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# Modern molecular era of the mycobacterial world: Insights into diagnosis and transmission of mycobacteria and associated diseases

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# Modern molecular era of the mycobacterial world: Insights into diagnosis and transmission of mycobacteria and associated diseases

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# Editorial: Modern molecular era of the mycobacterial world: Insights into diagnosis and transmission of mycobacteria and associated diseases

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KEYWORDS

**mycobacteria, transmission, genotyping, drug resistance, immune response, treatment**

Editorial on the Research Topic

Modern molecular era of the mycobacterial world: Insights into diagnosis and transmission of mycobacteria and associated diseases

Mycobacterium is a genus of actinobacteria with its own family, the Mycobacteriaceae. There are 265 mycobacterial species in this genus that have been identified thus far (www.bacterio.net/mycobacterium.html accessed on 17th Feb 2023). The pathogens in the Mycobacterium (*M*) genus, including *M. TB* complex, *M. leprae*, slow-growing mycobacteria (SGM), and rapid-growing mycobacteria (RGM), are known to cause infections in humans (1–3).

Non-tuberculous mycobacteria (NTM) are widely distributed in the environment and can infect animals and humans, according to the published literature (4–6). Even though saprophytes make up the majority of NTM, about one-third of NTM have been linked to illnesses that affect humans (Ratnatunga et al.). It is still unclear exactly how these mycobacterial infections are transmitted. Understanding the dynamics of transmission of mycobacterial infections can help to plan a strategy to lessen the burden of mycobacteria and stop the spread of mycobacterial infection.

It has been noted that 10 million people fall ill with tuberculosis (TB) worldwide, and a total of 1.6 million people die from TB (7); thus, drug-resistant TB remains a public health crisis. *M. tuberculosis* is proficient in altering the immune response for its own survival, leading to disease or latent infection. The molecules and mechanisms utilized to accomplish the survival of bacteria inside the host are not fully understood. Early and effective treatment of these infections is very important to prevent transmission within the population. There is an urgent need for fast and reliable point-of-care (POC) diagnostic methods for effective case management. Currently, the used methods for screening and diagnosis are clinical, immunological, microscopic, radiographic, and bacterial culture. In

addition, recent advances in molecular diagnostic methods, including loop-mediated isothermal amplification (LAMP), line probe assay (LPA), cartridge-based nucleic acid amplification test (CBNAAT), fully automated (GeneXpert) platform, and whole-genome sequencing (WGS), have been employed to diagnose and characterize tuberculosis and non-tuberculosis infections. Another technique for classifying and identifying mycobacteria uses the mass spectrometer (MS) and matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) technologies.

The other main mycobacterial disease affecting humans is leprosy caused by *M. leprae*. The transmission of leprosy has not been understood fully. In recent advancements in molecular biology, the molecular marker has been used for the detection of the viability of *M. leprae* and transmission in the community.

This Research Topic presents original research articles and case studies that focused on recent developments in molecular biology and immunology in respect to diagnostic techniques, the transmission aspect of mycobacteria, the use of medication to treat mycobacterial infection, and the evaluation of drug resistance patterns, as well as the understanding of immune responses in the host against the bacteria, cell subsets at the local site, and how immune responses can stimulate the outcome of disease.

For NTM, Zhou et al. reported the first case of M. senegalense infection in a human after laparoscopic cholecystectomy was observed in China. M. senegalense infection in humans is extremely rare, especially in immunocompetent individuals. It is also difficult to detect M. senegalense infection because its symptoms are non-specific, and routine diagnostic tests are less sensitive. By using a molecular PCR followed by next-generation sequencing (NGS), the DNA sequences of M. senegalense in patient tissue samples were identified. M. senegalense infection was treated with quadruple therapy with clarithromycin, moxifloxacin, rifampicin, and oxycycline for 60 days, and it was noted that patient's wound healed remarkably.

In the field of tuberculosis, Mtafya et al. prospectively assessed the monitoring of the treatment response of a tuberculosis (TB) patient with TB bacteriology and novel tuberculosis–molecular bacterial load assay (TB-MBLA). The authors assessed bacteriologically confirmed cases for TB and assessed the symptoms and bacteriological resolution using smear microscopy, culture, and TB-MBLA over a six-month treatment. A decrease in TB-MBLA positivity reflected a fall in the bacillary load. Low-baseline bacillary load patients were more likely to be bacteriologically negative by month 2 and 6 of treatment, respectively. Mtafya et al. showed a high percentage agreement of clinical symptoms with bacteriological positivity for TB at diagnosis, which weakens rapidly during the early weeks of anti-TB therapy. These findings provide new evidence that relying solely on TB symptoms for diagnosis or monitoring may mislead the treatment decisions of some patients and the final treatment outcomes. These findings advocate for more investment in bacteriological tests to improve the accuracy of TB diagnosis and treatment monitoring in routine healthcare settings.

Wang et al. reported that prisons are regarded as a hotspot for TB disease and transmission, emphasizing that prevention strategies must focus on this hotspot to achieve the global target of ending TB (8). The present study observed a significant burden of TB in an incarcerated population in Qingdao, where 3.36% of inmates had pulmonary TB. The prevalence of TB found in this study was six times higher than the estimated prevalence for the general population (0.5% in 2010) (9). It is not only a risk to other prisoners and prison staff, but it also has the potential to put communities in danger of TB disease after their release from prison.

Worku et al. evaluated the drug sensitivity of M. tuberculosis and its association with the bacterial genotype and evaluated the performance of Xpert MTB/RIF (Xpert) in detecting resistance to rifampicin (RIF). The authors reported that the SIT 149 (T3-ETH) spoligotype was significantly associated with resistance to one or more drugs and multidrug resistance to TB, which was widespread in Ethiopia. The Xpert assay was observed to have high sensitivity and specificity in detecting rifampicin-resistant M. tuberculosis. Hence, this test can be applied widely.

Zhuang et al. analyzed the clinical characteristics of patients with central airway stenosis due to tuberculosis. The authors reported that despite receiving mostly adequate anti-tuberculosis chemotherapy, patients with TB can present with CASTB, involving severe scarring stenosis, bronchial occlusion, tracheobronchomalacia, and even destroyed lungs.

Sharma et al. evaluated the immune responses at the local site of infection and in the peripheral blood to improve the understanding of the immunological mechanisms involved in the containment and progression of TB. Significantly higher intracellular calcium levels, phosphorylation levels of ZAP-70, Erk1/2, and p-38 in CD3 and CD28 induced cells of pleural fluid as compared to the blood cells of the same patient with tuberculous pleural effusion (TPE). An alteration in the activation of the same events after stimulation with ESAT-6 and Ag85A was noted.

Adepoju et al. observed that frontline TB providers in private hospitals in Lagos struggled with assigning correct treatment outcomes for TB patients based on the NTBLCP guideline. The huge proportion of TB patients with missing end-of-treatment, month six follow-up results is of great concern. The result was a huge discrepancy between the reported and actual cure and completion rate data. Increased access to all the periodic follow-up AFB tests for TB patients on treatment and availability of the National TB Guideline for referencing could potentially improve the adherence of private TB service providers while assigning TB treatment outcomes.

In the field of leprosy, Turankar et al. reported that the molecular epidemiology of leprosy is important to study leprosy transmission dynamics and to enhance the understanding of leprosy in endemic areas by utilizing the molecular typing method. In this study, the authors observed the presence of viable M. leprae in inhabitant areas of leprosy patients. These viable bacilli might survive in the environment and might help to cause leprosy disease after repeated exposure to a susceptible host. A similar genotype in clinical and environmental samples indicates that the

environment could possibly act as a source of infection. The SNP and VNTR combination showed the M. leprae strain similarities and their differentiation in certain blocks of Purulia. Such studies with the combination of genetic markers may provide a tool to track the transmission link in the community.

## Conclusion

The collection of papers in this Research Topic provides insight into the challenges associated with the diagnosis, prevention, and management of mycobacterial diseases. The importance of recent molecular techniques used for the rapid diagnosis and identification of specific species and the transmission aspect of mycobacterial disease is highlighted, from the individual to the community level. The completion of treatment is essential to stop the transmission of the disease and to facilitate its proper management. The advancement in molecular technology and recognition of the molecular marker in the *M. leprae* genome has added to the value of tracking community transmission.

## Author contributions

RT and IS wrote this editorial and interpreted all the data, followed by analysis. ML and UG reviewed and edited the draft. All authors contributed to the article and approved the submitted version.

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## 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.

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## References

1. Brites D, Gagneux S. The nature and evolution of genomic diversity in the mycobacterium tuberculosis complex. *Adv Exp Med Biol* (2017) 1019:1–26. doi: 10.1007/978-3-319-64371-7_1

2. Porvaznik I, Solovič I, Mokrý J. Non-tuberculous mycobacteria: Classification, diagnostics, and therapy. *Adv Exp Med Biol* (2017) 944:19–25. doi: 10.1007/5584_2016_45

3. Euzéby JP. List of bacterial names with standing in nomenclature: A folder available on the internet. *Int J Syst Bacteriol* (2021) 47:590–2. doi: 10.1099/00207713-47-2-590

4. Malama S, Munyeme M, Mwanza S, Muma JB. Isolation and characterization of non-tuberculous mycobacteria from humans and animals in namwala district of Zambia. *BMC Res Notes* (2014) 7:622. doi: 10.1186/1756-0500-7-622

5. Singh V, Turankar RP, Goel A. Molecular characterization of environmental mycobacterial species from leprosy endemic tribal regions of north purulia district, West Bengal. *Int J Mycobacteriol* (2019) 8(4):381–9. doi: 10.4103/ijmy.ijmy_120_19

6. Turankar RP, Lavania M, Darlong J, Siva Sai KSR, Sengupta U, Jadhav RS. Survival of mycobacterium leprae and association with acanthamoeba from environmental samples in the inhabitant areas of active leprosy cases: A cross sectional study from endemic pockets of purulia, West Bengal. *Infect Genet Evol* (2019) 72:199–204. doi: 10.1016/j.meegid.2019.01.014

7. World Health Organization (WHO). *Global tuberculosis report*. Geneva: WHO/HTM/TB (2021).

8. Cords O, Martinez L, Warren JL, O'Marr JM, Walter KS, Cohen T, et al. Incidence, and prevalence of tuberculosis in incarcerated populations: A systematic review and meta-analysis. *Lancet Public Health* (2021) 6(5):e300–8. doi: 10.1016/S2468-2667(21)00025-6

9. Wang L, Zhang H, Ruan Y, Chin DP, Xia Y, Cheng S, et al. Tuberculosis prevalence in china 1990-2010; A longitudinal analysis of national survey data. *Lancet* (2014) 383(9934):2057–64. doi: 10.1016/S0140-6736(13)62639-2

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CASE REPORT
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# Case Report: *Mycobacterium senegalense* Infection After Cholecystectomy

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**Background:** *Mycobacterium senegalense* is a non-tuberculous mycobacterium and is found everywhere in the environment. However, *M. senegalense* infection in human is extremely rare, especially in immunocompetent individuals. It is difficult to detect *M. senegalense* infection because its symptoms are non-specific, and routine diagnostic tests are less sensitive. It is also resistant to commonly used antibiotics. Here, we report the first case of *M. senegalense* infection after laparoscopic cholecystectomy in China.

