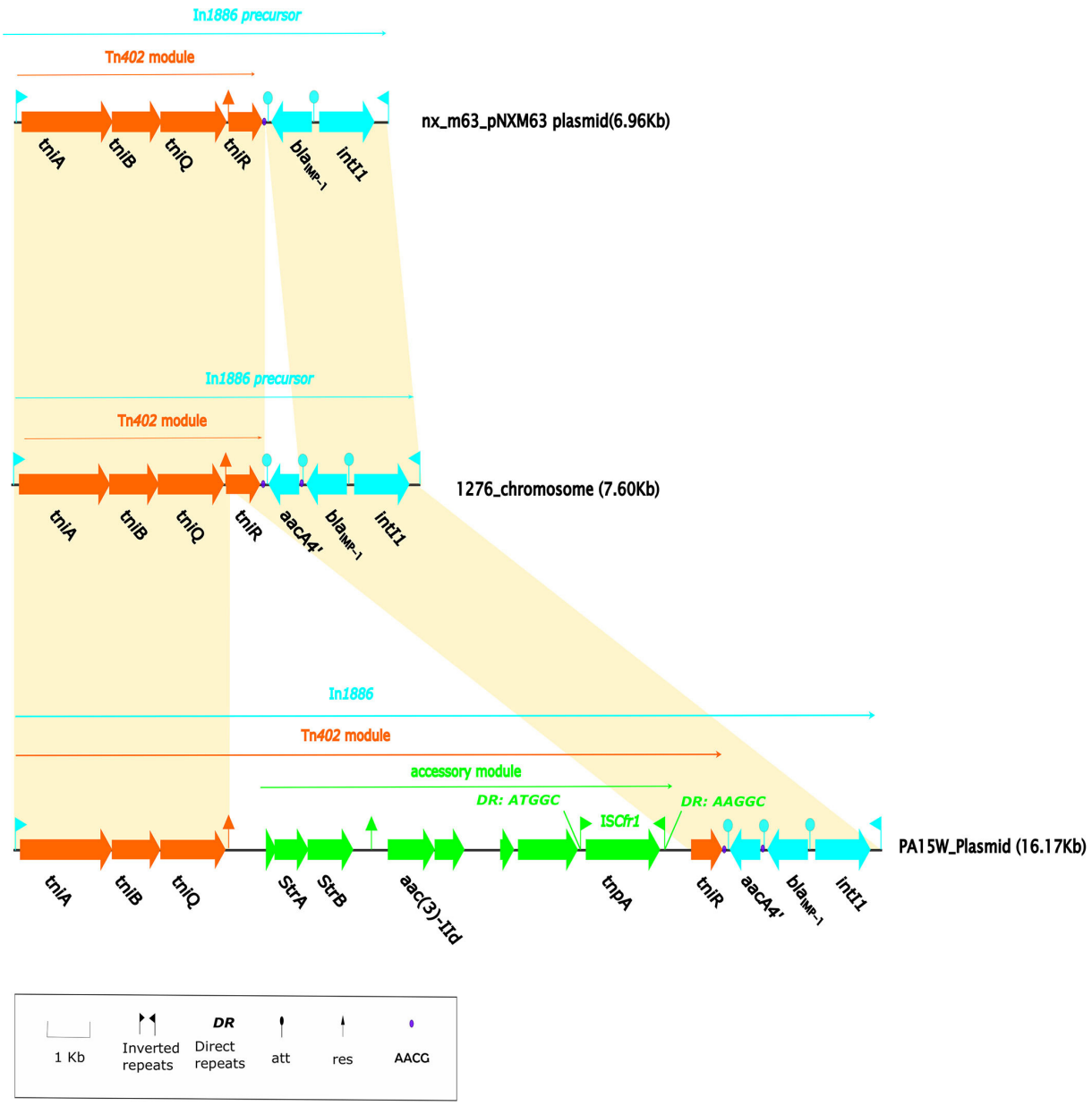


FIGURE 4
Linear alignment map of In*1886*. The Tn*402* transposition module is orange-red, the 3′-CS of the integron is blue, and the exogenous insertion element is green. The solid purple dot is the AACG sequence that is recognized by the gene cassette *att* and forms two copies in *In1886* due to the insertion of the *aacA4′* gene cassette. The shaded region represents a homologous region (>90% nucleotide identity). The pNXM63 (MW150990) from *Morganella morganii* nx_m63 contains an *intI1*-bla_{IMP-1}-*tniABQR* structure. When this integron was compared with In*1886*, except for the gene cassette *aacA4′*, all of the bases were completely identical, and there was no missing base. At the same time, the downstream region of the gene cassette bla_{IMP-1} in this integron contained the AACG site, which was present on both sides of the gene cassette *aacA4′* in In*1886*. In addition, In*1886* was also present in the plasmid (GenBank accession no.: MN961673) in *P. aeruginosa* PA15W. However, a 7.45-kb exogenous insertion structure was inserted in the res_site_r1 recombination site of the Tn*402* mobile module in the Tn*402* of PA15W-In*1886*, causing res_site_r1 to be truncated (Supplementary Table 2). This exogenous insertion structure contained eight open reading frames (ORFs), including a complete ISC*fr1* (IS1182 family), aminoglycoside resistance genes *strA* and *strB*, and *aac(3)-IId*.

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: https://www.ncbi.nlm.nih.gov/genbank/, CP091311.

Author contributions

All strains were provided by China-Japan Union Hospital, Jilin University. PC, XG, JC, JL, and LZhu conceived, directed, and carried out the study. ML, GL, JJ, YW, XZ, and BJ prepared samples for sequence analysis. JG, SS, and LZhe acquired samples and analyzed the data. All authors have read and approved the final manuscript.

Funding

Funding for study design, data collection, data generation, and publication costs was provided by the National Science and Natural Science Foundation of China (Grant agreement 31872486).

Acknowledgments

We are grateful to the members of the China-Japan Union Hospital, Jilin University.

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.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.929800/full#supplementary-material

References

Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., Tsang, K. K., Bouchard, M., Edalatmand, A., et al. (2020). CARD: antibiotic resistance surveillance with the Comprehensive Antibiotic Resistance Database. *Nucl. Acids Res.* 48, D517–D525. doi: 10.1093/nar/gkz935

Boratyn, G. M., Camacho, C., Cooper, P. S., Coulouris, G., Fong, A., Ma, N., et al. (2013). BLAST: a more efficient report with usability improvements. *Nucl. Acids Res.* 41, W29–33. doi: 10.1093/nar/gkt282

Bortolaia, V., Kaas, R. F., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., et al. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrobial Chemother.* 75, 3491–3500. doi: 10.1093/jac/dkaa345

Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., et al. (2015). RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep.* 5:8365. doi: 10.1038/srep08365

Brovedan, M. A., Marchiaro, P. M., Diaz, M. S., Faccone, D., Corso, A., Pasteran, F., et al. (2021). *Pseudomonas putida* group species as reservoirs of mobilizable Tn*402*-like class 1 integrons carrying *bla*VIM-2 metallo-β-lactamase genes. *Infect Genet Evol.* 96:105131. doi: 10.1016/j.meegid.2021.105131

CLSI (2020). *Performance Standards for Antimicrobial Susceptibility Testing.* Wayne, PA: Clinical and Laboratory Standards Institute.

Diene, S. M., and Rolain, J. M. (2014). Carbapenemase genes and genetic platforms in Gram-negative bacilli: *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species. *Clin. Microbiol. Infect.* 20, 831–838. doi: 10.1111/1469-0691.12655

Edwards, U., Rogall, T., Blöcker, H., Emde, M., and Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucl. Acids Res.* 17, 7843–53. doi: 10.1093/nar/17.19.7843

Gillings, M. R. (2017). Class 1 integrons as invasive species. *Curr. Opin. Microbiol.* 38, 10–15. doi: 10.1016/j.mib.2017.03.002

Grindley, N. D., and Reed, R. R. (1985). Transpositional recombination in prokaryotes. *Annu. Rev. Biochem.* 54, 863–896. doi: 10.1146/annurev.bi.54.070185.004243

Johnson, C. M., and Grossman, A. D. (2015). Integrative and Conjugative Elements (ICEs): what they do and how they work. *Annu. Rev. Genet.* 49, 577–601. doi: 10.1146/annurev-genet-112414-055018

Kaas, R. S., Leekitcharoenphon, P., Aarestrup, F. M., and Lund, O. (2014). Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS ONE* 9:e104984. doi: 10.1371/journal.pone.0104984

Labbate, M., Case, R. J., and Stokes, H. W. (2009). The integron/gene cassette system: an active player in bacterial adaptation. *Methods Mol. Biol.* 532, 103–125. doi: 10.1007/978-1-60327-853-9_6

Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., et al. (2019). ICEberg 2.0: an updated database of bacterial integrative and conjugative elements. *Nucl. Acids Res.* 47, D660–D665. doi: 10.1093/nar/gky1123

Mizuno, S., Ogawa, M., Yano, H., Kasahara, K., and Mikasa, K. (2020). Molecular and epidemiological characteristics of carbapenemase-producing *Klebsiella pneumoniae* clinical isolates in Japan. *mSphere* 5, e00490–e00420. doi: 10.1128/mSphere.00490-20

Morimoto, Y., Tohya, M., Aibibula, Z., Baba, T., Daida, H., Kirikae, T., et al. (2020). Re-identification of strains deposited as *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas putida* in GenBank based on whole genome sequences. *Int. J. Syst. Evol. Microbiol.* 70, 5958–5963. doi: 10.1099/ijsem.0.004468

Nandi, T., Ong, C., Singh, A. P., Boddey, J., Atkins, T., Sarkar-Tyson, M., et al. (2010). A genomic survey of positive selection in *Burkholderia pseudomallei* provides insights into the evolution of accidental virulence. *PLoS Pathog.* 6:e1000845. doi: 10.1371/journal.ppat.1000845

Peix, A., Ramírez-Bahena, M. H., and Velázquez, E. (2018). The current status on the taxonomy of Pseudomonas revisited: an update. *Infect. Genet. Evol.* 57, 106–116. doi: 10.1016/j.meegid.2017.10.026

Rowland, S. J., Boocock, M. R., Burke, M. E., Rice, P. A., and Stark, W. M. (2020). The protein-protein interactions required for assembly of the Tn3 resolution synapse. *Mol. Microbiol.* 114, 952–965. doi: 10.1111/mmi.14579

Tohya, M., Watanabe, S., Teramoto, K., Shimojima, M., Tada, T., Kuwahara-Arai, K., et al. (2019). *Pseudomonas juntendi* sp. nov., isolated from patients in Japan and Myanmar. *Int. J. Syst. Evol. Microbiol.* 69, 3377–3384. doi: 10.1099/ijsem.0.003623

Varani, A. M., Siguier, P., Gourbeyre, E., Charneau, V., and Chandler, M. (2011). ISsaga: an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. *Genome Biol.* 12:R30. doi: 10.1186/gb-2011-12-3-r30

Walsh, T. R., Toleman, M. A., Poirel, L., and Nordmann, P. (2005). Metallo-beta-lactamases: the quiet before the storm? *Clin. Microbiol Rev.* 18, 306–325. doi: 10.1128/CMR.18.2.306-325.2005

Xiang, G., Lan, K., Cai, Y., Liao, K., Zhao, M., Tao, J., et al. (2021). Clinical molecular and genomic epidemiology of *Morganella morganii* in China. *Front. Microbiol.* 12:744291. doi: 10.3389/fmicb.2021.744291

Yano, H., Miyakoshi, M., Ohshima, K., Tabata, M., Nagata, Y., Hattori, M., et al. (2010). Complete nucleotide sequence of TOL plasmid pDK1 provides evidence for evolutionary history of IncP-7 catabolic plasmids. *J. Bacteriol.* 192, 4337–4347. doi: 10.1128/JB.00359-10

Yoon, S. H., Ha, S. M., Lim, J. M., Kwon, S. J., and Chun, J. (2017). A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie van Leeuwenhoek* 110, 1281–1286. doi: 10.1007/s10482-017-0844-4

Zhu, Z., Yang, H., Yin, Z., Jing, Y., Zhao, Y., Fu, H., et al. (2021). Diversification and prevalence of the quinolone resistance crpP genes and the crpP-carrying Tn6786-related integrative and conjuative elements in *Pseudomonas aeruginosa*. *Virulence* 12, 2162–2170. doi: 10.1080/21505594.2021.1962160

Zong, Z. (2014). The complex genetic context of bla_{PER-1} flanked by miniature inverted-repeat transposable elements in *Acinetobacter johnsonii*. *PLoS ONE* 9:e90046. doi: 10.1371/journal.pone.0090046

frontiers | Frontiers in Microbiology

REVIEW
published: 01 August 2022
doi: 10.3389/fmicb.2022.808744

Mobile Tigecycline Resistance: An Emerging Health Catastrophe Requiring Urgent One Health Global Intervention

Madubuike Umunna Anyanwu[1], Obichukwu Chisom Nwobi[2], Charles Odilichukwu R. Okpala[3]* and Ifeoma M. Ezeonu[4]*

[1] Microbiology Unit, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria, [2] Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria, [3] Department of Functional Food Products Development, Faculty of Biotechnology and Food Science, Wrocław University of Environmental and Life Sciences, Wrocław, Poland, [4] Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria

OPEN ACCESS

Edited by:
Ziad Daoud, Central Michigan University, United States

Reviewed by:
Yuan Liu, Yangzhou University, China
Chong Chen, Yangzhou University, China

***Correspondence:**
Madubuike Umunna Anyanwu
madubuike.anyanwu@unn.edu.ng
Charles Odilichukwu R. Okpala
charlesokpala@gmail.com

Specialty section:
This article was submitted to Frontiers in Microbiology Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *03 November 2021*
Accepted: *24 May 2022*
Published: *01 August 2022*

Citation:
Anyanwu MU, Nwobi OC, Okpala COR and Ezeonu IM (2022) Mobile Tigecycline Resistance: An Emerging Health Catastrophe Requiring Urgent One Health Global Intervention. Front. Microbiol. 13:808744. doi: 10.3389/fmicb.2022.808744

Mobile tigecycline resistance (MTR) threatens the clinical efficacy of the salvage antibiotic, tigecycline (TIG) used in treating deadly infections in humans caused by superbugs (multidrug-, extensively drug-, and pandrug-resistant bacteria), including carbapenem- and colistin-resistant bacteria. Currently, non-mobile *tet*(X) and mobile plasmid-mediated transmissible *tet*(X) and resistance-nodulation-division (RND) efflux pump *tmexCD-toprJ* genes, conferring high-level TIG (HLT) resistance have been detected in humans, animals, and environmental ecosystems. Given the increasing rate of development and spread of plasmid-mediated resistance against the two last-resort antibiotics, colistin (COL) and TIG, there is a need to alert the global community on the emergence and spread of plasmid-mediated HLT resistance and the need for nations, especially developing countries, to increase their antimicrobial stewardship. Justifiably, MTR spread projects One Health ramifications and portends a monumental threat to global public and animal health, which could lead to outrageous health and economic impact due to limited options for therapy. To delve more into this very important subject matter, this current work will discuss why MTR is an emerging health catastrophe requiring urgent One Health global intervention, which has been constructed as follows: (a) antimicrobial activity of TIG; (b) mechanism of TIG resistance; (c) distribution, reservoirs, and traits of MTR gene-harboring isolates; (d) causes of MTR development; (e) possible MTR gene transfer mode and One Health implication; and (f) MTR spread and mitigating strategies.

Keywords: health threat, mobile tigecycline resistance, One Health, tetracycline overuse, antimicrobial resistance

INTRODUCTION

Antimicrobial resistance (AMR) continues to be a growing threat to the global public health (Silvester et al., 2021). Due to the promiscuous nature of plasmids, the resistance mechanism (mediated by a plasmid) are understood to spread horizontally/laterally among bacteria with lightning speed. It is no longer news in medicine that globally, estimated human deaths due

to infections by superbugs (multi-, extensively-, and pandrug-resistant bacteria), including carbapenem- and colistin-resistant bacteria, stand at about 700,000, eventually projected to reach 10 million by 2050 (O'Neill, 2014). Each year across Europe and the US alone, superbugs are responsible for the loss of more than 50,000 lives (O'Neill, 2014). In Africa, AMR-related annual deaths by 2050 are poised to reach about 4,000,000 (O'Neill, 2014). AMR is a One Health global problem that imposes enormous financial burden on countries irrespective of income level (Collignon et al., 2018).

By 2030, AMR could force up to an estimate of 24 million people into extreme poverty (World Health Organization [WHO], 2019) and by 2050, it could cause a global loss of US$100 trillion, which would decrease the (global) domestic product by 3.5% (O'Neill, 2014). In US alone, the challenge of AMR causes direct healthcare costs of about US$20 billion, excluding annual productivity loss of about US$35 billion (Dagostar, 2019). Developing countries [that is low-and middle-income countries (LMIC) in Asia, Africa and South America] remain the development/emergence hotspots of AMR—characterized by poor health infrastructure, poor hospital seeking habit, inadequate antimicrobial stewardship programs, unrestricted access to antibiotics, poor record-keeping, and weak or poor governance and regulatory framework (Ayukekbong et al., 2017; Anyanwu et al., 2021a). Accordingly, the AMR impacts greatly on poor nations that lack disease prevention and affordable treatments facilities (Silvester et al., 2021). For example, Thailand has estimated that superbugs are associated with respective annual death and economic loss of over 38,000 people and US $1.3 billion (Thamlikitkul et al., 2015). Traveling and trade facilitates the dissemination of AMR; thus the increasing population of most LMICs coupled with war and unstable governments, cause huge migration of the people thereby making it easy for resistant organisms to spread from these regions to other parts of the world (Yam et al., 2019; Bunduki et al., 2020).

Despite the increasing rate of development and spread of plasmid-mediated resistance against the two last-resort antibiotics, colistin (COL) and tigecycline (TIG), it is terrifying that there is no new safe and effective antimicrobial class coming to the market at least in the foreseeable future (Theuretzbacher et al., 2020). Essentially, this situation of emergence and spread of plasmid-mediated high-level TIG (HLT) resistance is very crucial and must not be taken for granted. Indeed, there is need to alert the global community to increase their antimicrobial stewardship, particularly the developing nations. Justifiably, MTR spread projects One Health ramifications and portends a monumental threat to global public and animal health, which could lead to outrageous health and economic impact due to limited options for therapy. To delve more into this very important subject matter, this current work will discuss why MTR is an emerging health catastrophe requiring urgent One Health global intervention, which has been constructed as follows: (a) antimicrobial activity of TIG; (b) mechanism of TIG resistance; (c) distribution, reservoirs, and traits of MTR gene-harboring isolates; (d) causes of MTR development; (e) possible MTR gene transfer mode and One Health implication; and (f) MTR spread and mitigating strategies.

ANTIMICROBIAL ACTIVITY OF TIGECYCLINE

Tigecycline is a third-generation tetracycline (belonging to glycycline class/category) antibiotic considered a salvage drug for treating deadly infections caused by superbugs, including carbapenem- and COL-resistant bacteria in humans. It was the first glycycline derived in the 1990s from the semisynthetic minocycline in an effort to overcome TET resistance (Zhanel et al., 2004). TIG has strong antibacterial activity against most Gram-positive and Gram-negative bacteria except *Proteus* and *Pseudomonas* (Yu R. et al., 2021), and it is approved only for treating complicated skin and skin structure infections (cSSTI) with the exclusion of diabetes foot infection, complicated intra-abdominal infections (cIAI) and community-acquired pneumonia (CAP) (Chen et al., 2020; Cui et al., 2020; Yaghoubi et al., 2021). TIG inhibits bacterial protein synthesis by reversibly binding to the 16S rRNA, hindering amino-acyl tRNA molecules from entering the A site of the ribosome and inhibiting the elongation of peptide chains (Zhang et al., 2020a; Yaghoubi et al., 2021). Chemical modification of TIG at the C-9 position of ring D led to enhanced binding to the target and more effective evasion of common TET resistance mechanisms, when compared to older TETs (tetracycline, doxycycline, and minocycline) (Zhang et al., 2020a).

First-generation TETs consisting of tetracycline, chlortetracycline and oxytetracycline were put into clinical practice in 1952 (Nelson and Levy, 2011) while the second-generation TETs made up of doxycycline and minocycline were put into use in 1976 (Fang et al., 2020). These antibiotics have been incorporated into animal feed to improve growth and feed efficiency (Zhang et al., 2021). They exert a bacteriostatic effect (inhibiting bacterial protein synthesis) against a wide variety of Gram-positive and Gram-negative bacteria as well as atypical/intracellular organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites by binding reversibly to the bacterial 30S and to some extent 50S ribosomal subunit and blocking incoming aminoacyl tRNA from binding to the ribosome acceptor site (Chopra and Roberts, 2001; Roberts, 2003). In addition, they could alter the cytoplasmic membrane causing intracellular components to leak from bacterial cells (Roberts, 2003). Although approved for human medicine by the United States FDA in 2005 and then in Europe in 2006 (Aminov, 2013) and China in 2010 (Zhang et al., 2020a), TIG has never been approved/used in livestock husbandry anywhere around the globe (He et al., 2019; Cui et al., 2020).

MECHANISM OF TIGECYCLINE RESISTANCE

TIG resistance similar to older tetracyclines, particularly before 2013, was understood as mutationally-acquired and chromosomally-encoded, as mediated by overexpression of chromosomal efflux pump regulator genes such as *ramAR*, *acrAB*, *OqxR,* and others (Sun S. et al., 2020; Wang et al., 2021b; Yaghoubi et al., 2021). The *tet*(X)—a gene encoding tetracycline

destructases considered flavin-dependent monooxygenases [Class A flavin monooxygenases (FMOs) requiring oxygen to function/confer resistance], capable of degrading all TETs, including TIG, as well as the recently FDA-approved fourth-generation TETs eravacycline and omadacycline, was deemed chromosomally-mediated and vertically transmitted only among the environmental and commensal microbiota (Sun J. et al., 2019; Fang et al., 2020; Aminov, 2021; Zhang et al., 2021). Therefore, TIG resistance might pose no serious risk because pathogenic organisms have not evolved *tet*(X) and the chromosome-mediated mechanisms, including chromosome-borne *tet*(X) orthologs (genes)—*tet*(X), *tet*(X1), and *tet*(X2), are only vertically transmitted, conferring resistance to low-level TIG and thus self-limiting by their very nature. Structurally, Tet(X) comprises a substrate-binding domain, coenzyme flavin adenine dinucleotide (FAD)-binding domain and C-terminal α-helix. Site-directed mutagenesis confirmed that the resistance phenotype/activity (capacity to mediate low-, moderate-, or high-level TIG resistance) of *tet*(X) variants is dependent on the residues in the TETs binding region and sites in the FAD binding region (Cui et al., 2021a,b,c). Thus, the accumulation of beneficial mutations results in increased activity (ability to exert moderate or HLT resistance) of *tet*(X) variants (Cui et al., 2021a). The *tet*(X1) is inert and inactive, but non-self-transmissible chromosome-borne *tet*(X2) possibly with accumulated beneficial mutations was recently shown to confer a rare HLT resistance (MIC $\geq$ 16 mgL^{-1}) only in *Flavobacteriaceae*, believed to be the ancestral source of *tet*(X) gene (Zhang et al., 2020a; Cui et al., 2021a,c). The knowledge about the active sites that lead to high-level resistance and about the evolutionary path of Tet(X) remains limited (Cui et al., 2021c). Nevertheless, a Sierra Leone study conducted in 2013 detected the *tet*(X) in a high proportion (21% of 52) of human clinical isolates belonging to diverse families (Leski et al., 2013). This finding signaled that *tet*(X) has been acquired by pathogenic bacteria probably from commensal/environmental microbiota through plasmid-mediated horizontal gene transfer (HGT) (Aminov, 2021). Hence, the increasing research interest in TIG resistance conferred by the *tet*(X) gene.

Unfortunately, as the globe grappled with the plasmid-mediated mobile COL resistance (*mcr*) gene [which had been discovered in the late 2015 and able to confer resistance to colistin (COL), a last resort antibiotic], in May 2019, the plasmid-encoded transmissible *tet*(X), *tet*(X3), and *tet*(X4) genes conferring HLT resistance were discovered in bacterial isolates from food animals, meat and environment in China (He et al., 2019). This discovery suggested the clinical efficacy of TIG is being threatened by the mobile TIG resistance (MTR). Retrospective analysis showed that MTR possibly emerged in 2016, thus there is speculation it is a recent event unlike *mcr* that evolved in the 1980s but detected approximately 40 years later (Sun C. et al., 2019). Nevertheless, the paradigm version of *tet*(X), *tet*(X0) gene was discovered on an R plasmid from a *Bacteroides fragilis* isolate (an anaerobe hence no effect on it) as early as the 1980s suggesting that plasmid-encoded *tet*(X) is not a recent event (Cui et al., 2021a). Since the discovery of mobile *tet*(X) genes in China, other reports are rapidly emerging regards the diverse ecological niches worldwide (Ding et al., 2020; Fang et al., 2020; Pan et al., 2020; Aminov, 2021; Marathe et al., 2021; Mohsin et al., 2021). Currently, *tet*(X) genes (both mobile and non-mobile) have been detected in at least 30 countries in 6 continents except Antarctica (Ding et al., 2020; Pan et al., 2020; Mohsin et al., 2021; Soliman et al., 2021; Usui et al., 2021; Wang et al., 2021f). To worsen the situation, barely few months (in April 2020) after the discovering of mobile *tet*(X) genes, a plasmid-encoded transmissible resistance-nodulation-division (RND) efflux pump gene, *tmexCD1-toprJ1* also conferring HLT resistance, was discovered in *Klebsiella pneumoniae* isolates from chickens in China (Lv et al., 2020). However, the mechanistic evolution of MTR is yet to be fully understood (Yongchang et al., 2019). Notably, very recently, plasmid-mediated TIG resistance-related gene, *tet*(Y) causing a decreased TIG susceptibility and transmitted across isolates by transposable element Tn*5393*, was detected in a clinical *Acinetobacter baumannii* isolate from China (Wang et al., 2021i). Additionally, mutations in plasmid-encoded *tet*(A) and *tet*(L) caused increased TIG resistance in virulent *K. pneumoniae* under selective pressure (Zhang et al., 2020a; Xu et al., 2021; Sun et al., 2022). The rapid transfer and acquisition of mobile *tet*(X) and *tmexCD1-toprJ1* genes is because they are carried by highly promiscuous mobile genetic elements like plasmids containing transposons, insertion sequences, and integrons (Aminov, 2021; Cui et al., 2021a,b). Thus, with MTR fast-spreading globally, it poses a monumental threat to antimicrobial therapy since TIG is a last-ditch drug for treating deadly infections in humans. Sadly, there remains this dilemma that not one specific antimicrobial can be used to treat the deadly infections especially those associated with TIG-resistant (TIG-r) bacteria. These organisms are potentially pandrug-resistant, so infections caused by them often result in fatality. Hence, we put to light the danger posed by the rapidly emerging and spreading plasmid-mediated HLT resistance and the need for the global community to halt injudicious use of TETs/TIG to protect global public health.

DISTRIBUTION, RESERVOIRS AND TRAITS OF MOBILE TIGECYCLINE RESISTANCE GENE-HARBORING ISOLATES

Ecological niches in which plasmid-encoded TIG resistance *tet*(X) genes have been detected are shown in **Table 1**. Clearly, there is increasing evidence of plasmid-encoded TIG resistance *tet*(X) genes reported in countries across Europe, Asia, United States, South America, and Africa involving humans, food animals, including chickens, pigs, ducks, pigeons, geese, and cattle, as well as companion animals, food animal products (meats), aquaculture and environment such as soil, livestock-farm water, wastewaters, sewages, and wildlife as reservoirs of MTR (Bai et al., 2019; Chen et al., 2019a,b, 2020, 2021; He et al., 2019; Sun C. et al., 2019, 2020; Sun J. et al., 2019; Cao et al., 2020; Cui et al., 2020, 2021b; Ding et al., 2020; Du et al., 2020; He T. et al., 2020; Li et al., 2020, 2021f; Ma et al., 2020; Pan et al., 2020; Ruan et al., 2020; Song et al., 2020; Wang et al.,

TABLE 1 | Ecological niches in which plasmid-encoded tigecycline resistance *tet*(X) genes have been detected.

Region	Country	Source of mobile *tet*(X) gene-harboring organism	Mobile *tet*(X) gene detected	*tet(X)*-carrying organism	*tet(X)*-associated plasmid	Additional resistance factors	Sequence types (Virulence genes/phylogrooup)	Contain genes encoding resistance against other last-resort antimicrobials	References
Asia	Japan	Pig	*tet*(X6)	*E. coli*	IncW	*aadA1*, *aadA2*, *cmlA1*, *dfrA1*, *sul1*, and *sul3*, Tn*As3,* and IS*Vsa3*	ST9222 (*capU*, *fyuA*, *gad*, *iha*, *sitA*, *terC,* and *traT*)	No	Usui et al., 2021
	Vietnam	Urban drainage	*tet*(X)	*Klebsiella aerogenes*	IncC-IncX	bla_{NDM-4}, $bla_{CTX-M-14}$, *qnrS1*, *aac(6′)-Ib-cr*, *cfr*, IS*26,* and IS*Vsa3*	–	Yes	Hirabayashi et al., 2021a
	Taiwan, China	Humans	*tet*(X6)	*Acinetobacter baumannii*	112 kb CMCVTAb1-Ab59 and 9 kb pAB-NCGM253	bla_{OXA-72}, bla_{OXA-66}, *ant(30)-IIa*, *aph(30)-Ib*, *aph(6)-Id*, *aph(30)-Ia*, *tet(Y)*, *abaQ*, ΔIS*CR2*, IS*CR2*, IS*26,* and Tn3	ST793 and ST723	Yes	Hsieh et al., 2021
	Singapore	Humans	*tet*(X4)	*E. coli*, *K. pneumoniae* and *Enterobacter clocae*	IncI1	***mcr-1***, bla_{TEM-1}, $bla_{CTX-M65}$, *aac(3)-IV*, *aph-(4)-Ia*, *floR*, *fosA*, IS*CR2,* and ΔIS*CR2*	ST515 and others	Yes	Ding et al., 2020
	Pakistan	Human, chickens, chicken meat, and environment (wild bird and slaughterhouse wastewater)	*tet(X4)*	*E. coli*	IncFII, IncHI2 and IncQ	***mcr-1***, *aadA1*, *aadA2*, *aadA8*, *aph(3′)-Ib*, *aph(6)-Id*, *aph(3′)-Ia*, *aph(3″)-Ib*, *aadA22*, *aadA5*, *aac(3)-IIe*, *aac(6′)-Ib-cr*, *aadA5*, *aac(3)-IId*, *arr-2*, bla_{EC-18}, bla_{EC-15}, $bla_{TEM-215}$, bla_{TEM-1B}, $bla_{TEM-215}$, bla_{CMY-2}, $bla_{CTX-M-15}$, $bla_{TEM-176}$, bla_{TEM-1}, bla_{OXA-1}, *cmlA1*, *dfrA1*, *dfrA12*, *arr-2*, *dfrA14*, *dfrA15*, *dfrA17*, *floR*, *osA3*, *fosA4*, *mph(A)*, *qnrS1*, *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*, *qnrS1*, *qnrS2*, *lnu(F)*, *mdf(A)*, *sul1*, IS*CR2,* and ΔIS*CR2*	ST6726 and ST224, ST6751, ST351, ST694, ST69, ST155, ST2253, ST2099, ST189, ST6706, ST1638, ST2847, ST2207, ST2234, ST48, and ST410 (*astA*, *cea*, *cma*, *capU*, *gad*, *hra*, *iss*, *iucC*, *iutA*, *lpfA*, *neuC*, *ompT*, *papA_F19*, *sitA*, *gad*, *terC,* and *traT*)	Yes	Li et al., 2021a,b,c,d; Mohsin et al., 2021

(Continued)

TABLE 1 | (Continued)

Region	Country	Source of mobile *tet*(X) gene-harboring organism	Mobile *tet*(X) gene detected	*tet(X)*-carrying organism	*tet(X)*-associated plasmid	Additional resistance factors	Sequence types (Virulence genes/phylogrooup)	Contain genes encoding resistance against other last-resort antimicrobials	References
	China	Humans, food animals (cattle, chickens, pigs, geese, pigeons, and ducks), food-animal products (pork, beef, and chicken meat), environment (farm water, soil, sewage, slaughterhouse environment, animal farm environment, and migratory wild birds), aquatic/ aquaculture system (shrimps)	*tet*(X3), *tet*(X4), *tet*(X5), *tet*(X5.2, *tet*(X5.3), *tet*(X6), *tet*(X7), and *tet*(X18)	*Escherichia, Klebsiella pneumoniae*, *Acinetobacter*, *Proteus*, *Salmonella*, *Citrobacter*, *Enterobacter cloacae*, *Rauoultella ornithinolytica, Riemeriella*, *Empedobacter,* and *S. multivorum*	IncX, IncX1, IncX4, IncI2, IncQ1, IncFIB, IncFII, IncFI, IncFIA, IncHIA ColE. ColRNA1, and IncHIB IncHI1A, IncHI1B, IncA/C2, IncX1-IncN, IncX1-IncR, IncX1-IncFIA/B-IncY, IncX1-IncFIA/B-IncHI1A/B IncFIA/B-IncHI1A/B, IncFIA18-IncFIB-IncX	***mcr*-1**, bla_{NDM-58}, bla_{NDM-5}, bla_{NDM-1}, bla_{TEM-1C}, bla_{TEM-1A}, $bla_{TEM-290}$, bla_{SHV-12}, $bla_{OXA-282}$, $bla_{CTX-M-14}$, bla_{TEM-30}, $bla_{CTX-M-65}$, $bla_{CTX-M-27}$, $bla_{CTX-M-122}$, $bla_{TEM-150}$, bla_{OXA-10}, bla_{OXA-83}, bla_{LAP-2}, bla_{CMH-3}, $bla_{EBR-1-like}$, $bla_{CTX-M-55}$, bla_{TEM-1B}, bla_{OXA-58}, bla_{CMY2}, bla_{DHA-1}, *ble*, bla_{CARB}, bla_{CARB-2}, *qacE1*, *qacL*, *erm*(42), *erm*(B), *lnu*(G), *lnu*(F), *qnrS2, qnrS1, qnrVC6*, *qnrD*, *oqxA*, *oqxB*, *strA*, *strB*, *aac(6′)-Ib*, *aac(6′)-Ib-cr*, *ant(3″)-Ia*, *aac(3)*, *aph*, *aadA2, aadA22, aadA1, aac(3)-IId, aph(3′)-Ia, acc(3)-Iva*, *aph(4)-Ia*, *aac(3)-IIa*, *aph(3′)-Ib*, *ant(3′)-Ih*, *aph(6″)-Id*, *aph(3′)-Via*, *aac(3)-Via*, *qacE1*, *aadS, arr-3, dfrA14, dfrA1*, *dfrA12*, *dfrA3b*, *dfrA20*, *dfrA5*, *dfrA16*, *cmlA1*, *cmlA6*, *cmlB1*, *cat*, *catB8*, *catB3*, *cfr*, *floR, fosA, mph*(A), *mph*(E), *msr*(E), *mef*(B), *mdf*(A), *sul1*, *sul2*, *sul3, tet*(A), *tet*(B), *tet*(M), *tet*(H), *tet*(Y), *tet*(J), *copA-D*, *cusR/S*, *czcA-D*, class 1 integrase *Intl*1, Tn*6451*, TnpA; IS*Vsa3*, IS*CR2*, IS*26*, IS*Aba1*, IS*4351*, IS*1R* and IS*1380*	ST3997, ST2325, ST3944, ST1421, ST515, ST10120, ST761, ST206, ST847, ST877, ST773, ST8302, ST871, ST10170, ST4656, ST2035, ST10392, ST4429, ST1602, ST10, ST744, ST761, ST746, ST2345, ST8504, ST189, ST1437, ST112, ST515, ST10115, ST641, ST1196, ST1638, ST10671, ST195, ST101, ST4704, ST6775, ST218, ST6833, ST2144, ST795, ST58, ST284, ST215, ST165, ST2065, ST789, ST540, ST1196, ST2064, ST642, ST1308, ST4156, ST295, ST761, ST34, ST278, ST453, ST6704, ST4541, ST972, ST7450, ST410, ST767, ST29 and ST48 (*irp2*, *fyuA*, *ybtA*, *astA*, *espX4*, *espX5*, *espY1*, *fik iuc*, *ybT*, and *ibeB*/A, B1and E) *K. pneumoniae*: ST37, ST534 and ST1418 *Salmonella*: ST8300 *Acinetobacter baumanii*: ST355 (*silP*)	Yes	Bai et al., 2019; Chen et al., 2019a,b, 2021; He et al., 2019; Sun J. et al., 2019; Sun C. et al., 2019; Cao et al., 2020; Chen et al., 2020; Cui et al., 2020; Du et al., 2020; He T. et al., 2020; Li et al., 2020; Ma et al., 2020; Pan et al., 2020; Ruan et al., 2020; Song et al., 2020; Sun S. et al., 2020; Wang et al., 2020a,b; Zhang et al., 2020a,b; Zheng et al., 2020; Cheng et al., 2021a,b; Cui et al., 2021b; Feng et al., 2021; Li et al., 2021a,c,d,e,f; Lu et al., 2021; Sun et al., 2021a,b; Tang et al., 2021; Wang et al., 2021c,f,g; Xu et al., 2021, Yu Y. et al., 2021; Zhang et al., 2021; Wu et al., 2022; Zhai et al., 2022
Africa	Egypt	Chickens	*tet*(X7)	*E. coli*	IncHI2, IncHI2A, IncFIA, IncFIB, and IncFIC (FII)	***mcr*-1.1**, *erm(B)*, *aph(3″)-Ib*, *aph(6)-Id*, *aac(3)-IIa*, *aph(3′)-Ia*, *aadA1*, *sul1*, *fosA*, *sul3*, *dfrA7*, *dfrA14*, *dfrA1*, *arr-2*, $bla_{CTX-M-15}$, bla_{TEM-1B}, *mph(A)*, *ere(A)*, *erm(B)*, *tet(A)*, *cmlA1,* and *floR*	ST155 and ST10 (*sitABCD*, *iucABCD,* and *iutA*)	Yes	Soliman et al., 2021
Europe	Norway	Wastewater	*tet*(X4)	*E. coli*	IncFII and IncI1-I and IncX4	$bla_{CTX-M-14}$, *erm(B)*, *erm(42)*, *qnrS1*, *dfrA12*, *sul2*, *tet(M)*, *aph(6)-Id*, *aadA1*, *aadA2*, *ant(2″)-Ia*, *aph(3″)-Ib,* and *cml*	ST167	Yes	Marathe et al., 2021
	United Kingdom	Pig	*tet*(X4)	*E. coli*	IncX1-IncY	bla_{TEM-1b}, *floR*, *aadA2,* and *linG*	ST1140	No	Martelli et al., 2021

Sequence type (ST): Warwick multilocus sequence type of tet(X)-positive E. coli isolates except otherwise stated; ST for Acinetobacter baumannii is based on Pasteur and Oxford schemes.