**Case Presentation:** A 55-year-old man was admitted because of repeated infections at multiple incision sites for more than 1 year. Although routine diagnostic test results were negative, metagenomic next-generation sequencing (mNGS) identified DNA sequences of *M. senegalense* in tissue samples from incision sites. The presence of *M. senegalense* was further confirmed by polymerase chain reaction and capillary electrophoresis. After 60 days of quadruple therapy with clarithromycin, moxifloxacin, rifampicin, and oxycycline, the patient's wound healed.

**Conclusion:** We believe the case findings contribute to the limited amount of knowledge about *M. senegalense* infection and raises awareness that this infection can result in poor wound healing, even in an immunocompetent host. Owing to a lack of early, precise diagnosis, it is difficult to treat *M. senegalense* infections. Based on our findings, mNGS is a sensitive diagnostic test for *M. senegalense* infections.

**Keywords:** ***Mycobacterium senegalense*, infection, non-tuberculous mycobacterium, metagenomic next-generation sequencing, case report**

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## INTRODUCTION

*Mycobacterium senegalense* is a non-tuberculous mycobacterium (NTM) that was first reported to cause bovine farcy in sub-Saharan Africa, exhibiting chronic granulomatous inflammation of skin lymphatics and draining lymph nodes of zebu cattle (1). However, human infections with *M. senegalense* are rare, and its zoonotic potential is unknown.

NTM is a complex pathogen that is usually a commensal or saprophytic organism. It is extensively found in the environment such as in soil and water sources, leading to high human–pathogen exposure (2), although only a few species infect humans. NTM is an opportunistic

pathogen that usually infects immunocompromised patients with intractable conditions such as those with underlying lung diseases, human immunodeficiency virus infections, and cancers, as well as those receiving chemotherapy (3). NTM can be divided into two groups based on how long they take to grow in a culture: rapid-growing and slow-growing species. The most common pathogen among the slow-growing NTM species is *M. avium-intracellulare*, which primarily causes pulmonary infection (4). *M. fortuitum* is the most common pathogen among the rapid-growing NTM species, which usually causes colonized or transient infections in the respiratory tract, in addition to disseminated disease, lymphadenitis, and skin and soft tissue infections (5, 6). Although *M. senegalense* belongs to the same group as *M. fortuitum*, it rarely infects humans.

So far, only seven cases of humans being infected by *M. senegalense* have been described; however, none of them appear to have occurred in China. In this study, we report the first case of *M. senegalense* infection after laparoscopic cholecystectomy in China. mNGS test identified that the patient was infected with *M. senegalense*, even though routine diagnostic tests were negative. The patient's condition improved, and the wound healed after quadruple therapy with clarithromycin, moxifloxacin, rifampicin, and doxycycline.

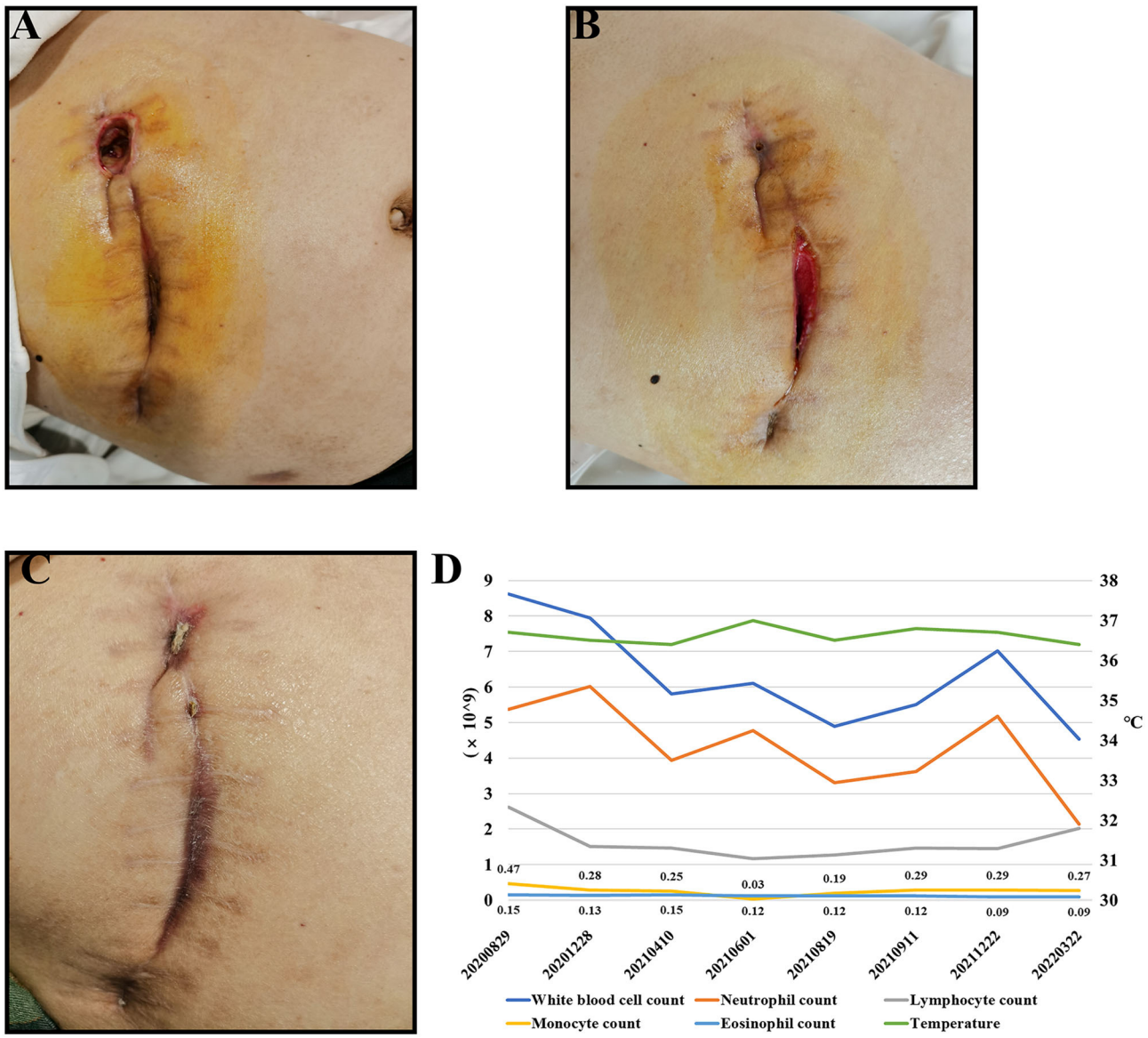


**FIGURE 1 |** Condition of the patient's incisions. **(A)** It shows the incisions of the patient on admission. **(B)** It shows the incisions of the patient after second surgery. **(C)** It shows the patient's incisions after 60 days of quadruple therapy. **(D)** The patient's temperature and routine blood test results.


**FIGURE 2 | (A)** Abdominal B-ultrasound results show the formation of sinus tract and empyema. **(B)** Chest computed tomography images of the lung window show no sign of tuberculosis-related diseases.

# MANUSCRIPT FORMATTING

## Case Presentation

A 55-year-old male patient, who present to our hospital in August 2021, because of repeated infection at multiple incisions for more than 1 year after laparoscopic cholecystectomy surgery done for gallstone complicated with cholangitis. On examination, the incisions under the xiphoid process were red and swollen, the incisions were dehiscent, local tenderness, high skin temperature and a little suppuration (**Figure 1A**).

The patient had multiple incisions infections and repeated purulent exudates after surgery, with an average recurrence about 25 days, but the blood routine results shown no abnormality. According to the B-ultrasound results (**Figure 2A**), considering the formation of sinus tract under xiphoid process. In September 2021, laparoscopic re-surgery and abdominal exploration were performed, the sinus trace was cut and drainage of abscess. Then given piperacillin sodium and tazobactam sodium anti-infection treatment, but the effect was still poor after a period of time. We discovered the surgical incisions still were not healing, the skin surrounding the incisions was red, swollen and painful, and suppuration was present (**Figure 1B**).

Since admission, the patient's body temperature and blood routine results were normal (**Figure 1D**). Additionally, regular bacterial culture and acid-fast bacillus (AFB) staining were negative, but blood samples for the tubercle bacillus antibody (TB-Ab) test were positive. Combined with the patient's chest CT (**Figure 2B**), tuberculosis related examination and the patient's clinical symptoms, they did not support tuberculosis-related diseases. In order to further resolve the question and determine the pathogens, the mategenomic sequencing technology covering the pathogen is used to accurately identify pathogens.

On November 29, 2021, the tissue from the incisions of the patient was sampled for DNA metagenomic next-generation sequencing (mNGS) (KingMed Diagnostics, Changsha, China). It detected 53 sequences that could be mapped to *M. senegalense* in a total of 113 sequences, and the coverage was 0.09%, making up 58.76% of the total microbe sequences (**Table 1**). Targeted PCR of *M. senegalense* using two pairs of primers was applied: 16S RNA forward 5′-AGCG GCGGAGCATGTGGATTA-3′, reverse 5′-GCTGATCTGCGA TTACTAGCGACTC-3′ (GenBank: DQ145802.1); rpoB forward 5′-TGCGTGCCATCTTCGGTGAGA-3′, reverse 5′-GTCG ATGTTCCAGCCTGCCTTG-3′ (GenBank: JF706631.1). The primers were designed and verified using Primer-BLAST based on the reference genome sequence of *M. senegalense* in NCBI. Subsequently, the capillary electrophoresis technique (Qsep 100TM; Bioptic) also curtained the *M. senegalense* infection (**Figure 3**).

According to the results, the patient was initiated with oral clarithromycin (500 mg, twice daily), moxifloxacin (400 mg, once daily), rifampicin (450 mg, once daily) and doxycycline (100 mg, once every 12 hours). Then the swelling gradually subsided and there was no obvious purulent exudation. After 20 days, the patient's incisions healed well, and there was no sign of recurrence in the 60th day after quadruple therapy (**Figure 1C**).

# DISCUSSION

We present a unique case of a patient with chronic incision site infections who underwent laparoscopic cholecystectomy and re-surgery, after which he was diagnosed as having an infection caused by an unusual NTM species.

Rapid-growing NTM species, such as *M. fortuitum,* have increasingly gained recognition during the last two decades because of their ability to thrive even in the harshest environments. In an immunosuppressed patient, these pathogens cause serious infections, such as bacteremia (5). *M. senegalense* is a rapid-growing NTM, belongs to the same group as *M. fortuitum*, and was first isolated from a bovine source (6). Infection with *M. senegalense* affects the sinus tracts and is

**TABLE 1 |** mNGS results of the tissue and exudate from the punctures in this case.

| Genus | | | Species | |
|---|---|---|---|---|
| **Generic name** | **Relative abundance (%)** | **Number of sequences** | **Species name** | **Number of sequences** |
| *Mycobacterium* | 58.76% | 113 | *Mycobacterium senegalense* | 53 |

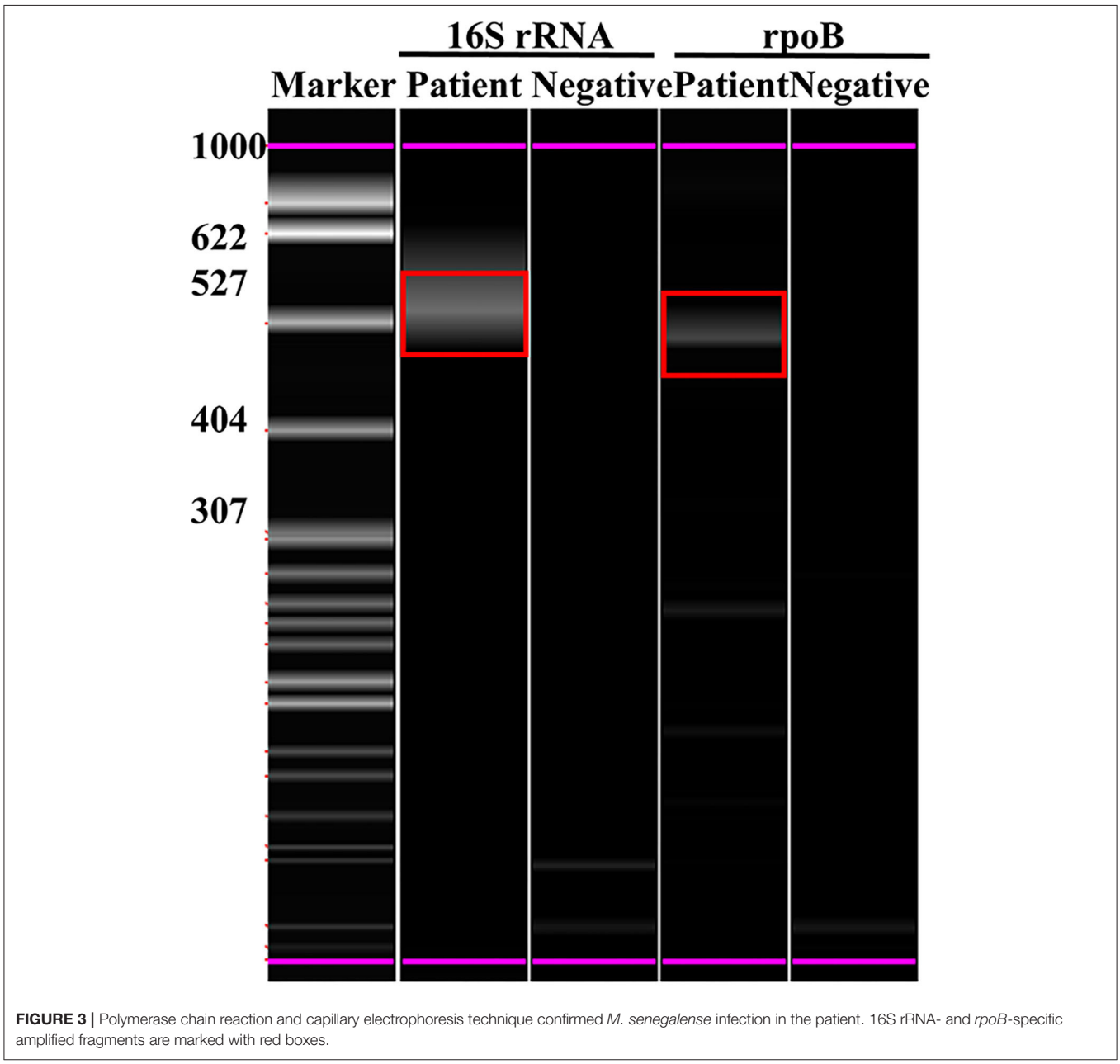


**FIGURE 3 |** Polymerase chain reaction and capillary electrophoresis technique confirmed *M. senegalense* infection in the patient. 16S rRNA- and *rpoB*-specific amplified fragments are marked with red boxes.

characterized by multiple abscesses and granuloma formation. Only 7 cases about human infection with *M. senegalense* have been reported, the recent report found it to cause skin infections in immunocompetent patients (7). Most patients had minimal history of contact with animals and travel to areas where *M. senegalense* infection was more commonly endemic (8–10). And most of those cases were identified by various sequencing techniques. The first case of human infection by

*M. senegalense* was described in 2005 (11). The patient had non-Hodgkin's lymphoma and was treated with R-CHOP. Her physical examination revealed high fever of 39.8°C, although she had no contact with any animal- or bovine-specific sources. Although it was difficult to diagnose, 16S rRNA gene, *rpoB*, and 16S-23S rRNA gene internal transcribed spacer sequence analyses finally revealed *M. senegalense* infection. Another case reported *M. senegalense* infection in a healthy girl with no prior history of serious illness or hospitalization (12). The patient was scratched by fish tank debris, following which the wound was sterilized and sutured. After 2 weeks, the wound failed to heal and appeared reddened and indurated. 16S gene sequencing and biochemical testing was used to diagnose the *M. senegalense* infection, which was the first infection reported in humans without immunodeficiency. This also indicated that immunocompetent individuals were also at a risk of infection.

It is worth mentioning that the patient had no history of contact with animals, and the patient's body temperature, routine blood test results, and other data indicated that infection was absent. These indicators were not present in previous cases, suggesting that *M. senegalense* may not cause changes in infection indicators. According to current literature, the prevalence and incidence of NTM infections continue to increase (11, 13). Rapid identification and subsequent drug susceptibility testing are essential for sleeting appropriate antibiotics against NTM. In addition, it is necessary to be vigilant about NTM infections, even if individuals have no contact history with animals, no travel history, and no infection-negative routine blood test results with recurrent infections at laparoscopic incision sites.

Because of the scarcity of literature on *M. senegalense*, we draw on treatment guidelines for NTM infections (14, 15). From the literature review, we found that the management of NTM infections is quite difficult. First, NTM infections possess several internal antimicrobial resistance mechanisms such as an impermeable cell wall and formation of granulomas, which reduces antimicrobial influx. Second, the long-term treatment of NTM infections leads to the development of drug resistance (16). According to the American Thoracic Society, Infectious Diseases Society of America, and Clinical and Laboratory Standards Institute, the latest recommended NTM drugs include macrolides, clofazimine, quinolones, sulfonamides, linezolid, aminoglycosides, bedaquiline, and tetracyclines (17). Additionally, the success rate of treatment for NTM infection is species specific. Usually, a combination of three or four drugs are administered to avoid the development of resistance to monotherapy. Based on previous experience combined with this patient's condition, we administered clarithromycin (500 mg, twice daily), moxifloxacin (400 mg, once daily), rifampicin (450 mg, once daily), and doxycycline (100 mg, once every 12 h). With this combination of therapy, the patient achieved good clinical efficacy. The case report provides a reference for the treatment of *M. senegalense*.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

HY, FLG, and SLZ collected and analyzed patient data. HLZ, HY, YFY, and HDL wrote the manuscript. HDL and JJL provided supervision. All authors reviewed, edited, and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

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## REFERENCES

1. Hamid ME. Current Perspectives on Mycobacterium farcinogenes and Mycobacterium senegalense, the Causal Agents of Bovine Farcy. *Vet Med Int.* (2014) 2014:247906. doi: 10.1155/2014/247906
2. Falkinham JO 3rd. Environmental sources of nontuberculous mycobacteria. *Clin Chest Med.* (2015) 36:35–41. doi: 10.1016/j.ccm.2014.10.003
3. Falkinham JO 3rd. Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *J Appl Microbiol.* (2009) 107:356–67. doi: 10.1111/j.1365-2672.2009.04161.x
4. Daley CL, Iaccarino JM, Lange C, Cambau E, Wallace RJ Jr, Andrejak C, et al. Treatment of nontuberculous mycobacterial pulmonary disease: an official ATS/ERS/ESCMID/IDSA clinical practice guideline. *Eur Respir J.* (2020) 56:2000535. doi: 10.1183/13993003.00535-2020
5. Schinsky MF, McNeil MM, Whitney AM, Steigerwalt AG, Lasker BA, Floyd MM, et al. Mycobacterium septicum sp. nov., a new rapidly growing species associated with catheter-related bacteraemia. *Int J Syst Evol Microbiol.* (2000) 50 (Pt 2):575–81. doi: 10.1099/00207713-50-2-575
6. Mohan K. Mycobacterium senegalense from bovines in Eastern Nigeria. *J Appl Bacteriol.* (1985) 59:277–81. doi: 10.1111/j.1365-2672.1985.tb01789.x