TABLE 2 | Specific reservoirs in which plasmid-borne transmissible *tet*(X) and *tmexCD-toprJ* genes have been detected.

Reservoir	References	
	***tet*(X)**	***tmexCD-toprJ***
Humans	He et al., 2019; Wang et al., 2019, 2020a,b, 2021f,g; Chen et al., 2020; Ding et al., 2020; Pan et al., 2020; Zhang et al., 2020a; Cui et al., 2021b; Hsieh et al., 2021; Li et al., 2021a,b; Zeng et al., 2021	Lv et al., 2020; He et al., 2021; Hirabayashi et al., 2021a; Qin et al., 2021; Wang et al., 2021b,h; Yang et al., 2021; Liu et al., 2022; Xiao et al., 2022; Zhai et al., 2022
Chickens	He et al., 2019; Li et al., 2019; Sun J. et al., 2019; Sun C. et al., 2020; Zheng et al., 2020; Chen et al., 2021; Fu et al., 2021; Li et al., 2021c,d,f; Mohsin et al., 2021; Sun et al., 2021a; Wang et al., 2021a; Xu et al., 2022	Lv et al., 2020; Sun S. et al., 2020; Li et al., 2021e; Wang et al., 2022; Xu et al., 2022
Pigeons	Cui et al., 2020; Wang et al., 2020b; Lu et al., 2022	–
Ducks	Cui et al., 2020; Ji et al., 2020; Pan et al., 2020; Wang et al., 2020b; Sun et al., 2021a; Yu Y. et al., 2021	–
Geese	Cui et al., 2020; Ji et al., 2020; Wang et al., 2021g	
Pigs	Bai et al., 2019; He et al., 2019; Sun C. et al., 2019; Sun J. et al., 2019; Chen et al., 2020; He T. et al., 2020; Li et al., 2020; Ma et al., 2020; Sun S. et al., 2020; Song et al., 2020; Cheng et al., 2021a,b; Feng et al., 2021; Fu et al., 2021; Martelli et al., 2021; Peng et al., 2021; Tang et al., 2021; Usui et al., 2021; Wang et al., 2021c; Xu et al., 2021; Li et al., 2022; Wu et al., 2022	Peng et al., 2021
Cattle	He et al., 2019; Zhang et al., 2020b; Fu et al., 2021	–
Companion animals	Sun et al., 2021b	–
Meat	Bai et al., 2019; Du et al., 2020; Li et al., 2021c,e; Sun et al., 2021a	Peng et al., 2021
Aquatic food	Li et al., 2019	–
Soil	Cao et al., 2020; Ji et al., 2020; Li et al., 2020, 2021f; Yu Y. et al., 2021	
Livestock farm water	Li et al., 2021f	–
Wastewaters	Li et al., 2020; Marathe et al., 2021	–
Sewages	Chen et al., 2019b; Mohsin et al., 2021	Peng et al., 2021; Wan et al., 2021
Urban sludge	–	Hirabayashi et al., 2021b
Wildlife (wild migratory birds)	Chen et al., 2019a, 2020; Cao et al., 2020; Fang et al., 2020; Ji et al., 2020	–
Water	Ji et al., 2020	–

–, no available report.

2020a,b; Zhang et al., 2020b, 2021; Zheng et al., 2020; Cheng et al., 2021a,b; Feng et al., 2021; Hirabayashi et al., 2021a,b; Hsieh et al., 2021; Li et al., 2021e,f; Lu et al., 2021; Marathe et al., 2021; Martelli et al., 2021; Mohsin et al., 2021; Soliman et al., 2021; Sun et al., 2021a,b; Tang et al., 2021; Wang et al., 2021f,g; Xu et al., 2021; Yu Y. et al., 2021; Wu et al., 2022; Zhai et al., 2022). We further demonstrate this, through the specific reservoirs in which plasmid-borne transmissible *tet*(X) and *tmexCD-toprJ* genes have been detected as shown in **Table 2**. Publications involving *tet*(X) genes appear to be more in humans, chickens and pigs, whereas those for *tmexCD-toprJ* genes appear to be more in humans and chickens. It is important to reiterate that from these ecological niches, the various MTR genes, such as *tet*(X3/3.2), *tet*(X4), *tet*(X5/5.2/5.3), *tet*(X6), *tet*(X7), and *tet*(X18), have been detected in a diversity of bacteria, which include *Escherichia*, *Rauoltella*, *Myroides*, *Enterobacter*, *Citrobacter*, *Klelbsiella*, *Shingobacterium multivorum*, *Proteus*, *Acinetobacter*, *Salmonella*, *Riemerella anatipestifer,* and *Empedobacter brevis* (Bai et al., 2019; He et al., 2019; Sun C. et al., 2019; Yongchang et al., 2019; Li et al., 2020, 2021a,c,d, 2022; Liu et al., 2020; Wang et al., 2020a, 2021f; Zhang et al., 2020a,b; Chen et al., 2021; Cheng et al., 2021a,b; Soliman et al., 2021; Xu et al., 2021). Notably, sequence alignment results revealed that *tet*(X5.2/5.3) genes shared 96.3–100% nucleotide sequence identity with *tet*(X6) and its variant (Chen et al., 2020; He D. et al., 2020; Zheng et al., 2020).

The drivers of these *tet*(X) genes in the isolates include promiscuous plasmids with various incompatibilities such as IncX1, IncQ1, IncW, IncA/C2, ColE2-like, IncFII, IncFIA, IncFIB, IncFIC, IncHIA, IncHIB, IncQ1-IncY hybrid, and many others, insertion sequences (IS*Vsa3*, IS*CR2*, IS*Aba1*, IS*26*, IS*4351*, and IS*1380*), transposon (Tn*As3*) and class one integrons (Bai et al., 2019; Sun C. et al., 2019; Sun J. et al., 2019; Li et al., 2020; Pan et al., 2020; Zheng et al., 2020; Soliman et al., 2021; Tang et al., 2021; Usui et al., 2021; **Table 1**). Of concern is that some *tet*(X)-bearing plasmids has become megaplasmid having acquired > 10 AMR genes conferring resistance against diverse classes of antimicrobial agents (Cui et al., 2020; Soliman et al., 2021; Sun et al., 2021a,b; **Table 1**). These megaplasmids poses a grave danger to the public and animal health as these plasmids are transmissible to other organisms and thereby jeopardizes antimicrobial therapy. Notably, a recent metagenomic study of human microbiome from 5 continents except Africa and Antarctica, showed that *Bacteroidaceae* has been an important reservoir and mutational incubator for mobile *tet*(X) orthologs in the human microbiome (Zhang et al., 2021). Furthermore, data mining from the NCBI database showed that mobile *tet*(X3/4/7)

genes are already circulating in United States, South America (Colombia), Middle East (Qatar), Asia (Thailand and India), and Africa (Cote d'Ivoire) (Fang et al., 2020; Soliman et al., 2021; Wang et al., 2021f).

The recently discovered transmissible RND efflux pump gene, *tmexCD1-toprJ* also confers HLT resistance as well as resistance to antimicrobials in many other classes (Lv et al., 2020). Thus, it poses a threat to public and animal health. The ecological niches in which plasmid-mediated HLT resistance efflux pump gene have been detected are shown in **Table 3**. The *tmexCD1-toprJ1* was first variant of the mobile RND efflux pump gene discovered in China in 2019. It was carried by IncFIB, IncFII, and IncFIA plasmids in *Klebsiella* isolates from humans, food animals (chickens and pigs), meat and environment (sewage from food market and slaughterhouse environment) (**Tables 2**, **3**). This occurrence in different ecological niches indicates that *tmexCD1-toprJ1* has disseminated to human, animal and environmental ecosystems and thus has One health ramifications. Other genetic elements driving *tmexCD1-toprJ* gene, including plasmids (IncX4, IncHI1B, IncQ, IncR, IncHI, plasmid hybrids, and others), insertion sequences (IS*26*, IS*903B*, IS*Kpn47*, IS*Kpn8,* and others), transposon Tn*5393*, and integrons have also been reported (He et al., 2021; Peng et al., 2021; Wan et al., 2021; Xu et al., 2022; **Table 3**). Very recently, variants of the plasmid-encoded transmissible RND efflux pump gene such as *tmexCD1-toprJ2* in *Rauoltella* isolate from a sick Chinese patient (Wang et al., 2021b) and *tmexCD1-toprJ3* in *Klebsiella* from urban sludge in Vietnam (Hirabayashi et al., 2021a) were reported. This distribution in different countries indicates that plasmid-mediated RND efflux mechanism of HLT resistance, is rapidly emerging and spreading in diverse ecological niches across the globe. A novel transmission pattern by which *tmexCD3-toprJ3* on integrative conjugative element (ICE) get integrated into other chromosomes of different bacterial hosts through cointegration of the circular intermediate was recently described (Wang et al., 2021e). Another transferrable novel efflux pump gene, *tnfxB3-tmexCD3-toprJ1b* cocarried with *tet*(X6) by ICE on the chromosome of *Proteus cibarius* and *Pseudmonas aeruginosa* isolates from chickens was also recently reported (Wang et al., 2021a). These findings point to the fact that ICE plays a huge role in the non-clonal restricted-dissemination of TIG resistance. So far, only Asian (Chinese and Vietnamese) studies have reported plasmid-encoded *tmexCD1-toprJ* (**Table 3**). It is worth noting that novel chromosomal-borne $tet(L)_{FSBL}$ flanked by IS*257* and plasmid-encoded $tet(L)_{A117V}$ efflux pump variants conferring resistance to TIG and eravacycline, were recently detected in *Staphylococcus* species isolated from pigs in China (Wang et al., 2021d).

Nevertheless, the NCBI database revealed that *tmexCD1-toprJ* gene is already spreading in Europe (United Kingdom), North America (United States), South America (Mexico), Africa (Kenya), and other parts of Asia (Pakistan and India) (Wang et al., 2021e). For now, the oldest isolate harboring plasmid-encoded *tmexCD1-toprJ* is a *Klebsiella pneumoniae* isolated in 2012 from the urine sample of a 21 year-old woman hospitalized for 21 days because of urinary tract infection (He et al., 2021). Therefore, mobile *tmexCD1-toprJ* likely emerged since at least a decade ago. But it seems to had remained restricted to *Klebsiella and Rauoltella* probably because it imposes a fitness cost, carried by plasmids with narrow host range or unstable in other *Enterobcaterales* (Lv et al., 2020). Nevertheless, the insertion sequence IS*26*, which likely transposed *tmexCD1-toprJ1* from chromosome (of *Pseudomonas*) to plasmid has the capacity to rapidly disperse *tmexCD1-toprJ* in *Enterobacterales* (Lv et al., 2020). The possession of both mobile *tet*(X) and RND efflux pumps could make diseases associated with such organism untreatable. Unfortunately, cocarriage of mobile *tet*(X) and *tmexCD3-toprJ3* has been detected (Hirabayashi et al., 2021b). High concentrations of *tmexCD1-toprJ1*, and *tet*(X3/4) was simultaneously detected in fecal samples of food-producing animals (Fu et al., 2021). Clonal dissemination and persistence of both *tet*(X) and RND efflux pumps gene is ensured since some of the MTR genes [e.g., *tet*(X3/4/5/6, *tmexCD1-toprJ1* and *tmexCD3-toprJ3*)] have been detected on chromosomes of bacteria (Chen et al., 2019a, 2020; Li et al., 2020; Liu et al., 2020; Peng et al., 2020; Zhang et al., 2020b; Wang et al., 2021e; Zheng et al., 2022). Both plasmid-borne and chromosomally-encoded *tet*(X) have been detected concurrently (Chen et al., 2020; Cheng et al., 2021a; Li et al., 2021a). Similarly, *tmexCD1-toprJ1* have also been simultaneously found on both plasmid and chromosome (Wang et al., 2021b).

More worrisome is that MTR tends to occur in conjunction with resistance to many other antimicrobial agents (including heavy metals), making the organism multi- to pandrug-resistant (**Table 1**). This coresistance further leads to limited options for therapy. Moreover, this situation can be exacerbated when an incriminated organism possesses an MTR mechanism [plasmid-encoded *tet*(X) or efflux pump] and gene coding resistance to other last-resort antimicrobials such as COL (conferred by *mcr*), fluoroquinolones, extended-spectrum cephalosporins and fosfomycin. Regrettably, such superbugs have already been isolated from humans (He et al., 2019; Ding et al., 2020; Ruan et al., 2020; Li et al., 2021a), food animals (He et al., 2019; Sun C. et al., 2019; He T. et al., 2020; Li et al., 2020, 2021c; Xu et al., 2021, 2022; Yu Y. et al., 2021), meat (Bai et al., 2019; Du et al., 2020; Mohsin et al., 2021; Soliman et al., 2021), and environment (Chen et al., 2019a; Cui et al., 2020; Mohsin et al., 2021; Yu Y. et al., 2021). Sadly, *tet*(X) even with genes coding resistance to other last-resort antibiotics, are horizontally/laterally transferred/acquired at a high frequency rate intra/inter-species/genus (Chen et al., 2019a; Sun C. et al., 2019; Cui et al., 2020; Ding et al., 2020; Du et al., 2020; Marathe et al., 2021; Soliman et al., 2021; Zhang et al., 2021). Likewise, the plasmid-encoded *tmexCD1-toprJ* with other resistance genes, including those encoding resistance to last-resort antimicrobials such as *mcr*, extended-spectrum β-lactamase and carbapenemase genes, has also been proven to be horizontally/laterally transferred at high frequency rate intra/inter-species/genera (Lv et al., 2020; Sun S. et al., 2020; Hirabayashi et al., 2021a; Qin et al., 2021; Wan et al., 2021; **Table 3**). Noteworthy, coexistence of *mcr*-1 and *tet*(X) genes, *tet*(X6), and *tet*(X7), on the same plasmid have been reported (Wang et al., 2021f; Xu et al., 2021). Also noteworthy,

TABLE 3 | Ecological niches in which plasmid-mediated high-level tigecycline resistance efflux pump gene have been detected.

Country	Source of mobile tigecycline efflux pump gene-harboring organism	Mobile tigecycline efflux pump gene detected (organism)	Organism (sequence type)	Associated plasmid	Additional resistance factors	Contain genes encoding resistance against other last-resort antimicrobials	References
Vietnam	Humans and environment (urban sludge)	*tmexCD3- toprJ3* (*Klebsiella aerogenes*) and *tmexCD1-toprJ1* (*K. pneumoniae*)	ST4 and ST273	IncFIB, IncHI1B and IncC-IncX1 and IS*26*	bla_{NDM-4}, $bla_{CTX-M-14}$, *qnrS1*, *aac(6′)-Ib-cr* and *cfr*	Yes	Hirabayashi et al., 2021a,b
China	Humans, food animal (chickens), food animal product (pork), and environment (chicken manure, chicken farm environment, slaughterhouse and sewage of food market)	*tmexCD1-toprJ1* (*K. pneumoniae* and *K. quasipneumoniae*) and *tmexCD2-toprJ2* (*Raoultella ornithinolytica* and *Klebsiella pnenumoniae*)	ST3447, ST37, ST1, ST11, ST180, ST236, ST1326, ST3332 ST967, ST147, ST896 and ST726 (*mrkABCDFHIJ*, *kfuABC*)	IncFIB, IncFII, IncX4, IncFIA, IncHI5 IncHI1B, and IncHI1B-FIB	***mcr*-1.1, *mcr*-8.1, *mcr*-8.5, *mcr*-10.1**, bla_{DHA-1}, $bla_{CTX-M-3}$, bla_{IMP-4}, bla_{NDM-1}, $bla_{OXY-1-3}$, $bla_{CTX-M-15}$, bla_{TEM-1B}, $bla_{CTX-M-55}$, $bla_{CTX-M-27}$, $bla_{CTX-M-9}$, $bla_{SHV-182}$, bla_{SHV-27}, bla_{SHV-11}, bla_{SHV-12}, $bla_{SHV-182}$, $bla_{SHV-110}$, $bla_{SHV-160}$, bla_{SHV-60}, $bla_{CTX-M-14}$, bla_{IMP-8} bla_{OXA-1}, bla_{NDM-1}, *strA*, *strB*, *armA*, *aac(6′)-Ib-cr*, *aadA5*, *aac(6″) aac(6′)-Ib4*,*-IId*, *aph(3′)-Ia*, *aadA16*, *aac(3′)-IId*, *aph(6)-Id-cr*, *aph (6)-Id*, *aph(39)-IId*, *aph(39)-Ia*, *aph(3)-Ib*, *aac(3)-IVa*, *aph(6)-Ib*, *aph(4)-Ia*, *amrA*, *aph(3)-Ia*, *ant(3″)-Ia*, *aadA1*, *aadA2*, *aadA2b*, *arr3*, *ant(2″)-Ia*, *aph(39)-Ib*, *armA*, *aph(6)-Id*, *aph(39)-Ia*, *afrA27*, *mph*(E), *mph(A)*, *msr*(E), *cmlA1*, *catB*, *qnrB4*, *qnrS1*, *oqxA*, *oqxB*, *qnrS1*, *qnrB52*, *qnrB2*, *sul1*, *sul2*, *sul3*, *dfrA1*, *dfrA14*, *dfrA12*, *dfrA27*, *dfrB4*, *cfr*, *rmtB*, *floR*, *fosA*, *fosA6*, *tet*(A), *cmlA1*, *catB3*, *qacE*, IS*Ec28*, IS*Ec29*, IS*Ec59*, IS*903*, IS*CR1*, IS*1*, IS*26*, IS*903B*, IS*Kpn47*, IS*Kpn8*, Tn*5393*, Tn*As1*, *Int*1, *Int*2 and *rmtC*, metal resistance gene clusters (*silP-copG-cusA-cusB-cusF-cusC-cusR-cusS* and *copABCD*)	Yes	Lv et al., 2020; Sun S. et al., 2020; He et al., 2021; Peng et al., 2021; Qin et al., 2021; Wan et al., 2021; Wang et al., 2021b,g, 2022; Yang et al., 2021; Liu et al., 2022; Xiao et al., 2022; Xu et al., 2022

ST, Warwick multilocus sequence type of Klebsiella isolates.

coexistence of HLT efflux pump genes (*tmexCD1-toprJ1* and *tmexCD2-toprJ2*) with carbapenem determinants (bla_{IMP-4}, and bla_{NDM-1}) on the same plasmid in clinical *Klebsiella* isolates have been reported (Wang et al., 2021h; Xiao et al., 2022). While conjugation (bacterial mating) is the major horizontal gene transfer (HGT) mechanism involved in the spread of MTR [*tet*(X) and HLT efflux pump] genes (Aminov, 2021; Cui et al., 2021a), transfer of these genes by other HGT mechanisms such as transformation (bacterial picking of naked DNA from environment) and transduction (prophage-mediated transfer) whereby viruses utilize the host's (bacterial) genetic machinery for division and subsequently spreading the integrated genes to other infected bacteria have also been described (Cui et al., 2021a; Li et al., 2021f).

Conjugative plasmid-borne *tet*(X) is rapidly transferred and acquired to/from bacteria intra/interspecies/genera by conjugation as these plasmids are highly promiscuous mobile genetic elements (MGEs) possessing conjugation machineries like transposons, integrons and type IV secretion system (T4SS) that capture genes from the donor and readily releases it to recipient organisms (Virolle et al., 2020). However, some chromosomal mobile elements like ICEs and transposons, also contribute to the transfer of *tet*(X). For example, the mobility of chromosome-borne *tet*(X15) was associated with IS*Aba1*-bound composite transposon Tn*6866* (Li et al., 2021b,e,f), *tet*(X4) was flanked by IS*91* family transposase genes in chromosome of *Shewanella xiamensis* (Dao et al., 2022), and the location of transferable *tet*(X6) in ICE have also been reported

(He D. et al., 2020; Wang et al., 2021c; Li et al., 2022). ICE are modular MGEs integrated into a host genome and are passively propagated during chromosomal replication and cell division. TIG use-selection pressure can induce the expression of *tet*(X) in ICE leading to excision and production of the conserved conjugation machinery (a T4SS), thereby possibly promoting genetic transfer to recipients (Zhang et al., 2020a). Moreover, MTR gene-bearing [especially *tet*(X)-bearing] plasmids are stably maintained and increase the growth rate of recipient organism (Cheng et al., 2021b), thus posing a challenge to antimicrobial therapy.

Remarkably, *tet*(X4)-plasmid remained stable in recipient organism for more than 100 generations in the presence of TIG selection pressure and up to 30 generations in the absence of TIG pressure (Li et al., 2021d). Thus, suggesting that elimination of MTR genes from the environment would be difficult if not practically impossible even after the withdrawal of TETs/TIG. The involvement of plasmid-encoded histone-like nucleoid-structuring (H-NS) protein in modulating the fitness of *tet*(X4)-bearing plasmids (such as IncX1) and ensuring the persistence of the gene have been reported (Cai et al., 2021). More critical is the situation where the transfer of *tet*(X4) resulted to increased size of transconjugant's plasmid, thus, making it increasingly bigger than plasmids of the parental strain (Li et al., 2020, 2021d). This situation would suggest that the plasmid homologous recombination may occur in the recipient organisms following the acquisition of mobile *tet*(X). The homologous plasmid recombination (of IS*26*) has been speculated to involve the horizontal transfer (conjugation) of *tet*(X4) borne on a small non-self-transmissible plasmid possessing T4SS, whereby the small plasmid incorporated into bigger and broader-host plasmids that enabled *tet*(X4) transmission to recipient organism (Zhai et al., 2022). Notably, the *tet*(X4)-IncF family plasmids appear commonly observed in the form of hybrid plasmid (Du et al., 2020; Li et al., 2020; Sun C. et al., 2020; Zhang et al., 2020a; Zhai et al., 2022). This understanding may explain why *tet*(X4)—a gene proven to possess a high HGT frequency (Li et al., 2021c), appears to breach the biological boundaries and able to spread onto different bacterial genus and species. Feasibly, mobile *tet*(X) genes would be transferrable to other organisms and through diverse routes, thus posing a huge emerging threat to both clinical and empirical antimicrobial therapy.

Furthermore, the population structure of MTR gene-harboring organisms is diversified without clonal restriction. Lamentably, a considerable proportion of these organisms also harbor virulence-associated genes (VAGs), while some belong to the established virulent epidemic clones. For example, the mobile *tet*(X) was harbored by *E. coli* isolates belonging to zoonotic high-risk pandemic extraintestinal pathogenic *E. coli* (ExPEC) clones ST10, ST410, ST95, ST58, and ST167 (Manges et al., 2019; Sun C. et al., 2020; Li et al., 2021d; Marathe et al., 2021; Soliman et al., 2021; **Tables 1**, **3**). Other pathogenic strains of *Enterobacterales* carrying mobile *tet*(X) such as *Proteus*, *Rauoltella* and *Acinetobacter* (**Table 1**) are major causes of infections especially in immunocompromised individuals (de Champs et al., 2000; Nakasone et al., 2015; Ayoub Moubareck and Hammoudi Halat, 2020). Essentially, virulent *K. pneumoniae* harboring mobile *tmexCD1-toprJ* isolated from microbial enumerations of hospitalized patients have been reported (Sun S. et al., 2020; He et al., 2021; Qin et al., 2021; Yang et al., 2021; Liu et al., 2022), particularly recovered from diverse ecological niches from different world regions (**Tables 1**, **3**). Additionally, the presence of VAGs in the MTR gene-bearing strains could confer a fitness advantage to the organisms (Jiang et al., 2021).

CAUSES OF MOBILE TIGECYCLINE RESISTANCE DEVELOPMENT

Antibiotics are well established to induce selective pressure in the bacteria, which prompts them to acquire the resistance genes (Anyanwu et al., 2021a). Globally, besides being massively used as prophylactics, TETs serve as a growth enhancer and for metaphylaxis in livestock, including aquaculture sectors (Aminov, 2021). The older TETs have been overused in both human and veterinary settings worldwide for the past 60 years (Sun C. et al., 2019), especially in developing countries (Umar et al., 2021). Specifically, factors like the low cost, broad-spectrum activity against Gram-positive, Gram-negative, and atypical bacteria appears to contribute to this overuse of older TETs, together with their ability to be administered orally and intravenously (Li et al., 2020). Notably, the use of TETs as feed additive in Europe and United States was, respectively, banned in 2006 and 2017 (Aminov, 2013). The opposite appears to be so in the developing nations, where TETs remain among the over-the-counter (OTC) medications, procured without prescription by individuals, as well as administered to livestock. For instance, in China, TETs serve as antimicrobial agents mostly for prophylaxis, and growth enhancement in animal husbandry, from 2010 to 2015 with 2770 tons of TETs consumed in 2017 in livestock (He et al., 2019; Umar et al., 2021).

TETs are still being administered in sub-therapeutic doses for prophylaxis and growth enhancement, especially developing countries. Undoubtedly, such situation would be exerting selective pressure for developing TET-/TIG-r organisms. Counterfeit/substandard TETs appear to be scattered in the markets in developing countries, which contributes to exacerbate the challenges associated with the subtherapeutic antimicrobial concentration (Ayukekbong et al., 2017). For the reason that TIG has never been licensed/used in veterinary medicine/animal husbandry, the overuse of older-generation TETs would continue to strengthen the selective pressure, which would bring about the emergence of TIG resistance (Sun C. et al., 2019; Lv et al., 2020; Aminov, 2021; Umar et al., 2021). Some workers showed that clinical samples from a person who had consumed doxycycline but not received the TIG could bring about a mobile *tet*(X)-positive *E. coli* (Wang et al., 2020a). Therefore, increasing the clinical use of TIG in humans would contribute to the TIG selective pressure (Wang et al., 2019; Zhang et al., 2020a). Moreover, the consumption of other antimicrobials, particularly the phenicols, cephalosporins and fluoroquinolones, would equally exert such selective pressure for TIG resistance (Chen et al., 2020; Lv et al., 2020). Besides, the frequent administration

of these antibiotics, which are usually administered in their subtherapeutic concentrations for prophylaxis and growth-enhancement in livestock, would potentially exert selective pressure. This would, therefore, prompt the rapid acquisition of resistance genes that possibly often involves the capture of MTR genes (especially by naturally competent organisms scavenging DNA fragments from the environment), by a single event of HGT. In fact, the use of florfenicol and tiamulin in livestock are believed to positively correlate with increasing concentration of mobile *tet*(X) and HLT efflux pump genes in animal fecal samples (Fu et al., 2021). Nevertheless, some resistance plasmids can capture resistance genes even in the absence of antibiotics selective pressure (Lopatkin et al., 2016).

POSSIBLE MOBILE TIGECYCLINE RESISTANCE GENE TRANSFER MODE AND ONE HEALTH IMPLICATION

AMR emerging from one ecosystem could easily move to another, thus having One Health ramifications (Anyanwu et al., 2020, 2021c). At the human-animal interface, individuals in close contact with animals such as animal handlers, animal health workers and owners/caretakers of animals, can easily acquire MTR. For example, *tet*(X3/4)-positive *E. coli* was isolated from the gut of live poultry market (LPM) workers and the LPM environment (Wang et al., 2019), suggesting that the workers possibly contracted the organisms following the handling of the birds, contaminated fomites or from environment. In fact, phylogenetic analysis revealed that *tet*(X4)-positive *E. coli* from human patients and animal origin were closely related indicating cross-sectorial clonal transmission of the organism between humans, animals and farm environment (Cui et al., 2021b). In addition, *tet*(X3/4) was present in the gut microbiomes of the LPM workers and surrounded environment (Wang et al., 2020a). The gut of humans and animals is conducive ("melting pot") for the exchange of resistance genes by HGT (Shterzer and Mizrahi, 2015). Notably, the LPM workers worked with gloves and mask and did not take antibiotics for the previous 3 months (Wang et al., 2019), thus implying that improper removal of personal protective equipment (PPE) and inadequate infection prevention and control (IPC) practices like hand hygiene even after the use of PPE, could result in infection of humans by TIG-r organism from the animal environment. Thus, human-to-human transmission of MTR could occur following direct contact with individuals with poor basic IPC practices like hand hygiene or indirectly from them through contact with contaminated fomites such as farm equipment and farm workers' paraphernalia. In essence, those in contact with animals (including veterinarians) are potential disseminators of MTR gene-containing organism into the public and veterinary hospital environments where these organisms have become a major nosocomial threat. Unfortunately, the organisms in ESKAPE (*Enterobacter*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas aeruginosa,* and *Escherichia coli*) group which are the most troubling pathogens associated with nosocomial and community infections worldwide (De Oliveira et al., 2020), have been reported as traffickers of MTR genes (He et al., 2019; Wang et al., 2019; Cui et al., 2020; Hsieh et al., 2021). However, the transmission of MTR at the human-animal interface is of more concern in developing countries because humans, especially those in rural areas, live in close contact with livestock (Alonso et al., 2017). Since *tet*(X3/4/5)-harboring organisms have been isolated from the gut of humans (**Table 2**), animals can be infected by TIG-r organisms following consumption of feed and/or drinking water contaminated (by feces) at the source by humans or by unhygienic animal caretakers and feed manufacturers. This mode of human to animal transmission of MTR is also more worrisome in developing countries because, they have poor environmental sanitation and many individuals/households have poor personal hygiene (Anyanwu et al., 2021b,c).

Since food animals have been shown to be reservoirs of MTR gene-harboring organisms (**Tables 1–3**), they are potential source for farm-to-plate transmission of MTR even in human population without antimicrobial use. For example, *tet*(X4)-positive Enterobacteriaceae was isolated from feces of healthy individuals who had never taken antibiotics (Ding et al., 2020; Ruan et al., 2020), indicating a possible acquisition from livestock/food animal products and circulation within the community. Slaughterhouses are critical points of contamination of animal products with MTR gene-harboring bacteria. For example, *tet*(X4)-positive *E. coli* and *tmexCD1-toprJ1*-positive *Klebsiella pneumoniae*/*Proteus mirabilis* have been isolated from slaughterhouses/slaughterhouse wastewaters (Sun C. et al., 2019; Li et al., 2020; Mohsin et al., 2021) and meats (pork, beef, chicken, and duck meat) for human consumption (Bai et al., 2019; Du et al., 2020; Mohsin et al., 2021). Thus, handling and consumption of raw/half-cooked meat/associated products is a route for the acquisition of TIG-r bacteria. It also means that individuals working in slaughterhouses can acquire these organisms from the slaughterhouse environment and contaminated fomites. Meat contamination by resistant organism is easy in developing countries due to unhygienic slaughtering methods and an unsanitary slaughterhouse environment (Anyanwu et al., 2021a). For instance, high prevalence of TIG and COL resistance has been reported in humans in a region with no history of COL/TIG use in Nigeria (Ugah and Udeani, 2021). Thus, indicating the acquisition from livestock, possibly through contact and/or farm-to-plate transmission since TETs and COL are used massively in livestock in Nigeria (Anyanwu et al., 2021b).

Since healthy and sick human fecal and clinical samples yielded *tet*(X3/4)-positive *E. coli* and *Acinetobacter* and *tmexCD1-toprJ1*-positive *Klebsiella* (He et al., 2019; Wang et al., 2019; Ding et al., 2020; Zeng et al., 2021), and these organisms were also recovered from human sewage (Cui et al., 2020), wastewaters (Marathe et al., 2021), animal feces (Sun C. et al., 2019; Cui et al., 2020; Li et al., 2020; Yu Y. et al., 2021) and slaughterhouse wastewaters/sewage (Mohsin et al., 2021), TIG-r organism/MTR can enter environmental ecosystem through anthropogenic (domestic, laboratory, and hospital) and agricultural (farm and slaughterhouse) sewages and wastewaters (**Table 2**). Asides improper disposal of wastes into environmental surface waters, rainfall run-offs could carry TIG-r organisms from wastes/sewages/sewage-spills on

land/landfills into these surface waters. For example, TIG-r *E. coli* carrying *mcr*-1 gene was recovered from sea water polluted with anthropogenic (domestic, hospital, and industrial wastes) and agricultural wastes in Algeria (Drali et al., 2018). Animals (such as straying, scavenging, wild/urban animals like water birds and pigeons, amphibians, snails, and so on) that depend on environmental surface waters for sustenance, could easily acquire these organisms. For example, *tet*(X3)-positive *Acinetobacter* has been isolated from waterfowls (geese and ducks) and pigeons and their neighboring environment (Cui et al., 2020). Waterfowls especially ducks, have been shown to be the natural host of the bacterium *Riemeriella anatipestifer*—the ancestral source/reservoir of diverse *tet*(X) genes (Umar et al., 2021; Zhang et al., 2021; Li et al., 2022). China has the largest waterfowl breeding industry in the world, and massive antibiotics (especially TET) is inevitably being used in the breeding process (Cui et al., 2021b). Large-scale use of TETs in the Chinese waterfowl breeding industry (partly to prevent septicemia *anserum exsudativa*—an economically important diseases caused by *Riemerilla anatipestifer*) is possibly the major cause of emergence and spread of MTR in China (Aminov, 2021; Cui et al., 2021b; Umar et al., 2021). Environmental surface water pollution is also of more concern in developing countries because, in most of these nations, poor environmental sanitation with practices like improper disposal of human/agricultural sewage, human open air defeacation and nomadic animal rearing allowing animals to discharge feces indiscriminately on land and surface waters, are still common (Anyanwu et al., 2021a,c). Furthermore, straying and scavenging animals, especially dogs/cats, are common in most developing countries. These companion animals can acquire TIG-r organisms from improperly disposed-off human excreta through coprophagic behavior (Leite-Martins et al., 2015) and then transfer the organisms to their caretakers.

Slaughterhouse wastes such as animal manure and internal organs are often used as economical mode in feeding of fish and companion carnivores (dogs and cats) in developing nations (Anyanwu et al., 2021c). Thus, a potential route through which TIG-r bacteria can enter aquaculture and companion animals. Furthermore, integrated farms common in developing countries, could facilitate the exchange of TIG-r organisms between food animals and aquaculture since excretions from livestock that may contain TIG-r organisms/MTR gene-containing organisms and un-metabolized antimicrobials, serve as food to fish that receives little or no supplementation (Anyanwu et al., 2020). There is also a possibility that TIG-r organism in anthropogenic/agricultural sewage/wastewater could escape treatment and get discharged into surface waters or end up on land/farm soil as organic manure. Escape of TIG-r organisms from sewage/wastewater treatment plants (WWTPs) into surface waters could lead to infection/colonization of feral aquatic animals/aquaculture (Cherak et al., 2021). For example, *tet*(X4)-positive *E. coli* has been isolated from shrimps captured for human consumption (Li et al., 2019). Thus, coming in contact with and consumption of contaminated/colonized aquatic foods, and the use of aquatic animals like fish/shrimps in making fish meal (which serves as protein source in livestock feed) is a route by which MTR can enter human and animal ecosystems from the aquaculture sector. Infected/colonized persons could release these organisms into the environment (land/soil) even in surface waters during swimming and bathing as well as from ships. Unfortunately, in developing countries, these waters are used (especially by those living at coastal areas) for various purposes (such as cooking, bathing, laundry, processing of food, etc.) potentially creating opportunities for the exchange of TIG-r organisms between humans and aquatic environments (Anyanwu et al., 2021c). Moreover, the water currents would also serve as the transport of these dangerous organisms to different parts of the world (Anyanwu et al., 2020).

Human/agricultural sewage and animal manure used as organic fertilizer on land, are a potential source for the dissemination of TIG-r organisms/MTR into soil and botanical (plant) ecosystems (Sun J. et al., 2019). For example, *tet*(X3/4)-harboring organisms (*E. coli* and *Acinetobacter*) and high abundance of these genes as well as *tmexCD1-toprJ1*, have been detected in farm soil and soil within livestock farms (Sun J. et al., 2019; Cao et al., 2020; Li et al., 2020; Cui et al., 2021b; Yu Y. et al., 2021). Garbage/synathropic flies that feed/breed on animal manure can pick these organisms from the soil and transport them to other ecological niches. Rainfall drops could raise TIG-r organisms from the soil to contaminate/colonize plants from where the MTR is re-incorporated into the food chain. Individuals who handle and eat plant products (fruits, vegetables), especially when raw, are likely to be infected by these organisms (Anyanwu et al., 2020). Grazing animals could easily acquire resistant organisms from contaminated plants (grasses and herbages). Encouragingly, composting or anaerobic digestion of animal manure before use as organic fertilizer in agricultural farms reduces the dissemination of AMR from animal to environmental ecosystem (Gao et al., 2019). It is worth noting that the soil contains TET-inactivating enzymes that detoxify only naturally occurring first-generation TETs, such as chlortetracycline and oxytetracycline, but fail to oxidize D-ring substituted analogs, including TIG, eravacycline and omadacycline (Gasparrini et al., 2020).