7. Cheng AY, Lee CH. Skin infection by Mycobacterium farcinogenes-senegalense group in an immunocompetent patient: a case report. *BMC Infect Dis.* (2022) 22:445. doi: 10.1186/s12879-022-07409-z
8. Bugeja A, Hae R, Rajda E, Clark EG, Akbari A, Fairhead T, et al. A living donor kidney transplant recipient with mycobacterium senegalense bacteremia: a case report. *Transpl Infect Dis.* (2021) 23:e13596. doi: 10.1111/tid.13596
9. Santos-Perez JL, Delgado-Mainar P, Toro-Rueda C, Baquero-Artigao F. Surgical site infection by Mycobacterium senegalense in a pediatric patient. *Enferm Infecc Microbiol Clin (Engl Ed).* (2021) 39:259–61. doi: 10.1016/j.eimce.2021.02.004
10. Carretero O, Reyes C, San-Juan R, Chaves F, Lopez-Roa P. Mycobacterium senegalense infection after implant-based breast reconstruction, Spain. *Emerg Infect Dis.* (2020) 26:611–3. doi: 10.3201/eid2603.190230
11. Oh WS, Ko KS, Song JH, Lee MY Ryu SY, Taek S, et al. Catheter-associated bacteremia by Mycobacterium senegalense in Korea. *BMC Infect Dis.* (2005) 5:107. doi: 10.1186/1471-2334-5-107
12. Talavlikar R, Carson J, Meatherill B, Desai S, Sharma M, Shandro C, et al. Mycobacterium senegalense tissue infection in a child after fish tank exposure. *Can J Infect Dis Med Microbiol.* (2011) 22:101–3. doi: 10.1155/2011/206532
13. Furuuchi K, Morimoto K, Yoshiyama T, Tanaka Y, Fujiwara K, Okumura M, et al. Interrelational changes in the epidemiology and clinical features of nontuberculous mycobacterial pulmonary disease and tuberculosis in a referral hospital in Japan. *Respir Med.* (2019) 152:74–80. doi: 10.1016/j.rmed.2019.05.001
14. Quang NT, Jang J. Current molecular therapeutic agents and drug candidates for mycobacterium abscessus. *Front Pharmacol.* (2021) 12:724725. doi: 10.3389/fphar.2021.724725
15. Kumar K, Daley CL, Griffith DE, Loebinger MR. Management of Mycobacterium avium complex and Mycobacterium abscessus pulmonary disease: therapeutic advances and emerging treatments. *Eur Respir Rev.* (2022) 31:210212. doi: 10.1183/16000617.0212-2021
16. Tarashi S, Siadat SD, Fateh A. Nontuberculous mycobacterial resistance to antibiotics and disinfectants: challenges still ahead. *Biomed Res Int.* (2022) 2022:8168750. doi: 10.1155/2022/8168750
17. Brown-Elliott BA, Nash KA, Wallace RJ Jr. Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. *Clin Microbiol Rev.* (2012) 25:545–82. doi: 10.1128/CMR.05030-11


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# Drug sensitivity of clinical isolates of *Mycobacterium tuberculosis* and its association with bacterial genotype in the Somali region, Eastern Ethiopia

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**Background:** Drug resistance is becoming a major bottleneck for tuberculosis (TB) control programs in countries with high TB burdens. Although several studies were conducted on the drug sensitivity of *Mycobacterium tuberculosis* (*M. tuberculosis*) in central Ethiopia, there is a lack of data on the drug sensitivity of *M. tuberculosis* in the peripheral regions of the country including in the Somali region. Therefore, the objective of this study was to evaluate the drug sensitivity of *M. tuberculosis* and its association with bacterial genotype and evaluate the performance of Xpert MTB/RIF (Xpert) in detecting resistance to rifampicin (RIF).

**Methods:** A total of 302 *M. tuberculosis* were tested using the BD BACTEC-Mycobacteria Growth Indicator Tube 960 (MGIT 960) system for their drug sensitivity to the first-line anti-TB drugs. Besides, the drug sensitivity of 10 multidrug-resistant (MDR) *M. tuberculosis* isolates was evaluated for the second-line anti-TB drugs. Additionally, 177 of the 302 isolates were tested for genotypic drug resistance using Xpert. Chi-square and Fisher's exact tests were used for the evaluation of the association between variables and drug sensitivity.

**Results:** The overall prevalence of resistance to at least one drug was 11.6% (95% CI: 7.9–15.2%), while the prevalence of MDR was 3.3% (95% CI: 1.3–5.3%). Two of the 10 MDR isolates were resistant to capreomycin. The spoligotype Shared International Type (SIT) 149 was significantly associated with either monoresistance or MDR ($p < 0.05$). Of the 177 isolates tested by Xpert, 6.2% (11/177) were RIF-resistant. Discordant between Xpert and MGIT 960 was observed in one isolate and linked with probe-binding delay ($\Delta$CT max = 5.8).

The sensitivity and specificity of the Xpert assay were 100 and 99.4%, respectively, while its positive and negative predictive values were 90.9 and 100%, respectively.

**Conclusion:** The magnitude of MDR *M. tuberculosis* in the Somali region of Ethiopia was higher than the national prevalence of MDR-TB warranting the strengthening of the TB control program in the Somali region. Besides, drug resistance was associated with SIT 149 spoligotype (genotype). The Xpert assay was observed to have high sensitivity and specificity in detecting RIF-resistant *M. tuberculosis*, which is encouraging for its application widely.

KEYWORDS

**bacterial genotype, drug sensitivity, mycobacterium tuberculosis, performance of Xpert, Somali region of Ethiopia**

## Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* complex (MTBC) and affects all parts of the body although it primarily affects the lungs. TB is a communicable disease that is a major cause of illness and one of the leading causes of death worldwide; it used to cause the largest death from a single infectious agent until the coronavirus 2019 (COVID-19) pandemic. According to the latest report of WHO, there were 9.9 million cases and 1.5 million deaths due to TB in 2020 (1).

Tuberculosis is both curable and preventable, and as such a 6-month anti-TB drug regimen can successfully treat 85% of patients with TB (1). Drug resistance to anti-TB drugs emerged shortly after the introduction of the first drug streptomycin (STM) in clinical use (2). In recent years, resistance to isoniazid (INH) and rifampicin (RIF), the two most effective first-line treatments, is of particular concern; resistance to both drugs is defined as multidrug-resistant (MDR)-TB. MDR-TB and RIF-resistant TB (RR-TB) both require second-line treatment. Extensively drug-resistant TB (XDR-TB) is defined as MDR-TB strains, which are resistant to any fluoroquinolones and at least one additional Group A drug [levofloxacin (LEV), moxifloxacin, bedaquiline, and linezolid (LZD)] (3). Pre-extensively drug-resistant TB (Pre-XDR TB) is the MDR-TB strain that is resistant to any fluoroquinolones (3). In 2020, WHO estimated that of the 132,222 cases of MDR-TB, 25,681 were either XDR or pre-XDR-TB (1). The proportion of patients with MDR who were diagnosed with TB for the first time was around 3–4%, while for those who had previously been treated for TB was around 18–21% (1).

Although TB can be treated, detecting the disease is not always easy, and drug resistance is becoming more common (1). The development of rapid and accurate TB identification methods, particularly for smear-negative TB and MDR-TB, is critical for improving cure rates, reducing drug resistance and treatment failure, and preventing TB transmission (4). The nucleic acid amplification technique (NAAT) is now widely available to detect MTBC and drug resistance mutations from culture isolate or direct patient specimens. In 2010, the WHO endorsed the use of the GeneXpert platform Xpert MTB/RIF (Xpert) (Cepheid, Sunnyvale, CA, United States) assay, a modern, rapid, automated, cartridge-based NAAT that can simultaneously identify TB and RIF resistance, which is a surrogate marker for MDR-TB in resource-limited settings where phenotypic drug sensitivity tests (DSTs) are available only in reference laboratories (5, 6).

Genotyping techniques have greatly improved our understanding of TB epidemiology and have been used to guide disease control efforts. Based on molecular epidemiology technologies, some studies have found that drug resistance in *M. tuberculosis* is associated with a particular genotype (7). Several studies suggested that *M. tuberculosis* Beijing lineage may be associated with drug-resistant TB compared with the strain of other lineages (8–11). It is also been speculated that this association might be circumstantial since Beijing strains are more prevalent in places of the world where MDR-TB has emerged as a result of inadequate TB treatment. Furthermore, if Beijing strains spread more quickly than other lineages, it appears like they have more easily acquired mutations in MDR-TB hotspots (12).

In Ethiopia, TB remains one of the leading public health problems claiming the lives of thousands of Ethiopians every year. Ethiopia is among the 30 high TB burden and 30 high TB/HIV burden countries in the world. In the WHO global report from 2021, Ethiopia has been removed from the list of countries with a high MDR-TB burden (1). The first study performed on resistance to anti-TB drugs was in 1981 in Addis Ababa. In that study, there was no MDR-TB, while the first MDR-TB in Ethiopia was reported in 1986 with a prevalence of 1.1% (13). The first national anti-TB drug resistance study was conducted in 2005; MDR-TB was detected in 1.6 % of the newly diagnosed and 11.8 % of previously treated TB cases

(14). According to the 2020 WHO global report, Ethiopia was among the high MDR-TB burden countries, with an estimated incidence of MDR/RR TB in 0.71% of new cases and 12% of previously treated cases in 2019 (15). The majority of TB drug resistance studies were conducted in only a few areas of the country and did not include marginalized pastoral areas such as the Somali region. Nonetheless, the less developed health infrastructure in pastoralist communities and the poor compliance with treatment due to patients' nomadic lifestyles contribute to the occurrence of MDR-TB in the Somali region (16). Therefore, the objective of this study was to evaluate the drug sensitivity profiles of *M. tuberculosis* strains using Xpert and MGIT 960 and to assess the association between drug resistance and the genetics of *M. tuberculosis* in the Somali region, Ethiopia.

# Methodology

## Study area and setting

This study was carried out in Jigjiga City, the capital of the Somali region, Ethiopia, the second largest region in the Federal Democratic Republic of Ethiopia. More than 83% of the population is rural and leads a pastoral or agro-pastoral way of life, with livestock serving as their primary source of income (17). The Somali region has long been facing unrest and instability that has severely hampered the government's capacity to provide basic social services to rural communities (18).

Patients with TB were recruited from three health facilities including Abilelie Health Center, Karamara Regional Hospital, and Jigjiga University Shiek Hassan Yabare Referral Hospital. These facilities were chosen as they represent the region's largest centers for TB diagnosis and treatment. Basic TB diagnostic facilities, such as smear microscopy, X-ray, and Xpert, were available in these facilities; however, only smear microscopy was available in the health center, and pathology laboratory service was only available at Jigjiga University Shiek Hassan Yabare Referral Hospital.