Antibiotics are not used in wildlife, but sadly, *tet*(X3/4/5)-positive *E. coli* and *Acinetobacter* has been isolated from wild birds (Chen et al., 2019a, 2020; Cao et al., 2020; Mohsin et al., 2021). This implies that MTR has already disseminated into the wild and it is of anthropogenic/agricultural origin. Wild animals can acquire TIG-r organisms/MTR following contact with anthropogenic (market, farm, slaughterhouse, home, and laboratory) wastes/sewages, drinking of contaminated water/wastewaters in the wild or environment with anthropogenic activity, feeding on carrion, and consumption of lower animals/plant products (fruits, vegetable, and grasses) contaminated by insects (on perching) or excreta of overflying birds (Anyanwu et al., 2020). The *tmexCD1-toprJ1* and mobile *tet*(X) genes have been detected in isolates from sewage of food market environment and slaughterhouses (Mohsin et al., 2021; Wan et al., 2021). Therefore, wild mammals/birds could easily acquire MTR from these ecological niches. Wildlife infected/colonized by TIG-r bacteria could disseminate these organisms to places (markets, playing grounds, etc.) frequented

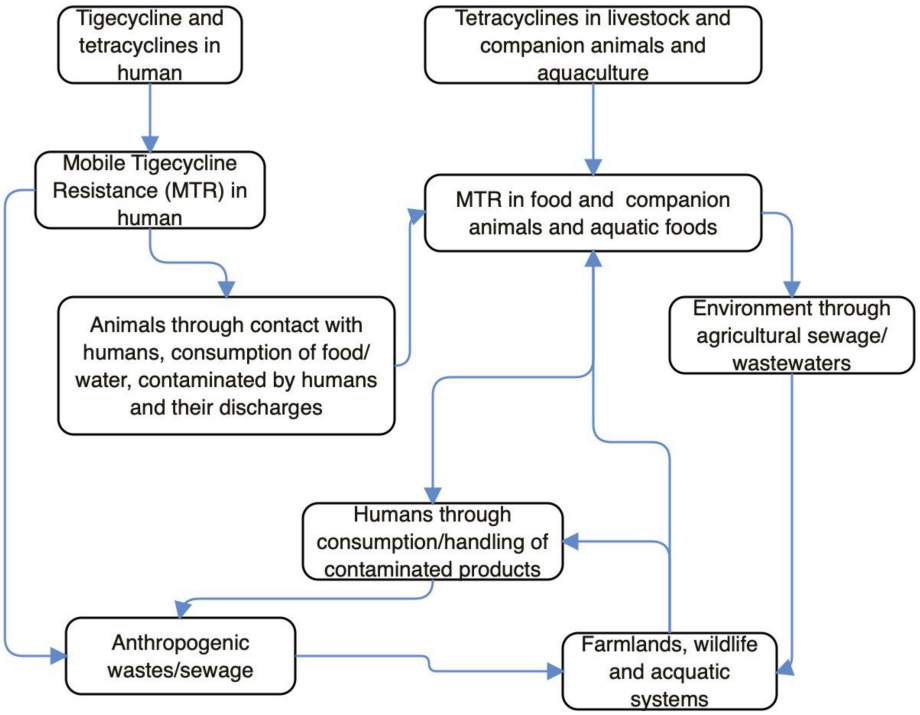


FIGURE 1 | Potential pathways through which mobile tigecycline resistance (MTR) emerges and builds up in human, animal, and environmental ecosystems.

by humans (Anyanwu et al., 2020). Since *tet*(X3/4/5)-positive Enterobacteriaceae has been recovered from wild migratory birds (Cao et al., 2020; **Tables 1**, **2**), it means that migratory wild birds can transport MTR (even following discharge of organisms in water bodies) from one country/continent to another (Anyanwu et al., 2020; Cao et al., 2020; Wan et al., 2021). Migratory wild birds always live far from dense human population and are not exposed to antibiotics directly; however, they can have access to animal and animal waste containing TIG-r organism/MTR genes (Cao et al., 2020; Umar et al., 2021). Thus, the presence of resistance gene in these birds is evidence of anthropization (Ewbank et al., 2021). The illustration of potential pathways through which MTR emerges and builds up in human, animal and environmental ecosystems can be seen in **Figure 1**. Besides tigecycline and tetracycline moving via human, livestock/companion animals and aquaculture, the MTR in both above mentioned two scenarios would combine and penetrate into the environment through agricultural sewage/wastewater. This eventually cycles back to human again, whether anthropogenically and or farmland/ aquaculture systems. In other words, through the consumption/handling of contaminated products, the agricultural sewage, farmlands/wildlife/aquatic systems, anthropogenic wastes/sewage/wastewater and humans strengthen the MTR's existence within the environment.

As the world is becoming one village, TIG-r organisms can be transported from one location to another through international/domestic food animal/meat/wildlife trade and travel (Bai et al., 2019; Wang et al., 2020b; Wan et al., 2021). Considering the fact that some of the MTR gene-containing isolates (especially *E. coli*, *Klebsiella,* and *Acinetobacter baumanii*) belonged to the high-risk zoonotic pandemic extraintestinal pathogenic multiresistant clones and contains virulence genes (Bai et al., 2019; Sun J. et al., 2019; Marathe et al., 2021; Qin et al., 2021; **Tables 1**, **3**), there could be outbreak of difficult-to-treat or untreatable diseases with outrageous health and economic impacts to the human and animal sectors. Already, virulent multidrug-resistant *tet*(X5)-harboring organisms have been isolated from sick animals (Chen et al., 2020). Notably, MTR is a monumental threat to global food safety/security and public health. Lamentably, the treatment of diseases associated with MTR is difficult due to limited/unavailable therapeutic option (He et al., 2019).

MOBILE TIGECYCLINE RESISTANCE SPREAD AND MITIGATING STRATEGIES

Globally, MTR has an immense One Health ramification and therefore warrants urgent intervention. Since older-generation TETs have been used for a long time, routine testing of isolates from diverse sectors (humans, animals—food and companion, environment, and aquaculture) for TIG resistance, is urgently warranted (Chen et al., 2019a, 2020; Yongchang et al., 2019). Retrospective screening (if possible, by whole genome sequencing) of archived TET-resistant/TIG-resistant isolates for plasmid-encoded *tet*(X) and *tmexCD1-toprJ* genes, is important to understand the evolution and spread of MTR. Metagenomic

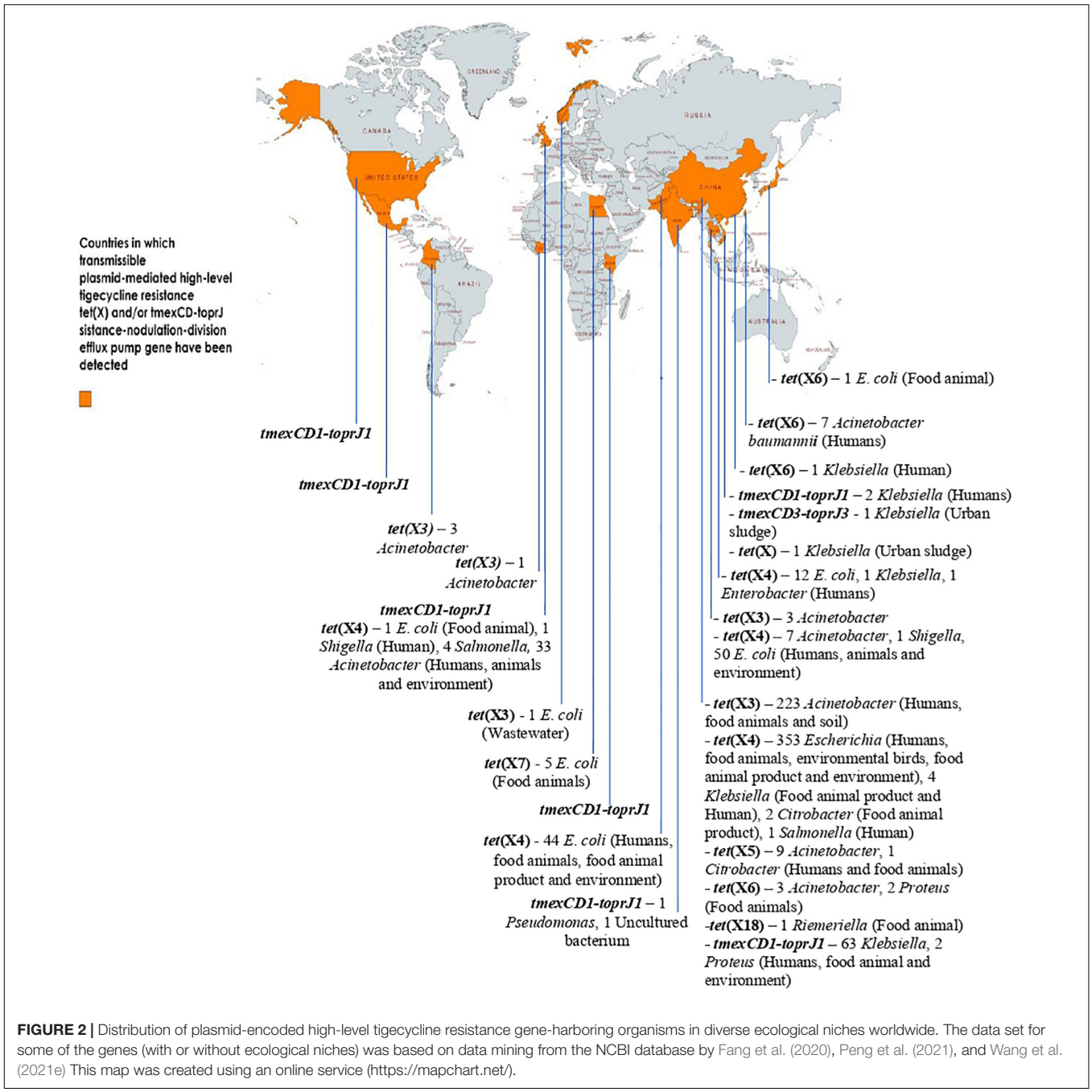


FIGURE 2 | Distribution of plasmid-encoded high-level tigecycline resistance gene-harboring organisms in diverse ecological niches worldwide. The data set for some of the genes (with or without ecological niches) was based on data mining from the NCBI database by Fang et al. (2020), Peng et al. (2021), and Wang et al. (2021e) This map was created using an online service (https://mapchart.net/).

studies could be useful in determining the magnitude of MTR mechanisms in different ecological niches (Fang et al., 2020). There is a need for developing affordable rapid test kits capable of detecting the diverse MTR genes, including those genes yet to emerge (Fang et al., 2020). A consideration should be given for pre-slaughter examination of food animals for MTR and post-slaughter testing of processed meat/associated products prior to distribution to retail shops, consumers or export. Quarantine and examination of individuals returning from TET/TIG resistance/*tet*(X)/*tmexCD1-toprJ* endemic regions and imported food/companion/wild animals capable of serving as vectors for the transportation of *tet*(X)/*tmexCD1-toprJ* gene-containing organisms. The decolonization of carriers of these organisms/genes is important to reduce their spread from one place to another. Intensified global surveillance (involving and supporting TIG resistance researches in developing countries) of MTR in diverse ecosystems is urgently needed to devise a holistic One Health approach in curtailing the spread of MTR before it gets out of hand (Okpala et al., 2021).

As new effective antimicrobial agent that is capable of destroying superbugs is not yet available, there is need for increased use of non-antibiotic agents such as antimicrobial

peptides and vaccines in order to reduce the use of antibiotics and development of MTR. Rational antimicrobial use should be promoted as this will inhibit the formation and evolution of cointegrate plasmids harboring emerging novel resistance genes (such as *mcr* and MTR genes) thereby reducing the transmission of these genes among bacteria (Lu et al., 2021). Hence, continuous surveillance of the emergence of *mcr*-carrying MDR plasmids and MTR gene-bearing MDR plasmids in bacterial isolates is warranted (Lu et al., 2021). The sale and use of TETs and florfenicol, especially in developing countries, should urgently be reconsidered (Peng et al., 2021; Wang et al., 2021b). For now, TIG is not available in most developing countries. Strict regulation is crucial to prevent the flooding of markets in these nations with counterfeit TIG. This salvage antibiotic should be strictly guarded by not licensing it for use in veterinary medicine. Finally, there is an urgent need for countries to improve on their antimicrobial stewardship emphasizing massive education of the stakeholders in human and animal medicine (physicians, veterinarians and allied health workers, policy makers, and clinical microbiologists) and the public, on the dangers associated with MTR as well as basic IPC practices like hand hygiene (which breaks transmission cycle of resistant organisms/pathogens), and environmental sanitation (Wan et al., 2021). It is also crucial to lay emphasis on prompt/adequate diagnosis and antimicrobial susceptibility testing as some of the isolates in this review showed susceptibility to some available antibiotics (Hsieh et al., 2021).

CONCLUDING REMARKS

In this current work, we have reviewed why MTR is an emerging health catastrophe requiring urgent One Health global intervention. We have discussed the antimicrobial activity of TIG and its mechanism of resistance. We have also discussed the distribution, reservoirs, and traits of MTR gene-harboring isolates, and the causes of MTR development. It is important to reiterate that the possible MTR gene transfer mode and its One Health implication, is very important, prior to understanding MTR spread, and how to mitigate it. The fact that MTR threatens the clinical efficacy of the salvage antibiotic, there is need to understand the position of TIG usage in treating deadly infections in humans, especially those that have been caused by superbugs, including carbapenem- and colistin-resistant bacteria. It is key to emphasize that TIG despite being a third-generation TET (belonging to glycycline) antibiotic, has never been used in animal husbandry anywhere around the globe. The regulation on the use of older-generation TETs particularly in developed countries, further demonstrates why this issue is very important. Some promising areas that provide the basis for further investigations in the future were identified in this review. More so, the danger posed by the rapidly emerging plasmid-mediated HLT resistance cannot be overemphasized. Thus, the global community needs to rise, to improve their antimicrobial stewardship and halt the injudicious use of antimicrobial agents especially TETs/TIG, to protect human and animal health.

Due to the overuse of TETs/other antimicrobial agents in human and animal sector as well as inappropriate use of TIG in humans, especially in developing countries, a fast-spreading MTR mediated by horizontally/laterally transmissible *tet*(X)- and *tmexCD1-toprJ* RND efflux pump gene-bearing plasmids, are jeopardizing the clinical usefulness of the last-ditch antibiotic TIG. For now, *tet*(X3) to *tet*(X7), and *tet*(X18) are the plasmid-encoded HLT resistance *tet*(X) gene variants known. Others such as *tet*(X14) and *tet*(X15) that also confer HLT resistance are chromosome-borne yet mobilizable by ICEs and composite transposons. Plasmid-borne *tet*(X) genes are harbored by a diversity of bacteria in different families but predominantly *Enterobacterales*. Three variants of *tmexCD-toprJ* RND efflux pump gene (*tmexCD1-toprJ1*, *tmexCD2-toprJ2,* and *tmexCD3-toprJ3*) with specific host range and limited plasmid vector have been described. Importantly, the distribution of plasmid-encoded HLT resistance gene-harboring organisms in diverse ecological niches worldwide is depicted in **Figure 2**. Clearly, the MTR appears strongly emerging and spreading rapidly in the human, animal and environmental ecosystems, including wildlife, worldwide. Thus, MTR is a potential health catastrophe in the making, if aggressive efforts to curb its spread is not implemented immediately. An aggressive One Health surveillance/monitoring and intervention approach to tackle this rapidly emerging global health threat, is urgently warranted.

AUTHOR CONTRIBUTIONS

MUA and OCN: conceptualization, methodology, investigation, formal analysis, supervision, funding acquisition, and writing—original draft. CORO and IME: formal analysis, visualization, resources, writing—review and editing, and project administration. All authors contributed to the intellectual content and agreed to the final submitted version.

FUNDING

The APC is financed/co-financed by Wrocław University of Environmental and Life Sciences. Publication was funded by the Wrocław University of Environmental and Life Sciences, Wrocław, Poland. Publication financed by the project UPWR 2.0: international and interdisciplinary program of development of Wrocław University of Environmental and Life Sciences, co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062/18 of June 4, 2019.

ACKNOWLEDGMENTS

CORO acknowledges financial support from Wrocław University of Environmental and Life Sciences, Wrocław, Poland.

REFERENCES

Alonso, C. A., Zarazaga, M., Ben Sallem, R., Jouini, A., Ben Slama, K., Torres, C., et al. (2017). Antibiotic resistance in *Escherichia coli* in husbandry animals: the African perspective. *Lett. Appl. Microbiol.* 64, 318–334. doi: 10.1111/lam.12724

Aminov, R. I. (2013). Evolution in action: dissemination of tet(X) into pathogenic microbiota. *Front. Microbiol.* 4:192. doi: 10.3389/fmicb.2013.00192

Aminov, R. I. (2021). Acquisition and spread of antimicrobial resistance: a tet(X) case study. *Int. J. Mol. Sci.* 22:3905. doi: 10.3390/ijms22083905

Anyanwu, M. U., Jaja, I. F., and Nwobi, O. C. (2020). Occurrence and characteristics of mobile colistin resistance (mcr) gene-containing isolates from the environment: a review. *Int. J. Environ. Res. Public Health* 17:1028. doi: 10.3390/ijerph17031028

Anyanwu, M. U., Jaja, I. F., Oguttu, J. W., Jaja, C. J., Chah, K. F., and Shoyinka, V. S. (2021a). Is Africa ready for mobile colistin resistance threat? *Infect. Ecol. Epidemiol.* 11:1962781. doi: 10.1080/20008686.2021.1962781

Anyanwu, M. U., Okpala, C. O. R., Chah, K. F., and Shoyinka, S. V. (2021c). Prevalence and traits of mobile colistin resistance gene harbouring isolates from different ecosystems in Africa. *Biomed. Res. Int.* 2021:6630379. doi: 10.1155/2021/6630379

Anyanwu, M. U., Marrollo, R., Paolucci, M., Brovarone, F., Nardini, P., Chah, K. F., et al. (2021b). Isolation and characterization of colistin-resistant Enterobacterales from chickens in Southeast Nigeria. *J. Glob. Antimicrob. Resist.* 26, 93–100. doi: 10.1016/j.jgar.2021.04.030

Ayoub Moubareck, C., and Hammoudi Halat, D. (2020). Insights into *Acinetobacter baumannii*: a review of microbiological, virulence, and resistance traits in a threatening nosocomial pathogen. *Antibiotics* 9:119. doi: 10.3390/antibiotics9030119

Ayukekbong, J. A., Ntemgwa, M., and Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries: causes and control strategies. *Antimicrob. Resist. Infect. Control* 6:47. doi: 10.1186/s13756-017-0208-x

Bai, L., Du, P., Du, Y., Sun, H., Zhang, P., Wan, Y., et al. (2019). Detection of plasmid-mediated tigecycline- resistant gene tet(X4) in *Escherichia coli* from pork, Sichuan and Shandong Provinces, China. *Euro Surveill.* 24:1900340. doi: 10.2807/1560-7917.ES.2019.24.25.1900340

Bunduki, G. K., Katembo, J. M., and Kamwira, I. S. (2020). Antimicrobial resistance in a war-torn country: lessons learned in the Eastern Democratic Republic of the Congo. *One Health* 9:1001203. doi: 10.1016/j.onehlt.2019.100120

Cai, W., Tang, F., Jiang, L., Li, R., Wang, Z., and Liu, Y. (2021). Histone-Like nucleoid structuring protein modulates the fitness of tet(X4)-bearing IncX1 plasmids in Gram-negative bacteria. *Front. Microbiol.* 12:763288. doi: 10.3389/fmicb.2021.763288

Cao, J., Wang, J., Wang, Y., Wang, L., Bi, Y., Zhu, B., et al. (2020). Tigecycline resistance tet(X3) gene is going wild. *Biosaf. Health* 2, 9–11. doi: 10.1016/j.bsheal.2020.01.002

Chen, C., Cui, C. Y., Zhang, Y., He, Q., Wu, X. T., Li, G., et al. (2019a). Emergence of mobile tigecycline resistance mechanism in *Escherichia coli* strains from migratory birds in China. *Emerg. Microbes Infect.* 8, 1219–1222. doi: 10.1080/22221751.2019.1653795

Chen, C., Chen, L., Zhang, Y., Cui, C. Y., Wu, X. T., He, Q., et al. (2019b). Detection of chromosome-mediated tet(X4)-carrying Aeromonas caviae in a sewage sample from a chicken farm. *J. Antimicrob. Chemother.* 74, 3628–3630. doi: 10.1093/jac/dkz387

Chen, C., Cui, C., Yu, J., He, Q., Wu, X., He, Y., et al. (2020). Genetic diversity and characteristics of high-level tigecycline resistance Tet(X) in Acinetobacter species. *Genome Med.* 12:111. doi: 10.1186/s13073-020-00807-5

Chen, C., Cui, C.-Y., Wu, X.-T., Fang, L.-X., He, Q., He, B., et al. (2021). Spread of tet(X5) and tet(X6) genes in multidrug-resistant *Acinetobacter baumannii* strains of animal origin. *Vet. Microbiol.* 253:108954. doi: 10.1016/j.vetmic.2020.108954

Cheng, Y., Chen, Y., Liu, Y., Song, J., Chen, Y., Shan, T., et al. (2021a). Detection of a new tet(X6)-encoding plasmid in Acinetobacter towneri. *J. Glob. Antimicrob. Resist.* 25, 132–136. doi: 10.1016/j.jgar.2021.03.004

Cheng, Y., Liu, Y., Chen, Y., Huang, F., Chen, R., Xiao, Y., et al. (2021b). Global dissemination of tet(X3) and tet(X6) among livestock-associated Acinetobacter is sporadic and mediated by highly diverse plasmidomes. *bioRxiv [Preprint]* doi: 10.1101/2021.08.02.454847

Cherak, Z., Loucif, L., Moussi, A., and Rolain, J. M. (2021). Epidemiology of mobile colistin resistance (mcr) genes in aquatic environments. *J. Glob. Antimicrob. Resist.* 27, 51–62.

Chopra, I., and Roberts, M. C. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65, 232–260.

Collignon, P., Beggs, J. J., Walsh, T. R., Gandra, S., and Laxminarayan, R. (2018). Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis. *Lancet Planet. Health* 2, e398–e405. doi: 10.1016/S2542-5196(18)30186-4

Cui, C.-Y., Chen, C., Liu, B.-T., He, Q., Wu, X.-T., Sun, R.-Y., et al. (2020). Co-occurrence of plasmid-mediated tigecycline and carbapenem resistance in Acinetobacter spp. from waterfowls and their neighboring environment. *Antimicrob. Agents Chemother.* 64, e2502–e2519. doi: 10.1128/AAC.02502-19

Cui, C., Chen, Q., He, Q., Chen, C., Zhang, R., Feng, Y., et al. (2021a). Transferability of tigecycline resistance: characterization of the expanding Tet(X) family. *Wires Mech. Dis.* 14:e1538. doi: 10.1002/wsbm.1538

Cui, C. Y., Li, X., Chen, C., Wu, X. T., He, Q., Jia, Q. L., et al. (2021b). Comprehensive analysis of plasmid-mediated tet(X4)-positive *Escherichia coli* isolates from clinical settings revealed a high correlation with animals and environments-derived strains. *Sci. Total Environ.* 28:150687. doi: 10.1016/j.scitotenv.2021.150687

Cui, C.-Y., He, Q., Jia, Q.-L., Li, C., Chen, C., Wu, X.-T., et al. (2021c). Evolutionary trajectory of the Tet(X) family: critical residue changes towards high-level tigecycline resistance. *mSystems* 6:e00050–21. doi: 10.1128/mSystems.00050-21

Dagostar, P. (2019). Antimicrobial resistance: implications and costs. *Infect. Drug Resist.* 12, 3903–3910.

Dao, T. D., Kasuga, I., Hirabayashi, A., Nguyen, D. T., Tran, H. T., Vu, H., et al. (2022). Emergence of mobile tigecycline resistance gene tet(X4)-harbouring Shewanella xiamenensis in a water environment. *J. Glob. Antimicrob. Resist.* 28, 140–142. doi: 10.1016/j.jgar.2021.12.022

de Champs, C., Bonnet, R., Sirot, D., Chanal, C., and Sirot, J. (2000). Clinical relevance of *Proteus mirabilis* in hospital patients: a two year survey. *J. Antimicrob. Chemother.* 45, 537–539. doi: 10.1093/jac/45.4.537

De Oliveira, D., Forde, B. M., Kidd, T. J., Harris, P., Schembri, M. A., Beatson, S. A., et al. (2020). Antimicrobial resistance in ESKAPE pathogens. *Clin. Microbiol. Rev.* 33:e00181–19. doi: 10.1128/CMR.00181-19

Ding, Y., Saw, W., Tan, L. W. L., Moong, D. K. N., Nagarajan, N., Teo, Y. Y., et al. (2020). Emergence of tigecycline- and eravacycline-resistant tet(X4)-producing *Enterobacteriaceae* in the gut microbiota of healthy Singaporeans. *J. Antimicrob. Chemother.* 75, 3480–3484. doi: 10.1093/jac/dkaa372

Drali, R., Berrazeg, M., Zidouni, L. L., Hamitouche, F., Abbas, A. A., Deriet, A., et al. (2018). Emergence of mcr-1 plasmid-mediated colistin-resistant *Escherichia coli* isolates from seawater. *Sci. Total Environ.* 642, 90–94. doi: 10.1016/j.scitotenv.2018.05.387

Du, P., Liu, D., Song, H., Zhang, P., Li, R., Fu, Y., et al. (2020). Novel IS26-mediated hybrid plasmid harboring tet(X4) in *Escherichia coli*. *J. Glob. Antimicrob. Resist.* 21, 162–168. doi: 10.1016/j.jgar.2020.03.018

Ewbank, A. C., Esperón, F., Sacristán, C., Sacristán, I., Krul, R., Cavalcanti, E. M., et al. (2021). Seabirds as anthropization indicators in two different tropical biotopes: a one health approach to the issue of antimicrobial resistance genes pollution in oceanic islands. *Sci. Total Environ.* 754:142141. doi: 10.1016/j.scitotenv.2020.142141

Fang, L.-X., Chen, C., Cui, C.-Y., Li, X.-P., Zhang, Y., Liao, X.-P., et al. (2020). Emerging high-level tigecycline resistance: novel tetracycline destructases spread via the mobile Tet(X). *Bioessays* 42:e2000014. doi: 10.1002/bies.202000014

Feng, J., Ma, J., Li, K., Su, M., Li, R., Bai, L., et al. (2021). Extensive spread of tet(X4) in multidrug-resistant *Escherichia coli* of animal origin in western China. *Vet. Microbiol.* 269:109420. doi: 10.1016/j.vetmic.2022.109420

Fu, Y., Chen, Y., Liu, D., Yang, D., Liu, Z., Wang, Y., et al. (2021). Abundance of tigecycline resistance genes and association with antibiotic residues in Chinese livestock farms. *J. Hazard. Mater.* 409:124921. doi: 10.1016/j.jhazmat.2020.124921

Gao, Y., Lu, C., Shen, D., Liu, J., Ma, Z., Yang, B., et al. (2019). Elimination of the risks of colistin resistance gene (mcr-1) in livestock manure during composting. *Environ. Int.* 126, 61–68.

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.* 3:241. doi: 10.1038/s42003-020-0966-5

He, D., Wang, L., Zhao, S., Liu, L., Liu, J., Hu, G., et al. (2020). A novel tigecycline resistance gene, tet(X6), on an SXT/R391 integrative and conjugative element in a Proteus genomospecies 6 isolate of retail meat origin. *J. Antimicrob. Chemother.* 75, 1159–1164. doi: 10.1093/jac/dkaa012

He, R., Yang, Y., Wu, Y., Zhong, L. L., Yang, Y., Chen, G., et al. (2021). Characterization of a plasmid-encoded resistance-nodulation-division efflux pump in *Klebsiella pneumoniae* and Klebsiella quasipneumoniae from patients in China. *Antimicrob. Agents Chemother.* 65, e02075–20. doi: 10.1128/AAC.02075-20

He, T., Wang, R., Liu, D., Walsh, T. R., Zhang, R., Lv, Y., et al. (2019). Emergence of plasmid-mediated high-level tigecycline resistance genes in animals and humans. *Nat. Microbiol.* 4, 1450–1456.

He, T., Wei, R., Li, R., Zhang, L., Sun, L., Bao, H., et al. (2020). Co-existence of tet(X4) and mcr-1 in two porcine *Escherichia coli* isolates. *J. Antimicrob. Chemother.* 75, 764–766. doi: 10.1093/jac/dkz510

Hirabayashi, A., Ha, V. T. T., Van Nguyen, A., Nguyen, S. T., Shibayama, K., and Suzuki, M. (2021a). Emergence of a plasmid-borne tigecycline resistance in *Klebsiella pneumoniae* in Vietnam. *J. Med. Microbiol.* 70. doi: 10.1099/jmm.0.001320

Hirabayashi, A., Dao, T. D., Takemura, T., Hasebe, F., Trang, L. T., Thanh, N. H., et al. (2021b). A transferable IncC-IncX3 hybrid plasmid cocarrying blaNDM-4, tet(X), and tmexCD3-toprJ3 confers resistance to carbapenem and tigecycline. *mSphere* 6:e0059221. doi: 10.1128/mSphere.00592-21

Hsieh, Y.-C., Wu, J.-W., Chen, Y.-Y., Quyen, T. L. T., Liao, W.-C., Li, S.-W., et al. (2021). An outbreak of tet(X6)-carrying tigecycline-resistant *Acinetobacter baumannii* isolates with a new capsular type at a hospital in Taiwan. *Antibiotics* 10:1239. doi: 10.3390/antibiotics10101239

Ji, K., Xu, Y., Sun, J., Huang, M., Jia, X., Jiang, C., et al. (2020). Harnessing efficient multiplex PCR methods to detect the expanding Tet(X) family of tigecycline resistance genes. *Virulence* 11, 49–56. doi: 10.1080/21505594.2019.1706913

Jiang, L., Cai, W., Tang, F., Wang, Z., and Liu, Y. (2021). Characterization of fitness cost caused by tigecycline-resistance gene tet(X6) in different host bacteria. *Antibiotics* 10:1172. doi: 10.3390/antibiotics10101172

Leite-Martins, L., Mahú, M. I., Costa, A. L., Bessa, L. J., Vaz-Pires, P., Loureiro, L., et al. (2015). Prevalence of antimicrobial resistance in faecal enterococci from vet-visiting pets and assessment of risk factors. *Vet. Rec.* 176:674. doi: 10.1136/vr.102888

Leski, T. A., Bangura, U., Jimmy, D. H., Ansumana, R., Lizewski, S. E., Stenger, D. A., et al. (2013). Multidrug-resistant tet(X)-containing hospital isolates in Sierra Leone. *Int. J. Antimicrob. Agents* 42, 83–86. doi: 10.1016/j.ijantimicag.2013.04.014

Li, R., Jiang, Y., Peng, K., Wang, Y., Wang, M., Liu, Y., et al. (2022). Phenotypic and genomic analysis reveals Riemerella anatipestifer as the potential reservoir of tet(X) variants. *J. Antimicrob. Chemother.* 77, 374–380. doi: 10.1093/jac/dkab409

Li, R., Liu, Z., Peng, K., Liu, Y., Xiao, X., and Wang, Z. (2019). Cooccurrence of two tet(X) variants in an Empedobacter brevis strain of shrimp origin. *Antimicrob. Agents Chemother.* 63, e01636–19.

Li, R., Lu, X., Peng, K., Liu, Z., Li, Y., Liu, Y., et al. (2020). Deciphering the structural diversity and classification of the mobile tigecycline resistance gene tet(X)-bearing plasmidome among bacteria. *mSystems* 5, e00134–20.

Li, R., Mohsin, M., Lu, X., Abdullah, S., Munir, A., and Wang, Z. (2021a). Emergence of plasmid mediated resistance genes tet(X) and mcr-1 in *Escherichia coli* clinical isolates from Pakistan. *mSphere* 6:e0069521. doi: 10.1128/mSphere.00695-21

Li, R., Peng, K., Xiao, X., Wang, Y., and Wang, Z. (2021b). Characterization of novel ISAba1-bounded tet(X15)-bearing composite transposon Tn6866 in Acinetobacter variabilis. *J. Antimicrob. Chemother.* 76, 2481–2483. doi: 10.1093/jac/dkab182

Li, R., Lu, X., Munir, A., Abdullah, S., Liu, Y., Xiao, X., et al. (2021c). Widespread prevalence and molecular epidemiology of tet(X4) and mcr-1 harboring *Escherichia coli* isolated from chickens in Pakistan. *Sci. Total Environ.* 806:150689. doi: 10.1016/j.scitotenv.2021.150689

Li, R., Li, Y., Peng, K., Yin, Y., Liu, Y., He, T., et al. (2021d). Comprehensive genomic investigation of tigecycline resistance gene tet(X4)-bearing strains expanding among different settings. *Microbiol. Spectr.* 9:e0163321.

Li, Y., Peng, K., Yin, Y., Sun, X., Zhang, W., Li, R., et al. (2021e). Occurrence and molecular characterization of abundant tet(X) variants among diverse bacterial species of chicken origin in Jiangsu, China. *Front. Microbiol.* 12:751006. doi: 10.3389/fmicb.2021.751006

Li, Y., Wang, Q., Peng, K., Liu, Y., Xia, X., Mohsin, M., et al. (2021f). Distribution and genomic characterization of tigecycline resistant tet(X4)-positive *Escherichia coli* of swine farm origin. *Microb. Genom.* 7:000667. doi: 10.1099/mgen.0.000667

Liu, C., Wu, Y., Fang, Y., Sang, Z., Huang, L., Dong, N., et al. (2022). Emergence of an ST1326 (CG258) Multi-Drug Resistant *Klebsiella pneumoniae* Co-harboring mcr-8.2, ESBL genes, and the resistance-nodulation-division efflux pump gene cluster tmexCD1-toprJ1 in China. *Front. Microbiol.* 13:800993. doi: 10.3389/fmicb.2022.800993

Liu, D., Zhai, W., Song, H., Fu, Y., Schwarz, S., He, T., et al. (2020). Identification of the novel tigecycline resistance gene tet(X6) and its variants in Myroides, Acinetobacter and Proteus of food animal origin. *J. Antimicrob. Chemother.* 75, 1428–1431. doi: 10.1093/jac/dkaa037

Lopatkin, A. J., Huang, S., Smith, R. P., Srimani, J. K., Sysoeva, T. A., Bewick, S., et al. (2016). Antibiotics as a selective driver for conjugation dynamics. *Nat. Microbiol.* 1:16044. doi: 10.1038/nmicrobiol.2016.44

Lu, X., Du, Y., Peng, K., Zhang, W., Li, J., Wang, Z., et al. (2022). Coexistence of tet(X4), mcr-1, and blaNDM-5 in ST6775 *Escherichia coli* Isolates of animal origin in China. *Microbiol. Spectr.* 10, e0019622. doi: 10.1128/spectrum.00196-22

Lu, X., Xiao, X., Liu, Y., Li, R., and Wang, Z. (2021). Emerging opportunity and destiny of *mcr*-1- and *tet*(X4)-coharboring plasmids in *Escherichia coli*. *Microbiol. Spectr.* 9:e0152021. doi: 10.1128/Spectrum.01520-21

Lv, L., Wan, M., Wang, C., Gao, X., Yang, Q., Partridge, S. R., et al. (2020). Emergence of a plasmid encoded resistance-nodulation-division efflux pump conferring resistance to multiple drugs, including tigecycline, in *Klebsiella pneumoniae*. *mBio* 11, e02930–19. doi: 10.1128/mBio.02930-19

Ma, J., Wang, J., Feng, J., Liu, Y., Yang, B., Li, R., et al. (2020). Characterization of three porcine Acinetobacter towneri strains Co-Harboring tet(X3) and blaOXA-58. *Front. Cell. Infect. Microbiol.* 10:586507. doi: 10.3389/fcimb.2020.586507

Manges, A. R., Geum, H. M., Guo, A., Edens, T. J., Fibke, C. D., and Pitout, J. D. D. (2019). Global extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. *Clin. Microbiol. Rev.* 32, e00135–18. doi: 10.1128/CMR.00135-18

Marathe, N. P., Svanevik, C. S., Ghavidel, F. Z., and Grevskott, D. H. (2021). First report of mobile tigecycline resistance gene tet(X4)-harboring multidrug-resistant *Escherichia coli* from wastewater in Norway. *J. Glob. Antimicrob. Resist.* 27, 37–40. doi: 10.1016/j.jgar.2021.07.019

Martelli, F., AbuOun, M., Cawthraw, S., Storey, N., Turner, O., Ellington, M., et al. (2021). Detection of the transferable tigecycline resistance gene *tet*(X4) in *Escherichia coli* from pigs in the United Kingdom. *J. Antimicrob. Chemother.* 77, 846–848.

Mohsin, M., Hassan, B., Martins, W. M. B. S., Li, R., Abdullah, S., Sands, K., et al. (2021). Emergence of plasmid-mediated tigecycline resistance tet(X4) gene in *Escherichia coli* isolated from poultry, food and the environment in South Asia. *Sci. Total Environ.* 787, 147613. doi: 10.1016/j.scitotenv.2021.147613

Nakasone, E. S., Kaneshiro, R., Min, K., and Tokeshi, J. (2015). Emergence of Raoultella ornithinolytica on O'ahu: a case of community-acquired R. ornithinolytica urinary tract infection. *Hawaii J. Med. Public Health* 74, 174–175.