## Study design and study subjects

A health facility-based cross-sectional study was conducted on 336 patients with pulmonary and 156 patients with extrapulmonary TB visiting three selected health institutions in Jigjiga town between October 2018 and December 2019. The study subjects were bacteriologically confirmed patients with pulmonary TB and clinically suspected patients with extrapulmonary TB aged $\geq$15 years. Each patient provided informed consent to be included in this study. Patients who were unable to produce sputum and were less than 15 years were excluded from this study. Sputum and fine needle aspirates were collected and cultured from all patients with pulmonary TB and extrapulmonary TB, respectively. Culture positivity was confirmed in 323 samples of which 302 were successfully tested for drug sensitivity. Additionally, 177 of the 302 isolates were tested by Xpert, as part of a routine diagnosis for TB and RIF sensitivity test. In connection, the performance of Xpert in detecting RIF resistance was evaluated using MGIT 960 system as the gold standard.

## Sample collection and processing

Sputum samples were collected from each study participant by trained laboratory personnel, as recommended by WHO (19), and acid-fast bacilli or Xpert positive was stored in the refrigerator at $-20^{\circ}$C at the sample collection site. Fine needle aspirates (FNAs) were collected and examined for cytology by an experienced pathologist under an aseptic procedure. The cytological diagnosis was performed on the first few drops of the aspirates, and the rest was stored in the refrigerator at $-20^{\circ}$C at the sample collection site. All sputum specimens and FNA were transported in a packed ice box to the TB laboratory at the Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University (AAU) at $+4^{\circ}$C and stored at $-80^{\circ}$C until further processing for culture. A structured questionnaire was used to collect patient variables including socio-demographic factors. Questionnaires were filled by trained nurses and laboratory technologists at each health institute.

## Xpert MTB/RIF

Xpert was performed at Karamara Hospital according to the manufacturer's instructions. A volume of 3–4 ml of sputum was mixed with sample reagent buffer (included in the kit) in 1:2 ratio, the tubes were vigorously agitated two times, and the tubes were left for 15 min at room temperature. The inactivated mixture was then transferred to the test cartridge with a sterile disposable pipette. The GeneXpert assay instrument was loaded with cartridges and generated results in $<$2 h. The trained laboratory personnel operated the Xpert modules and cartridges, which included specimen processing, invalid results handling, and standard protocol interpretation (5, 20).

The Xpert assay amplifies the 81-base pair portion of the *rpoB* (RNA polymerase enzyme $\beta$-subunit gene known as Rifampicin Resistance Determining Region (RRDR) and uses five probes (labeled A–E) to detect amplification. To signal "MTB detection," at least two of five probes must be positive within a certain cycle threshold (Ct) window. Xpert assay additionally signals RR when probe dropout (no hybridization) or delayed Ct ($\Delta$Ct max is $>$4.0) of one to three probes occurs. The $\Delta$Ct max was calculated using the difference between the earliest (first) and latest (last) Ct across the five probes (21).

## Culture and identification

All samples were processed and cultured at ALIPB, AAU according to the Petroff technique (22). The specimens were decontaminated using an equal amount of 4% NaOH stock solution and sample and centrifuged at 3,000 rpm for 15 min. Then, the supernatant was discarded, and the sediment was neutralized with 2N HCl and finally inoculated onto two Löwenstein–Jensen (LJ) egg slant medium containing 0.6% sodium pyruvate and the other 0.75 % glycerol. The slants were incubated for at least 8 weeks with weekly observation for the presence of mycobacterial colonies. *M. tuberculosis* was identified from other members of the MTBC species using region of difference (RD)-9 polymerase chain reactions (PCR) from heat-killed cells (23). Spoligotyping was performed to characterize all isolates, as described previously by Kamerbeek (24). In brief, the primers DRa (biotinylated at the 5'end) and DRb were used to amplify the direct repeat (DR) region using the PCR machine. The amplified products were hybridized with a series of 43 immobilized oligonucleotides covalently bound to a membrane, each of which corresponds to a distinct spacer DNA sequence found within the DR locus of MTBC. Hybridized DNA was identified using the enhanced chemiluminescence technique after incubation with streptavidin-peroxidase, which binds to the biotin label on the PCR product, and exposure to X-ray film in accordance with the manufacturer's instructions. The presence and absence of spacers were represented on the X-ray film by squares that were black or white, respectively. *M. tuberculosis* H37Rv and *Mycobacterium bovis* strains were used as positive controls, while distilled water was used as the negative control. The spoligotype patterns were converted into binary and octal formats and entered into the online spoligotype database to determine SIT number, and the results were compared with already existing designations in the international spoligotyping database (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/).

## Phenotypic drug sensitivity tests

A drug sensitivity test was performed at National Tuberculosis Reference Laboratory, Ethiopian Public Health Institute (EPHI). The BD BACTEC-MGIT 960 system was used to test four first-line drugs using the SIRE kit: STM, INH, RIF, and ethambutol (EMB). All MDR-TB isolates were tested to second-line DST using MGIT 960 systems. The primary isolates were diluted to 1:100 before DST was conducted, and DST was performed within 1–5 days of a positive growth result. The final drug concentrations for first-line drugs STM, INH, RIF, and EMB were 1.0, 0.1, 1, and 5.0 μg/ml, respectively. The final drug concentration for second-line drugs were amikacin (AMK) 1.0 μg/ml, kanamycin (KAN) 2.5 μg/ml, capreomycin (CAP) 2.5 μg/ml, ofloxacin (OFX) 2 μg/ml, LEV 1.5 μg/ml, clofazimine (CFZ) 1 μg/ml, and LZD 1 μg/ml. A growth control tube was used for each isolate, containing a growth supplement but no drug. The MGIT 960 system's software algorithm determined the relative growth ratio between the drug-containing tube and the drug-free growth control tube. Then, the instrument automatically determines the final interpretation and susceptibility results. If 1% or more of the test population grows in the presence of the critical concentration of the drug, the isolate is considered resistant (25).

## Line probe assay

Line probe assay (LPA) (Genotype MTBDR*plus*) was performed to further investigate the discordant result between Xpert and MGIT 960 according to the manufacturer's instructions in the EPHI laboratory (26).

## Data analysis

All components of the data were entered and analyzed using SPSS 20 computer software. Descriptive statistics were used to depict the socio-demographic features. The relationship between drug resistance and bacterial and host-related factors was analyzed using Pearson's $\chi^2$ test or Fisher's exact test as appropriate. MedCalc Statistical Software version 20.106 was used to calculate the test performance of Xpert. Statistical significance was determined at $p < 0.05$.

# Results

## Magnitude of drug resistance and its association with demographic/clinical characteristics of the patients

The prevalence of drug resistance to at least one first-line anti-TB drug was 11.6% (95% CI: 7.9–15.2), and MDR was observed in 3.3% (95% CI: 1.7–5.6) of the isolates. The results of sensitivity tests for first-line anti-TB drugs are shown in Table 1. Of the 10 MDR *M. tuberculosis* isolates tested for the second-line DST, only two isolates were found to be resistant to CAP. Thus, according to the new WHO publication (3), none of the 10 MDR isolates was either pre-XDR TB or XDR TB.

A total of 323 MTBC were isolated from 249 sputum 249 and 74 FNA samples during the study period. But following sub-culturing on MGIT for DST, 2.8% (9/323) of the isolate lost viability while 3.7% (12/323) of the isolates were contaminated. Thus, these two groups were excluded from the final analysis. Therefore, 302 isolates (232 from pulmonary TB cases and 70 from extrapulmonary TB cases) were tested for drug sensitivity. The 302 patients who were the source of the

TABLE 1 Drug resistance profiles of new and previously treated patients with tuberculosis (TB) in the Somali Region, Ethiopia ($n = 302$).

| | Previously treated cases, $n = 12$ | New cases, $n = 290$ | Total, $n = 302$ |
|---|---|---|---|
| First line drugs | *n* (%) | *n* (%) | *n* (%) |
| Resistant to at least one drug | 6 (50) | 29 (10) | 35 (11.6) |
| **Resistant to any of the drugs** | | | |
| STM | 2 (16.7) | 21 (7.2) | 23 (7.6) |
| INH | 4 (33.3) | 12 (4.1) | 16 (5.3) |
| RIF | 2 (16.7) | 8 (2.8) | 10 (3.3) |
| EMB | 0 | 0 | 0 |
| **Poly resistance** | | | |
| STM + INH | 0 | 2 (0.7) | 2 (0.7) |
| RIF + INH | 2 (16.7) | 6 (2.1) | 8 (2.6) |
| STM + INH + RIF | 0 | 2 (0.7) | 2 (0.7) |
| All MDR | 2 (14.3) | 8 (2.8) | 10 (3.3) |
| **Monoresistance** | | | |
| STM | 2 (16.7) | 17 (5.9) | 19 (6.3) |
| INH | 2 (16.7) | 2 (0.7) | 4 (1.3) |

STM, streptomycin; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; MDR, multidrug-resistant.

isolates comprised 64.9% (196/302 male, 60.3% (182/302) urban residents, and 76.8% (232/302) pulmonary TB patients. Among extrapulmonary specimens 95.7% (67/70) were collected from lymph nodes [with cervical lymph node 88.6% (62/70)], 2.9% (2/70) from skin lesions, and 1.4% (1/70) from breast abscess. The median age of the patients was 28 years (range: 15 to 80 years) and 81.8% (247/302) were in the age group of 15–44 years.

The socio-demographic characteristics of the study participants are summarized in Table 2. Previous anti-TB drug treatment was associated with resistance to one or more drugs ($\chi^2 = 17.9$, $p < 0.05$), and MDR ($\chi^2 = 6.9$, $p = 0.055$). The proportion of drug-resistant strains for one or more drugs was similar in extrapulmonary TB (12.9%) and pulmonary TB (11.2%) ($\chi^2 = 0.14$, $p = 0.705$), and no MDR-TB strains were isolated from extrapulmonary TB ($\chi^2 = 3.12$, $p = 0.124$). Table 2 shows the relationship between drug sensitivity and demographic and clinical features of study participants.

## Association between drug resistance and bacterial genotype

The associations between drug resistances with isolated bacterial genotypes are presented in Table 3. The SIT 149 (T3-ETH) spoligotype was significantly associated with resistance to one or more drugs ($\chi^2 = 13.9$, $p < 0.05$) and MDR ($\chi^2 = 12.4$, $p < 0.05$) when compared with the other spoligotypes. The 10 MDR isolates were grouped as SIT 149 (T3-ETH), SIT 37 (T3), and SIT 21 (CAS1-Kili), which accounted for 70% (7/10), 20% (2/10), and 10% (1/10) of the isolates, respectively. One of the MDR isolates was identified during household contact investigation according to the Ethiopian TB guidelines (1, 27) and had the same spoligotype pattern as the index case SIT 149 (T3-ETH). Both clustered MDR SIT 37 isolates were resistant to the second-line anti-TB drug CAP.

## Genotypic drug resistance detection by Xpert MTB/RIF

Xpert was performed on 177 isolates of the 232 isolates recovered from pulmonary TB cases as a part of routine diagnosis. RIF resistance was detected in 6.2% (11/177) of the isolates, while the remaining 93.8% (166/177) were RIF sensitive. RIF indeterminate results were not found in any of the isolates. RIF resistance mutations result in complete dropout (no hybridization) on probe E (codons 529–533) in nine isolates and complete dropout on probe D (codons 523–529) in one isolate. RIF discordant result occurred between Xpert and MGIT 960 in one isolate and was associated with probe-binding delay ($\Delta C_T$ max = 5.8). On further analysis using the LPA, RR mutation was detected. But it is not due to hybridization with the known mutant probes (MUT1, MUT2A, MUT2B, and MUT3) rather by the absence of hybridization with the wild-type probe 8 (WT8) (codons 530–533).

## Sensitivity and specificity of Xpert MTB/RIF for the detection of RIF resistance

Of the 177 isolates tested by Xpert assay, 6.2% (11/177) were RIF-resistant. Discordant between Xpert and MGIT 960 was observed in one isolate and linked with probe-binding delay ($\Delta C_T$ max = 5.8). The sensitivity and specificity of the Xpert assay were 100 and 99.4%, respectively, while its positive and negative predictive values were 90.9 and 100%, respectively (Table 4).

# Discussion

This study was conducted to evaluate the drug sensitivity profile of 302 *M. tuberculosis* isolated from the Somali region of eastern Ethiopia. Both phenotypic and genotypic methods were used in this study. This could be considered important as it is the first study that was conducted on the evaluation of drug resistance in *M. tuberculosis*, which is critical for successful TB treatment and future healthcare planning.

TABLE 2 Demographic characteristics and their relationship with drug resistance in patients with TB in Somali region, Ethiopia ($n = 302$).

| Characteristics | | Resistant to one or more drugs *n* (%) | 95% CI | $\chi^2$ test | *p*-value | MDR *n* (%) | 95% CI | $\chi^2$ test | *p*-value |
|---|---|---|---|---|---|---|---|---|---|
| Sex | Female | 15 (14.2) | 7.5 −21.7 | 1.06 | 0.306 | 4 (3.8) | 0.9 −7.5 | 0.109 | 0.745 |
| | Male | 20 (10.2) | 6.1−14.3 | | | 6 (3.1) | 1–5. | | |
| Residence | Rural | 16 (13.3) | 7.5–20 | 0.591 | 0.442 | 2 (1.7) | 0–4.2 | 1.682 | 0.325 |
| | Urban | 19 (10.4) | 6–15.4 | | | 8 (4.4) | 1.6–7.7 | | |
| Contact History | Yes | 7 (11.9) | 5.1–22 | 0.005 | 0.941 | 4 (6.8) | 1.7–13.6 | 2.755 | 0.109 |
| | No | 28 (11.5) | 7.8–15.6 | | | 6 (2.5) | 0.8–4.5 | | |
| Type of TB | Extra-pulmonary TB | 9 (12.9) | 5.7–21.4 | 0.143 | 0.705 | 0 | 0 | 3.121 | 0.124 |
| | pulmonary TB | 26 (11.2) | 7.3–15.5 | | | 10 (4.3) | 1.7–6.9 | | |
| History of Treatment | Previously Treated | 6 (50) | 25–75 | 17.9 | 0.001* | 2 (16.7) | 0–41.7 | 6.962 | 0.055 |
| | New | 29 (10) | 6.6–13.8 | | | 8 (2.8) | 1–4.8 | | |
| Age Group | 15–24 | 12 (10.2) | 5.1–16.1 | 2.488 | 0.477 | 4 (3.4) | 0.8–6.8 | 0.674 | 0.914 |
| | 25–34 | 12 (15) | 7.5–23.8 | | | 3 (3.8) | 0–8.8 | | |
| | 35–44 | 7 (14.3) | 4.1–24.5 | | | 2 (4.1) | 0–10.2 | | |
| | 45 | 4 (7.3) | 1.8–14.5 | | | 1 (1.8) | 0–5.5 | | |

CI, confidence interval; MDR, multidrug-resistant.
*Statistically significant.

TABLE 3 Genotype of *Mycobacterium tuberculosis* and its association with drug resistance in the Somali region, Ethiopia ($n = 302$).

| Characteristics | | Resistant to one or more drug *n* (%) | 95% CI | $\chi^2$ test | *p*-value | MDR *n* (%) | 95% CI | $\chi^2$ test | *p*-value |
|---|---|---|---|---|---|---|---|---|---|
| Dominant SIT | SIT 149 | 13 (24.1) | 13–35.2 | | | 7 (13) | 3.7–22.2 | | |
| | SIT 21 | 6 (14.6) | 4.9–26.8 | 13.9 | **0.005*** | 1 (2.4) | 0–7.3 | 12.42 | **0.005*** |
| | SIT 26 | 2 (7.4) | 0–18.5 | | | 0 | 0 | | |
| | SIT 53 | 4 (19) | 4.8–38.1 | | | 0 | 0 | | |
| | Others | 10 (6.3) | 2.5–10.7 | | | 2 (1.3) | 0–3.1 | | |
| Clustering | Clustered | 32 (12.3) | 8.8–16.1 | 0.845 | 0.443 | 10 (3.8) | 1.5–6.5 | 1.625 | 0.368 |
| | Unique | 3 (7.3) | 0–17.1 | | | 0 | 0 | | |

SIT, Shared International Type; MDR, multidrug-resistant.
*statistically significant.

The prevalence of drug resistance recorded in new patients was comparable with the results of studies conducted in Gondar (28) and South Omo (29); however, it was relatively higher than the prevalence reported in northern Ethiopia (30). Higher prevalence values were also reported from southwestern Ethiopia (31), central Ethiopia (32), eastern Ethiopia (33), and south Ethiopia (34). The differences in the prevalence of drug resistance reported from the different regions of Ethiopia could be caused by different factors. It could be due to variations in the strength of the TB control programs at the study sites, treatment compliance of the patients, the economic status (nutritional status) of the patients, the immunological status of the patients, and variation in the virulence of the bacterial strain.

The prevalence of MDR in the new and previously treated patient was similar to the prevalence of MDR reported by studies conducted in northwest Ethiopia (35); but higher than the prevalence values reported from different parts of Ethiopia (29, 31, 32), although a very high prevalence (61.9%) of MDR was reported from Addis Ababa (36).

Regarding mono-resistance, STM mono-resistance was the most prevalent and is comparable with the results of previous studies in Ethiopia (33, 37), while some other studies reported found a lower percentage of STM mono-resistance (31, 38). Nonetheless, a relatively high prevalence of STM mono-resistance was reported using a large-scale Ethiopian community survey (39) and from the Amhara region (40). INH mono-resistance was lower than those reported by others in the

TABLE 4 Diagnostic performance of Xpert MTB/RIF for the detection of rifampicin resistance as compared with gold standard MGIT 960.

| Performance | Value | 95% CI |
|---|---|---|
| Sensitivity | 100.0% | 69.2–100 |
| Specificity | 99.4% | 96.7–99.9 |
| PPV | 90.9% | 58.6–98.6 |
| NPV | 100.0% | |
| Accuracy | 99.4% | 96.9–99.9 |

NPV, negative predictive value; PPV, positive predictive value; CI, confidence interval.

country (31, 33, 36, 37). Mono-resistance to RIF and EMB was not identified in our study. A low prevalence of RIF and EMB resistance was observed previously in Ethiopia (30, 40–42). In contrast to the reports of the previous studies conducted in Ethiopia (36, 43), no XDR cases and pre-XDR were detected in this study. The possible reasons for the differences in either mono-resistance of MDR are indicated above and related to either patient factors, TB control program, and/or pathogen factors.

The SIT 149 that belongs to lineage four exhibited a high frequency of drug resistance, and the association between this spoligotype and drug resistance was significant. It is established that Beijing (lineage 2) is associated with MDR globally although the lineage is less frequent in Ethiopia (7, 44, 45). The observation of an association between SIT 149 and drug resistance was also observed by other studies (43, 46, 47). SIT 149 is the most frequently isolated spoligotype from different regions of Ethiopia. So, the observation association between SIT 149 and drug resistance could be due to its frequent occurrence in Ethiopia which can also be reflected in the drug-resistant isolates. Such observation was reported by a review that was published by Panwalkar et al. (48). It can also reflect the real association between bacterial genetics and drug resistance, which could be due to mutation in the genes of isolates with SIT 149 spoligotype that guarantees them drug resistance.

The prevalence of drug resistance was comparable between extrapulmonary TB and pulmonary TB in this study. But no MDR-TB was in the isolates of extrapulmonary TB in this study. So far, no extensive study was conducted on the comparison of drug resistance in pulmonary TB and extrapulmonary isolates in Ethiopia while studies from India (49) and Korea (50) indicated that MDR-TB was less common in patients with extrapulmonary TB than in patients with pulmonary TB.

The observation of seven isolates with SIT 149 spoligotype pattern and two isolates with SIT 37 spoligotype pattern means that the observation of clustered patterns could suggest recent transmission of MDR-TB in the study area. Substantiating this hypothesis, in this study, it was observed that one of the MDR isolates was isolated from the same household as the index case although the discriminating power of spoligotyping is generally low (51).