Nelson, M. L., and Levy, S. B. (2011). The history of the tetracyclines. *Ann. N.Y. Acad. Sci.* 1241, 17–32.

O'Neill, J. (2014). *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*. Geneva: WHO.

Okpala, C. O. R., Anyanwu, M. U., Łukańko, S., Nwobi, O. C., Korzeniowska, M., and Ezeonu, I. M. (2021). Animal-food-human antimicrobial resistance fundamentals, prevention mechanisms and global surveillance trends: a terse review. *Appl. Food Biotechnol.* 8, 89–102.

Pan, Y., Awan, F., Zhenbao, M., Zhang, X., Zeng, J., Zeng, Z., et al. (2020). Preliminary view of the global distribution and spread of the tet(X) family

of tigecycline resistance genes. *J. Antimicrob. Chemother.* 75, 2797–2803. doi: 10.1093/jac/dkaa284

Peng, K., Li, R., He, T., Liu, Y., and Wang, Z. (2020). Characterization of a porcine Proteus cibarius strain co-harbouring tet(X6) and cfr. *J. Antimicrob. Chemother.* 75, 1652–1654. doi: 10.1093/jac/dkaa047

Peng, K., Wang, Q., Yin, Y., Li, Y., Liu, Y., Wang, M., et al. (2021). Plasmids shape the current prevalence of tmexCD1-toprJ1 among *Klebsiella pneumoniae* in food production chains. *mSystems* 6:e0070221. doi: 10.1128/mSystems.00702-21

Qin, S., Peng, J., Deng, R., Peng, K., Yan, T., Chen, F., et al. (2021). Identification of two plasmids coharboring carbapenemase genes and *tmexCD1-toprJ1* in clinical *Klebsiella pneumoniae* ST2667. *Antimicrob. Agents Chemother.* 65, e00625–21.

Roberts, M. C. (2003). Tetracycline therapy: update. *Clin. Infect. Dis.* 36, 462–467.

Ruan, Z., Jia, H., Chen, H., Wu, J., He, F., and Feng, Y. (2020). Co-existence of plasmid-mediated tigecycline and colistin resistance genes *tet*(X4) and *mcr-1* in a community-acquired *Escherichia coli* isolate in China. *J. Antimicrob. Chemother.* 75, 3400–3402.

Shterzer, N., and Mizrahi, I. (2015). The animal gut as a melting pot for horizontal gene transfer. *Can. J. Microbiol.* 61, 603–605. doi: 10.1139/cjm-2015-0049

Silvester, R., Madhavan, A., Kokkat, A., Parolla, A., Adarsh, B. M., Harikrishnan, M., et al. (2021). Global surveillance of antimicrobial resistance and hypervirulence in *Klebsiella pneumoniae* from LMICs: an in-silico approach. *Sci. Total Environ.* 802:149859. doi: 10.1016/j.scitotenv.2021.149859

Soliman, A. M., Ramadan, H., Zarad, H., Sugawara, Y., Yu, L., Sugai, M., et al. (2021). Coproduction of Tet(X7) conferring high-level tigecycline resistance, fosfomycin FosA4, and Colistin Mcr-1.1 in *Escherichia coli* strains from chickens in Egypt. *Antimicrob. Agents Chemother.* 65, e02084–20. doi: 10.1128/AAC.02084-20

Song, H., Liu, D., Li, R., Fu, Y., Zhai, W., Liu, X., et al. (2020). Polymorphism existence of mobile tigecycline resistance gene tet(X4) in *Escherichia coli*. *Antimicrob. Agents Chemother.* 64, e01825–19. doi: 10.1128/AAC.01825-19

Sun, C., Cui, M., Zhang, S., Liu, D., Fu, B., Li, Z., et al. (2020). Genomic epidemiology of animal-derived tigecycline-resistant *Escherichia coli* across China reveals recent endemic plasmid-encoded tet(X4) gene. *Commun. Biol.* 3:412. doi: 10.1038/s42003-020-01148-0

Sun, C., Cui, M., Zhang, S., Wang, H., Song, L., Zhang, C., et al. (2019). Plasmid-mediated tigecycline-resistant gene tet(X4) in *Escherichia coli* from food-producing animals, China, 2008–2018. *Emerg. Microbes Infect.* 8, 1524–1527. doi: 10.1080/22221751.2019.1678367

Sun, H., Wan, Y., Du, P., Liu, D., Li, R., Zhang, P., et al. (2021a). Investigation of tigecycline resistant *Escherichia coli* from raw meat reveals potential transmission among food-producing animals. *Food Control* 121:107633.

Sun, H., Zhai, W., Fu, Y., Li, R., Du, P., and Bai, L. (2021b). Co-occurrence of plasmid-mediated resistance genes tet(X4) and blaNDM-5 in a multidrug-resistant *Escherichia coli* isolate recovered from chicken in China. *J. Glob. Antimicrob. Resist.* 24:415–417. doi: 10.1016/j.jgar.2021.02.010

Sun, J., Chen, C., Cui, C.-Y., Zhang, Y., Liu, X., Cui, Z. H., et al. (2019). Plasmid-encoded tet(X) genes that confer high-level tigecycline resistance in *Escherichia coli*. *Nat. Microbiol.* 4, 1457–1464. doi: 10.1038/s41564-019-0496-4

Sun, L., Xu, G., Nan-Meng, Li, G. L., Wang, Z. Y., Mei, C. Y., et al. (2022). Emergence of carbapenem- and tigecycline-resistant *Klebsiella pneumoniae* ST617. *J. Glob. Antimicrob. Resist.* 29, 278–280. doi: 10.1016/j.jgar.2022.04.003

Sun, S., Gao, H., Liu, Y., Jin, L., Wang, R., Wang, X., et al. (2020). Co-existence of a novel plasmid-mediated efflux pump with colistin resistance gene mcr in one plasmid confers transferable multidrug resistance in *Klebsiella pneumoniae*. *Emerg. Microbes Infect.* 9, 1102–1113. doi: 10.1080/22221751.2020.1768805

Tang, Y., Lai, Y., Kong, L., Wang, X., Li, C., Wang, Y., et al. (2021). Characterization of three porcine *Escherichia coli* isolates co-harbouring tet(X4) and cfr. *J. Antimicrob. Chemother.* 76, 263–264. doi: 10.1093/jac/dkaa384

Thamlikitkul, V., Rattanaumpawan, P., Boonyasiri, A., Pumsuwan, V., Judaeng, T., Tiengrim, S., et al. (2015). Thailand antimicrobial resistance containment and prevention program. *J. Glob. Antimicrob. Resist.* 3, 290–296.

Theuretzbacher, U., Outterson, K., Engel, A., and Karlén, A. (2020). The global preclinical antibacterial pipeline. *Nat. Rev. Microbiol.* 18, 275–285. doi: 10.1038/s41579-019-0288-0

Ugah, U. I., and Udeani, T. K. (2021). High prevalence of phenotypic resistance to colistin, tigecycline and netilmicin in a region with no history of colistin administration in Nigeria. *Clin. Lab.* 67. doi: 10.7754/Clin.Lab.2020.200405

Umar, Z., Chen, O., Tang, B., Xu, Y., Wang, J., Zhang, H., et al. (2021). The poultry pathogen Riemerella anatipestifer appears as a reservoir for Tet(X) tigecycline resistance. *Environ. Microbiol.* 23, 7465–7482. doi: 10.1111/1462-2920.15632

Usui, M., Fukuda, A., Suzuki, Y., Nakajima, C., and Tamura, Y. (2021). Broad-host-range IncW plasmid harbouring tet(X) in *Escherichia coli* isolated from pigs in Japan. *J. Glob. Antimicrob. Resist.* 28, 97–101. doi: 10.1016/j.jgar.2021.12.012

Virolle, C., Goldlust, K., Djermoun, S., Bigot, S., and Lesterlin, C. (2020). Plasmid transfer by conjugation in gram-negative bacteria: from the cellular to the community level. *Genes* 11:1239. doi: 10.3390/genes11111239

Wan, M., Gao, X., Lv, L., Cai, Z., and Liu, J.-H. (2021). IS26 mediates the acquisition of tigecycline resistance gene cluster tmexCD1-toprJ1 by IncHI1B-FIB plasmids in *Klebsiella pneumoniae* and Klebsiella quasipneumoniae from food market sewage. *Antimicrob. Agents Chemother.* 65, e02178–20. doi: 10.1128/AAC.02178-20

Wang, C. Z., Gao, X., Lv, L. C., Cai, Z. P., Yang, J., and Liu, J. H. (2021a). Novel tigecycline resistance gene cluster tnfxB3-tmexCD3-toprJ1b in Proteus spp. and *Pseudomonas aeruginosa*, co-existing with tet(X6) on an SXT/R391 integrative and conjugative element. *J. Antimicrob. Chemother.* 11:dkab325. doi: 10.1093/jac/dkab325

Wang, C. Z., Gao, X., Yang, Q. W., Lv, L. C., Wan, M., Yang, J., et al. (2021b). A novel transferable resistance-nodulation-division pump gene cluster, tmexCD2-toprJ2, confers tigecycline resistance in Raoultella ornithinolytica. *Antimicrob. Agents Chemother.* 65, e02229–20. doi: 10.1128/AAC.02229-20

Wang, J., Wu, H., Mei, C.-Y., Wang, Y., Wang, Z.-Y., Lu, M.-J., et al. (2021c). Multiple mechanisms of tigecycline resistance in *Enterobacteriaceae* from a pig farm, China. *Microbiol. Spectr.* 9:e0041621. doi: 10.1128/Spectrum.00416-21

Wang, L., Liu, D., Lv, Y., Cui, L., Li, Y., Li, T., et al. (2019). Novel plasmid-mediated tet(X5) gene conferring resistance to tigecycline, eravacycline, and omadacycline in a clinical *Acinetobacter baumannii* isolate. *Antimicrob. Agents Chemother.* 64, e01326–19. doi: 10.1128/AAC.01326-19

Wang, N., Li, D., Schwarz, S., Qin, S., Yao, H., and Du, X. D. (2021d). Novel Tet(L) efflux pump variants conferring resistance to tigecycline and eravacycline in Staphylococcus Spp. *Microbiol. Spectr.* 9:e0131021. doi: 10.1128/Spectrum.01310-21

Wang, Q., Peng, K., Liu, Y., Xiao, X., Wang, Z., and Li, R. (2021e). Characterization of TMexCD3-TOprJ3, an RND-type efflux system conferring resistance to tigecycline in *Proteus mirabilis*, and its associated integrative conjugative element. *Antimicrob. Agents Chemother.* 65:e0271220. doi: 10.1128/AAC.02712-20

Wang, X., Sun, N., Liu, X., Li, F., Sun, J., Huang, J., et al. (2022). Small clone dissemination of tmexCD1-toprJ1-carrying *Klebsiella pneumoniae* isolates in a chicken farm. *J. Glob. Antimicrob. Resist.* 29, 105–112. doi: 10.1016/j.jgar.2022.02.012

Wang, Y., Liu, F., Xu, X., Huang, H., Lyu, N., Ma, S., et al. (2021f). Detection of plasmid-mediated tigecycline resistance gene tet(X4) in a *Salmonella enterica* serovar Llandoff Isolate. *Infect. Microbes Dis.* 3, 198–204.

Wang, Y., Liu, F., Zhu, B., and Gao, G. F. (2020a). Discovery of tigecycline resistance genes tet(X3) and tet(X4) in live poultry market worker gut microbiomes and the surrounded environment. *Sci. Bull.* 65, 340–342.

Wang, Y., Liu, F., Zhu, B., and Gao, G. F. (2020b). Metagenomic data screening reveals the distribution of mobilized resistance genes tet (X), mcr and carbapenemase in animals and humans. *J. Infect.* 80, 121–142. doi: 10.1016/j.jinf.2019.09.003

Wang, Y., Lyu, N., Liu, F., Liu, W. J., Bi, Y., Zhang, Z., et al. (2021g). More diversified antibiotic resistance genes in chickens and workers of the live poultry markets. *Environ. Int.* 153:106534. doi: 10.1016/j.envint.2021.106534

Wang, Y., Zhu, B., Liu, M., Dong, X., Ma, J., Li, X., et al. (2021h). Characterization of IncHI1B plasmids encoding efflux pump TmexCD2-ToprJ2 in carbapenem-resistant Klebsiella variicola, Klebsiella quasipneumoniae, and Klebsiella michiganensis Strains. *Front. Microbiol.* 12:759208. doi: 10.3389/fmicb.2021.759208

Wang, Z., Li, H., Zhang, J., Wang, X., Zhang, Y., and Wang, H. (2021i). Identification of a novel plasmid-mediated tigecycline resistance-related gene, tet(Y), in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 77, 58–68. doi: 10.1093/jac/dkab375

World Health Organization [WHO] (2019). *New Report Calls for Urgent Action to Avert Antimicrobial Resistance Crisis*. Geneva: WHO.

Wu, Y., He, R., Qin, M., Yang, Y., Chen, J., Feng, Y., et al. (2022). Identification of plasmid-mediated tigecycline-resistant gene *tet*(X4) in *Enterobacter cloacae* from pigs in China. *Microbiol. Spectr.* 1:e0206421.

Xiao, T., Peng, K., Chen, Q., Hou, X., Huang, W., Lv, H., et al. (2022). Coexistence of tmexCD-toprJ, blaNDM-1, and blaIMP-4 in One Plasmid Carried by Clinical Klebsiella spp. *Microbiol. Spectr.* 10:e0054922. doi: 10.1128/spectrum.00549-22

Xu, L., Wan, F., Fu, H., Tang, B., Ruan, Z., Xiao, Y., et al. (2022). Emergence of colistin resistance gene mcr-10 in Enterobacterales isolates recovered from fecal samples of chickens, slaughterhouse workers, and a nearby resident. *Microbiol. Spectr.* 10:e0041822. doi: 10.1128/spectrum.00418-22

Xu, Y., Liu, L., Zhang, H., and Feng, Y. (2021). Co-production of Tet(X) and MCR-1, two resistance enzymes by a single plasmid. *Environ. Microbiol.* 23, 7445–7464. doi: 10.1111/1462-2920.15425

Yaghoubi, S., Zekiy, A. O., Krutova, M., Gholami, M., Kouhsari, E., Sholeh, M., et al. (2021). Tigecycline antibacterial activity, clinical effectiveness, and mechanisms and epidemiology of resistance: narrative review. *Eur. J. Clin. Microbiol. Infect. Dis.* 41, 1003–1022. doi: 10.1007/s10096-020-04121-1

Yam, E., Hsu, L., Yap, E. H., Yeo, T. W., Lee, V., Schlundt, J., et al. (2019). Antimicrobial resistance in the asia pacific region: a meeting report. *Antimicrob. Resist. Infect. Control* 8:202. doi: 10.1186/s13756-019-0654-8

Yang, X., Ye, L., Chan, E. W., Zhang, R., and Chen, S. (2021). Characterization of an IncFIB/IncHI1B Plasmid encoding efflux pump TMexCD1-TOprJ1 in a clinical tigecycline- and carbapenem-resistant *Klebsiella pneumoniae* strain. *Antimicrob. Agents Chemother.* 65, e02340–20. doi: 10.1128/AAC.02340-20

Yongchang, X., Liu, L., Sun, J., and Feng, Y. J. (2019). Limited distribution and mechanism of the TetX4 tetracycline resistance enzyme. *Sci. Bull.* 64, 1478–1481.

Yu, R., Zhang, Y., Xu, Y., Schwarz, S., Li, X.-S., Shang, Y.-H., et al. (2021). Emergence of a tet(M) variant conferring resistance to Tigecycline in Streptococcus suis. *Front. Vet. Sci.* 8:709327. doi: 10.3389/fvets.2021.709327

Yu, Y., Cui, C.-Y., Kuang, X., Chen, C., Wang, M.-G., Liao, X.-P., et al. (2021). Prevalence of tet(X4) in *Escherichia coli* from duck farms in Southeast China. *Front. Microbiol.* 12:716393. doi: 10.3389/fmicb.2021.716393

Zeng, Y., Lu, J., Liu, C., Ling, Z., Sun, Q., Wang, H., et al. (2021). A method for screening tigecycline-resistant gene tet(X) from human gut. *J. Glob. Antimicrob. Resist.* 24, 29–31. doi: 10.1016/j.jgar.2020.11.010

Zhai, W., Tian, Y., Lu, M., Zhang, M., Song, H., Fu, Y., et al. (2022). Presence of mobile tigecycline resistance gene *tet*(X4) in clinical *Klebsiella pneumoniae*. *Microbiol. Spectr.* 10:e0108121.

Zhanel, G. G., Homenuik, K., Nichol, K., Noreddin, A., Vercaigne, L., Embil, J., et al. (2004). The glycylcyclines: a comparative review with the tetracyclines. *Drugs* 64, 63–88.

Zhang, R., Dong, N., Shen, Z., Zeng, Y., Lu, J., Liu, C., et al. (2020a). Epidemiological and phylogenetic analysis reveals Flavobacteriaceae as potential ancestral source of tigecycline resistance gene tet(X). *Nat. Commun.* 11:4648. doi: 10.1038/s41467-020-18475-9

Zhang, R., Dong, N., Zeng, Y., Shen, Z., Lu, J., Liu, C., et al. (2020b). Chromosomal and plasmid-borne tigecycline resistance genes tet(X3) and tet(X4) in dairy cows in a Chinese farm. *Antimicrob. Agents Chemother.* 64, e00674–20. doi: 10.1128/AAC.00674-20

Zhang, R., Sun, J., Sun, R., Wang, M., Cui, C., Fang, L., et al. (2021). Source tracking and global distribution of the mobilized tigecycline resistant gene tet(X). *bioRxiv [Preprint]* doi: 10.1101/2021.07.29.454411

Zheng, X. R., Zhu, J. H., Zhang, J., Cai, P., Sun, Y. H., Chang, M. X., et al. (2020). A novel plasmid-borne tet(X6) variant co-existing with blaNDM-1 and blaOXA-58 in a chicken *Acinetobacter baumannii* isolate. *J. Antimicrob. Chemother.* 75, 3397–3399. doi: 10.1093/jac/dkaa342

Zheng, X., Ma, J., Lu, Y., Sun, D., Yang, H., Xia, F., et al. (2022). Detection of tet(X6) variant-producing Proteus terrae subsp. cibarius from animal cecum in Zhejiang, China. *J. Glob. Antimicrob. Resist.* 29, 124–130. doi: 10.1016/j.jgar.2022.02.011

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 Anyanwu, Nwobi, Okpala and Ezeonu. 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 Microbiology

TYPE Original Research
PUBLISHED 04 August 2022
DOI 10.3389/fmicb.2022.869538

Check for updates

OPEN ACCESS

EDITED BY
Ziad Daoud,
Central Michigan University,
United States

REVIEWED BY
Hui Zhang,
Jiangsu Academy of Agricultural Sciences (JAAS), China
Dipak Kathayat,
Cornell University, United States

*CORRESPONDENCE
Mahdi Ghanbari
mahdi.ghanbari@dsm.com

†These authors share first authorship

SPECIALTY SECTION
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

RECEIVED 04 February 2022
ACCEPTED 08 July 2022
PUBLISHED 04 August 2022

CITATION
Temmerman R, Ghanbari M, Antonissen G, Schatzmayr G, Duchateau L, Haesebrouck F, Garmyn A and Devreese M (2022) Dose-dependent impact of enrofloxacin on broiler chicken gut resistome is mitigated by synbiotic application.
Front. Microbiol. 13:869538.
doi: 10.3389/fmicb.2022.869538

COPYRIGHT
© 2022 Temmerman, Ghanbari, Antonissen, Schatzmayr, Duchateau, Haesebrouck, Garmyn and Devreese. 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.

Dose-dependent impact of enrofloxacin on broiler chicken gut resistome is mitigated by synbiotic application

Robin Temmerman[1†], Mahdi Ghanbari[2*†], Gunther Antonissen[1], Gerd Schatzmayr[2], Luc Duchateau[3], Freddy Haesebrouck[1], An Garmyn[1] and Mathias Devreese[1]

[1]Department of Pathobiology, Pharmacology and Zoological Medicine, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [2]DSM - BIOMIN Research Center, Tulln, Austria, [3]Faculty of Veterinary Medicine, Biometrics Research Center, Ghent University, Merelbeke, Belgium

Fluoroquinolone agents are considered critical for human medicine by the World Health Organization (WHO). However, they are often used for the treatment of avian colibacillosis in poultry production, creating considerable concern regarding the potential spread of fluoroquinolone resistance genes from commensals to pathogens. Therefore, there is a need to understand the impact of fluoroquinolone application on the reservoir of ARGs in poultry gut and devise means to circumvent potential resistome expansion. Building upon a recent dose optimization effort, we used shotgun metagenomics to investigate the time-course change in the cecal microbiome and resistome of broiler chickens receiving an optimized dosage [12.5 mg/kg body weight (bw)/day], with or without synbiotic supplementation (PoultryStar®, BIOMIN GmbH), and a high dosage of enrofloxacin (50 mg/kg bw/day). Compared to the high dose treatment, the low (optimized) dose of enrofloxacin caused the most significant perturbations in the cecal microbiota and resistome of the broiler chickens, demonstrated by a lower cecal microbiota diversity while substantially increasing the antibiotic resistance genes (ARGs) resistome diversity. Withdrawal of antibiotics resulted in a pronounced reduction in ARG diversity. Chickens receiving the synbiotic treatment had the lowest diversity and number of enriched ARGs, suggesting an alleviating impact on the burden of the gut resistome. Some Proteobacteria were significantly increased in the cecal metagenome of chickens receiving enrofloxacin and showed a positive association with increased ARG burden. Differential abundance (DA) analysis revealed a significant increase in the abundance of ARGs encoding resistance to macrolides-lincosamides-streptogramins (MLS), aminoglycosides, and tetracyclines over the period of enrofloxacin application, with the optimized dosage application resulting in a twofold higher number of affected ARG compared to high dosage application. Our results provide novel insights into the dose-dependent effects of clinically important enrofloxacin application in shaping the broiler gut resistome, which was mitigated by a synbiotic application. The contribution to

ameliorating the adverse effects of antimicrobial agents, that is, lowering the spread of antimicrobial resistance genes, on the poultry and potentially other livestock gastrointestinal microbiomes and resistomes merits further study.

KEYWORDS

broiler chicken, enrofloxacin, gut resistome, gut microbiome, synbiotic

Introduction

Antimicrobial resistance (AMR) accounts for an annual death toll of approximately 700,000 people and is a major threat to global health (World Health Organisation (WHO), 2014). There is a general consensus that the alarming levels of AMR are strongly related to the overuse and misuse of antimicrobial agents in human and animal medicine, as well as in agriculture and livestock production systems (Anomaly, 2009; Chantziaras et al., 2014; Raboisson et al., 2020), where antimicrobial use is projected to increase further due to the growth of human and livestock populations (Van Boeckel et al., 2015). Fluoroquinolones are of critical importance in human medicine for the treatment of *Campylobacter*, *Salmonella*, and *Shigella* infections. These infections are zoonotic and limited therapeutic options are available (WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2019). Due to their crucial status and the development of AMR in human medicine, fluoroquinolone use in the veterinary sector is under scrutiny. In the broiler industry, fluoroquinolone chemotherapeutics such as enrofloxacin are still frequently administered via drinking water for the treatment of colibacillosis (an infection that is caused by the enigmatic avian pathogenic *Escherichia coli*) in Europe and Asia (Persoons et al., 2012; Joosten et al., 2019; Roth et al., 2019b), which creates a considerable concern regarding the potential spread of fluoroquinolone resistance genes from commensal bacteria found in livestock to pathogenic organisms in humans (Dheilly et al., 2012; Gregova and Kmet, 2020; Temmerman et al., 2021). However, there is limited published information on the impact of enrofloxacin treatment on the poultry gut microbiome and, more importantly, no information on the development of resistome [the reservoir of antibiotic resistance genes (ARGs)] in poultry gut, leaving a considerable knowledge gap that limits the thorough evaluation and optimization of such critically important antibiotics in broiler production. In fact, the judicious use of antibiotics is extremely important to preserve the efficacy of antimicrobials while mitigating resistance development (World Health Organization (WHO), 2015; Foditsch et al., 2019). Using the 16S rRNA amplicon sequencing approach, previous studies have shown that treatment with enrofloxacin causes significant shifts in intestinal microbiota of chickens while hindering recovery from *Salmonella* Typhimurium and selecting for fluoroquinolone resistance in coliforms (Li et al., 2017a,b; Ma et al., 2020).

With regard to the use of enrofloxacin in the poultry sector, the European Medicines Agency's Committee for Veterinary Medicinal Products urged to revise the currently approved dose of 10 mg/kg bw/day (European Medicines Agency, 2018, 2021), considering the development of resistance, as well as the notoriously large inter-animal variability in drinking behavior (Soraci et al., 2014; Ferran and Roques, 2019; Ferran et al., 2020), which can lead to both under- (treatment failure and resistance selection) and overexposure (adverse effects) in animals. In our recent efforts to optimize the dose of enrofloxacin for the treatment of colibacillosis in broiler chickens, using a novel drinking behavior pharmacokinetic (DBPK) model, we found a dosage of 12.5 mg/kg per day to be efficacious in treating colibacillosis caused by strains without acquired resistance mechanisms (Temmerman et al., 2021). However, *in vivo* evaluation of such optimized doses in broiler chickens is still lacking. The impact of other antimicrobials on the residential microbiota in broilers has been investigated (Xiong et al., 2018; Luiken et al., 2019; Turcotte et al., 2020), showing a range of effects, including reduction of overall diversity of the bacterial communities, microbiome metabolic shifts, increasing gut susceptibility to colonization, and stimulation of the development of AMR. In medical research, there are indications that alternatives to antibiotics, including dietary prebiotics, probiotics, and synbiotics (products that combine the simultaneous and potentially synergistic use of pre- and probiotics) (Gibson et al., 2004; Markowiak and Śliżewska, 2017) can reverse the perturbations induced by antimicrobial administration (Korpela et al., 2018; Abd El-Hack et al., 2020).

Building on our previous findings, we performed a shotgun metagenomics analysis to provide insights into the effects of enrofloxacin treatment on the broiler cecal microbiome and antimicrobial resistance gene (ARG) reservoir (resistome), applied at the optimized (12.5 mg/kg bw/day, OPT group) or high dose (50 mg/kg bw/day, HIGH group). Given the importance of studying the potential means to circumvent resistome expansion, we also evaluated the change in the cecal microbiome and resistome of broiler chickens supplemented with a synbiotic in conjunction with

the administration of enrofloxacin (OPT_PS group). The findings of this study have important implications for poultry production and public health, as there is little research on the effects of therapeutic doses of enrofloxacin and synbiotics in conjugation with the application of enrofloxacin on the gut microbiome and resistome.

Materials and methods

Animals and experimental design

The animal trial was reviewed and approved by the ethical committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University (EC 2020-016). The care and treatment of the birds were in full compliance with the national and European legislation concerning animal welfare (European Commission., 2010; Royal Decree., 2013). Throughout the study, feed and drinking water were provided *ad libitum*.

Eighty-four healthy 1-day-old male broiler chicks (Ross 308), with similar body weights (bws) of approximately 55 g were randomly allocated to one of four treatments [$n = 7$ cages (3 chickens each) per treatment]: (1) standard commercial feed (**Table 1**, Farm mash 1&2, Versele-Laga, Deinze, Belgium) for 37 days (control group), 2) antibiotic-treated group 1 [OPT, 12.5 mg/kg bw/day Baytril® 10% oral solution (Bayer, Diegem, Belgium) consecutively via drinking water from day 21 to day 23 of age], (3) antibiotic-treated group 2 (HIGH, 50 mg/kg bw/day Baytril® 10% oral solution consecutively via drinking water from day 21 to day 23 of age), and (4) synbiotic supplemented group (OPT_PS), in which chickens received the commercial feed supplemented with a synbiotic product (PoultryStar®, BIOMIN GmbH, Austria) during their lifetime, in conjunction with receiving enrofloxacin at a dose of 12.5 mg/kg bw/day via drinking water from day 21 to day 23 of age. The age of the birds was selected to reflect the real commercial setting and scenario for antimicrobial use, since colibacillosis frequently occurs in the second half of the broiler growing period. Additionally, the 3-day treatment duration was in compliance with the label of Baytril. The synbiotic supplement was composed of 2×10^8 CFU/g multi-species probiotic: *Enterococcus faecium* (1.20×10^8 CFU/g), *Bifidobacterium animalis* (6×10^7 CFU/g), *Lactobacillus salivarius* (2×10^7 CFU/g), and prebiotic inulin. The amount of mL enrofloxacin that needed to be added in the drinking water was calculated using the following formula (Equation 1) (Temmerman et al., 2021):

$$\frac{Dose\ \left(\frac{mg}{kg}\right) * average\ BW\ (kg) * number\ of\ animals\ per\ group * amount\ of\ water(L)}{Average\ water\ uptake\ per\ group\ in\ 24h\ (L)} \quad (1)$$

Cecal sampling, DNA extraction, library preparation and sequencing

Immediately before (day 20) and after enrofloxacin treatment (day 24), and 2 weeks after antibiotic withdrawal (day 37), one bird per replicate was sacrificed and digesta samples from the cecum were collected in Eppendorf cups, immediately snap frozen (liquid N2), and stored at −20°C. The animals were euthanized with pentobarbital IV (sodium pentobarbital 20%, Kela, Hoogstraten, Belgium) dosed at approximately 100 mg/kg.

DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Antwerp, Belgium), which as shown in our recent studies, has minimum effects on the microbial community structure and promising results in terms of the DNA integrity for chicken cecum samples (Wegl et al., 2021). The purity of the DNA was assessed by evaluating the 260/280 and 260/230 absorbances using NanoDrop® (ND-1000, Thermo Fisher Scientific, Merelbeke, Belgium), while Quantus® (Promega, Leiden, The Netherlands) was used to estimate the DNA concentration. Finally, DNA integrity was visually validated by gel electrophoresis. The DNA was sent to LGC Genomics GmbH, Berlin, Germany, where paired-end sequencing libraries were constructed using the Illumina Nextera XT Library Preparation Kit (Illumina Inc., San Diego, CA) followed by sequencing on the Illumina NextSeq 500 platform using high-output chemistry (2×150 bp) according to the manufacturer's instructions.

Sequenced data processing and statistical analysis

After demultiplexing and adaptor trimming (trimmomatic v. 0.39) (Bolger et al., 2014) taxonomic profiles of the demultiplexed reads and taxa relative abundance estimates were generated using Kraken2 (Wood et al., 2019) and Bracken (Lu et al., 2017), with kraken2 confidence set to 0.05 and bracken threshold to t −10. The abundances of ARGs were quantified by mapping the reads against the hand-curated AMR database MEGARes v2.0 (Doster et al., 2020) using USEARCH (v10) (Edgar, 2010). In MEGARes 2.0, the nodes of the acyclic hierarchical ontology included four antimicrobial compound types, 57 classes, 220 mechanisms of resistance, and 1,345 gene groups that classified the 7,868 accessions. High confidence matches to the sequence in the MEGARes database were obtained by considering the entire coverage of the query reads against ARG genes with an identity threshold of 90% (parameters were set as "-usearch-global -id 0.9, maxaccepts 1, threads 50") (Ghanbari et al., 2019). The variance explained by each variable included in the trial was quantified by omnibus testing on Bray–Curtis dissimilarity matrices from metagenome data, using PERMANOVA with the adonis function in the R package Vegan (Dixon, 2003). Ordination was performed with

TABLE 1 Composition of the broiler diet (Farm mash 1&2, Versele-Laga).

Composition
Wheat, soya feed (produced from genetically modified soya), maize, wheat gluten feed, palm oil, calcium carbonate, sunflower seed feed, maize gluten feed, beet molasses, monocalcium phosphate, sodium chloride, sodium bicarbonate
Analytical constituents
Crude protein 18.0%, crude fat 5.0%, crude ash 5.5%, crude fiber 3.0%, lysine 0.97%, methionine 0.45%, calcium 0.95%, phosphorus 0.53%, sodium 0.10%
Additives/kg
Nutritional additives
3a672a vitamin A 10000 IU, 3a671 vitamin D3 2500 IU, 3a700 Vitamin E (all-rac-alpha-tocopheryl acetate) 80 mg, 3b103 iron (ferrous sulfate, monohydrate) 50 mg, 3b202 iodine (calcium iodate, anhydrous) 2.14 mg, 3b405 copper (cupric sulfate, pentahydrate) 10 mg, 3b502 manganese (manganous oxide) 75 mg, 3b603 zinc (zinc oxide) 70 mg, 3b802 selenium (coated granulated sodium selenite) 0.30 mg
Zoo technical additives
4a1617 endo-1,4-β-xylanase (EC 3.2.1.8) 1050 EPU, 4a16 6-phytase (EC 3.1.3.26) 250 FTU
Technological additives
Antioxidants
Coccidiostats and histomonostats
E763 lasalocid sodium 125 mg

log-transformed normalized reads on two dimensions with the "prcomp" ordinate function from the package "stats" v3.6.2 and visualized using the "fviz_pca" function from the "factoextra" function from the FaceFacefactoextra package v1.0.7.

Linear regression analysis was performed considering random differences between the treatment groups at the first sampling (for the control and enrofloxacin treatments) as covariate terms in the model if the richness (the number of unique taxa) and inverse Simpson diversity (the number and relative abundance of unique taxa) were affected by the different treatments. The Inverse Simpson diversity index was selected due to its biological interpretation aspect and since it does not tend to be as affected by sampling effort as other diversity indexes. The Mann–Whitney test was used for statistical comparisons between the pairs of groups at each sampling time point. Log-fold changes in abundance (of taxa and ARGs) between groups were determined by a negative binomial generalized linear model using DESeq2 version 1.26.0. (Love et al., 2014) in R, considering the statistical significance at BH-corrected $P \leq 0.10$. In all statistical analyses, the cage was considered as the experimental unit.

Results

Synbiotic administration resulted in a significantly higher body weight in broiler chicken

The average weights per treatment group (7 animals per group, 28 in total) and associated variability at the end of the animal trial are shown in **Figure 1**. The broiler weight ranges at day 37 were 1.25–1.95, 1.37–2.20, 1.25–2.00, and 1.58–2.00 kg for the control, HIGH, OPT, and OPT_PS groups, respectively. The corresponding averages are 1.59, 1.82, 1.54, and 1.83 kg, respectively. Chickens that received the highest enrofloxacin dose (HIGH group) and the optimized enrofloxacin dose supplemented with the synbiotic (OPT_PS group) had higher average weights at 37 days of age when compared to the control group, although only the chickens in the OPT with synbiotic supplementation (OPT_PS) group showed a much higher homogeneity in weight gain and a significant difference in weight group ($p = 0.034$, **Figure 1**). Interestingly, the difference between the optimized dosing (OPT) and OPT_PS was statistically significant ($p = 0.026$), suggesting an important role of synbiotic application in restoring physiological balance after perturbation by antibiotics and, consequently, improving the weight gain.

Enrofloxacin and synbiotic applications were significantly associated with microbiota and resistome profiles variation in broiler chicken

Shotgun metagenomics of the 84 cecum samples generated over 600 million paired-end sequence reads with a mean number of eight million reads per sample (see **Supplementary File 1**, which contains detailed information on the samples, including library size). On average, 23% of the reads per sample were classified to the species level using Kraken2 with a confidence threshold of 5%. Using the MEGARes v2.0 database with a 90% gene cut-off fraction, 1,281,069 reads were aligned to 1,000 antimicrobial resistance genes across

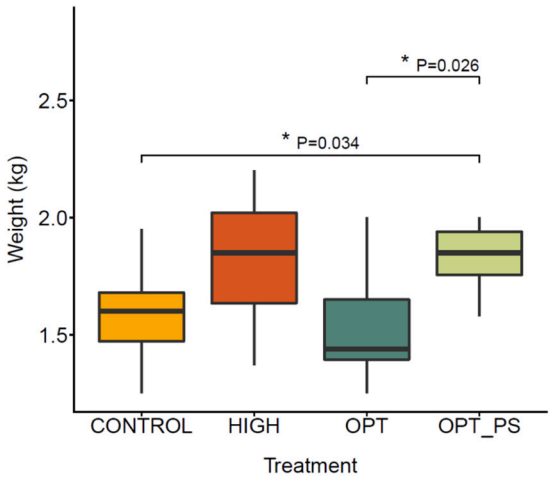


FIGURE 1
Box plot of the final body weight distribution of broiler chickens in different groups at 37 days of age. Control group (CONTROL); higher dose (HIGH): 50 mg/kg bw/day enrofloxacin; optimized dosing (OPT): 12.5 mg/kg bw/day enrofloxacin; optimized dosing with synbiotic supplementation (OPT_PS): 12.5 mg/kg bw/day enrofloxacin administration with synbiotic supplementation). FDR-corrected *p*-value from the Mann–Whitney statistical analysis is shown (*$P < 0.05$).

treatments. Permutational Multivariate Analysis of Variance (PERMANOVA) statistical analysis calculated on Bray–Curtis dissimilarity matrices from metagenome data showed that the age of the birds (sampling time points: days 20, 24, and 37) was the largest driver of variation in both cecum microbiota and ARG resistome profiles, with antibiotic and synbiotic application capturing less variation (Figure 2A). In line with this, principal component analysis (PCA) revealed a clear separation of the sample based on the day of sampling (age) (Figures 2B,C). Regarding the effect of antibiotic administration on global diversity, the lower, optimized dosage of enrofloxacin (OPT) resulted in a higher variation compared to the higher dosage (HIGH) on the composition and structure of the taxonomical (Adonis R2, 5.9% vs. 3.6%) and the ARG resistome profiles (Adonis R2, 3.9% vs. 3.1%) in broiler chickens. Synbiotic application (OPT_PS) significantly explained 3.1 and 4.6% of the variation (Adonis R2) of the cecum microbiota and ARG resistome profiles, respectively (Figure 2A).