The results of culture-based phenotypic drug susceptibility testing procedures can take weeks to months. Molecular-based testing such as Xpert, in contrast, can provide results within 2 h. Therefore, many countries have incorporated Xpert into their routine TB diagnostic algorithms. Xpert is one of the most useful tests for detecting RIF resistance, which acts as a proxy for MDR-TB and predicts poor treatment outcomes. The assays detect mutations in the 81-base-pair *rpoB* gene, which is linked to RIF resistance in 95–98% of cases (5). Detecting RR by Xpert is a critical element of TB care for both the individual patient and the public health since it starts a sequence of events that includes further DST, contact investigation, and patient referral for potentially toxic and less effective MDR therapy (52). The Xpert has high sensitivity and specificity for identifying RR in patients with pulmonary TB. Similar results were reported by a meta-analysis, which reported a pooled sensitivity of 96% and a specificity of 98% (53).

In this study, 11 RIF-resistant isolates on Xpert were also resistant to INH on the MGIT 960 phenotypic test, indicating that the Xpert test is a useful test for detecting MDR-TB in resource-limited settings. However, one isolate was susceptible to RIF on MGIT 960 phenotypic test. This discordant (false positive) result was due to probe delay evident in this study ($\Delta$Ct = 5.8). To rule out the possibility that low bacterial load affected the initial Xpert assay results by delaying Probe E binding (54), we retested the Xpert assay from the MGIT test's growth control, and the results were similar. This isolate was further evaluated by LPA, and unknown RIF-resistance mutations were detected owing to the absence of WT8 hybridization rather than hybridization with known mutation probes. This false positive RIF-resistant molecular tests result might be due to a silent mutation rather than real resistance, which could explain the delayed probe E binding on Xpert and the absence of binding to known mutation probes on LPA (55). Alternatively, the unusual occurrence of false positive results might be attributed to the detection of RR strains by Xpert but not by the current reference standard and the phenotypic culture-based DST (MGIT 960) (4). In this study, the most prevalent mutation (9 out of 10) of the MDR isolates was found in the codons 529–533 (probe E), and the rest (1 out of 10) was detected in codons 523–529 (probe D). A similar observation was made earlier in Ethiopia (56). Furthermore, similar results were reported from Nigeria, Uganda, and Pakistan (57–59).

## Conclusion

The magnitude of MDR isolates of *M. tuberculosis* in the Somali region of Ethiopia was higher than the national prevalence of MDR-TB warranting the strengthening of the TB control program in the Somali region of Ethiopia. Particularly, drug resistance was common in isolates with the spoligotype SIT 149, which was widespread in Ethiopia. The Xpert assay

was observed to have high sensitivity and specificity in detecting RIF-resistant *M. tuberculosis*, which is encouraging for its application widely.

## Data availability statement

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

## Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of the Aklilu Lemma Institute of Pathobiology, Addis Ababa University Ref No. ALIPB/IRB/002/2017/18. Permission was requested from Ethiopian Somali Regional State Health bureau and each study sites. The study was explained to the patients, and consent for participation was obtained prior to collecting the specimens. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

GW contributed to designing this study, data collection, analysis, drafting of the manuscript, analysis of and interpretation of the result, and edition of the manuscript. BG supervised the study and edited the manuscript. MG, BM, GD, GS, MG, MA, WS, and WA contributed to the field data collection, culturing of samples, and drug sensitivity testing. RT and LC contributed to the edition of the manuscript. GA contributed to conceptualizing and designing this study, leading, and supervising. All authors contributed to this study and approved the submitted version.

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## 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.

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## Supplementary material

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## References

1. Organization WH. *Global Tuberculosis Report 2021*. Geneva: World Health Organization (2021).

2. Crofton J, Mitchison DA. Streptomycin resistance in pulmonary tuberculosis. *Br Med J.* (1948) 2:1009–15. doi: 10.1136/bmj.2.4588.1009

3. World Health Organization.*Meeting Report of the WHO Expert Consultation on the Definition of Extensively Drug-Resistant Tuberculosis*. Geneva: World Health Organization, (2021) 27–29 October 2020.

4. Organization WH. *Xpert MTB/RIF Implementation Manual: Technical and Operational 'How-To'; Practical Considerations*. Geneva: World Health Organization (2014).

5. Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, et al. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. *J Clin Microbiol.* (2010) 48:229–37. doi: 10.1128/JCM.01463-09

6. Organization WH. *Automated Real-Time Nucleic Acid Amplification Technology for Rapid and Simultaneous Detection of Tuberculosis and Rifampicin Resistance: Xpert MTB/RIF Assay for the Diagnosis of Pulmonary and Extrapulmonary TB in Adults and Children: Policy Update*. Geneva: World Health Organization (2013).

7. Brown T, Nikolayevskyy V, Velji P, Drobniewski F. Associations between mycobacterium tuberculosis strains and phenotypes. *Emerg Infect Dis.* (2010) 16:272–80. doi: 10.3201/eid1602.091032

8. Almeida D, Rodrigues C, Ashavaid TF, Lalvani A, Udwadia ZF, Mehta A. High incidence of the Beijing genotype among multidrug-resistant isolates of Mycobacterium tuberculosis in a tertiary care center in Mumbai, India. *Clin Infect Dis.* (2005) 40:881–6. doi: 10.1086/427940

9. Cox HS, Kubica T, Doshetov D, Kebede Y, Rüsch-Gerdess S, Niemann S. The Beijing genotype and drug resistant tuberculosis in the Aral Sea region of Central Asia. *Respir Res.* (2005) 6:134. doi: 10.1186/1465-9921-6-134

10. Park YK, Shin S, Ryu S, Cho SN, Koh WJ, Kwon OJ, et al. Comparison of drug resistance genotypes between Beijing and non-Beijing family strains of Mycobacterium tuberculosis in Korea. *J Microbiol Methods.* (2005) 63:165–72. doi: 10.1016/j.mimet.2005.03.002

11. Zhang J, Mi L, Wang Y, Liu P, Liang H, Huang Y, et al. Genotypes and drug susceptibility of Mycobacterium tuberculosis Isolates in Shihezi, Xinjiang Province, China. *BMC Res Notes.* (2012) 5:309. doi: 10.1186/1756-0500-5-309

12. Hanekom M. Gey van Pittius NC, McEvoy C, Victor TC, Van Helden PD, Warren RM. Mycobacterium tuberculosis Beijing genotype: a template for success. *Tuberculosis.* (2011) 91:510–23. doi: 10.1016/j.tube.2011.07.005

13. Fanta BS, Abay SM, Subas CD. Anti-tuberculosis drug resistance in ethiopia. a mata-analysis. *J Drug Del Therap.* (2014) 4:154–63. doi: 10.22270/jddt.v4i3.863

14. World Health Organization. *Anti-Tuberculosis Drug Resistance in the World Global Report Number 4* Geneva: World Health Organization (2008).

15. Organization WH. *Global Tuberculosis Report 2020*. Geneva: World Health Organization (2020).

16. Khogali M, Zachariah R, Reid T, Alipon SC, Zimble S, Mahama G, et al. Self-administered treatment for tuberculosis among pastoralists in rural Ethiopia: how well does it work? *Int Health.* (2014) 6:112–7. doi: 10.1093/inthealth/ihu008

17. CSA E. *Population Projection of Ethiopia for All Regions at Wereda Level From 2014–2017*. Addis Ababa: Central Statistical Agency of Ethiopia (2013).

18. Gele AA, Bjune GA. Armed conflicts have an impact on the spread of tuberculosis: the case of the Somali Regional State of Ethiopia. *Conflict Health.* (2010) 4:1. doi: 10.1186/1752-1505-4-1

19. Organization WH. Guidelines for surveillance of drug resistance in tuberculosis- 5th ed. 2015.

20. Blakemore R, Story E, Helb D, Kop J, Banada P, Owens MR, et al. Evaluation of the analytical performance of the Xpert MTB/RIF assay. *J Clin Microbiol.* (2010) 48:2495–501. doi: 10.1128/JCM.00128-10

21. Berhanu RH, Schnippel K, Kularatne R, Firnhaber C, Jacobson KR, Horsburgh CR, et al. Discordant rifampicin susceptibility results are associated with Xpert® MTB/RIF probe B and probe binding delay. *Int J Tubercul Lung Dis.* (2019) 23:358–62. doi: 10.5588/ijtld.17.0837

22. Organization WH. *Laboratory Services in Tuberculosis Control*. Part III Culture Geneva, Switzerland: WHO (1998).

23. Berg S, Firdessa R, Habtamu M, Gadisa E, Mengistu A, Yamuah L, et al. The burden of mycobacterial disease in ethiopian cattle: implications for public health. *PLoS ONE.* (2009) 4:e5068. doi: 10.1371/annotation/f7240b30-f202-45c5-aacb-4fda2efb8b3d

24. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. *J Clin Microbiol.* (1997) 35:907–14. doi: 10.1128/jcm.35.4.907-914.1997

25. Springer B, Lucke K, Calligaris-Maibach R, Ritter C, Böttger EC. Quantitative drug susceptibility testing of Mycobacterium tuberculosis by use of MGIT 960 and EpiCenter instrumentation. *J Clin Microbiol.* (2009) 47:1773–80. doi: 10.1128/JCM.02501-08

26. GGenotype MTBDRplus V. 2.0 instruction for use. *Testing Instruction*. Germany: Hain Life Sciences (2012).

27. FMOH. *National Guidelines for TB, DR-TB and Leprosy in Ethiopia*. Addis Ababa: Federal Ministry of Health (2017).

28. Biadglegne F, Tessema B, Sack U, Rodloff AC. Drug resistance of Mycobacterium tuberculosis isolates from tuberculosis lymphadenitis patients in Ethiopia. *Indian J Med Res.* (2014) 140:116–22.