Further, pairwise *post hoc* comparisons[1] showed a significant difference in the profiles of the relative abundance of taxa and ARGs (with the exception of the optimized dosage effect on the taxa profiles) between the enrofloxacin (both HIGH and OPT) and control treatment at day 24 of sampling. No significant differences were seen for the sample taken at day 20 or for those taken 2 weeks after the withdrawal of enrofloxacin at day 37 (Table 2), indicating the resilience of the bacterial communities carrying ARGs from antibiotic perturbation. On the other hand, OPT_PS showed a significant difference with the control groups in the relative abundance of taxonomy (with the exception of day 20) and resistome profiles at days 20 and 37 (Table 2). On day 24, immediately after an optimized dosage of enrofloxacin was applied in combination with the synbiotic product, the microbiota and ARG resistome profiles of the samples clearly diverged from the control group (P = 0,006), suggesting a synergistic effect on the chicken cecal metagenome (Table 2).

1 https://rdrr.io/github/GuillemSalazar/EcolUtils/man/adonis.pair.html

Enrofloxacin application resulted in a lower cecal microbiota diversity while substantially increasing antibiotic resistance gene resistome diversity

Linear regression analysis, taking into account the random differences between the high and optimized doses of enrofloxacin with control treatments on day 20, revealed a significant association of enrofloxacin application with the reduction of the microbiota Observed index from days 20 to day 24 (HIGH vs. control coefficient estimate day 24 = −41.143 with P = $2.55e^{-05}$, OPT vs. control coefficient estimate day 24 = −33.857 with P = 0.000414, and OPT_PS vs. control observed index coefficient estimate day 24 = −41.857 with $P = 1.91e^{-05}$). Two weeks after the withdrawal of the antibiotic, although the microbial community richness recovered to some extent (HIGH vs. control CE day 37 = −26.429 with $P = 0.00507$, OPT vs. control EC day 37 = −26.000 with P = 0.005795, and OPT_PS vs. control CE day 37 = −20.143, with P = 0.030770), the cecum microbiota Observed index was still significantly lower in all groups than in the control. For OPT_PS at day 20, the observed index for the cecum microbiota was higher compared to those in the control (187 ± 9 vs. 183 ± 6), although the difference was not statistically significant.

Calculation of the Inverse Simpson diversity index revealed that while the cecum microbiota diversity in the animals in the control group increased (not significantly) from day 20 (inverse Simpson 11.248 ± 1.819) to day 24 of age (12.842 ± 2.617), for the broiler chickens receiving the HIGH and OPT dosage, there was either no change (HIGH group, IS 12.048 ± 4.256–12.210 ± 4.0189) or a reduction (not significant; OPT group, IS 11.909 ± 3.059–10.536 ± 3.024) in microbial diversity (Figure 3A). For OPT_PS, the reduction in diversity was less pronounced (Figure 3A). At day 20 of age, OPT_PS showed generally higher diversity compared to the control (IS 12.960 ± 3.259 vs. 11.248 ± 1.819, respectively). While the withdrawal of enrofloxacin application did not seem to have a large impact on the recovery of the diversity for the animals in the HIGH and OPT_PS groups, it resulted in a significant increase in the cecum microbiota diversity in the optimized dosage group (Figure 3A).

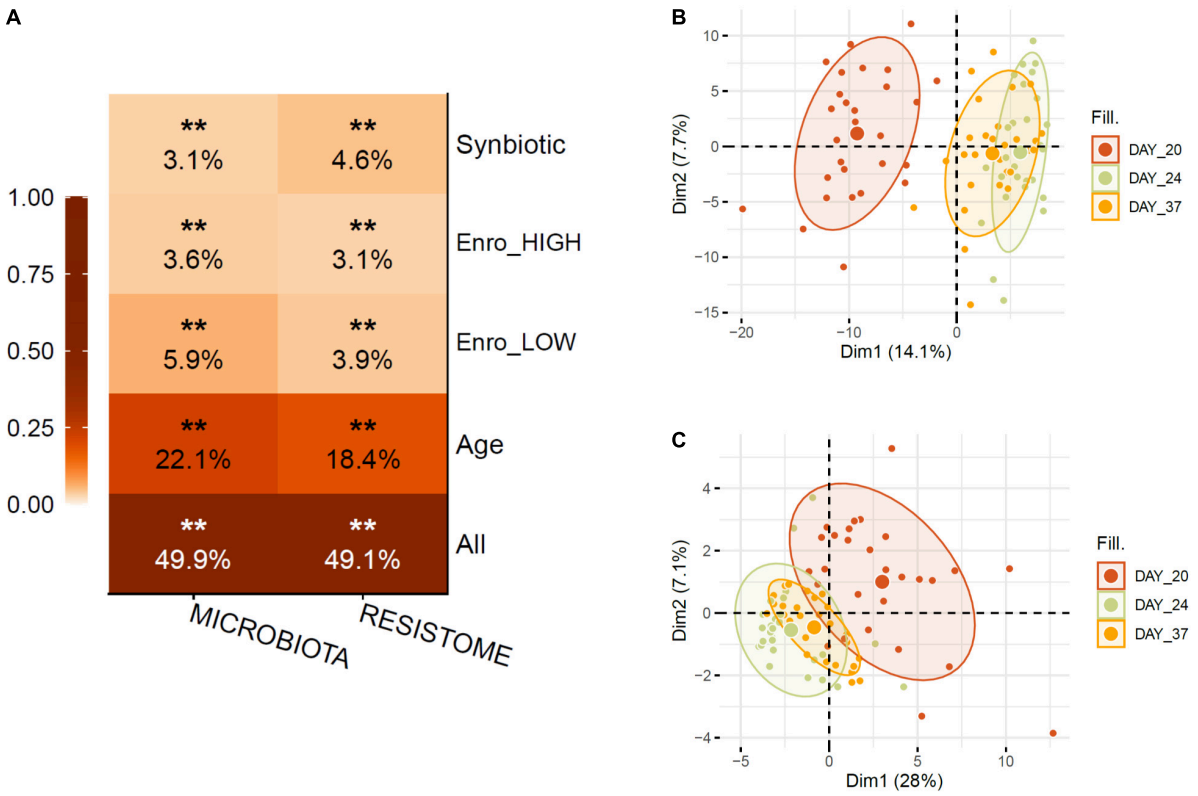


FIGURE 2
(A) PERMANOVA shows that the animal age had the largest effect on variation for both measurement types followed by the low/optimized dosage of enrofloxacin application. Stratified tests (Enro-HIGH/OPT (LOW), Synbiotic) consider only samples within the indicated category. Stars show FDR-corrected statistical significance (FDR $^{**}P \leq 0.01$). Variance is estimated for each feature independently, except for "All," for which all metadata are included in the model. **(B)** Principle component analysis showing the changes in taxonomical and **(C)** ARG resistome profiles due to the effect of age. Ellipses indicate 95% confidence intervals of around centroids of the groupings with treatments as factor.

A significant association of enrofloxacin application with the change in the diversity of ARGs was revealed [linear regression analysis, $F_{(11,\ 72)} = 2.258$, $p = 0.02016$ with an R2 of 0.26]. Over the course of the enrofloxacin application, that is, days 21–23, the diversity of ARGs in the cecum metagenome of the birds receiving enrofloxacin significantly increased ($P < 0.05$), regardless of dosage (Figure 3B). While OPT_PS had the lowest ARG diversity at day 20, the application of the optimized dosage of enrofloxacin on the top of the synbiotic supplement resulted in the largest increase ($P < 0.01$) on the diversity of ARGs from days 20 to 24 compared to all other treatments (Figure 3B). Yet, the cecum ARG diversity of birds in the OPT group (17.393 ± 0.727) at day 20 was significantly higher than that in the control (16.477 ± 1.651, $P = 0.620$), HIGH (16.176 ± 1.412, $P = 0.097$), and OPT_PS (16.089 ± 0.617, $P = 0.011$) groups, suggesting an alleviation effect of synbiotic application on the expansion of broiler cecal resistome (Figure 3B). Withdrawal of antibiotic application resulted in a pronounced reduction in ARG diversity, with OPT_PS showing significantly lower diversity of ARGs compared to the control group ($P = 0.011$) at day 37 (Figure 3B).

Proteobacteria were significantly affected by the enrofloxacin application and are associated with an increased antibiotic resistance genes burden

Taxonomic profiling of the cecum samples in terms of the most abundant microbial phyla and species over the course of the study is shown in Figure 4. In total, 1,573 different microbial species were observed, of which species belonging to Firmicutes, followed by species belonging to Actinobacteria and Proteobacteria, were the most dominant members of the cecal microbiota (Figure 4).

Differential abundance (DA) analysis of the species-level taxonomic assignments using the DESeq2 tool revealed a significant effect of enrofloxacin application on shaping the cecal microbiota over the course of antibiotic application. Among the different groups of the impacted taxa, species belonging to the Proteobacteria phylum were among the most affected taxa due to enrofloxacin application followed

TABLE 2 Pairwise comparisons for permutational multivariate analysis of variance (PERMANOVA) using bray-cutis distance matrices.

		Sums of Sqs	Mean Sqs	F-Model	R2	*P*-value	*q*-value*
DAY_20CONTROL VS. DAY_20HIGH	Microbiota	0.041	0.041	0.794	0.062	0.583	0.631
	Resistome	0.012	0.012	0.589	0.047	0.905	0.905
DAY_20CONTROL VS. DAY_20OPT	Microbiota	0.094	0.094	1.508	0.112	0.127	0.186
	Resistome	0.023	0.023	1.265	0.095	0.239	0.258
DAY_20CONTROL VS. DAY_20OPT_PS	Microbiota	0.066	0.066	1.259	0.095	0.236	0.311
	Resistome	0.037	0.037	1.562	0.115	0.085	**0.098**
DAY_24CONTROL VS. DAY_24HIGH	Microbiota	0.109	0.109	2.598	0.178	0.005	**0.026**
	Resistome	0.043	0.043	3.988	0.249	0.005	**0.009**
DAY_24CONTROL VS. DAY_24OPT	Microbiota	0.087	0.087	1.456	0.108	0.146	0.209
	Resistome	0.034	0.034	2.277	0.159	0.003	**0.007**
DAY_24CONTROL VS. DAY_24OPT_PS	Microbiota	0.090	0.090	2.472	0.171	0.006	**0.026**
	Resistome	0.042	0.042	4.810	0.286	0.003	**0.007**
DAY_37CONTROL VS. DAY_37HIGH	Microbiota	0.032	0.032	0.707	0.056	0.740	0.763
	Resistome	0.031	0.031	1.639	0.120	0.090	0.101
DAY_37CONTROL VS. DAY_37OPT	Microbiota	0.028	0.028	0.569	0.045	0.897	0.897
	Resistome	0.014	0.014	1.024	0.079	0.444	0.457
DAY_37CONTROL VS. DAY_37OPT_PS	Microbiota	0.048	0.048	1.104	0.084	0.324	0.381
	Resistome	0.032	0.032	2.942	0.197	0.012	**0.017**

*FDR-corrected *P*-value. Bold values denote statistical significance.

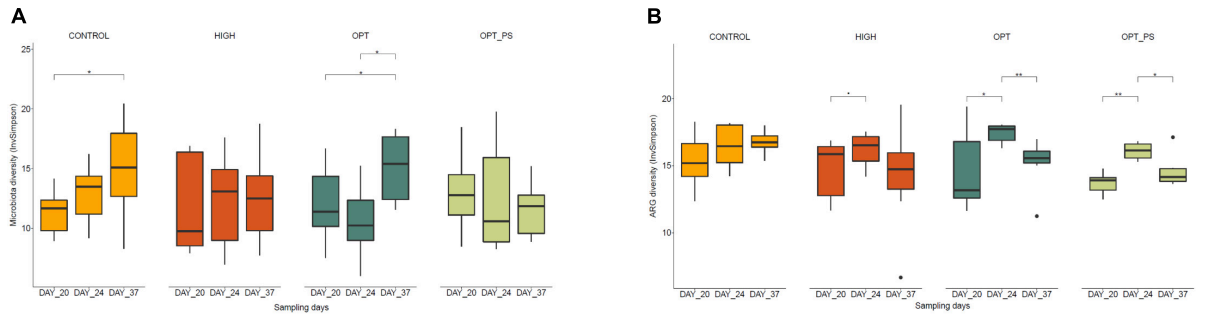


FIGURE 3
Diversity analysis (based on Inverse Simpson index) of the **(A)** microbial taxa and **(B)** ARGs in chicken cecal metagenome. FDR-corrected *p*-value from the Mann–Whitney statistical analysis is shown. *$P < 0.05$, **$P < 0.01$, respectively.

by species from Firmicutes and Actinobacteria (Figure 5A and Supplementary Data 1). In fact, over the course of the enrofloxacin application (days 21–23), both high and optimized dose application resulted in a significant (FDR-corrected *p*-value < 10%) increase in the abundance of 17 proteobacterial species, while significantly reducing the abundance of 15 proteobacterial species (Figure 5B and Supplementary Data 1). Two weeks after the last day of antibiotic application, the number of affected microbial species belonging to the Proteobacteria decreased, with only four and five species showing a significantly higher abundance in the HIGH and OPT groups compared to the control, respectively (Figure 5B and Supplementary Data 1). Synbiotic application significantly (FDR-corrected *p*-value < 10%) reduced the abundance of 15 proteobacterial species, including *Escherichia coli* and *Campylobacter* spp., on day 20 (Supplementary Data 1).

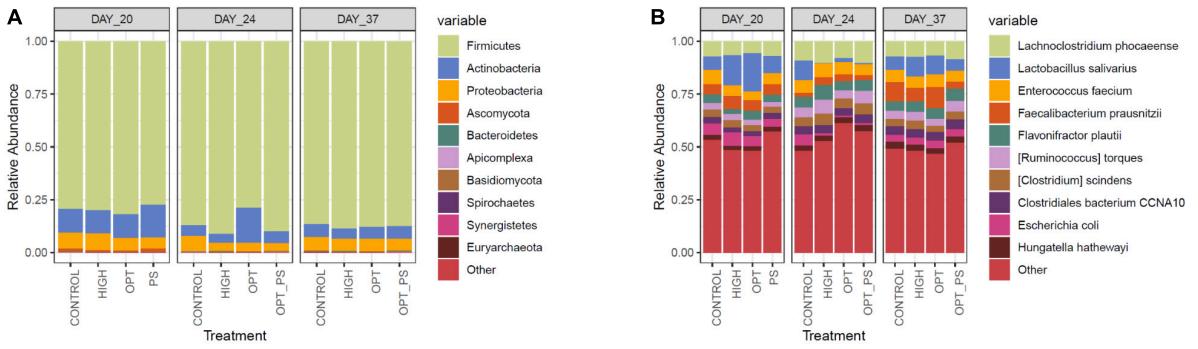


FIGURE 4
The 10 most abundant taxa in the bacterial communities at the phylum **(A)** and species **(B)** level, according to Kraken2 analysis.

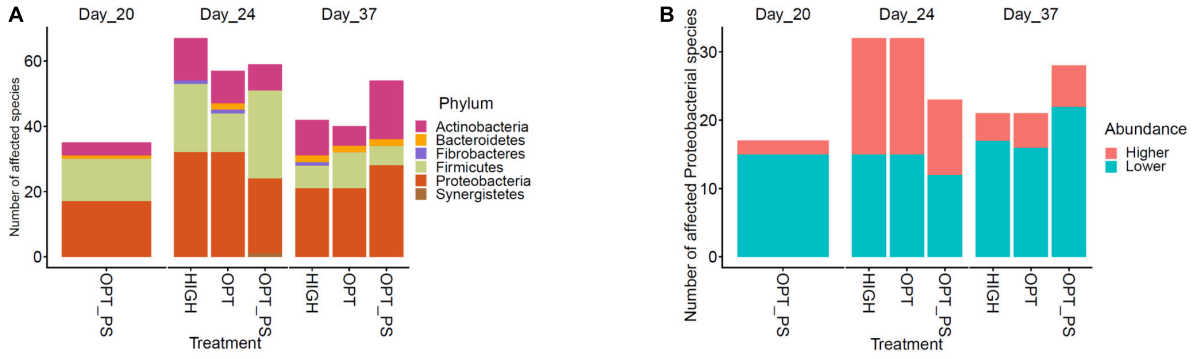


FIGURE 5
Impact of synbiotic and/or enrofloxacin application on differential abundances of microbiota. The bar plot shows the number of affected taxa **(A)** and Proteobacterial members (either increased or decreased) **(B)** based on differential abundance analysis in the chicken cecum.

Interestingly, the OPT_PS group, showed a lower number of significantly increased Proteobacteria species compared to the OPT group (11 vs. 17, Figure 5B and Supplementary Data 1). Furthermore, with regard to the Firmicutes phylum, while species from the *Lactobacillus*, *Bacillus*, and *Anaerobutyricum* showed a significant increase (FDR-corrected p-value $< 10\%$) in the cecum microbiota of chickens in OPT_PS compared to the control, several microbial pathogenic species such as *Enterococcus cecorum*, *Enterococcus hirae*, and *Enterococcus gallinarum* were significantly decreased (FDR-corrected p-value $< 10\%$) (Supplementary Data 1). At day 37, 2 weeks after the withdrawal of antibiotics, the highest number of significantly decreased Proteobacteria was observed in the broiler chickens in OPT_PS (Figure 3).

Antibiotic resistome profiling of the cecal metagenome showed that, regardless of antibiotic medication, all samples harbored a diverse range of ARGs (Figure 6A). Genes encoding MLS (mean = 29.64 ± 0.045 across all samples) and tetracycline (Mean = 27.70 ± 0,051 across all samples) resistance were the most predominant classes of ARGs to which the reads were aligned, followed by oxazolidinone and aminoglycoside resistance genes (Figure 6A). Correlation analysis showed that among the different dominant groups of the microbial phyla, Proteobacteria had the highest positive correlation (Spearman correlation, $R = 0.7$, $P = 3.1e^{-13}$, Figure 6B) with the diversity of ARGs in the cecal metagenome of the broiler chickens (Figure 7B), with Actinobacteria showing a weak positive correlation (Spearman correlation $R = 0.29$, $P = 0.0085$) and Firmicutes showing a strong negative correlation (Spearman correlation $R = -0.6$, $P = 1.2e^{-10}$).

In line with the observation that several members of Proteobacteria significantly increased in the cecal metagenome of broiler chickens receiving enrofloxacin, DA analysis revealed a significant increase in the abundance of ARGs encoding resistance to antibiotics belonging to different classes of antibiotics (mainly MLS, aminoglycosides, and tetracyclines) in these two groups from days 20 to 24, with the optimized dosage application resulting in a twofold higher number of DA ARGs compared to high dose application (32 DA ARGs vs. 17 DA ARGs, respectively; Figures 7, 8 and Supplementary Data 1). Examples of the increased ARGs in the samples from the HIGH and OPT treatments taken at day 24 include

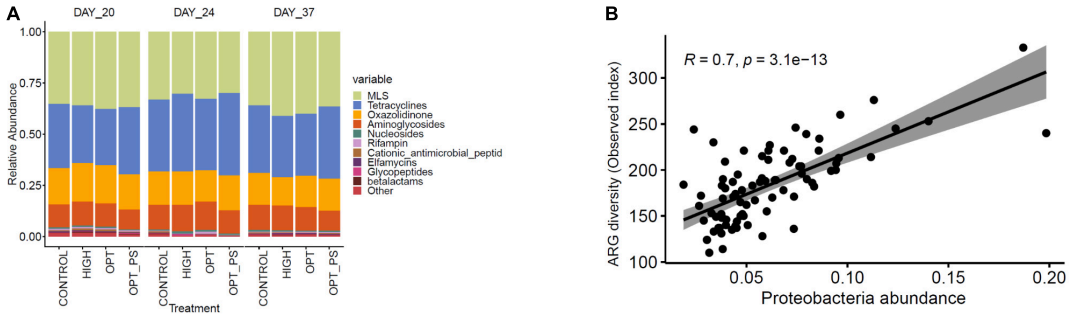


FIGURE 6
(A) Relative abundances of the top 10 classes of ARGs in the cecum digesta of medicated and non-medicated broiler chickens at different time points. **(B)** Scatterplots showing a strong Spearman's rho correlation coefficient (R) between the ARG resistome load and the abundance of Proteobacteria in broiler chicken cecum metagenome samples.

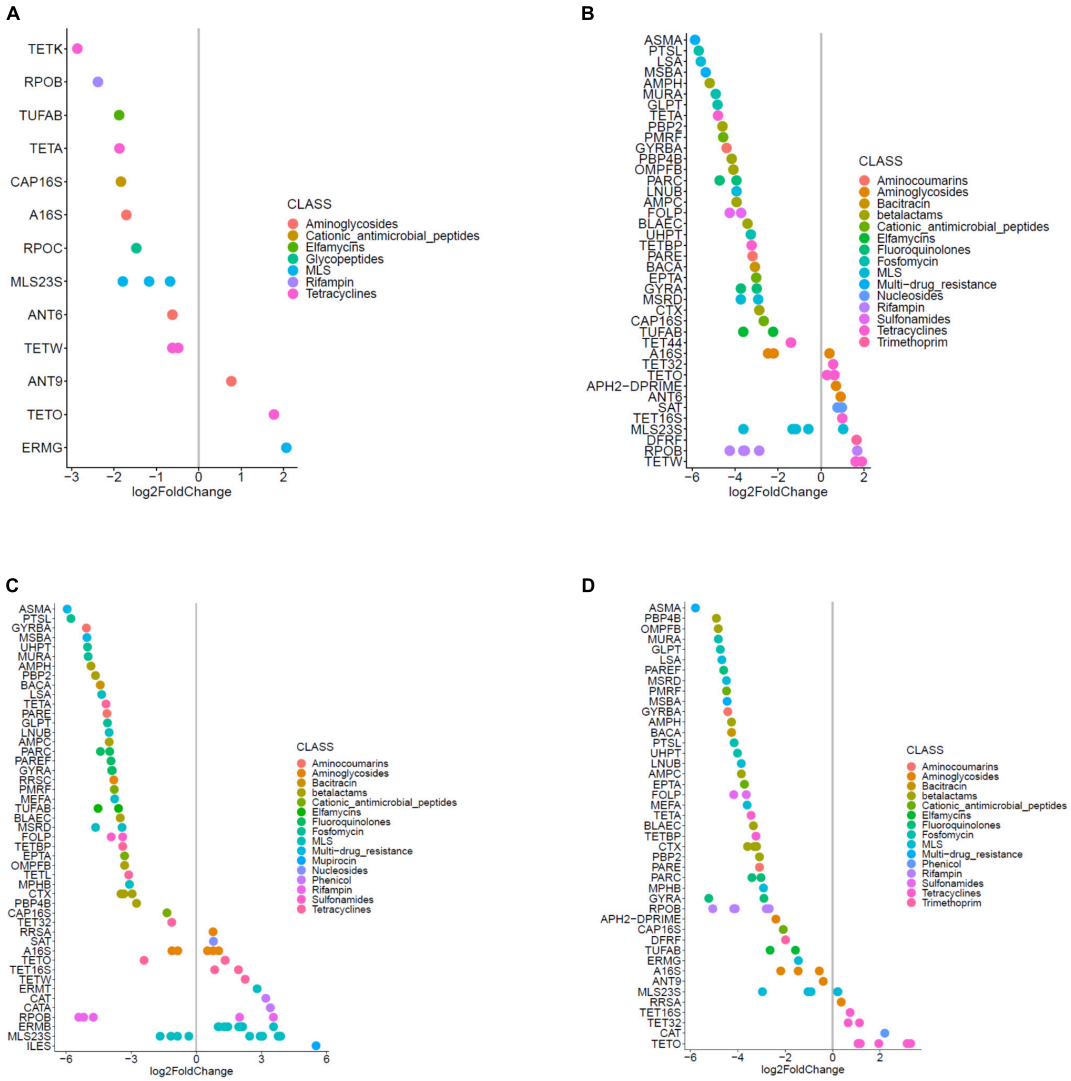


FIGURE 7
Significant ($q < 0.10$) log-fold changes in the abundances of ARG hits in **(A)** samples from OPT_PS taken at day 20, **(B)** samples from High group taken at day 24, **(C)** samples from OPT group taken at day 24, and **(D)** samples from OPT_PS group taken at day 24.

cat and *catA* (encoding chloramphenicol acetyltransferases), *ermB* (encoding 23S rRNA methyl-transferases), and MLS23S (macrolide-resistant 23S rRNA mutation) (**Figures 7B,C**). The most strongly diminished ARGs were the *asmA* (multi-drug RND efflux regulator) and *ptsL* (fosfomycin target mutation) genes, with an approximately −6 log2 fold change (**Figures 7B,C**). Other decreased genes included those encoding *gyrA*, *pbp2* (Penicillin binding protein2), and *CTX* (class A beta-lactamases), as well as *tetA*, *tetBP*, and *tet32* (**Figures 7B,C**). Withdrawal of antibiotic administration seems to result in the recovery of ARG resistome to the same level as the control group, as there were almost no DA ARGs in the cecum metagenomes of broiler chickens between the high/optimized dosage and those in the control group (**Figure 8**). For the samples taken from the OPT_PS group at day 20, where only synbiotic products were applied, the abundance of 13 ARGs was significantly lower than that in the control group, with three ARGs showing a significantly higher abundance in the chicken cecum metagenomes (**Figures 7A**, **8** and **Supplementary Data 1**). At day 24, for OPT_PS, an alleviating effect of synbiotic application on the ARG burden was observed (**Figures 7D**, **8** and **Supplementary Data 1**). Fifty-three ARGs (vs. 32 ARGs in OPT) were significantly lower in abundance in this group than in the control group, and only 13 genes (vs. 32 ARGs in OPT), mainly belonging to tetracycline resistance, showed significantly higher abundance compared to the control group (**Figure 7D** and **Supplementary Data 1**). No DA ARGs were found in the animals in this group at day 37 compared to the control group.

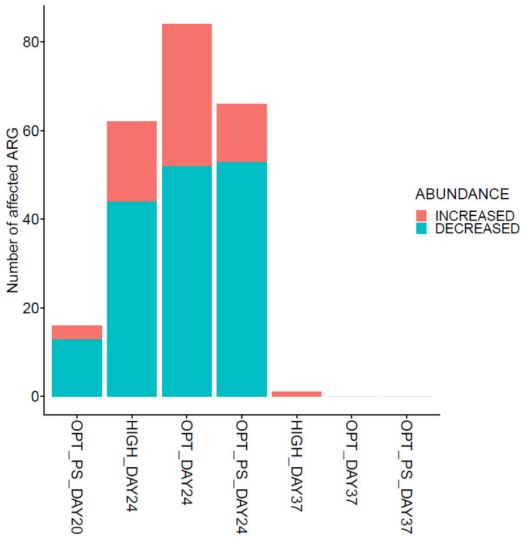


FIGURE 8
Impact of synbiotic and/or enrofloxacin application on differential abundances of ARGs. The bar plot shows the number of significantly affected ARGs (either increased or decreased) based on differential abundance analysis in broiler chicken cecum metagenomes.

Discussion

In this study, we characterized the impact of the critically important fluoroquinolone enrofloxacin following a lower, optimized (12.5 mg/kg bw/day) or high dosage (50 mg/kg bw/day) and its withdrawal on the broiler chicken cecal microbiota and associated resistome by applying shotgun metagenomics. In addition, as a unique aspect of our study, the effect of synbiotic administration in conjunction with the application of enrofloxacin on the cecal microbiota and its ARG reservoir was characterized.

Regarding the gallinaceous cecal microbiota, our study found Firmicutes abundance levels of approximately 80–90% on the three sample days (prior and post enrofloxacin treatment). This is in agreement with the previous observation that Firmicutes are the dominant group of microbial taxa in cecal microbiota over the 6-week lifespan of broilers (Oakley et al., 2014; Oakley and Kogut, 2016; Shang et al., 2018; Wegl et al., 2021). While Firmicutes bacteria are important members of both mammals and birds, there is inter-species variation. For example, the relative abundance of the Firmicutes phylum in the human cecum was comparable to that in broilers (Sun et al., 2019). However, in piglets, the dominance of Firmicutes was much less clear and was rivaled by the phylum Bacteriodetes (Ghanbari et al., 2019; Tan et al., 2019). Because of the similar basic composition of the cecal microbiota when compared with humans (Rychlik, 2020), the chicken cecum and residential microbiota can potentially function as a model for investigating microbiome and associated resistome dynamics in humans following different interventions, when validated with functional microbiome comparisons.

We report a significant dose-dependent impact of enrofloxacin application, with a high dose of the antibiotic causing a much higher perturbation of the diversity, structure, and composition of the cecal microbiota. This can be explained by the stronger bactericidal action of high-dose enrofloxacin compared to the lower dose. In line with this, it has been previously reported that enrofloxacin applied at a dose of 100 mg/kg bw completely eradicated *Salmonella* Typhimurium from chicken gut digesta immediately after administration (Li et al., 2017b) and was shown to have a significant effect on the chicken fecal and cecal microbiota structure evaluated by 16S amplicon sequencing in two independent studies (Li et al., 2017a; Ma et al., 2020).

Similar to previous observations in broiler chickens (Morales-Barrera et al., 2016; Li et al., 2017a; Ma et al., 2020), the Proteobacteria phylum was the most affected taxa by enrofloxacin application in the current study. The observed increase in the abundance of several Proteobacteria members in the medicated animals in the present study might be due to the potential of these taxa to carry ARGs. This is in accordance with

previous reports that ARGs are more enriched in Proteobacteria and this phylum expands following antimicrobial treatment, thereby increasing the ARG burden (Young and Schmidt, 2004; Antonopoulos et al., 2009; Hu et al., 2016). Indeed, we observed a significant short-term expansion in the diversity and structure of ARG reservoirs in the cecum of the enrofloxacin-treated chickens, which was mitigated by the synbiotic supplementation in the group of chickens receiving the optimized dosage of enrofloxacin concomitantly with the synbiotic product. We believe that this beneficial effect can in fact be explained through ecological effects of the synbiotic supplement on the microbiome, such as its pronounced effect against strains that likely carry the expanded ARGs in the cecal microbiota. This is supported by the observation that, by day 20 of sampling, where all animals but those in the synbiotic group (OPT_PS) received no synbiotic supplement, a significant inhibition of several important pathobionts belonging to Proteobacteria, such as *E. coli* and *Campylobacter* spp., was observed in the cecal microbiota of the OPT_PS group. Furthermore, some other important pathobionts that may carry genes encoding acquired resistance to β-lactams and aminoglycoside antibiotics (Li et al., 2017a) and have potential negative impacts on chicken gut integrity, such as *Enterococcus cecorum*, *Enterococcus hirae*, and *Enterococcus gallinarum*, showed a lower abundance in the cecal microbiota of the OPT_PS group when they were receiving the optimized dosage of enrofloxacin (i.e., days 21–23) on the top of the synbiotic product. Such antagonistic effects have been previously observed and reported for the synbiotic product used in the current study (Ghareeb et al., 2012; Luoma et al., 2017; Roth et al., 2019a; Shanmugasundaram et al., 2019; Deng et al., 2020), which can explain the significantly higher bw gain of the OPT_PS group compared to the control. Interestingly, animals subjected to a HIGH dose of enrofloxacin (50 mg/kg bw/day) also achieved higher weights compared to the OPT dosage and control groups. Since fluoroquinolones are concentration-dependent antimicrobial agents (Levison and Levison, 2009), a possible explanation could be a stronger bactericidal action following the higher dose and subsequent larger shift in the microbiota, leading to a more pronounced growth promotion effect (Feighner and Dashkevicz, 1987; Butaye et al., 2003).

These findings raise the possibility that synbiotic-associated resistome expansion mitigation constitutes a unique observation stemming from our experimental design or the supplemented synbiotic product we utilized. Next, we aimed to determine whether other synbiotic studies reflected our findings. To our knowledge, there are no additional publicly available datasets displaying shotgun metagenomic data from the gastrointestinal microbiome of concomitantly synbiotic- and antibiotic-supplemented in either human or livestock species. However, in a recent study, probiotic colonization was associated with a reduced ARG load in endoscopic samples from 10 healthy individuals (Montassier et al., 2021). In contrast, the probiotic mix containing 11 strains from the *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Lactococcus* genera exacerbated the antibiotic-mediated resistome expansion in the lower gastrointestinal tract mucosa collected from 21 individuals. This observation may be due to the delay on microbiome recovery from antibiotics caused by probiotics, which consequently allowed for the expansion of species that likely carry the ARGs (Montassier et al., 2021). Additional studies are required to determine the effects of concomitant antibiotic and synbiotic application on the gut resistome.

Biodiversity analysis of the resistome results revealed the presence of a diverse range of resistance genes in the cecal microbiome of broiler chickens, even in the absence of antimicrobial pressure. However, there were substantial qualitative and quantitative differences between the cecal resistome investigated in this study and the results obtained from studies in pigs (Ghanbari et al., 2019), commercial feedlot cattle (Doster et al., 2018), poultry in other parts of the world (Guo et al., 2018), human feces (Pal et al., 2016), and environmental samples (Pal et al., 2016). This finding corroborates the assertion that ARGs are not randomly distributed in diverse environments but are the result of different selective pressures (Xiong et al., 2018; Ghanbari et al., 2019). The extent to which different enrofloxacin dosages affect resistance development in broiler chickens needs to be determined. We observed a detectable increase in the diversity and abundance of resistance genes, mainly macrolide resistance genes encoding rRNA methylases, followed by tetracycline resistance determinants in the cecum resistome of the chickens receiving the optimized dosage of enrofloxacin (i.e., 10 mg/kg bw/day), which was even higher than the observed increase (mainly tetracycline resistance determinants) in high-dose treated animals, although the gut resistome mainly recovered 2 weeks after cessation of treatment. Such collateral effects of antibiotic application (i.e., enrichment of ARGs not conferring resistance to the administered agent) have been observed before for fluoroquinolone (Sanders et al., 1984; Tolun et al., 2004; Macesic et al., 2014), oxytetracycline (Ghanbari et al., 2019), chlortetracycline, sulfamethazine, and penicillin (Looft et al., 2012) antibiotics, highlighting the need to carefully examine the patterns of multiple drug resistance that may occur simultaneously. While several enriched ARGs in the enrofloxacin-treated groups are based on resistance mutations of a chromosomal gene [e.g., A16S (Prammananan et al., 1998), MLS 23S rRNA (Sugiyama et al., 2017), *tet16S* (Ross et al., 1998)], the majority of enriched genes [*ermB* (Leclercq, 2002), *cat* (Shaw, 1983), *catA* (Shaw, 1983), *ileS* (Brown et al., 2003), *sat* (Shaw et al., 1993), *tetW/O/32* (Connell et al., 2003), *ant6* (Shaw et al., 1993), *aph2-prime* (Shaw et al., 1993), and *dfrF* (Gleckman et al., 1981)] are associated with mobile genetic elements, potentially explaining the observed collateral effects (Looft et al., 2012; Ghanbari et al., 2019). A high dose of enrofloxacin is likely

to provide a sufficient concentration of the active drug in the intestinal contents. This potentially results in stronger bactericidal activity, effectively killing many (susceptible) commensals that likely harbor a variety of intrinsic and acquired ARGs. Consistent with our findings, three rounds of 7-day treatment alternated with 7-day withdrawal of enrofloxacin at a high dose (100 mg/kg bw) was effective in treating *Salmonella* Typhimurium infection while selecting for less resistance in both *Salmonella* Typhimurium and coliforms in chickens, compared to either PK/PD optimized (4 mg/kg bw) or 0.1 mg/kg/bw (Li et al., 2017b). Further trials and experiments are required to confirm these observations by looking beyond metagenomics-based resistome profiling i.e., at effects on ARG expression and by showing that enriched ARGs can be horizontally transferred to other commensals or pathogens and confer phenotypic resistance. In fact, a recently reported study showed that only around 60% of ARGs identified in broiler gut microbiota were expressed (Wang et al., 2020) when an integrated approach shotgun metagenomics and metatranscriptomics was used.

Conclusion

In this study, we investigated the impact of synbiotics and clinically important fluoroquinolones on the cecal reservoir of ARGs in broiler chickens by applying a shotgun metagenomic sequencing approach. Our results provide novel insights into the dose-dependent effects of enrofloxacin application on shaping the broiler gut resistome, which was mitigated by a synbiotic application. The contribution to ameliorating the adverse effects of antibiotics, that is, lowering the spread of antimicrobial resistance genes, in poultry and other livestock gastrointestinal tracts merits further studies.

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/, PRJNA761967.

Ethics statement

The animal study was reviewed and approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University.

Author contributions

RT, MG, GA, LD, and MD designed the study. MG, GA, GS, FH, AG, and MD supervised the experiments. RT, MG, and GA performed the experiments. RT and MG analyzed the results and wrote the manuscript. All authors read, revised, and approved the final manuscript.

Funding

This research was supported by the Special Research Fund of Ghent University (grant no. BOF17/STA/014).

Acknowledgments

We thank Nikolaus Grabner, Andreas Koestelbauer, and Gertrude Wegl for their outstanding technical and analytical assistance.

Conflict of interest

MG and GS are employed by DSM Animal Nutrition & Health. DSM Animal Nutrition & Health is involved in natural feed additive development and research on natural alternatives to in-feed medication in livestock production.

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.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.869538/full#supplementary-material

References

Abd El-Hack, M. E., El-Saadony, M. T., Shafi, M. E., Qattan, S. Y. A., Batiha, G. E., Khafaga, A. F., et al. (2020). Probiotics in poultry feed: a comprehensive review. *J. Anim. Physiol. Anim. Nutr.* 104, 1835–1850. doi: 10.1111/jpn.13454

Anomaly, J. (2009). Harm to others: the social cost of antibiotics in agriculture. *J. Agric. Environ. Ethics* 22, 423–435. doi: 10.1007/s10806-009-9160-y

Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L., and Young, V. B. (2009). Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infect. Immun.* 77, 2367–2375. doi: 10.1128/IAI.01520-08

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/BIOINFORMATICS/BTU170

Brown, J. R., Gentry, D., Becker, J. A., Ingraham, K., Holmes, D. J., and Stanhope, M. J. (2003). Horizontal transfer of drug-resistant aminoacyl-transfer-RNA synthetases of anthrax and gram-positive pathogens. *EMBO Rep.* 4, 692–698. doi: 10.1038/sj.embor.embor881

Butaye, P., Devriese, L. A., and Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: Effects of less well known antibiotics on gram-positive bacteria. *Clin. Microbiol. Rev.* 16, 175–188. doi: 10.1128/CMR.16.2.175-188.2003

Chantziaras, I., Boyen, F., Callens, B., and Dewulf, J. (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J. Antimicrob. Chemother.* 69, 827–834. doi: 10.1093/jac/dkt443

Connell, S. R., Tracz, D. M., Nierhaus, K. H., and Taylor, D. E. (2003). Ribosomal Protection Proteins and Their Mechanism of Tetracycline Resistance. *Antimicrob. Agents Chemother.* 47, 3675–3681. doi: 10.1128/AAC.47.12.3675-3681.2003

Deng, W., Dittoe, D. K., Pavilidis, H. O., Chaney, W. E., Yang, Y., and Ricke, S. C. (2020). Current Perspectives and Potential of Probiotics to Limit Foodborne Campylobacter in Poultry. *Front. Microbiol.* 0:2989. doi: 10.3389/FMICB.2020.583429

Dheilly, A., Le Devendec, L., Mourand, G., Bouder, A., Jouy, E., and Kempf, I. (2012). Resistance gene transfer during treatments for experimental avian colibacillosis. *Antimicrob. Agents Chemother.* 56, 189–196. doi: 10.1128/AAC.05617-11

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–930. doi: 10.1111/J.1654-1103.2003.TB02228.X

Doster, E., Lakin, S. M., Dean, C. J., Wolfe, C., Young, J. G., Boucher, C., et al. (2020). MEGARes 2.0: a database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data. *Nucleic Acids Res.* 48, D561–D569. doi: 10.1093/NAR/GKZ1010

Doster, E., Rovira, P., Noyes, N. R., Burgess, B. A., Yang, X., Weinroth, M. D., et al. (2018). Investigating Effects of Tulathromycin Metaphylaxis on the Fecal Resistome and Microbiome of Commercial Feedlot Cattle Early in the Feeding Period. *Front. Microbiol.* 9:1715. doi: 10.3389/fmicb.2018.01715

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

European Commission. (2010). Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. *Off. J. Eur. Union* L 276, 33–79.

European Medicines Agency (2018). *Questions and answers on use of enrofloxacin-containing veterinary medicines administered via drinking water to chickens and turkeys*. Available online at: www.ema.europa.eu/contact (accessed November 29, 2019)

European Medicines Agency (2021). *Reflection paper on dose review and adjustment of established veterinary antibiotics in the context summary of product characteristic (SPC) harmonisation*. Available online at: https://www.ema.europa.eu/en/dose-review-adjustment-established-veterinary-antibiotics-context-summary-product-characteristic-spc (accessed April 12, 2021)

Feighner, S. D., and Dashkevicz, M. P. (1987). Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. *Appl. Environ. Microbiol.* 53:331. Available online at: /pmc/articles/PMC203661/?report=abstract (accessed July 14, 2021). doi: 10.1128/aem.53.2.331-336.1987

Ferran, A. A., Lacroix, M. Z., Bousquet-Mélou, A., Duhil, I., and Roques, B. B. (2020). Levers to improve antibiotic treatment of lambs via drinking water in sheep fattening houses: the example of the sulfadimethoxine/trimethoprim combination. *Antibiotics* 9:561. doi: 10.3390/antibiotics9090561

Ferran, A. A., and Roques, B. B. (2019). Can oral group medication be improved to reduce antimicrobial use? *Vet. Rec.* 185, 402–404. doi: 10.1136/vr.l5656

Foditsch, C., Pereira, R. V. V., Siler, J. D., Altier, C., and Warnick, L. D. (2019). Effects of treatment with enrofloxacin or tulathromycin on fecal microbiota composition and genetic function of dairy calves. *PLoS One* 14:e0219635. doi: 10.1371/JOURNAL.PONE.0219635

Ghanbari, M., Klose, V., Crispie, F., and Cotter, P. D. (2019). The dynamics of the antibiotic resistome in the feces of freshly weaned pigs following therapeutic administration of oxytetracycline. *Sci. Rep.* 9, 1–11. doi: 10.1038/s41598-019-40496-8

Ghareeb, K., Awad, W. A., Mohnl, M., Porta, R., Biarnés, M., Böhm, J., et al. (2012). Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of Campylobacter jejuni in broiler chickens. *Poult. Sci.* 91, 1825–1832. doi: 10.3382/ps.2012-02168

Gibson, G. R., Probert, H. M., Loo, J., Van, Rastall, R. A., and Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* 17, 259–275. doi: 10.1079/nrr200479

Gleckman, R., Blagg, N., and Joubert, D. W. (1981). Trimethoprim: mechanisms of action, antimicrobial activity, bacterial resistance, pharmacokinetics, adverse reactions, and therapeutic indications. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* 1, 14–19. doi: 10.1002/j.1875-9114.1981.tb03548.x

Gregova, G., and Kmet, V. (2020). Antibiotic resistance and virulence of *Escherichia coli* strains isolated from animal rendering plant. *Sci. Reports* 101, 1–7. doi: 10.1038/s41598-020-72851-5

Guo, X., Stedtfeld, R. D., Hedman, H., Eisenberg, J. N. S., Trueba, G., Yin, D., et al. (2018). Antibiotic resistome associated with small-scale poultry production in rural ecuador. *Environ. Sci. Technol.* 52, 8165–8172. doi: 10.1021/acs.est.8b01667

Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., et al. (2016). The bacterial mobile resistome transfer network connecting the animal and human microbiomes. *Appl. Environ. Microbiol.* 82, 6672–6681. doi: 10.1128/AEM.01802-16

Joosten, P., Sarrazin, S., Van Gompel, L., Luiken, R. E. C., Mevius, D. J., Wagenaar, J. A., et al. (2019). Quantitative and qualitative analysis of antimicrobial usage at farm and flock level on 181 broiler farms in nine European countries. *J. Antimicrob. Chemother.* 74, 798–806. doi: 10.1093/jac/dky498

Korpela, K., Salonen, A., Vepsäläinen, O., Suomalainen, M., Kolmeder, C., Varjosalo, M., et al. (2018). Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. *Microbiome* 6, 1–11. doi: 10.1186/s40168-018-0567-4

Leclercq, R. (2002). Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin. Infect. Dis.* 34, 482–492. doi: 10.1086/324626

Levison, M. E., and Levison, J. H. (2009). Pharmacokinetics and Pharmacodynamics of Antibacterial Agents. *Infect. Dis. Clin. North Am.* 23, 791–815. doi: 10.1016/j.idc.2009.06.008

Li, J., Hao, H., Cheng, G., Liu, C., Ahmed, S., Shabbir, M. A. B., et al. (2017a). Microbial shifts in the intestinal microbiota of *salmonella* infected chickens in response to enrofloxacin. *Front. Microbiol.* 0:1711. doi: 10.3389/FMICB.2017.01711

Li, J., Hao, H., Cheng, G., Wang, X., Ahmed, S., Shabbir, M. A. B., et al. (2017b). The effects of different enrofloxacin dosages on clinical efficacy and resistance development in chickens experimentally infected with *Salmonella* Typhimurium. *Sci. Reports* 71, 1–12. doi: 10.1038/s41598-017-12294-7

Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci.* 109, 1691–1696. doi: 10.1073/PNAS.1120238109

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 1512, 1–21. doi: 10.1186/S13059-014-0550-8

Lu, J., Breitwieser, F. P., Thielen, P., and Salzberg, S. L. (2017). Bracken: estimating species abundance in metagenomics data. *PeerJ Comput. Sci.* 3:e104. doi: 10.7717/PEERJ-CS.104

Luiken, R. E. C., Van Gompel, L., Munk, P., Sarrazin, S., Joosten, P., Dorado-García, A., et al. (2019). Associations between antimicrobial use and the faecal resistome on broiler farms from nine European countries. *J. Antimicrob. Chemother.* 74, 2596–2604. doi: 10.1093/jac/dkz235

Luoma, A., Markazi, A., Shanmugasundaram, R., Murugesan, G. R., Mohnl, M., and Selvaraj, R. (2017). Effect of synbiotic supplementation on layer production

and cecal *Salmonella* load during a *Salmonella* challenge. *Poult. Sci.* 96, 4208–4216. doi: 10.3382/ps/pex251

Ma, B., Mei, X., Lei, C., Li, C., Gao, Y., Kong, L., et al. (2020). Enrofloxacin shifts intestinal microbiota and metabolic profiling and hinders recovery from *salmonella enterica* subsp. enterica serovar typhimurium infection in neonatal chickens. *mSphere* 5:20. doi: 10.1128/msphere.00725-20

Macesic, N., Morrissey, C. O., Cheng, A. C., Spencer, A., and Peleg, A. Y. (2014). Changing microbial epidemiology in hematopoietic stem cell transplant recipients: increasing resistance over a 9-year period. *Transpl. Infect. Dis.* 16, 887–896. doi: 10.1111/tid.12298

Markowiak, P., and Śliżewska, K. (2017). Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients* 9:9091021. doi: 10.3390/NU9091021

Montassier, E., Valdés-Mas, R., Batard, E., Zmora, N., Dori-Bachash, M., Suez, J., et al. (2021). Probiotics impact the antibiotic resistance gene reservoir along the human GI tract in a person-specific and antibiotic-dependent manner. *Nat. Microbiol.* 2021, 1–12. doi: 10.1038/s41564-021-00920-0

Morales-Barrera, E., Calhoun, N., Lobato-Tapia, J. L., Lucca, V., Prado-Rebolledo, O., Hernandez-Velasco, X., et al. (2016). Risks involved in the use of enrofloxacin for *Salmonella* enteritidis or *Salmonella* heidelberg in commercial poultry. *Front. Vet. Sci.* 3:72. doi: 10.3389/fvets.2016.00072

Oakley, B. B., and Kogut, M. H. (2016). Spatial and temporal changes in the broiler chicken cecal and fecal microbiomes and correlations of bacterial taxa with cytokine gene expression. *Front. Vet. Sci.* 3:11. doi: 10.3389/fvets.2016.00011

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., et al. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiol. Lett.* 360, 100–112. doi: 10.1111/1574-6968.12608

Pal, C., Bengtsson-Palme, J., Kristiansson, E., and Larsson, D. G. J. (2016). The structure and diversity of human, animal and environmental resistomes. *Microbiome* 41, 1–15. doi: 10.1186/S40168-016-0199-5

Persoons, D., Dewulf, J., Smet, A., Herman, L., Heyndrickx, M., Martel, A., et al. (2012). Antimicrobial use in Belgian broiler production. *Prev. Vet. Med.* 105, 320–325. doi: 10.1016/j.prevetmed.2012.02.020

Prammananan, T., Sander, P., Brown, B. A., Frischkorn, K., Onyi, G. O., Zhang, Y., et al. (1998). A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in Mycobacterium abscessus and Mycobacterium chelonae. *J. Infect. Dis.* 177, 1573–1581. doi: 10.1086/515328

Raboisson, D., Ferchiou, A., Sans, P., Lhermie, G., and Dervillé, M. (2020). The economics of antimicrobial resistance in veterinary medicine: Optimizing societal benefits through mesoeconomic approaches from public and private perspectives. *One Heal.* 10:100145. doi: 10.1016/j.onehlt.2020.100145

Ross, J. I., Eady, E. A., Cove, J. H., and Cunliffe, W. J. (1998). 16S rRNA mutation associated with tetracycline resistance in a gram- positive bacterium. *Antimicrob. Agents Chemother.* 42, 1702–1705. doi: 10.1128/aac.42.7.1702

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., and Domig, K. J. (2019b). The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: a global overview. *Poult. Sci.* 98, 1791–1804. doi: 10.3382/ps/pey539

Roth, N., Hofacre, C., Zitz, U., Mathis, G. F., Moder, K., Doupovec, B., et al. (2019a). Prevalence of antibiotic-resistant E. coli in broilers challenged with a multi-resistant E. coli strain and received ampicillin, an organic acid-based feed additive or a synbiotic preparation. *Poult. Sci.* 98, 2598–2607. doi: 10.3382/ps/pez004

Royal Decree. (2013). Belgian Royal Decree of 29 May 2013 on the protection of animals used for scientific purposes. *Belg. Staatsbl.* 29:2013.

Rychlik, I. (2020). Composition and function of chicken gut microbiota. *Anim.* 103:103. doi: 10.3390/ANI10010103

Sanders, C. C., Sanders, W. E. Jr., Goering, R. V., and Werner, V. (1984). Selection of multiple antibiotic resistance by quinolones, beta-lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes. *Antimicrob. Agents Chemother.* 26:797. doi: 10.1128/AAC.26.6.797

Shang, Y., Kumar, S., Oakley, B., and Kim, W. K. (2018). Chicken gut microbiota: Importance and detection technology. *Front. Vet. Sci.* 5:254. doi: 10.3389/fvets.2018.00254

Shanmugasundaram, R., Mortada, M., Cosby, D. E., Singh, M., Applegate, T. J., Syed, B., et al. (2019). Synbiotic supplementation to decrease *Salmonella* colonization in the intestine and carcass contamination in broiler birds. *PLoS One* 14:e0223577. doi: 10.1371/JOURNAL.PONE.0223577

Shaw, K. J., Rather, P. N., Hare, R. S., and Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* 57, 138–163. doi: 10.1128/mr.57.1.138-163.1993

Shaw, W. V. (1983). Chloramphenicol acetyltransferase: Enzymology and molecular biology. *Crit. Rev. Biochem. Mol. Biol.* 14, 1–46. doi: 10.3109/10409238309102789

Soraci, A. L., Amanto, F., Tapia, M. O., de la Torre, E., and Toutain, P. L. (2014). Exposure variability of fosfomycin administered to pigs in food or water: Impact of social rank. *Res. Vet. Sci.* 96, 153–159. doi: 10.1016/j.rvsc.2013.12.003

Sugiyama, H., Yoshida, I., Ueki, M., Tanabe, K., Manaka, A., and Hiramatsu, K. (2017). In vitro antibacterial activity of α-methoxyimino acylide derivatives against macrolide-resistant pathogens and mutation analysis in 23S rRNA. *J. Antibiot.* 70, 264–271. doi: 10.1038/ja.2016.148

Sun, L., Jia, H., Li, J., Yu, M., Yang, Y., Tian, D., et al. (2019). Cecal gut microbiota and metabolites might contribute to the severity of acute myocardial ischemia by impacting the intestinal permeability, oxidative stress, and energy metabolism. *Front. Microbiol.* 10:1745. doi: 10.3389/fmicb.2019.01745

Tan, Z., Dong, W., Ding, Y., Ding, X., Zhang, Q., and Jiang, L. (2019). Changes in cecal microbiota community of suckling piglets infected with porcine epidemic diarrhea virus. *PLoS One* 14:219868. doi: 10.1371/journal.pone.0219868

Temmerman, R., Pelligand, L., Schelstraete, W., Antonissen, G., Garmyn, A., and Devreese, M. (2021). Enrofloxacin dose optimization for the treatment of colibacillosis in broiler chickens using a drinking behaviour pharmacokinetic model. *Antibiotics* 10:604. doi: 10.3390/antibiotics10050604

Tolun, V., Küçükbasmaci, O., Torümküney-Akbulut, D., Catal, C., Ang-Kucuker, M., and Ang, O. (2004). Relationship between ciprofloxacin resistance and extended-spectrum β-lactamase production in *Escherichia coli* and *Klebsiella pneumoniae* strains. *Clin. Microbiol. Infect.* 10, 72–75. doi: 10.1111/J.1469-0691.2004.00723.X

Turcotte, C., Thibodeau, A., Quessy, S., Topp, E., Beauchamp, G., Fravalo, P., et al. (2020). Impacts of short-term antibiotic withdrawal and long-term judicious antibiotic use on resistance gene abundance and cecal microbiota composition on commercial broiler chicken farms in québec. *Front. Vet. Sci.* 0:1067. doi: 10.3389/FVETS.2020.547181

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2015). Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. U. S. A.* 112, 5649–5654. doi: 10.1073/pnas.1503141112

Wang, Y., Hu, Y., Liu, F., Cao, J., Lv, N., Zhu, B., et al. (2020). Integrated metagenomic and metatranscriptomic profiling reveals differentially expressed resistomes in human, chicken, and pig gut microbiomes. *Environ. Int.* 138:105649. doi: 10.1016/j.envint.2020.105649

Wegl, G., Grabner, N., Köstelbauer, A., Klose, V., and Ghanbari, M. (2021). Toward best practice in livestock microbiota research: a comprehensive comparison of sample storage and dna extraction strategies. *Front. Microbiol.* 12:322. doi: 10.3389/FMICB.2021.627539

WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) (2019). *Critically Important Antimicrobials for Human Medicine 6th Re.vision 2018. Ranking of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use*. Available online at: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1 (accessed December 19, 2019).

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Gen. Biol.* 201, 1–13. doi: 10.1186/S13059-019-1891-0

World Health Organisation (WHO) (2014). *Antimicrobial resistance: global report on surveillance 2014*. Geneva: World Health Organisation.

World Health Organization (WHO) (2015). *Global Action Plan on Antimicrobial Resistance*. Geneva: World Health Organisation.

Xiong, W., Wang, Y., Sun, Y., Ma, L., Zeng, Q., Jiang, X., et al. (2018). Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. *Microbiome* 6:34. doi: 10.1186/s40168-018-0419-2

Young, V. B., and Schmidt, T. M. (2004). Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the fecal microbiota. *J. Clin. Microbiol.* 42, 1203–1206. doi: 10.1128/JCM.42.3.1203-1206.2004

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 06 September 2022
DOI 10.3389/fmicb.2022.898248

Check for updates

OPEN ACCESS

EDITED BY
Ziad Daoud,
Central Michigan University,
United States

REVIEWED BY
Ruichao Li,
Yangzhou University, China
Vijayalakshmi Selvakumar,
Kangwon National University,
South Korea

*CORRESPONDENCE
Xueshan Xia
oliverxia2000@aliyun.com
Zulqarnain Baloch
znbalooch@yahoo.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

RECEIVED 17 March 2022
ACCEPTED 05 July 2022
PUBLISHED 06 September 2022

CITATION
Aslam B, Chaudhry TH, Arshad MI, Muzammil S, Siddique AB, Yasmeen N, Khurshid M, Amir A, Salman M, Rasool MH, Xia X and Baloch Z (2022) Distribution and genetic diversity of multi-drug-resistant *Klebsiella pneumoniae* at the human–animal–environment interface in Pakistan. *Front. Microbiol.* 13:898248. doi: 10.3389/fmicb.2022.898248

COPYRIGHT
© 2022 Aslam, Chaudhry, Arshad, Muzammil, Siddique, Yasmeen, Khurshid, Amir, Salman, Rasool, Xia and Baloch. 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.

Distribution and genetic diversity of multi-drug-resistant *Klebsiella pneumoniae* at the human–animal–environment interface in Pakistan

Bilal Aslam[1†], Tamoor Hamid Chaudhry[1,2†], Muhammad Imran Arshad[3], Saima Muzammil[1], Abu Baker Siddique[1], Nafeesa Yasmeen[4], Mohsin Khurshid[1], Afreenish Amir[2], Muhammad Salman[2], Muhammad Hidayat Rasool[1], Xueshan Xia[5*] and Zulqarnain Baloch[5*]

[1]Department of Microbiology, Government College University Faisalabad, Faisalabad, Pakistan, [2]National Institute of Health, Islamabad, Pakistan, [3]Institute of Microbiology, University of Agriculture Faisalabad, Faisalabad, Pakistan, [4]National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou, China, [5]Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, China

Klebsiella pneumoniae is ubiquitous and known to be a notorious pathogen of humans, animals, and plant-based foods. *K. pneumoniae* is a recognized trafficker of antibiotic resistance genes (ARGs) between and from different ecological niches. A total of 775 samples (n = 775) were collected from September 2017 to August 2019 from humans, animals, and environmental sources by applying the random convenient sampling technique. A total of 120 (15.7%) samples were confirmed as *K. pneumoniae*. The distribution of *K. pneumoniae* among humans, the environment, and animals was 17.1, 12.38, and 10%, respectively. Isolates have shown significant resistance against all the subjected antibiotics agents except colistin. ARGs profiling revealed that the highest percentage prevalence (67.5%) of bla_{CTX-M} was estimated in the isolates, and various carbapenem resistance genes that were found in the study were bla_{NDM-1} (43.3%), bla_{OXA-48} (38%), and (1.67%) bla_{KPC-2}. Overall, 21 distinct sequence types (ST) and 13 clonal complexes (CCs) were found through the multi-locus sequence typing (MLST) analysis. Taking together, the distribution of multi-drug resistance (MDR) *K. pneumoniae* clones in the community and associated environment is alarming for the

health care system of the country. Health policymakers should consider the role of all the integral parts of humans, animals, and the associated environment intently to cope with this serious public and animal health concern.

KEYWORDS

transmission, antimicrobial resistance, human–animal–environment, MLST analysis, Pakistan

Introduction

Antimicrobial resistance (AMR) is a mounting health concern all over the world. The global expansion of the AMR is due to the excessive and irrational use of antibiotics in clinical settings and food-producing animals. Additionally, deprived sanitation facilities, overpopulation, and inadequate sewerage disposal systems are also vital contributing factors to the dissemination of AMR (Aslam et al., 2018). Globally AMR poses huge economic losses; only in the USA losses of $35 billion have been estimated (Cecchini et al., 2015; Ventola, 2015). By the year 2050, 444 million people of the world population would be affected by resistant infections and birth rates would be reduced significantly (Bartlett et al., 2013). In the absence of new antibiotics, AR poses a severe threat to human and animal health in association with their wider environment.

The interplay between various ecologies is critically significant regarding the AMR. There is a number of associated factors among humans, animals, and the wider environment which permit not only the dissemination of various pathogens but also facilitate the transmission of mobile genetic elements carrying the antibiotic resistance genes (ARGs). To develop pragmatic and useful predictions regarding AMR, we need to have adequate and comprehensive data about the dynamics of different antibiotics, multidrug-resistant (MDR) pathogens, and the presence of various resistance determinants in different hosts and the wide-ranging environment (Woolhouse et al., 2015).

The ecological range of *Klebsiella pneumoniae* in various hosts and environments has been well documented (Wyres and Holt, 2016). Since the 1970s, the ubiquitous distribution of *K. pneumoniae* has been reported, and it is known to be a contaminant of animal- and plant-based foods (Davis and Price, 2016). *K. pneumoniae* is a pathogenic Gram-negative bacterium, a member of the Enterobacterales. World Health Organization (WHO) declared the carbapenem-resistant Enterobacterales (CRE) as a health threat of critical importance (Ventola, 2015).

Population genomics of *K. pneumoniae* revealed that it is characterized by mass scale diversified and deep-branching clonal groups. There are approximately 5,500 genes present in *K. pneumoniae* genomes, 2,000 genes shared amongst different strains, and approximately, 3,500 genes that have a major part of the AMR genes, which are collected from a wide-ranging pool ($\geq$ 30,000) of protein-coding sequences. Only a few clonal groups from universally distributed *K. pneumoniae* proved dexterous to acquire ARGs (Wyres and Holt, 2016).

In Pakistan, available data are not significant, and routine surveillance at the national level has been overlooked. Lately, we reported the emergence of MDR *K. pneumoniae* in the hospital and veterinary settings (Chaudhry et al., 2019; Aslam et al., 2020; Chaudhry et al., 2020). Herein, the present study was designed to find out the distribution and genetic diversity of *K. pneumoniae* in humans, animals, and their associated environments.

Materials and methods

Sample collection

Using a random convenient sampling technique, a total of (n = 775) samples were collected by following standard aseptic conditions and microbiological procedures. In detail, clinical samples (n = 525) including urine samples (n = 122), tracheal aspirates (n = 89), wounds and pus (n = 158), and blood (n = 156) were collected from different hospitals. The environmental samples (n = 210), including wastewater (n = 39), were taken from hospital effluents and nearby communities, and other samples were collected from operation theater waste (n = 31), hospital sludge (n = 23), and ward waste (n = 19), and abattoir/farm wastewater (n = 65) and veterinary sludge/waste (n = 33) were collected from healthy and infected animals at public and private dairy farms and slaughterhouses. Veterinary source samples (n = 40) include milk (n = 18) and fecal samples (n = 22) from the animals.

Isolation and identification of *Klebsiella pneumoniae*

In brief, some of the samples including fecal swabs, abattoir samples, and ward and operation theater waste samples were dispensed into sterilized glass tubes containing 1 mL PBS followed by streaking on nutrient agar plates; on the other hand, the remaining samples including wastewater, clinical specimens, milk, and sludge samples were streaked directly on nutrient agar plates. Plates were incubated for 24 h at 37°C. Afterward, selective media named HiChrom Klebsiella Selective agar (Himedia®) was used for the isolation of *K. pneumoniae*. Biochemical characterization of the isolates was done by API 20E Kit and VITEK identification system (bioMérieux, France). For molecular identification of the isolates, 16S rDNA PCR was performed. A total of 35 PCR cycles were performed for the amplification after initial denaturation at 95°C for 3 min. The cycle specifications include: denaturation at 95°C for 35 s, annealing at 50°C for 30 s, extension at 72°C for 60 s, and a final extension was done at 72°C for 5 min. Subsequently, to visualize the amplified PCR product agarose (Thermo Fisher Scientific, United States) gel electrophoresis was performed and gel documentation system (Bio-Rad, United States) was used to read the PCR products.

Antibiotic susceptibility testing

For antibiotic resistance (AR) profiling of the isolates, different antibiotics with CLSI recommended concentrations were used (CLSI, 2018). Antibiotics used in the study were ampicillin 10 μg and piperacillin 100 μg, cefuroxime 30 μg, cefixime 5 μg, ceftriaxone 30 μg, and cefepime 30 μg, meropenem 10 μg, ciprofloxacin 5 μg, tetracycline 30 μg, and minocycline 30 μg, trimethoprim-sulfamethoxazole 1.25/23.75 μg, colistin 10 μg, and tigecycline 15 μg. *Escherichia coli* (ATCC-25922) was used as a quality control strain.

In addition to the disc diffusion method, the minimum inhibitory concentration (MIC) of all the antibiotics in the study was determined through broth microdilution assay as described previously (Chaudhry et al., 2020). Precisely, freshly grown *K. pneumoniae* was used to make 0.5 McFarland standards. Different ranges (0.06–256 μg/mL) of antibiotics dilutions were made. Micro-titration plate wells having growth were used to take inoculum, which was streaked on Petri plates containing nutrient agar and incubated for 24 h at 37°C. Results were interpreted according to the CLSI, 2018 recommendations.

Detection of antibiotic resistance genes

DNA extraction was done by using a DNA Extraction kit (Qiagen™, Hilden, Germany). DNA quantification was performed through NanoDrop 2000 (Thermo Scientific™, United States), and DNA ≥ 60 ng/μl concentration was used for the further experiment. Extracted DNA from freshly grown isolates was used for genetic screening and the detection of β-lactamase genes, including ESBL genes (bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, $bla_{CTX-M-14}$, and PAN_{CTX-M}), carbapenemases genes (bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{NDM-1}, and bla_{OXA-48}), fluoroquinolones (*qnr*A, *qnr*B, *qnr*S, *par*C, *gyr*A, and *gyr*B), tetracycline (*tet*A and *tet*B), and sulfonamides (*sul*1 and *sul*2) genes. The amplicons were sent for Sanger sequencing to Macrogen (Seoul, South Korea), and the obtained sequences were verified using the NCBI BLAST tool.

Multi-locus sequence typing

The MLST was carried out by following the Pasteur MLST scheme by the amplification of seven housekeeping genes of *K. pneumoniae*. The PCR was carried out by making up a 50 μl reaction mixture that comprises 25 μl of 2X PCR Master Mix (Thermo-Scientific, United States), and each primer (10 μM) was 1 and 2 μl sample DNA. The following PCR cycle conditions were set: initial denaturation at 94°C for 2 min and then 35 cycles of initial denaturation at 94°C for 20 s. The annealing temperature for all the genes were set at 50°C, except for *tonB* (45°C) and *gapA* (60°C), for 30 s and extension at 72°C for 30 s, and at the end, a final extension at 72°C for 5 min was performed in PCR Thermal Cycler (Bio-Rad Inc., United States) (Diancourt et al., 2005). The amplified product of respective genes was analyzed on ethidium bromide-stained 1% agarose gel, the band size was assessed by GeneRuler 1 kb plus DNA ladder (Thermo-Scientific™, United States), and the snapshots were taken by gel documentation system (ChemiDoc™ XRS + System, Bio-Rad, United States). The genes were sequenced with the same forward and reverse sequencing primers from Macrogen™. For the determination of sequence types (ST), the MLST database for *K. pneumoniae* was referred according to the pasture scheme.[1]

Conjugation assay

To assess the transferability of *K. pneumoniae* isolates, conjugation assay was performed. Isolates of the study were kept as a donor while *E. coli* cells (Thermo Fisher®, United States) were used as the recipient bacterial cells. Confirmation of the transconjugants was done by streaking on Luria-Bertani (LB) agar supplemented with meropenem (MEM), imipenem (IMP), and ertapenem (ETP). Colony PCR of the transconjugants was also performed by using specific primers of bla_{NDM-1} bla_{KPC} and bla_{OXA-48}. MICs of the transconjugants were determined according to CLSI (2018) guidelines.

1 https://bigsdb.pasteur.fr/klebsiella/

Results

Distribution of *Klebsiella pneumoniae* among different sample sources

Overall, out of (n = 775) the collected samples, a total of 120 isolates were confirmed as *K. pneumoniae.* From n = 525 clinical samples, a total of 90 isolates were *K. pneumoniae,* while from (n = 210) environmental samples, a total of 26 isolates were *K. pneumoniae,* and out of veterinary samples (n = 40), a total of 4 isolates were confirmed *K. pneumoniae* (Table 1).

Resistance profiling

All isolates (100%; $P < 0.05$) were found resistant to ampicillin and piperacillin, while 114 (95.0%; $P < 0.05$) isolates were found resistant to cefuroxime. Moreover, 97 (80.8%; $P < 0.05$) isolates were found resistant to the third generation cephalosporins named cefixime and ceftriaxone, while 71 (59.2%; $P < 0.05$) isolates were found resistant to cefepime. The resistance to carbapenems (meropenem) was found in 39 (32.5%) isolates. Moreover, 87 (72.5%) and 45 (37.5%) isolates were found resistant to moxifloxacin and ciprofloxacin, respectively, while 65 (54.2%; $P < 0.05$) and 72 (60.0%; $P < 0.05$) isolates were resistant to minocycline and tetracycline, respectively. Although 65 (54.2%; $P < 0.05$) isolates were resistant to cotrimoxazole and 18 (15.0%) isolates were found resistant to chloramphenicol, only 01 isolate was found resistant to tigecycline. Fortunately, all the isolates were found susceptible to colistin (Table 2).

Detection of antibiotic resistance genes

Molecular AR profiling of all (120) the isolates revealed that bla_{SHV} *was* detected in 92.5% followed by bla_{CTX-M} (67.5%; $P < 0.05$), while other ARGs which were detected in the isolates showed the percentage prevalence as follows: bla_{TEM} (57.5%; $P < 0.05$), $bla_{CTX-M-1}$ (64%; $P < 0.05$), $bla_{CTX-M-2}$, (11.6%), $bla_{CTX-M-9}$ (27.5%), $bla_{CTX-M-14}$ (49%), bla_{NDM-1} (43.3%), and bla_{OXA-48} (38%), and all the isolates were negative for bla_{VIM}, bla_{GIM}, and bla_{IMP}. One of the most significant findings of the study was the detection of the bla_{KPC-2} gene among the 02 isolates i.e., one isolate from a clinical source and one from the veterinary source were found positive for bla_{KPC-2} (Table 3). In addition to the ESBL and carbapenemases genes, various other ARGs which were included in the study showed the following distribution patterns among *K. pneumoniae* isolates: *tet*A (40%), *tet*B (45%), *qnr*S (36.6%), *qnr*B (44%), *sul*1 (39%), and *sul*2 (44%), respectively.

Selected isolates after molecular identification and ARG's detection were subjected to sequencing. Obtained sequences were used for BLAST analysis and submitted for NCBI GenBank accession numbers, which include MK620996.1, MK620997.1, MK620998.1, MK620999.1, MK621000.1, MK621001.1, MK621002.1, MK585512.1, MK585513.1, and MK458727.1.

Distribution of sequence types

The panel of 120 *K. pneumoniae* isolates revealed 21 distinct sequence types (ST), while a total of 13 clonal complexes (CCs) were observed (Figure 1). The distribution of STs in humans, animals, and the environment has been shown in Figure 2A. Various STs found from clinical samples were ST1, ST100, ST111, ST134, ST274, and ST412 (Figure 2BI). ST11 was a single ST found in veterinary (animal) sources (milk and diarrhea, Figure 2BII), whereas different STs which were found in the isolates of environmental sources were also observed in the isolates of clinical sources, these STs were as follows: ST48, ST15, ST231, CC147, ST431, ST580, ST859, ST1137, ST1488, ST1561, ST1709, and ST2167 (Figure 2BIII). While all the STs observed in the isolates of veterinary and veterinary environment sources were also observed in the isolates of clinical sources, these STs were as follows: ST11, ST29, and ST258 (Figure 2A).

TABLE 1 Details of all the collected samples and percentage prevalence of *K. pneumoniae* from various sample sources.

Sample type	Number of isolates(n)	Prevalence percentage (%)
Human		
Urine (n = 122)	13	10.6
Sputum (n = 89)	21	23.6
Wounds and Pus (n = 158)	21	13.3
Blood (n = 156)	35	22.4
Total (n = 525)	**90**	**17.1**
Chi sq-stat = 10.950; alph- chi-sq critical = 7.8147; p-value chi sq test = 0.0120		
Environment		
Ward waste (n = 19)	3	16
Operation theater waste (n = 31)	1	3
Wastewater (n = 39)	9	23
Hospital sludge (n = 23)	4	17
Abattoir/wastewater (n = 65)	7	11
Veterinary sludge/waste (n = 33)	2	6
Total (n = 210)	**26**	**12.38**
Chi sq-stat = 8.615; alph- chi-sq critical = 11.070; p-value chi sq test = 0.125		
Animals		
Milk (n = 18)	1	5
Fecal samples (n = 22)	3	13.6
Total (n = 40)	**4**	**10**
Chi sq-stat = 0.7182; alph- chi-sq critical = 3.8414; p-value chi sq test = 0.3967		

TABLE 2 Antibiotic resistance profile of indigenous *K. pneumoniae* isolates (n = 120) against all the antibiotics used in the study.

Antibiotics	Resistance	Intermediate	Susceptible
Ampicillin	120 (100%)	0 (0.0%)	0 (0.0%)
Piperacillin	120 (100%)	0 (0.0%)	0 (0.0%)
Cefuroxime	114 (95.0%)	0 (0.0%)	6 (5.0%)
Cefixime	97 (80.8%)	2 (1.7%)	21 (17.5%)
Ceftriaxone	97 (80.8%)	2 (1.7%)	21 (17.5%)
Cefepime	71 (59.2%)	0 (0.0%)	49 (40.8%)
Meropenem	39 (32.5%)	4 (3.3%)	77 (64.2%)
Ciprofloxacin	45 (37.5%)	5 (4.2%)	70 (58.3%)
Moxifloxacin	87 (72.5%)	0 (0.0%)	33 (27.5%)
Minocycline	65 (54.2%)	4 (3.3%)	51 (42.5%)
Tetracyclines	72 (60.0%)	3 (2.5%)	45 (37.5%)
Tigecycline	1 (0.8%)	3 (2.5%)	116 (96.7%)
Chloramphenicol	18 (15.0%)	0 (0.0%)	102 (85.0%)
Colistin	0 (0.0%)	0 (0.0%)	120 (100.0%)
Cotrimoxazole	65 (54.2%)	0 (0.0%)	55 (45.8%)

Thirteen isolates, comprising seven isolates from clinical and six isolates from veterinary sources, matched ST11 ($P < 0.05$). ST48 was the second most abundant sequence type corresponding to the 12 isolates and ST48 ($P < 0.05$) was the most commonly found ST in the human sources as the 11 clinical isolates. Both dominant STs were classified as MDR with high resistance profile against majorly used antibiotics. CC231 (ST231) was observed in 09 isolates. ST431 and ST231 STs were different from each other by single-locus variant (SLV), and ST15 was found to be the originator genotype of clonal complex CC15. Furthermore, the four diverse STs of CC147 including ST1137, ST1488, ST1561, and ST580 were also found to have double locus variants (DLV) to each other.

Another ST258 corresponded to the 1 + 1 isolate equally from sputum and veterinary sludge, which was designated as XDR with high resistance profile against 13 antibiotics and showed a marked presence of bla_{kpc-2}, bla_{NDM-1}, and bla_{OXA-48}. The presence of these carbapenems encoding genes was also evident in ST11, which is a SLV of ST258, but both belong to different CCs. There are few isolates of human and environmental origins that matched with ST1709 and ST2167, which were quite dissimilar to each other in allelic profiles, and all these isolates were found susceptible to all the antibiotics used in the study. Details of STs harboring carbapenem resistance genes are given in Table 4.

Conjugation validation

All the selected isolates displayed transferability, and colonies of the transconjugants were observed on LB agar plates containing MEM, IMP, and ETP. The MIC_{90} of these antibiotics (MEM, IMP, and ETP) against transconjugants were 32, 16, and 16 μg/mL, respectively. Various carbapenemases genes (bla_{NDM-1} bla_{KPC} and bla_{OXA-48}) were detected by colony PCR, which confirmed the transconjugants.

TABLE 3 Prevalence of all beta-lactamases genes found in isolates from different sources.

Beta-lactamases genes	Sequence	Reference	All Isolates n (%)	Human n (%)	Environment n (%)	Veterinary n (%)
bla_{TEM}	F-TCAACATTTCCGTGTCG R-CTGACAGTTACCAATGCTTA	(Chaudhry et al., 2020)	69 (58%)	47 (52%)	9 (53%)	13 (100%)
bla_{SHV}	F-ATGCGTTATATTCGCCTGTG R-AGATAAATCACCACAATGCGC		119 (99%)	88 (98%)	17 (100%)	13 (100%)
$bla_{CTX-M-1}$	F-GTTTCCGCTATTACAAACCGTTG R-GCCCATGGTTAAAAAATCACTGC		77 (64%)	54 (60%)	12 (71%)	11 (85%)
$bla_{CTX-M-2}$	F-TGGAAGCCCTGGAGAAAAGT R-CTTATCGCTCTCGCTCTGTT		14 (12%)	5 (6%)	7 (41%)	2 (15%)
$bla_{CTX-M-9}$	F-ATGGTGACAAAGAGAGTGCA R-CCCTTCGGCGATGATTCTC		33 (28%)	22 (24%)	7 (41%)	4 (31%)
$bla_{CTX-M-14}$	F-GAGAGTGCAACGGATGATG R-TGCGGCTGGGTAAAATAG		59 (49%)	41 (46%)	7 (41%)	11 (85%)
PANbla_{CTX-M}	F- GGATATCGTTGGTGGTGCCATA R-TTTGCGATGTGCAGTACCAGTAA		81 (68%)	55 (61%)	13 (76%)	13 (100%)
bla_{KPC-2}	F-TGCAGAGCCCAGTGTCAGTTT R-CGCTCTATCGGCGATACCA		2 (2%)	1 (1%)	0 (0%)	1 (8%)
bla_{IMP}	F-GGAATAGAGTGGCTTAATTCTC R-CCAAACCACTACGTTATC		0 (0%)	0 (0%)	0 (0%)	0 (0%)
bla_{VIM}	F-GATGGTGTTTGGTCGCATA R-CGAATGCGCAGCACCAG		0 (0%)	0 (0%)	0 (0%)	0 (0%)
bla_{GIM}	F-TCGACACACCTTGGTCTGAA R-AACTTCCAACTTTGCCATGC		0 (0%)	0 (0%)	0 (0%)	0 (0%)
bla_{NDM-1}	F-TGCCCAATATTATGCACCCGG R-CGAAACCCGGCATGTCGAGA		52 (43%)	31 (34%)	9 (53%)	12 (92%)
bla_{OXA-48}	F-TTGGTGGCATCGATTATCGG R-GAGCACTTCTTTTGTGATGGC		46 (38%)	29 (32%)	6 (35%)	11 (85%)

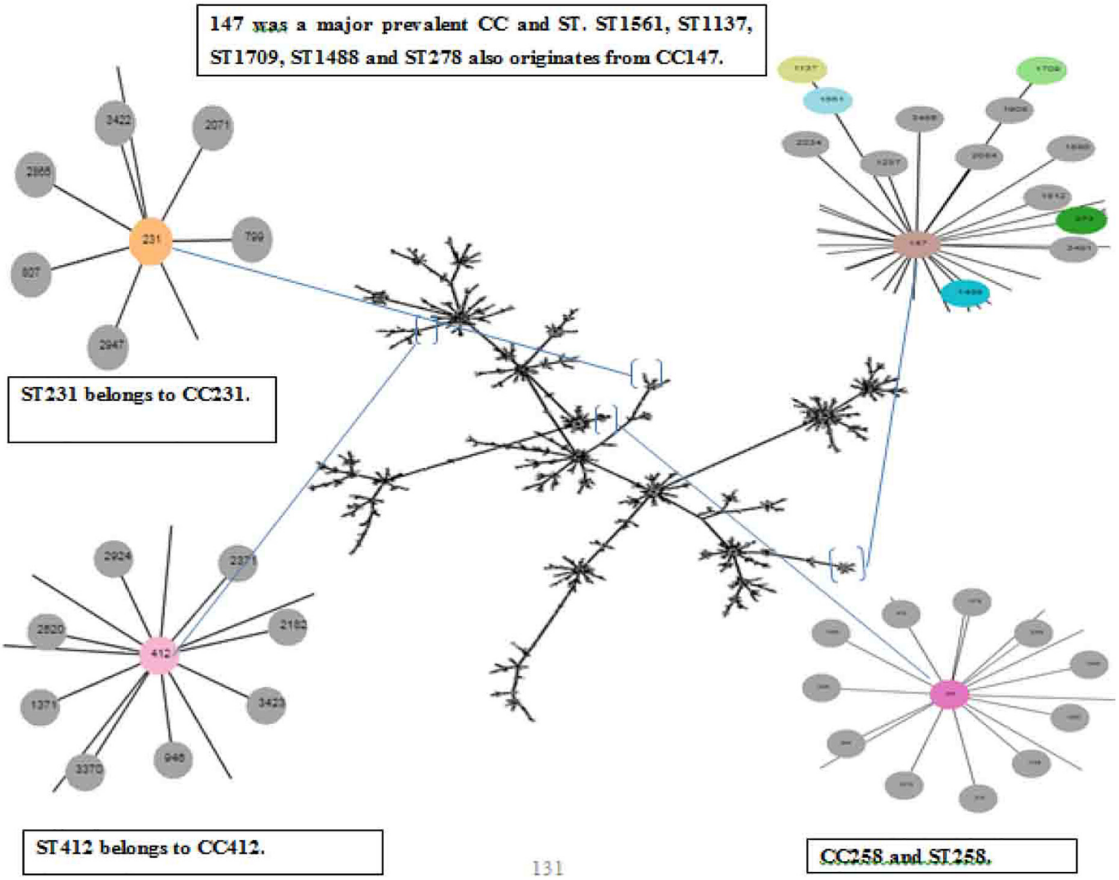


FIGURE 1
eBURST analysis (Phyloviz) of Klebsiella pneumoniae representing the STs and CCs, Partial snapshots of branches magnified and highlighted each CC and ST found in the study.

Discussion

The mounting crisis of AR is a global challenge that is affecting humans as well as animals in association with the wider environment. Irrational use of antibiotics in health care settings, veterinary and agricultural practices is a substantial contributing factor in the global expansion of AR. Moreover, poor sanitation and disposal systems associated with massive populations have a threatening impact on the dissemination of AR to the community (Adnan et al., 2017; Aslam et al., 2018; Baloch et al., 2018). Pakistan is one of the leading countries in the production of animal products, and the use of antibiotics in animals and veterinary practices is a routine matter in the country. Despite intense modernized farming, Pakistan still lacks sufficient data on the use and estimation of antibiotics in animals (Hassan Ali et al., 2010; Idrees et al., 2011; Khan et al., 2013; Adnan et al., 2017; Mohsin et al., 2017; Mohsin, 2019).

The findings of the present study showed that overall, 120 (15.7%) were confirmed *K. pneumoniae* isolated from various sources of humans, animals, and the environment (**Figure 2A**). It has been well established that *K. pneumoniae* can survive in different environments because of its large genome size (5.7 Mbps with 5,455 protein) than *E. coli* (5.1 Mbps with 4,915 genes) (Sudarwanto et al., 2015). A total of 90 (17.42%) *K. pneumoniae* isolates were obtained from clinical samples. Isolation of *K. pneumoniae* has been reported from various clinical specimens across Pakistan. Most of the isolates of the present study were obtained from respiratory samples, i.e., sputum (23.6%), blood samples (22.4%) followed by pus samples (13.3%) collected from various wounds and surgical sites, and urine samples (14.4%) collected from UTI-infected male and female patients. The is important and suggested that it may lead to hospital-acquired infection or put patients at risk of ventilator-associated pneumonia and bacteremia due to *K. pneumoniae*. Same findings have been reported from Aga Khan University Hospital, Karachi. Pakistan, where they also found comparable percentage prevalence in various sample sources, particularly blood samples (Khan et al., 2010b). The same study has also been reported from

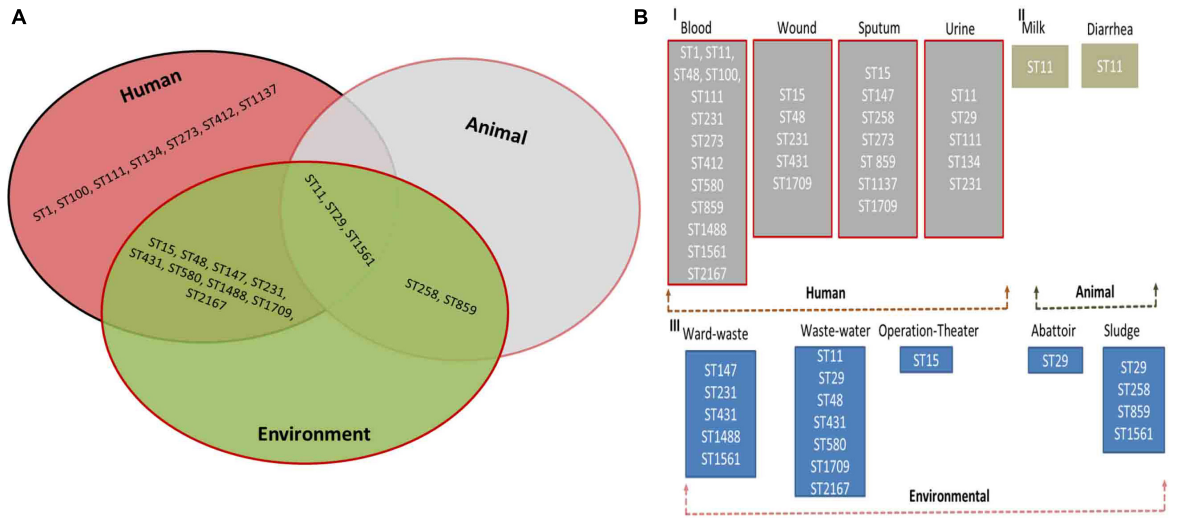


FIGURE 2
(A) Distribution of multilocus sequence types (STs) of Klebsiella pneumoniae in Human, Animal, and Environment. **(B)** Prevalence of multilocus sequence types (STs) in blood, wound, sputum, urine (Human) **(I)**, milk and fecal (Animal) **(II)** and ward-waste, wastewater, operation-theater, Abattoir, and sludge (Environment) **(III)**.

Lahore, Pakistan (Riaz et al., 2012), but they reported a low percentage prevalence of *K. pneumoniae* from various clinical samples; however, pus samples in this study were also the least possible clinical source of *K. pneumoniae*. Another study reported from Karachi Pakistan revealed comparable findings regarding the percentage prevalence (17%) of *K. pneumoniae* from urine and blood samples (Khan et al., 2010a).

In the present study, about 70% of the *K. pneumoniae* clinical isolates were found to be ESBL-producing *K. pneumoniae*, which is a very alarming health situation prevailing in Pakistan. Isolates exhibited various antibiotic resistance determinants (ARDs) e.g., bla_{TEM} and bla_{CTX-M}, which were associated with conferring the resistance against beta-lactam antibiotics. These findings were worrying because in local health settings most of the prescribed regimens are limited to a specific class of antibiotics. Same findings showing up to 70% ESBL producing *K. pneumoniae* have been reported by Shah et al. (2003), while another study reported 30% ESBL producing *K. pneumoniae* from Lahore (Ejaz et al., 2013). Comparable reports have been published from neighboring countries like India and Iran, where they found 56 and 59% ESBL producing *K. pneumoniae* from various samples, respectively (Jain and Mondal, 2007; Ghafourian et al., 2011). In a study from India, the prevalence of ESBL and carbapenemase-producing *K. pneumoniae* isolates were 47 and 17%, respectively (Wyres et al., 2020). In another study from India, 84% of *K. pneumoniae* isolates were ESBL producers whereas the carbapenem resistance phenotypes were observed in 66% of isolates (Bhaskar et al., 2019).

Additionally, a study conducted in the recent past found ESBL producing *K. pneumoniae* with a rate of 15.8% (Bukhari et al., 2016), the low percentage prevalence may be due to the small sampling fraction and a huge diversity of sample sources as compared to our studied samples. Worryingly, a significant percentage prevalence (32%) of carbapenem-resistant *K. pneumoniae* has also been detected in the present study. Isolates exhibited different ARGs which are responsible for the resistance against carbapenems e.g., bla_{KPC} (1.67%), bla_{OXA} (38.0%), and bla_{NDM-1} (43.3%), etc. It has been well characterized that *K. pneumoniae* displays carbapenem resistance by involving various mechanisms, like carbapenemases ARGs, outer membrane permeability alterations, and efflux pump system (Pitout et al., 2015). Few reports of carbapenem resistance in Pakistan have been published, and a recent study revealed the first blaOXA-181-producing carbapenem-resistant (CR) *K. pneumoniae* from Pakistan, which rang the alarm of pan-drug resistant (PDR) *K. pneumoniae* (Nahid et al., 2017). In a study from the largest tertiary care hospital in Bangladesh, the prevalence of carbapenem-resistant KP isolates was 30%. Most isolates were found to harbor bla_{NDM-1} i.e., 53% followed by bla_{NDM-5} (14%), $bla_{OXA-181}$ (12%), and $bla_{OXA-232}$ (10%) (Okanda et al., 2020).

From Pakistan, in livestock, there is barely any such report available to compare our findings. But the percentage prevalence estimated in the present study is closely relative to the findings of a study conducted in India, where they found a 6% percentage prevalence of Enterobacterales from various samples of veterinary sources, especially milk and

TABLE 4 The genetic diversity of indigenous *K. pneumoniae* isolates from different sources.

Sequence type (Pasteur Scheme)	Clonal complex	Allelic profile (*gapA, infB, mdh, pgi, phoE, rpoB, tonB*)	No. of isolates	Sources	No. of resistant antibiotics	MDR/XDR	Acquired carbapenem resistance mechanism
ST1	1	4 + 4 + 1 + 1 + 7 + 4 + 10	2	Clinical (2)	11	MDR	
ST11	11	3 + 3 + 1 + 1 + 1 + 1 + 4	13	Clinical (7) veterinary (6)	10	MDR	bla_{NDM-1} bla_{OXA-48}
ST15	15	1 + 1 + 1 + 1 + 1 + 1 + 1	9	Clinical (8) environmental (1)	10	MDR	–
ST29	29	2 + 3 + 2 + 2 + 6 + 4 + 4	9	Clinical (3) veterinary (6)	8	MDR	–
ST48	48	2 + 5 + 2 + 2 + 7 + 1 + 10	12	Clinical (11) environmental (1)	8	MDR	–
ST100	101	2 + 6 + 5 + 5 + 4 + 4 + 6	1	Clinical (1)	4	MDR	–
ST111	111	2 + 1 + 5 + 1 + 17 + 4 + 42	7	Clinical (7)	11–12	XDR (1) MDR (6)	bla_{NDM-1} bla_{OXA-48}
ST134	292	3 + 1 + 2 + 1 + 1 + 1 + 4	3	Clinical (3)	8	MDR	–
ST147	147	3 + 4 + 6 + 1 + 7 + 4 + 38	3	Clinical (2) environmental (1)	10	MDR	bla_{OXA-48}
ST231	231	2 + 6 + 1 + 3 + 26 + 1 + 77	10	Clinical (9) Environmental (1)	8–9	MDR	bla_{OXA-48}
ST258	258	3 + 3 + 1 + 1 + 1 + 1 + 79	2	Clinical (1) Veterinary (1)	13	XDR	bla_{NDM-1} bla_{OXA-48}
ST273	147	3 + 4 + 6 + 1 + 7 + 4 + 4	8	Clinical (8)	10	MDR	bla_{OXA-48}
ST412	412	2 + 1 + 2 + 1 + 9 + 1 + 112	2	Clinical (2)	9	MDR	–
ST431	15	2 + 1 + 1 + 1 + 1 + 1 + 1	3	Clinical (2) environmental (1)	9	MDR	–
ST580	147	3 + 4 + 6 + 1 + 9 + 4 + 38	4	Clinical (2) environmental (2)	4	MDR	–
ST859	11	3 + 3 + 1 + 1 + 1 + 1 + 1	6	Clinical (5) environmental (1)	9	MDR	bla_{NDM-1}
ST1137	147	3 + 4 + 6 + 4 + 7 + 4 + 38	6	Clinical (5) environmental (1)	5	MDR	–
ST1488	147	3 + 4 + 2 + 1 + 7 + 4 + 38	4	Clinical (3) environmental (1)	4	MDR	–
ST1561	147	3 + 4 + 6 + 1 + 7 + 1 + 38	5	Clinical (2) environmental (3)	6	MDR	–
ST1709	147	3 + 1 + 1 + 1 + 7 + 4 + 38	8	Clinical (6) environmental (2)	3	MDR	–
ST2167	37	2 + 9 + 2 + 1 + 13 + 1 + 35	3	Clinical (1) environmental (2)	5	MDR	–

fecal samples (Kar et al., 2015). The same sort of study has been conducted in England and Wales in 2011; they detected ESBLs-harboring bacteria in various samples of veterinary origin like fecal samples, farm premises samples, and waste milk (Randall et al., 2014). They also detected bla_{CTX-M} in waste milk, and they also suggested the key role of the farm environment in the dissemination of ARGs. A study has been reported from Germany, where they have detected the plasmid-mediated ESBLs producing gram-negative bacteria from cattle suffering from mastitis (Freitag et al., 2017). A significant prevalence of CR *K. pneumoniae* in poultry meat has been reported in China (Wu et al., 2016). Although they have identified a list of ARDs like the findings of the study, they have also reported bla_{CTX-M} as one of the most prevalent resistance genes in the samples. In a meta-analysis from India, the prevalence of ESBL-producing bacteria increased from 12% in 2013 to 33% in 2019 in veterinary settings. The species-wise prevalence of ESBL phenotypes was 5% in *Pseudomonas* spp., 9% in *E. coli*, and 10% in *K. pneumoniae,* which shows that the ESBL-producing

K. pneumoniae is the leading ESBL-producing bacterial species in India (Kuralayanapalya et al., 2019).

Overall, a total of 21 discrete STs with their specific allelic pattern were identified from all the isolates. The most abundant ST found in the study was ST11; a total of 13 (70%) isolates were assigned with ST11, and among them 07 isolates were clinical specimens and 06 isolates were from veterinary samples. Overall, due to the unavailability of data on *K. pneumoniae* STs, we do not have such studies to corroborate our findings. In a retrospective study from Zhejiang, China from 2008 to 2018, it is reported that the prevalence of carbapenem-resistant *K. pneumoniae* increased from 2.5% (2008) to 15.8% (2018). Interestingly, the ST11 was the most common sequence type and blaKPC-2 was the most prevalent carbapenem-resistant determinant (Hu et al., 2020). In the recent past, sporadic reports of *K. pneumoniae* ST11 have been published from Pakistan (Qamar et al., 2018). In contrast to the findings which were specifically designed for bla_{NDM-1}, the results of the present study revealed a comprehensive status of *K. pneumoniae* ST11 due to the molecular resistance pattern depicting various ARGs. The second most observed ST was the ST48; 12 isolates were allotted with the ST48, and among these isolates, 11 isolates belong to clinical origin while 01 sample was from an environmental source. ST11 and ST48 were found to be MDR as they showed a significant resistance pattern to almost all the antibiotics used in the study except colistin (Qamar et al., 2018).

To the best of our knowledge, this is the very first report describing the dissemination of bla_{KPC-2} producing *K. pneumoniae* ST258 at the human–environment interface from Pakistan. The global distribution of CG258 is well recognized and has been well reported. The findings of the study are partly in agreement with the available literature, as ST11, a group member of CG258 was the most abundant ST found in the study. Additionally, bla_{KPC-2} producing *K. pneumoniae* ST258 was also a novel finding from Pakistan. Globally, ST258 is known as a key etiology of MDR *K. pneumoniae,* especially carbapenemase-producing *K. pneumoniae* infections (Munoz-Price et al., 2013; Chen et al., 2014; Pitout et al., 2015).

Conclusion

In conclusion, the major findings of the present study strengthened the understanding of the vital resistant determinants of *K. pneumoniae* which are never reported from Pakistan. Additionally, genetic diversity among indigenous *K. pneumoniae* isolates from different sources enabled us to find out different STs from humans, animals, and the associated environment, which may be very helpful in the future to control the public and animal health menace caused by MDR *K. pneumoniae*. Although it is one of the comprehensive studies reported from Pakistan, it also has some limitations. Particularly, the present study described the genetic diversity in MDR *K. pneumoniae* based on MLST, while the genetic characterization based on whole genome sequence (WGS) analysis is lacking. In the future, a detailed investigation based on WGS surveillance of MDR *K. pneumoniae* could be a potential work plan to decipher the genetic relatedness of this pathogen at the human–animal–environment interface.

Data availability statement

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

Ethics statement

The study was conducted in a span of 2 years from September 2017 to August 2019; proper permission was granted by the Ethical Review Board (ERB) of Government College University, Faisalabad, Pakistan.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Funding

This study was supported by grants from the Major Science and Technology Special Project of Yunnan Province (No. 2019ZF004) and the First People's Hospital of Yunnan Province (202205AG070053-10 and KHBS-2022-008).

Acknowledgments

We thank all the participants involved in this study.

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.

References

Adnan, M., Khan, H., Kashif, J., Ahmad, S., Gohar, A., Ali, A., et al. (2017). Clonal expansion of sulfonamide resistant *Escherichia coli* isolates recovered from diarrheic calves. *Pak. Vet. J.* 37, 230–232.

Aslam, B., Chaudhry, T. H., Arshad, M. I., Alvi, R. F., Shahzad, N., Yasmeen, N., et al. (2020). The First bla(KPC) harboring *Klebsiella pneumoniae* ST258 strain isolated in Pakistan. *Microb. Drug Resist.* 26, 783–786. doi: 10.1089/mdr.2019.0420

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., et al. (2018). Antibiotic resistance: a rundown of a global crisis. *Infect. Drug Resist.* 11, 1645–1658. doi: 10.2147/IDR.S173867

Baloch, Z., Aslam, B., Muzammil, S., Khurshid, M., Rasool, M. H., and Ma, K. (2018). Selection inversion: a probable tool against antibiotic resistance. *Infect. Drug Resist.* 11, 1903–1905. doi: 10.2147/IDR.S176759

Bartlett, J. G., Gilbert, D. N., and Spellberg, B. (2013). Seven ways to preserve the miracle of antibiotics. *Clin Infect Dis* 56, 1445–1450. doi: 10.1093/cid/cit070

Bhaskar, B. H., Mulki, S. S., Joshi, S., Adhikary, R., and Venkatesh, B. M. (2019). Molecular characterization of extended spectrum β-lactamase and carbapenemase producing *Klebsiella pneumoniae* from a tertiary care hospital. *Indian J. Crit. Care Med.* 23, 61–66. doi: 10.5005/jp-journals-10071-23118

Bukhari, A., Arshad, M., Raza, S., Azam, M., and Mohsin, M. (2016). Emergence of extended spectrum beta-lactamases-producing strains belonging to cefotaxime-M-1 class from intensive care units patients and environmental surfaces in Pakistan. *Int. J. One Health* 2, 69–74. doi: 10.14202/IJOH.2016.69-74

Cecchini, M., Langer, J., and Slawomirski, L. (2015). *Antimicrobial resistance in G7 countries and beyond: economic issues, policies and options for action*. Paris: Organization for Economic Co-operation and Development, 1–75.

Chaudhry, T. H., Aslam, B., Arshad, M. I., Alvi, R. F., Muzammil, S., Yasmeen, N., et al. (2020). Emergence of bla (NDM-1) Harboring *Klebsiella pneumoniae* ST29 and ST11 in Veterinary Settings and Waste of Pakistan. *Infect. Drug Resist.* 13, 3033–3043. doi: 10.2147/IDR.S248091

Chaudhry, T. H., Aslam, B., Arshad, M. I., Nawaz, Z., and Waseem, M. (2019). Occurrence of ESBL-producing *Klebsiella pneumoniae* in hospital settings and waste. *Pak. J. Pharm. Sci.* 32, 773–778.

Chen, L., Mathema, B., Chavda, K. D., Deleo, F. R., Bonomo, R. A., and Kreiswirth, B. N. (2014). Carbapenemase-producing *Klebsiella pneumoniae*: molecular and genetic decoding. *Trends Microbiol.* 22, 686–696. doi: 10.1016/j.tim.2014.09.003

CLSI (2018). *performance standards for antimicrobial susceptibility testing: twenty-eighth informational supplement. CLSI document M100 (ISBN 1-56238-839-8)*. Malvern, PA: CLSI.

Davis, G. S., and Price, L. B. (2016). Recent research examining links among *klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. *Curr. Environ. Health Rep.* 3, 128–135. doi: 10.1007/s40572-016-0089-9

Diancourt, L., Passet, V., Verhoef, J., Grimont, P. A., and Brisse, S. (2005). Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J. Clin. Microbiol.* 43, 4178–4182. doi: 10.1128/JCM.43.8.4178-4182.2005

Ejaz, H., Ul-Haq, I., Mahmood, S., Zafar, A., and Mohsin Javed, M. (2013). Detection of extended-spectrum β-lactamases in *Klebsiella pneumoniae*: comparison of phenotypic characterization methods. *Pak. J. Med. Sci.* 29, 768–772.doi: 10.12669/pjms.293.3576

Freitag, C., Michael, G. B., Kadlec, K., Hassel, M., and Schwarz, S. (2017). Detection of plasmid-borne extended-spectrum β-lactamase (ESBL) genes in *Escherichia coli* isolates from bovine mastitis. *Vet. Microbiol.* 200, 151–156. doi: 10.1016/j.vetmic.2016.08.010

Ghafourian, S., Bin Sekawi, Z., Sadeghifard, N., Mohebi, R., Kumari Neela, V., Maleki, A., et al. (2011). The prevalence of ESBLs Producing *Klebsiella pneumoniae* isolates in some major hospitals, Iran. *Open Microbiol. J.* 5, 91–95. doi: 10.2174/1874285801105010091

Hassan Ali, N., Farooqui, A., Khan, A., Khan, A. Y., and Kazmi, S. U. (2010). Microbial contamination of raw meat and its environment in retail shops in Karachi, Pakistan. *J. Infect. Dev. Ctries* 4, 382–388. doi: 10.3855/jidc.599

Hu, Y., Liu, C., Shen, Z., Zhou, H., Cao, J., Chen, S., et al. (2020). Prevalence, risk factors and molecular epidemiology of carbapenem-resistant *Klebsiella pneumoniae* in patients from Zhejiang, China, 2008-2018. *Emerg. Microbes Infect.* 9, 1771–1779.doi: 10.1080/22221751.2020.1799721

Idrees, M., Shah, M. A., Michael, S., Qamar, R., and Bokhari, H. (2011). Antimicrobial resistant *Escherichia coli* strains isolated from food animals in Pakistan. *Pak. J. Zool.* 43, 303–310.

Jain, A., and Mondal, R. (2007). Prevalence & antimicrobial resistance pattern of extended spectrum beta-lactamase producing Klebsiella spp isolated from cases of neonatal septicaemia. *Indian J. Med. Res.* 125, 89–94.

Kar, D., Bandyopadhyay, S., Bhattacharyya, D., Samanta, I., Mahanti, A., Nanda, P. K., et al. (2015). Molecular and phylogenetic characterization of multidrug resistant extended spectrum beta-lactamase producing *Escherichia coli* isolated from poultry and cattle in Odisha, India. *Infect. Genet. Evol.* 29, 82–90. doi: 10.1016/j.meegid.2014.11.003

Khan, E., Schneiders, T., Zafar, A., Aziz, E., Parekh, A., and Hasan, R. (2010b). Emergence of CTX-M Group 1-ESBL producing Klebsiella pneumonia from a tertiary care centre in Karachi, Pakistan. *J. Infect. Dev. Ctries* 4, 472–476. doi: 10.3855/jidc.674

Khan, E., Ejaz, M., Zafar, A., Jabeen, K., Shakoor, S., Inayat, R., et al. (2010a). Increased isolation of ESBL producing *Klebsiella pneumoniae* with emergence of carbapenem resistant isolates in Pakistan: report from a tertiary care hospital. *J. Pak. Med. Assoc.* 60, 186–190.

Khan, G. A., Berglund, B., Khan, K. M., Lindgren, P. E., and Fick, J. (2013). Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities–a study in Pakistan. *PLoS One* 8:e62712. doi: 10.1371/journal.pone.0062712

Kuralayanapalya, S. P., Patil, S. S., Hamsapriya, S., Shinduja, R., Roy, P., and Amachawadi, R. G. (2019). Prevalence of extended-spectrum beta-lactamase producing bacteria from animal origin: a systematic review and meta-analysis report from India. *PLoS One* 14:e0221771. doi: 10.1371/journal.pone.0221771

Mohsin, M. (2019). The under reported issue of antibiotic-resistance in food-producing animals in Pakistan. *Pak. Vet. J.* 39, 323–328.doi: 10.29261/pakvetj/2019.037

Mohsin, M., Raza, S., Schaufler, K., Roschanski, N., Sarwar, F., Semmler, T., et al. (2017). High Prevalence of CTX-M-15-Type ESBL-Producing *E. coli* from migratory avian species in Pakistan. *Front. Microbiol.* 8:2476. doi: 10.3389/fmicb.2017.02476

Munoz-Price, L. S., Poirel, L., Bonomo, R. A., Schwaber, M. J., Daikos, G. L., Cormican, M., et al. (2013). Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect. Dis.* 13, 785–796.doi: 10.1016/S1473-3099(13)70190-7

Nahid, F., Zahra, R., and Sandegren, L. (2017). A blaOXA-181-harbouring multi-resistant ST147 *Klebsiella pneumoniae* isolate from Pakistan that represent an intermediate stage towards pan-drug resistance. *PLoS One* 12:e0189438. doi: 10.1371/journal.pone.0189438

Okanda, T., Haque, A., Koshikawa, T., Islam, A., Huda, Q., Takemura, H., et al. (2020). Characteristics of carbapenemase-producing *Klebsiella pneumoniae* isolated in the intensive care unit of the largest tertiary hospital in Bangladesh. *Front. Microbiol.* 11:612020. doi: 10.3389/fmicb.2020.612020

Pitout, J. D., Nordmann, P., and Poirel, L. (2015). Carbapenemase-producing *Klebsiella pneumoniae*, a key pathogen set for global nosocomial dominance. *Antimicrob. Agents Chemother.* 59, 5873–5884. doi: 10.1128/AAC.01019-15

Qamar, M. U., Saleem, S., Toleman, M. A., Saqalein, M., Waseem, M., Nisar, M. A., et al. (2018). In vitro and in vivo activity of Manuka honey against NDM-1-producing *Klebsiella pneumoniae* ST11. *Future Microbiol.* 13, 13–26.

Randall, L., Heinrich, K., Horton, R., Brunton, L., Sharman, M., Bailey-Horne, V., et al. (2014). Detection of antibiotic residues and association of cefquinome residues with the occurrence of Extended-Spectrum β-Lactamase (ESBL)-producing bacteria in waste milk samples from dairy farms in England and Wales in 2011. *Res. Vet. Sci.* 96, 15–24. doi: 10.1016/j.rvsc.2013.10.009

Riaz, S., Faisal, M., and Hasnain, S. (2012). Prevalence and comparison of Beta-lactamase producing *Escherichia coli* and Klebsiella spp from clinical and environmental sources in Lahore, Pakistan. *Afr. J. Microbiol. Res.* 6, 465–470.doi: 10.5897/AJMR11.1457

Shah, A. A., Hasan, F., Ahmed, S., and Hameed, A. (2003). Prevalence of extended-spectrum ss-Lactamases in Nosocomial and out-patients (Ambulatory). *Pak. J. Med. Sci.* 19, 187–191.

Sudarwanto, M., Akineden, Ö, Odenthal, S., Gross, M., and Usleber, E. (2015). Extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* in bulk tank milk from dairy farms in Indonesia. *Foodborne Pathog. Dis.* 12, 585–590. doi: 10.1089/fpd.2014.1895

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P T* 40, 277–283.

Woolhouse, M., Ward, M., Van Bunnik, B., and Farrar, J. (2015). Antimicrobial resistance in humans, livestock and the wider environment. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370:20140083.

Wu, K., Spiegelman, D., Hou, T., Albanes, D., Allen, N. E., Berndt, S. I., et al. (2016). Associations between unprocessed red and processed meat, poultry, seafood and egg intake and the risk of prostate cancer: A pooled analysis of 15 prospective cohort studies. *Int. J. Cancer* 138, 2368–2382. doi: 10.1002/ijc.29973

Wyres, K. L., and Holt, K. E. (2016). *Klebsiella pneumoniae* population genomics and antimicrobial-resistant clones. *Trends Microbiol.* 24, 944–956. doi: 10.1016/j.tim.2016.09.007

Wyres, K. L., Nguyen, T. N. T., Lam, M. M. C., Judd, L. M., Van Vinh Chau, N., Dance, D. A. B., et al. (2020). Genomic surveillance for hypervirulence and multi-drug resistance in invasive *Klebsiella pneumoniae* from South and Southeast Asia. *Genome Med.* 12:11. doi: 10.1186/s13073-019-0706-y

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 14 December 2022
DOI 10.3389/fmicb.2022.875775

Check for updates

OPEN ACCESS

EDITED BY
Ziad Daoud,
Central Michigan University,
United States

REVIEWED BY
Steven Lee Foley,
National Center for Toxicological Research (FDA), United States
Natalie Ring,
University of Edinburgh,
United Kingdom

*CORRESPONDENCE
Maria M. Konstantinovski
mmkonstant@gmail.com

SPECIALTY SECTION
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

RECEIVED 14 February 2022
ACCEPTED 08 November 2022
PUBLISHED 14 December 2022

CITATION
Konstantinovski MM, Schouls LM, Witteveen S, Claas ECJ, Kraakman ME, Kalpoe J, Mattson E, Hetem DJ, van Elzakker EPM, Kerremans J, Hira V, Bosch T and Gooskens J (2022) Livestock-associated methicillin-resistant *Staphylococcus aureus* epidemiology, genetic diversity, and clinical characteristics in an urban region.
Front. Microbiol. 13:875775.
doi: 10.3389/fmicb.2022.875775

COPYRIGHT
© 2022 Konstantinovski, Schouls, Witteveen, Claas, Kraakman, Kalpoe, Mattson, Hetem, van Elzakker, Kerremans, Hira, Bosch and Gooskens. 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.

Livestock-associated methicillin-resistant *Staphylococcus aureus* epidemiology, genetic diversity, and clinical characteristics in an urban region

Maria M. Konstantinovski[1,2]*, Leo M. Schouls[3], Sandra Witteveen[3], Eric C. J. Claas[1], Margriet E. Kraakman[1], Jayant Kalpoe[4], Eva Mattson[2], David J. Hetem[5], Erika P. M. van Elzakker[6], Jos Kerremans[7], Vishal Hira[8], Thijs Bosch[3] and Jairo Gooskens[1]

[1]Department of Medical Microbiology, Leiden University Medical Center, Leiden, Netherlands, [2]Department of Microbiology, Medical Laboratories, Reinier de Graaf Groep, Delft, Netherlands, [3]Center for Infectious Diseases Research, Diagnostics and Laboratory Surveillance, National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands, [4]Department of Medical Microbiology, Regional Laboratory of Public Health Kennemerland, Haarlem, Netherlands, [5]Department of Medical Microbiology, Haaglanden Medical Center, The Hague, Netherlands, [6]Laboratory of Medical Microbiology, HagaZiekenhuis, The Hague, Netherlands, [7]Department of Medical Microbiology, Alrijne Hospital, Leiderdorp, Netherlands, [8]Department of Medical Microbiology and Infection Prevention, Groene Hart Ziekenhuis, Gouda, Netherlands

Objectives: While Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), defined as CC398, is a well-known pathogen among those working with livestock, there are indications that LA-MRSA prevalence among the general population is increasing. However, the clinical impact in urban areas remains unknown. The aim of this study was to assess the genetic epidemiology and clinical characteristics of LA-MRSA in an urban area with a limited livestock population.

Methods: In this retrospective study, we evaluated LA-MRSA strains that were collected between 2014 and 2018 from patients who received clinical care in a single urban area in Netherlands. Patient files were assessed for livestock exposure data, clinical findings, and contact tracing information. Next-generation sequencing (NGS) analysis in combination with wgMLST was conducted to assess genetic diversity and relatedness and to detect virulence and resistance genes.

Results: LA-MRSA strains were cultured from 81 patients, comprising 12% of all the MRSA strains found in seven study laboratories between 2014 and 2018. No livestock link was found in 76% of patients (n =61), and 28% of patients (n =23) had an infection, mostly of the skin or soft tissue. Contact tracing had been initiated in 14 cases, leading to the identification of two hospital transmissions: a cluster of 9 cases and one of 2 cases. NGS data were available for 91% (n =75) of the patients. wgMLST confirmed the clusters detected *via*

contact tracing (n =2) and identified 5 additional clusters without a known epidemiological link. Relevant resistance and virulence findings included the PVL virulence gene (3 isolates) and tetracycline resistance (79 isolates).

Conclusion: LA-MRSA may cause a relevant burden of disease in urban areas. Surprisingly, most infections in the present study occurred in the absence of a livestock link, suggesting inter-human transmission. These findings and the presence of PVL and other immune evasive complex virulence genes warrant future surveillance and preventative measures.

KEYWORDS

cgMLST clustering, one-health, antimicrobial surveillance, LA-MRSA CC398, whole-genome sequencing, Staphylococcal infection/epidemiology, MRSA, *Staphylococcus aureus*

Introduction

The past two decades have shown a rapid increase in methicillin-resistant *Staphylococcus aureus* (MRSA) strains belonging to the clonal complex 398 (CC398) in livestock, the so-called Livestock-Associated MRSA (LA-MRSA). These strains pose a zoonotic risk, particularly for those working in close contact with pigs, veal calves, and poultry (Van Den Broek et al., 2009), as colonization rates of up to 63% have been reported (Cuny et al., 2009; Van Cleef et al., 2010; Bisdorff et al., 2012; van Cleef et al., 2014). However, recent data have shown that LA-MRSA is also an emerging and significant pathogen among people outside the farming industry, and emergence of LA-MRSA was associated with an increasing number of infections of skin, soft tissue, and bloodstream (Fitzgerald, 2012; Lekkerkerk et al., 2012; Köck et al., 2013; Larsen et al., 2015, 2017).

Previous studies reported the emergence of "human" LA-MRSA sub-lineages spreading independently of a livestock reservoir (McCarthy et al., 2012; Bosch et al., 2016). Such lineages could be better adapted to the human host due to the acquisition of virulence and host adaptation genes, which are commonly located on mobile genetic elements (Matuszewska et al., 2020). Such adaptations may increase the risk of human-to-human spread in healthcare settings and in communities in non-farming areas. The European Centre for Disease Prevention and Control (ECDC) highlighted the public health and veterinary importance of regarding LA-MRSA as a 'One Health' issue and recommended systematic surveillance to map potential reservoirs and transmission pathways in order to enable appropriate control measures (ECDC, 2013).

While there is an urgent need to understand the various aspects of the occurrence of LA-MRSA in non-farming areas, the impact of LA-MRSA in urban areas has not yet been studied. Hence, there is a paucity of data on the clinical and infection control burden of such infections.

The objectives of this retrospective study were to assess the clinical and infection control burden in an urban area with limited livestock in Netherlands, to gain more insight into the epidemiological characteristics and the genetic diversity using Whole-Genome Sequencing (WGS), and to identify potential transmission routes of LA-MRSA in urban settings.

Materials and methods

In this retrospective study, LA-MRSA isolates were included between 2014 and 2018 from patients who either resided in the study region or received clinical care there. The study region comprised mostly urban areas in the Mid-West region of the Netherlands (provinces Noord-Holland and Zuid-Holland). A map with the location of the laboratories and a background of rural versus urban areas as defined by the National Bureau of Statistics can be found in Figure 1 (Centraal Bureau van de Statistiek, n.d.). Seven laboratories from this study region collaborated in the study. These laboratories serve both hospitals and primary care patients.

National surveillance

The Dutch national MRSA surveillance is a voluntary surveillance system. *Via* this system, virtually all Dutch medical microbiology laboratories (MMLs) submit their MRSA isolates for characterization and archiving to the National Institute of Public Health and the Environment (RIVM). All MRSA isolates are submitted by the MMLs *via* the Type-Ned MRSA system and are subjected to MLVA, which also includes the detection of the genes for *mecA*, *mecC*, and the *lukF* gene, which is indicative for Panton-Valentine leukocidin (PVL) (Schouls et al., 2009).

Inclusion of LA-MRSA isolates

LA-MRSA was defined as MRSA belonging to the MLVA complex 398. Isolates submitted by six of the seven study laboratories were included through the national surveillance. One laboratory did not participate in the national surveillance, but

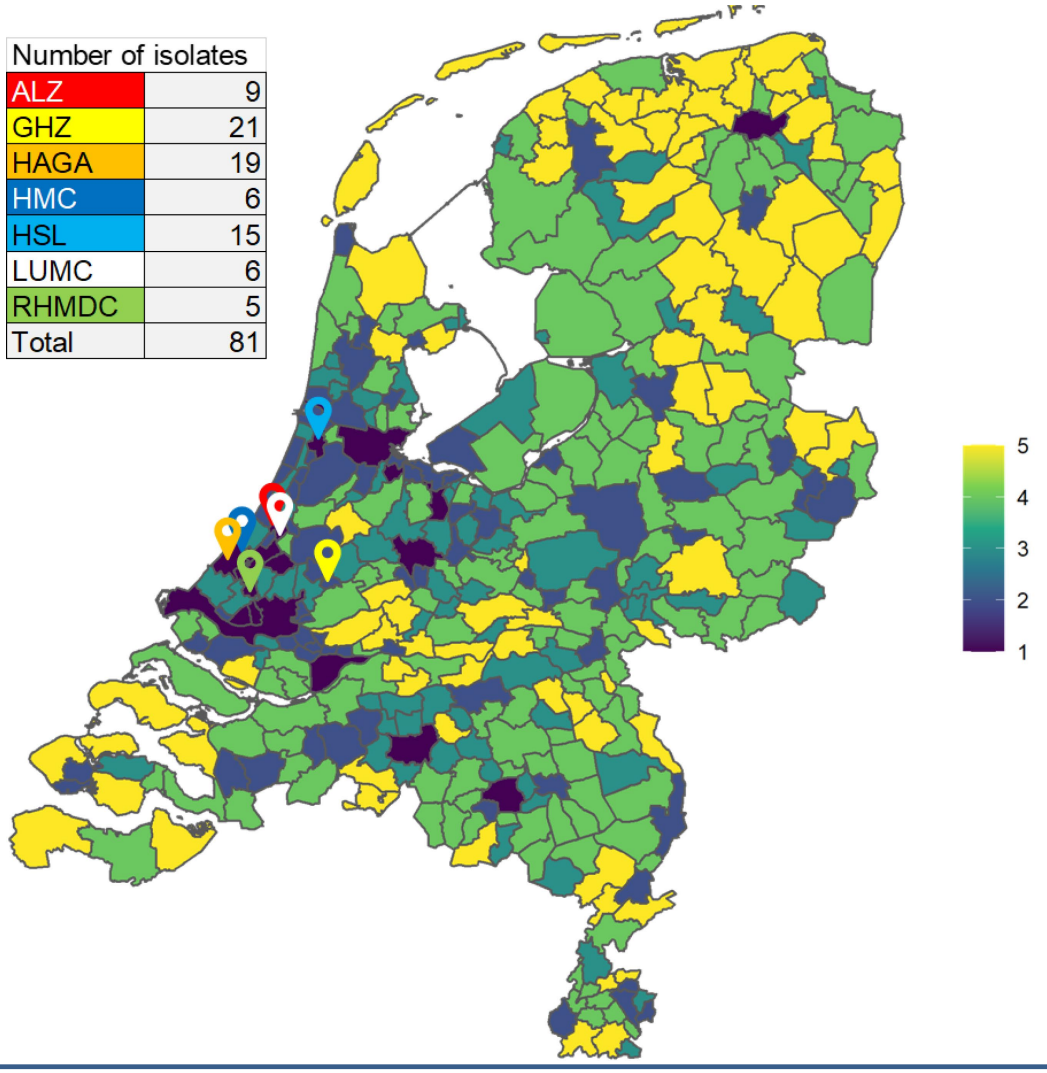


FIGURE 1
Map of Netherlands showing the urbanization level and the participating laboratories.

clinical MRSA isolates in this laboratory are stored at −80°C for future reference as part of routine procedures. Hence, we used areal-time PCR to select LA-MRSA isolates for the study as described by van Meurs et al. (2013). This PCR targets the ST398-specific sequence SAPIG2194 and produces a 124-bp fragment.

Clinical data and contact tracing

We retrospectively evaluated patient files to retrieve patient demographics, livestock exposure, and other MRSA risk factors as defined in the national guidelines. Clinical charts were

reviewed to determine if an MRSA infection was present and whether cultures had been taken for screening or for clinical purposes. In case of positive clinical cultures, patient files were assessed to differentiate between infection and colonization of the sample site. Patients with exclusively positive screening cultures and/or colonization were categorized as "carrier only." MRSA risk factors include having been an MRSA carrier in the past, having a known MRSA close contact, having received medical treatment abroad, international adoption, and livestock contact.

Baseline characteristics were compared to the data available from Type-Ned for non-LA-MRSA isolates from the participating laboratories and to nationwide data for LA-MRSA isolates. Infection control reports were assessed for results of contact tracing in the event of an unexpected MRSA finding. Data were entered into the Castor Electronic Data Capture system.

The study protocol approval and the need for patient consent were waived by the medical ethical committee of the Leiden University Medical Center (G18.021/SH/sh).

Whole-genome sequencing and analysis

Isolates for the study were recovered from −80°C storage and grown on a blood agar plate. Several colonies totaling 50–100 µl biomass volume per isolate were suspended in 450 µl Zymo DNA/RNA Shield buffer (Zymo Research, United States) for transport to the external NGS Laboratory (BaseClear, Leiden, Netherlands). DNA extraction was done using a standard molecular biology kit from Zymo Research. Study isolates were subjected to next-generation sequencing (NGS) analysis using the Illumina HiSeq 2500 after Genomic Nextera XT library preparation NGS data were used for *de novo* assembly using CLC Genomic Workbench v20.0.3 (Qiagen Bioinformatics, Aarhus, Denmark). Isolates were characterized using a whole-genome MLST (wgMLST) scheme based on the 1,861 core-genome and 706 accessory-genome scheme as developed by SeqSphere version v3.5.0 (Ridom GmbH, Münster, Germany) with the *S. aureus COL* (NC_002951.2, 10-JUN-2013) as a reference genome. The obtained wgMLST profiles were imported into BioNumerics to assess genetic relatedness of the isolates (*7.6.3,* Applied Maths, Sint-Martens). Genetic clusters were distinguished with the SeqSphere-defined cluster alert of 24 alleles (Leopold et al., 2014). Minimum spanning trees were drawn using BioNumerics using both study isolates and NGS data available from the national surveillance (2014 to 2018) that were submitted by non-study laboratories.

Resistance and virulence genes were determined using ResFinder (version 4.0) and VirulenceFinder (*version 2.04*) databases. If the presence of certain resistance or virulence genes varied within a genetic cluster, we additionally aligned the reference genome to the WGS data of the isolate using CLC Genomics to exclude minor errors in the sequencing and assembly process.

Results

A total of 81 patients with LA-MRSA were included in the study, which comprised 12% of all the MRSA isolates found in the study laboratories. The LA-MRSA and non-LA-MRSA isolates from the study laboratories made up 3.3% of all MRSA isolates in the national surveillance during the study period. MRSA isolates from the regional laboratories accounted for 1.6% of all nationwide LA-MRSA isolates and 3.8% of non-LA-MRSA isolates, respectively. Table 1 presents the baseline characteristics of the study patients compared to characteristics of the national LA-MRSA and regional non-LA-MRSA population.

Risk factors

Risk factors for MRSA carriage were present in 52 (66%) of the patients, 21 of whom had been in contact with livestock. Overall, 60 (76%) of the 81 included patients had no recorded livestock contact. In the 27 patients with no recorded MRSA risk factors, all LA-MRSA cultures were incidental findings that were either detected in clinical cultures ($n = 22$) or during pre-operative screenings for orthopedic joint implantation ($n = 5$). For 2 patients, there was a lack of risk factor data; however, the LA-MRSA had been cultured in an MRSA screening culture.

Clinical presentation

Infection was present in 23 patients (28%), and an additional three patients had positive clinical cultures without apparent infection. Cultures originated mostly from the skin and soft tissue

TABLE 1 Baseline characteristics of the study patients.

Characteristic	LA-MRSA urban ($n = 81$)	LA-MRSA nationwide ($n = 4,946$)	Urban non-LA-MRSA ($n = 640$)
Age in year	0–86	0–99	0–96
-mean	*48*	*50*	*43*
Female (%)	43%	38%	44%
Carriage only (%)	68%	80%	65%
Infection/clinical sample (%)	28%	16%	33%
MRSA risk factor data available (%)	98%	51%	78%
-of whom positive[1]	*66%*	*43%*	*41%*
-of whom had livestock contact	*26%*	*67%*	*0.2%*
-MUO[2]	*34%*	*24%*	*47%*
PVL-positive isolates	3.7%	2.5%	37%

Urban MRSA and Nationwide LA-MRSA data derived from Type-Ned.
[1]as defined in national guidelines.
[2]MUO: MRSA of unknown origin.
Due to missing data, not all the percentages add up to 100%.

($n = 20$), but pulmonary ($n = 5$) and urinary tract ($n = 1$) infections were also diagnosed. Five patients required some type of surgical intervention as part of the treatment. No deep-seated infections causing bacteremia occurred in the study population, while sporadic bacteremia cases ($n = 28$) did occur in the national population.

Carriage and eradication

For 51 of the 81 included patients, follow-up cultures were available. Carriage of LA-MRSA varied from a single positive finding to carriage of up to 72 months. Eradication was successful in 38 patients and failed in 13 patients. In the group with persistent carriage ($n = 13$), the recorded carriage duration varied from 4 to 72 months. Complicating circumstances for eradication that occurred among the patients included persistent exposure in farmers and their families, chronic skin defects, or persistent use of foreign materials, such as urinary catheters. One patient in whom eradication treatment failed was in possession of a feline pet that carried an LA-MRSA strain of an identical cluster type. Eradication was only successful after simultaneous treatment of both the pet and the patient.

Contact tracing

In 14 cases, a contact tracing investigation had been initiated by the Infection Control department. For 8 investigations, the number of screened contacts is known; these ranged from 1 to 103 contacts, with a total of 226 contacts. Nosocomial transmission was demonstrated in 2 investigations, namely Cluster 1 and 2, with eight confirmed secondary cases and two possible secondary cases (Table 2).

Next-generation sequencing analysis and wgMLST

MRSA NGS data were present for 74 study patients and one pet animal. For several reasons, NGS data of seven patients were missing. Five patients provided two isolates, hence NGS data for 80 isolates were included in the study.

A heterogenic genetic population was detected, in which many allelic differences were present (Figure 2). Analysis by means of wgMLST revealed 13 genetic clusters, which were clusters of nosocomial transmission, household members, unknown epidemiological links, and a control group of three isolates from one patient submitted by different laboratories. An overview of all the genetic clusters detected with wgMLST can be found in Table 2. An MST including nationwide LA-MRSA isolates is shown in Figure 3.

Virulence and resistance genes

All isolates carried *hlg* and *aur* genes. Eight isolates carried additional virulence factors. Three isolates carried PVL and other genes of the immune evasive complex and were not part of a genetic cluster but showed some genetic relation within the same branch. Four isolates carried *scn*, and one isolate carried *tst*. Of these 8 patients, only one had a known livestock contact and worked in a poultry slaughterhouse. Four patients had relocated from abroad or had been abroad recently, including two patients with isolates that lacked resistance genes for tetracycline that were detected during a routine adoption screening.

All isolates carried *mecA*, and 5 isolates missed *blaZ*. Tetracycline resistance genes were detected in 78 out of 80 isolates (*tetK* 47 isolates, *tetL* seven isolates, *tetM* 74 iolates, and *tetT* one isolate). A full overview of all virulence and resistance genes detected in the study isolates is provided in the Supplementary material.

Discussion

This study describes the molecular epidemiology of LA-MRSA in an urban region using genetic analysis based on whole-genome sequencing (WGS), a technique which has a high discriminatory power to distinguish closely related isolates in cases in which a

TABLE 2 Epidemiological background per cluster.

Genetic cluster	Epidemiological links/results of contact tracing
1	Outbreak in hospital with 9 out of 10 cases identified *via* contact tracing. The suspected index case was a nurse with atopic eczema. The positive cases detected *via* contact tracing were 6 patients and 2 of their household contacts. For one case, the route of transmission is unknown; this concerned a screening of an epidemiologically unrelated ICU patient waiting for an organ transplantation.
2	Nosocomial transmission, MRSA of index case detected in a screening prior to an orthopedic surgery and a positive contact identified *via* contact tracing. For the third patient, a dialysis patient who returned from a vacation abroad and was found to be colonized in a wound, the transmission route is unknown.
3, 5, 9	Isolates of same patient submitted by different healthcare institutions. Data are shown as a control group for the ability of cluster detection.
4, 8, 13	Epidemiological link and route of transmission unknown.
6, 10, 11, 12	Household members
7	Owner with feline pet animal. One unlinked case.

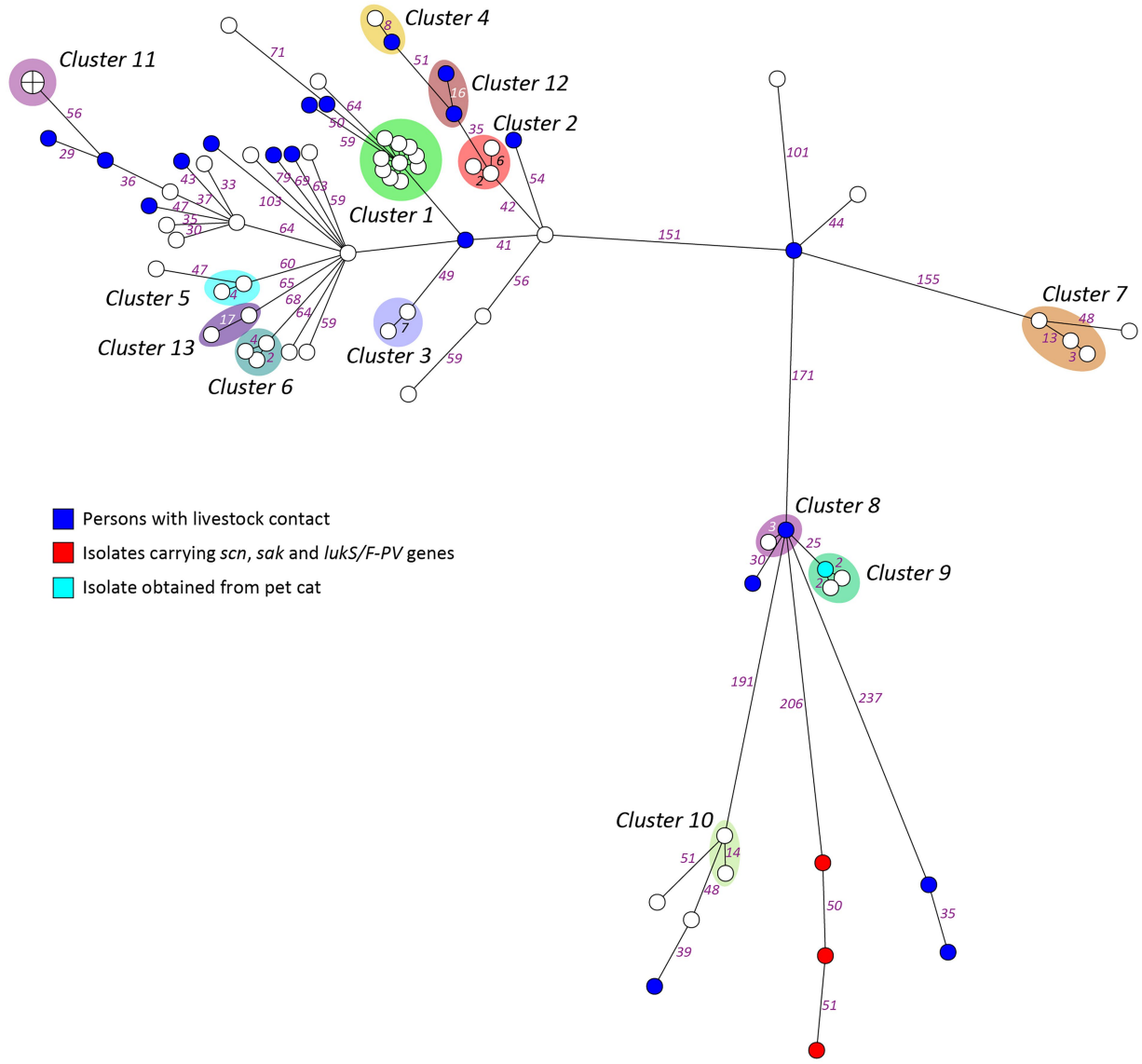


FIGURE 2
Minimum spanning tree based on wgMLST of the study isolates. Genetic distance is represented by the numbers on the connecting lines as the number of allelic differences in the wgMLST scheme.

single source or direct transmission is plausible. In our study population of 81 patients with LA-MRSA, 78% had no known livestock contact. The absence of this traditional risk factor led to a significant burden on Infection Control, with 14 contact tracing investigations due to unexpected MRSA findings, resulting in the identification of one large outbreak and a small cluster of nosocomial transmission. Three isolates carried genes coding for PVL production, and 98% of the isolates carried tetracycline resistance genes. Five genetic clusters included cases without a known epidemiological link.

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) carriage had a considerable impact on the patients included in our study. Almost a third of these patients had an LA-MRSA infection, and several of these infections ($n = 5$) required surgical intervention. Interestingly, the infection rate in our study population was higher than the infection rate in the national data. A limitation of the study is the missing data with regard to the presence of risk factors in the national surveillance, which makes comparison of risk factor occurrence difficult. The higher infection rate in the urban population may be due to selection bias, as patients with no risk factors will not receive screening cultures and thus remain unrecognized until an infection occurs. This would imply that the actual LA-MRSA occurrence might be even higher within the urban population than the incidence of 12% described in this study. Prolonged carriage of MRSA or failure of MRSA eradication was identified in 25% of the patients for whom data were available. More research is needed for a more

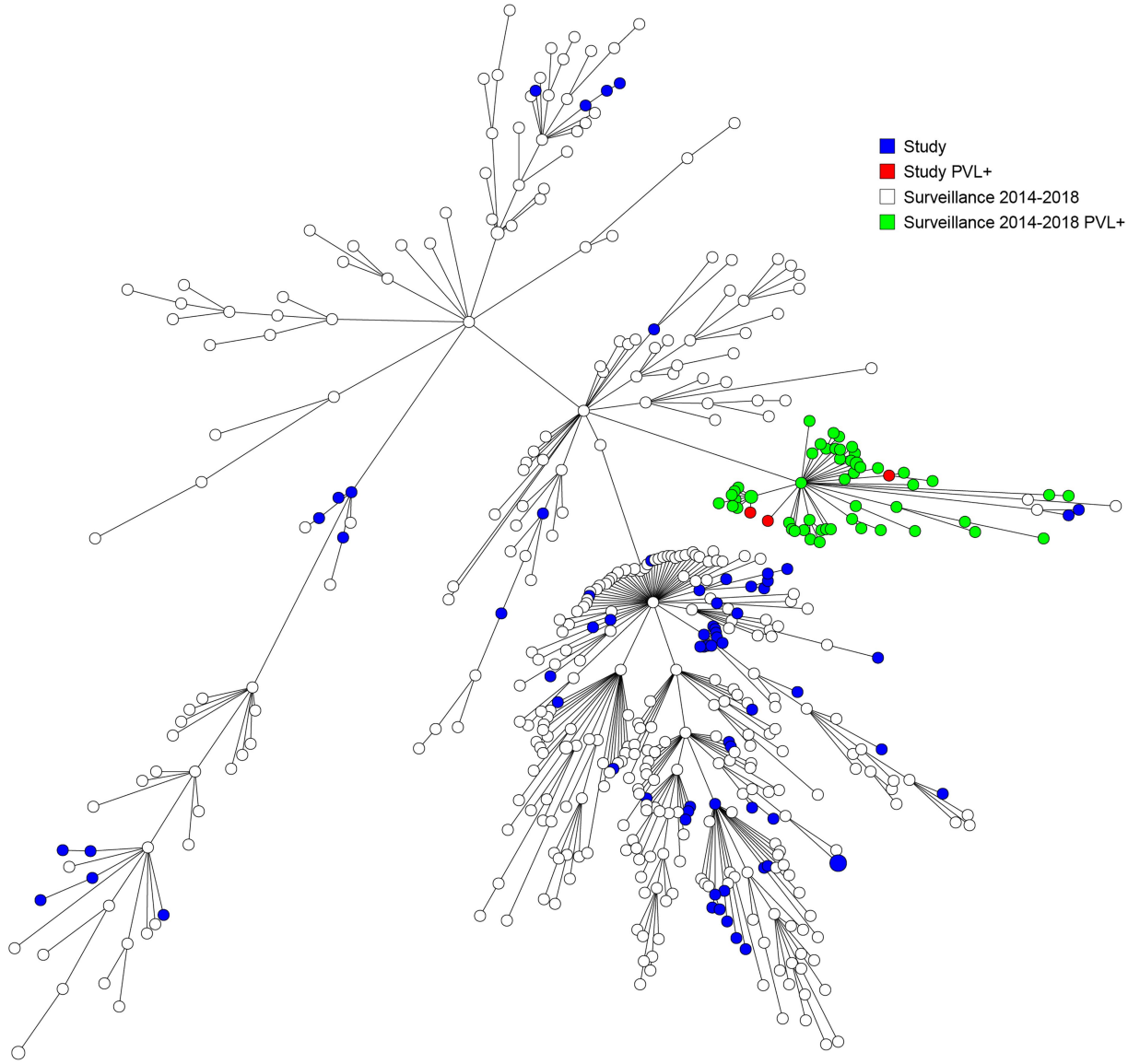


FIGURE 3
Minimum spanning tree based on wgMLST including both study and national surveillance isolates from 2014 till 2018.

complete understanding of carriage duration and eradication success rates, as this study was not designed to obtain complete follow-up data.

Whole-genome MLST identified 13 genetic clusters with allelic differences ranging from 0 to 17, and the cluster alert was set to 24. In our setting, the use of a cluster alert of up to 24 is informative. For the detection of transmission events within a short time span, such as outbreaks, other authors have suggested a threshold of up to eight allelic differences to define *S. aureus* transmission (Mellmann et al., 2016; Park et al., 2017). Indeed, most isolates in the large hospital outbreak genetic cluster in our study had 1 or 2 allelic differences, and only a single case had eight allelic differences. However, to understand the epidemiology of LA-MRSA, it is also relevant to detect transmission events over a prolonged period of time or indirect transmissions, which are likely to result in more allelic differences. We detected four genetic clusters above the threshold of eight allelic differences: three household clusters and one cluster with an unknown link. The slightly higher number of allelic differences could be attributed to the time that elapsed between the transmission event in the past and the moment of incidental discovery of carriage.

Our study population strains are a genetically diverse population that resembles the national LA-MRSA reservoir and shows no evidence of a dominant lineage. Our findings are in line with the outcomes of several studies in countries with intensive livestock farming that did not find any evidence that clonal expansion of specific sub-lineages is responsible for the increase of LA-MRSA cases among the general population. These authors suggested that the general population is consistently exposed to a random spillover of bacteria from livestock reservoirs, which are transmitted by human-to-human contact or *via*

environmental transmission routes (Lekkerkerk et al., 2015; Grøntvedt et al., 2016; Larsen et al., 2017; Sieber et al., 2018, 2019). It is of interest that in our study, half of the carriers of a specific so-called "human" and more virulent CC398 strain may have acquired it abroad, as four out of eight patients had a recent link to a foreign stay. However, more study is needed to establish if there is a genetic relatedness to strains such as the ones reported from Asia (Møller et al., 2019; Lu et al., 2021).

We found several instances of human-to-human transmission. This transmission route may explain the dissemination among the urban population, although the present study did not examine the potential role of environmental transmission routes. In view of the high percentage of LA-MRSA carriage in patients without a livestock link in our study, more research is needed to elucidate the exact transmission routes.

Conclusion

In the studied urban area in Netherlands, most of the detected LA-MRSA carriers did not have any livestock contact, while 12% of all MRSA strains were LA-MRSA as we observed human-to-human transmission, it does seem plausible that the livestock population acts as a reservoir of LA-MRSA and that continuous introduction of LA-MRSA to the human population with subsequent human-to-human transmission is the cause of urban human LA-MRSA acquisition. Contact tracings of incidental LA-MRSA findings revealed a hospital outbreak. Taking this into account, it is of concern that the present study identified PVL- and *scn/sak*-positive genetic lineages. To design potential interventions, it is important to closely monitor the occurrence of LA-MRSA and to further investigate transmission routes in urban areas. However, interventions may not be effective without eliminating the reservoir of LA-MRSA.

Data availability statement

The WGS data presented in the study are deposited in the European Nucleotide Archive repository. The accession numbers are included in the Supplementary material Data Sheet.

Author contributions

MMK, JG, TB, and LS: conception or design of the work. MMK, MK, SW, JaK, EM, DH, EE, JoK, VH, EC: data collection. MMK, LS, JG: data analysis and interpretation. MMK, LS: Drafting the article. Critical revision of the article and final approval of the version to be published. All authors contributed to the article and approved the submitted version.

Funding

Funding received from NL Ministerie van Volksgezondheid, Wetenschap en Sport for the costs involved in the laboratory analysis of the study.

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.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.875775/full#supplementary-material

References

Bisdorff, B., Scholhölter, J. L., Claußen, K., Pulz, M., Nowak, D., and Radon, K. (2012). MRSA-ST398 in livestock farmers and neighbouring residents in a rural area in Germany. *Epidemiol. Infect.* 140, 1800–1808. doi: 10.1017/S0950268811002378

Bosch, T., van Luit, M., Pluister, G. N., Frentz, D., Haenen, A., Landman, F., et al. (2016). Changing characteristics of livestock-associated meticillin-resistant Staphylococcus aureus isolated from humans-emergence of a subclade transmitted without livestock exposure, the Netherlands, 2003 to 2014. *Euro Surveill.* 21. doi: 10.2807/1560-7917.ES.2016.21.21.30236

Centraal Bureau van de Statistiek (n.d.). *Methodology—Degree of Urbanisation*. Available at: https://www.cbs.nl/en-gb/our-services/methods/definitions/degree-of-urbanisation (Accessed November 24, 2022).

Cuny, C., Nathaus, R., Layer, F., Strommenger, B., Altmann, D., and Witte, W. (2009). Nasal colonization of humans with methicillin-resistant Staphylococcus aureus (MRSA) CC398 with and without exposure to pigs. *PLoS One* 4:e6800. doi: 10.1371/journal.pone.0006800

ECDC (2013). *Livestock-associated Meticillin-resistant Staphylococcus aureus (LA-MRSA) Among MRSA From Humans Across the EU/EEA*. Sweden: ECDC Survey.

Fitzgerald, J. R. (2012). Livestock-associated Staphylococcus aureus: origin, evolution and public health threat. *Trends Microbiol.* 20, 192–198. doi: 10.1016/j.tim.2012.01.006

Grøntvedt, C. A., Elstrøm, P., Stegger, M., Skov, R. L., Skytt Andersen, P., Larssen, K. W., et al. (2016). Methicillin-resistant Staphylococcus aureus CC398 in humans and pigs in Norway: a "one health" perspective on introduction and transmission. *Clin. Infect. Dis.* 63, 1431–1438. doi: 10.1093/cid/ciw552

Köck, R., Schaumburg, F., Mellmann, A., Köksal, M., Jurke, A., Becker, K., et al. (2013). Livestock-associated methicillin-resistant Staphylococcus aureus (MRSA) as causes of human infection and colonization in Germany. *PLoS One* 8:e55040. doi: 10.1371/journal.pone.0055040

Larsen, J., Petersen, A., Larsen, A. R., Sieber, R. N., Stegger, M., Koch, A., et al. (2017). Emergence of livestock-associated methicillin-resistant Staphylococcus aureus bloodstream infections in Denmark. *Clin. Infect. Dis.* 65, 1072–1076. doi: 10.1093/cid/cix504

Larsen, J., Petersen, A., Sørum, M., Stegger, M., van Alphen, L., Valentiner-Branth, P., et al. (2015). *Meticillin-resistant Staphylococcus aureus CC398*

is an increasing cause of disease in people with no livestock contact in Denmark, 1999 to 2011. Eurosurveillance: bulletin europeen Sur les maladies transmissibles. *Euro. Surveill.* 20. doi: 10.2807/1560-7917.ES.2015.20.37.30021

Lekkerkerk, W. S., Sande-Bruinsma, N., van der Sande, M. A. B., Tjon-A-Tsien, A., Groenheide, A., Haenen, A., et al. (2012). Emergence of MRSA of unknown origin in the Netherlands. *Clin. Microbiol. Infect.* 18, 656–661. doi: 10.1111/j.1469-0691.2011.03662.x

Lekkerkerk, W. S., van Wamel, W. J. B., Snijders, S. V., Willems, R. J., van Duijkeren, E., Broens, E. M., et al. (2015). What is the origin of livestock-associated methicillin-resistant Staphylococcus aureus clonal complex 398 isolates from humans without livestock contact? An epidemiological and genetic analysis. *J. Clin. Microbiol.* 53, 1836–1841. doi: 10.1128/JCM.02702-14

Leopold, S. R., Goering, R. V., Witten, A., Harmsen, D., and Mellmann, A. (2014). Bacterial whole-genome sequencing revisited: portable, scalable, and standardized analysis for typing and detection of virulence and antibiotic resistance genes. *J. Clin. Microbiol.* 52, 2365–2370. doi: 10.1128/JCM.00262-14

Lu, H., Zhao, L., Si, Y., Jian, Y., Wang, Y., Li, T., et al. (2021). The surge of Hypervirulent ST398 MRSA lineage with higher biofilm-forming ability is a critical threat to clinics. *Front. Microbiol.* 12:636788. doi: 10.3389/fmicb.2021.636788

Matuszewska, M., Murray, G. G. R., Harrison, E. M., Holmes, M. A., and Weinert, L. A. (2020). The evolutionary genomics of host specificity in Staphylococcus aureus. *Trends Microbiol.* 28, 465–477. doi: 10.1016/j.tim.2019.12.007

McCarthy, A. J., van Wamel, W., Vandendriessche, S., Larsen, J., Denis, O., Garcia-Graells, C., et al. (2012). Staphylococcus aureus CC398 clade associated with human-to-human transmission. *Appl. Environ. Microbiol.* 78, 8845–8848. doi: 10.1128/AEM.02398-12

Mellmann, A., Bletz, S., Böking, T., Kipp, F., Becker, K., Schultes, A., et al. (2016). Real-Time Genome Sequencing of Resistant Bacteria Provides Precision Infection Control in an Institutional Setting. *J. Clin. Microbiol.* 54, 2874–2881. doi: 10.1128/JCM.00790-16

Møller, J. K., Larsen, A. R., Østergaard, C., Møller, C. H., Kristensen, M. A., and Larsen, J. (2019). International travel as source of a hospital outbreak with an unusual meticillin-resistant Staphylococcus aureus clonal complex 398, Denmark, 2016. *Euro Surveill.* 24. doi: 10.2807/1560-7917.ES.2019.24.42.1800680

Park, K. H., Greenwood-Quaintance, K. E., Uhl, J. R., Cunningham, S. A., Chia, N., Jeraldo, P. R., et al. (2017). Molecular epidemiology of Staphylococcus aureus bacteremia in a single large Minnesota medical center in 2015 as assessed using MLST, core genome MLST and spa typing. *PLoS One* 12:e0179003. doi: 10.1371/journal.pone.0179003

Schouls, L. M., Spalburg, E. C., van Luit, M., Huijsdens, X. W., Pluister, G. N., van Santen-Verheuvel, M. G., et al. (2009). Multiple-locus variable number tandem repeat analysis of Staphylococcus aureus: comparison with pulsed-field gel electrophoresis and spa-typing. *PLoS One* 4:e5082. doi: 10.1371/journal.pone.0005082

Sieber, R. N., Larsen, A. R., Urth, T. R., Iversen, S., Møller, C. H., Skov, R. L., et al. (2019). Genome investigations show host adaptation and transmission of LA-MRSA CC398 from pigs into Danish healthcare institutions. *Sci. Rep.* 9:18655. doi: 10.1038/s41598-019-55086-x

Sieber, R. N., Skov, R. L., Nielsen, J., Schulz, J., Price, L. B., Aarestrup, F. M., et al. (2018). Drivers and dynamics of methicillin-resistant livestock-associated Staphylococcus aureus CC398 in pigs and humans in Denmark. *MBio* 9. doi: 10.1128/mBio.02142-18

van Cleef, B. A. G. L., Broens, E. M., Voss, A., Huijsdens, X. W., Züchner, L., van Benthem, B. H. B., et al. (2010). High prevalence of nasal MRSA carriage in slaughterhouse workers in contact with live pigs in the Netherlands. *Epidemiol. Infect.* 138, 756–763. doi: 10.1017/S0950268810000245

van Cleef, B. A. G. L., van Benthem, B. H. B., Verkade, E. J. M., van Rijen, M., Kluytmans-van den Bergh, M. F. Q., Schouls, L. M., et al. (2014). Dynamics of methicillin-resistant Staphylococcus aureus and methicillin-susceptible Staphylococcus aureus carriage in pig farmers: a prospective cohort study. *Clin. Microbiol. Infect.* 20, O764–O771. doi: 10.1111/1469-0691.12582

van den Broek, I. V. F., van Cleef, B. A. G. L., Haenen, A., Broens, E. M., van der Wolf, P. J., van den Broek, M. J. M., et al. (2009). Methicillin-resistant Staphylococcus aureus in people living and working in pig farms. *Epidemiol. Infect.* 137, 700–708. doi: 10.1017/S0950268808001507

van Meurs, M. L., Schellekens, J. J. A., de Neeling, A. J., Duim, B., Schneeberger, P. M., and Hermans, M. H. A. (2013). Real-time PCR to distinguish livestock-associated (ST398) from non-livestock-associated (methicillin-resistant) Staphylococcus aureus. *Infection* 41, 339–346. doi: 10.1007/s15010-012-0319-5

Frontiers in Microbiology

Explores the habitable world and the potential of microbial life

The largest and most cited microbiology journal which advances our understanding of the role microbes play in addressing global challenges such as healthcare, food security, and climate change.

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