29. Wondale B, Medhin G, Abebe G, Tolosa S, Mohammed T, Teklu T, et al. Phenotypic and genotypic drug sensitivity of Mycobacterium tuberculosis complex isolated from South Omo Zone, Southern Ethiopia. *Infect Drug Resist.* (2018) 11:1581–9. doi: 10.2147/IDR.S165088

30. Tessema B, Beer J, Emmrich F, Sack U, Rodloff AC. First- and second-line anti-tuberculosis drug resistance in Northwest Ethiopia. *Int J Tuberc Lung Dis.* (2012) 16:805–11. doi: 10.5588/ijtld.11.0522

31. Abebe G, Abdissa K, Abdissa A, Apers L, Agonafir M. de-Jong BC, et al. Relatively low primary drug resistant tuberculosis in southwestern Ethiopia. *BMC Res Notes.* (2012) 5:1–6. doi: 10.1186/1756-0500-5-225

32. Bedewi Z, Mekonnen Y, Worku A, Medhin G, Zewde A, Yimer G, et al. Mycobacterium tuberculosis in central Ethiopia: drug sensitivity patterns and association with genotype. *New Microb New Infect.* (2017) 17:69–74. doi: 10.1016/j.nmni.2017.02.003

33. Seyoum B, Demissie M, Worku A, Bekele S, Aseffa A. Prevalence and drug resistance patterns of mycobacterium tuberculosis among new smear positive pulmonary tuberculosis patients in Eastern Ethiopia. *Tuberc Res Treat.* (2014) 2014:753492. doi: 10.1155/2014/753492

34. Merid Y, Hailu E, Habtamu G, Tilahun M, Abebe M, Hailu M, et al. Molecular Epidemiology of Mycobacterium tuberculosis strains isolated from pulmonary tuberculosis patients in south Ethiopia. *J Infect Dev Ctries.* (2021) 15:1299–307. doi: 10.3855/jidc.14742

35. Mekonnen F, Tessema B, Moges F, Gelaw A, Eshetie S, Kumera G. Multidrug resistant tuberculosis: prevalence and risk factors in districts of metema and west armachiho, Northwest Ethiopia. *BMC Infect Dis.* (2015) 15:461. doi: 10.1186/s12879-015-1202-7

36. Dagne B, Desta K, Fekade R, Amare M, Tadesse M, Diriba G, et al. The Epidemiology of first and second-line drug-resistance Mycobacterium tuberculosis complex common species: evidence from selected TB treatment initiating centers in Ethiopia. *PLoS ONE.* (2021) 16:e0245687. doi: 10.1371/journal.pone.0245687

37. Adane K, Ameni G, Bekele S, Abebe M, Aseffa A. Prevalence and drug resistance profile of Mycobacterium tuberculosis isolated from pulmonary tuberculosis patients attending two public hospitals in East Gojjam zone, northwest Ethiopia. *BMC Public Health.* (2015) 15:572. doi: 10.1186/s12889-015-1933-9

38. Lobie TA, Woldeamanuel Y, Asrat D, Beyene D, Bjørås M, Aseffa A. Genetic diversity and drug resistance pattern of Mycobacterium tuberculosis strains isolated from pulmonary tuberculosis patients in the Benishangul Gumuz region and its surroundings, Northwest Ethiopia. *PLoS ONE.* (2020) 15:e0231320. doi: 10.1371/journal.pone.0231320

39. Getahun M, Ameni G, Kebede A, Yaregal Z, Hailu E, Medihn G, et al. Molecular typing and drug sensitivity testing of Mycobacterium tuberculosis isolated by a community-based survey in Ethiopia. *BMC Public Health.* (2015) 15:751. doi: 10.1186/s12889-015-2105-7

40. Yimer SA, Agonafir M, Derese Y, Sani Y, Bjune GA, Holm-Hansen C. Primary drug resistance to anti-tuberculosis drugs in major towns of Amhara region, Ethiopia. *Apmis.* (2012) 120:503–9. doi: 10.1111/j.1600-0463.2011.02861.x

41. Bruchfeld J, Aderaye G, Palme IB, Bjorvatn B, Ghebremichael S, Hoffner S, et al. Molecular epidemiology and drug resistance of mycobacterium tuberculosis isolates from ethiopian pulmonary tuberculosis patients with and without human immunodeficiency virus infection. *J Clin Microbiol.* (2002) 40:1636–43. doi: 10.1128/JCM.40.5.1636-1643.2002

42. Demissie M, Gebeyehu M, Berhane Y. Primary resistance to anti-tuberculosis drugs in Addis Ababa, Ethiopia. *Int J Tuberc Lung Dis.* (1997) 1:64–7.

43. Agonafir M, Lemma E, Wolde-Meskel D, Goshu S, Santhanam A, Girmachew F, et al. Phenotypic and genotypic analysis of multidrug-resistant tuberculosis in Ethiopia. *Int J Tuberculosis and Lung Disease.* (2010) 14:1259–65.

44. Glynn JR, Kremer K, Borgdorff MW, Rodriguez MP, Soolingen Dv. *Beijing/W Genotype Mycobacterium Tuberculosis and Drug Resistance*. (2006).

45. Tulu B, Ameni G. Spoligotyping based genetic diversity of Mycobacterium tuberculosis in Ethiopia: a systematic review. *BMC Infect Dis.* (2018) 18:140. doi: 10.1186/s12879-018-3046-4

46. Diriba B, Berkessa T, Mamo G, Tedla Y, Ameni G. Spoligotyping of multidrug-resistant Mycobacterium tuberculosis isolates in Ethiopia. *Int J Tuberc Lung Dis.* (2013) 17:246–50. doi: 10.5588/ijtld.12.0195

47. Bekele S, Derese Y, Hailu E, Mihret A, Dagne K, Yamuah L, et al. Line-probe assay and molecular typing reveal a potential drug resistant clone of Mycobacterium tuberculosis in Ethiopia. *Trop Dis Travel Med Vaccines.* (2018) 4:15-. doi: 10.1186/s40794-018-0075-3

48. Panwalkar N, Chauhan DS, Desikan P. Spoligotype defined lineages of mycobacterium tuberculosis and drug resistance: merely a casual correlation? *Indian J Med Microbiol.* (2017) 35:27–32. doi: 10.4103/0255-0857.202327

49. Raveendran R, Oberoi JK, Wattal C. Multidrug-resistant pulmonary and extrapulmonary tuberculosis: a 13 years retrospective hospital-based analysis. *Indian J Med Res.* (2015) 142:575–82. doi: 10.4103/0971-5916.171285

50. Lee HY, Lee J, Lee YS, Kim M-Y, Lee H-K, Lee Y-M, et al. Drug-resistance pattern of Mycobacterium tuberculosis strains from patients with pulmonary and extrapulmonary tuberculosis during 2006 to 2013 in a Korean tertiary medical center. *Korean J Intern Med.* (2015) 30:325–34. doi: 10.3904/kjim.2015.30.3.325

51. Barnes PF, Cave MD. Molecular epidemiology of tuberculosis. *New Eng J Med.* (2003) 349:1149–56. doi: 10.1056/NEJMra021964

52. Osman M, Simpson JA, Caldwell J, Bosman M, Nicol MP, Tang YW. GeneXpert MTB/RIF version G4 for identification of rifampin-resistant

tuberculosis in a programmatic setting. *J Clin Microbiol.* (2014) 52:635–7. doi: 10.1128/JCM.02517-13

53. Horne DJ, Kohli M, Zifodya JS, Schiller I, Dendukuri N, Tollefson D, et al. Xpert MTB/RIF and Xpert MTB/RIF Ultra for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Sys Rev*. 2019 6:9593. doi: 10.1002/14651858.CD009593.pub4

54. Ngabonziza JCS, Decroo T, Migambi P, Habimana YM, Van Deun A, Meehan CJ, et al. Prevalence and drivers of false-positive rifampicin-resistant Xpert MTB/RIF results: a prospective observational study in Rwanda. *The Lancet Microbe*. (2020) 1:e74–83. doi: 10.1016/S2666-5247(20)30007-0

55. Alonso M, Palacios JJ, Herranz M, Penedo A, Menéndez A, Bouza E, et al. Isolation of Mycobacterium tuberculosis strains with a silent mutation in rpoB leading to potential misassignment of resistance category. *J Clin Microbiol.* (2011) 49:2688–90. doi: 10.1128/JCM.00659-11

56. Alemu A, Tadesse M, Seid G, Mollalign H, Eshetu K, Sinshaw W, et al. Does Xpert® MTB/RIF assay give rifampicin resistance results without identified mutation? Review of cases from Addis Ababa, Ethiopia. *BMC Infect Dis.* (2020) 20:1–6. doi: 10.1186/s12879-020-4817-2

57. Ochang EA, Udoh UA, Emanghe UE, Tiku GO, Offor JB, Odo M, et al. Evaluation of rifampicin resistance and 81-bp rifampicin resistant determinant region of rpoB gene mutations of Mycobacterium tuberculosis detected with XpertMTB/Rif in cross river State, Nigeria. *Int J Mycobacteriol.* (2016) 5:145. doi: 10.1016/j.ijmyco.2016.09.007

58. Mboowa G, Namaganda C, Ssengooba W. Rifampicin resistance mutations in the 81 bp RRDR of rpoB gene in Mycobacterium tuberculosis clinical isolates using Xpert® MTB/RIF in Kampala, Uganda: a retrospective study. *BMC Infect Dis.* (2014) 14:481. doi: 10.1186/1471-2334-14-481

59. Ullah I, Shah AA, Basit A, Ali M, Khan A, Ullah U, et al. Rifampicin resistance mutations in the 81 bp RRDR of rpoB gene in Mycobacterium tuberculosis clinical isolates using Xpert MTB/RIF in Khyber Pakhtunkhwa, Pakistan: a retrospective study. *BMC Infect Dis*. (2016) 16:413. doi: 10.1186/s12879-016-1745-2

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# Existence of viable *Mycobacterium leprae* in natural environment and its genetic profiling in a leprosy endemic region

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**Introduction:** Molecular epidemiology of leprosy is very important to study leprosy transmission dynamics and to enhance our understanding of leprosy in endemic areas by utilizing the molecular typing method. Nowadays our understanding of leprosy transmission dynamics has been refined by SNP typing and VNTR marker analysis of *M. leprae* strains.

**Objective:** This study was carried out to find out the presence of viable *M. leprae* in the soil and water samples from residing areas of leprosy patients staying in different blocks of Purulia district of West Bengal, understanding their genotypes and compared with that of *M. leprae* present in patients.

**Material and methods:** Slit-skin smear (SSS) samples (n=112) were collected from the active multibacillary leprosy patients from different blocks of leprosy endemic area. Soil samples (n=1060) and water samples (n=620) were collected from residing areas of leprosy patients. SNP subtyping was performed by PCR followed by sequencing. Multiplex PCR was performed using fifteen ML-VNTR loci and results were analysed.

**Results:** We observed high PCR positivity in soil samples (344 out of 1060; 32%) and water samples (140 out of 620; 23%). These PCR positive samples when further screened for viability, it was observed that 150 soil samples (44%) and 56 water samples (40%) showed presence of 16S rRNA. SNP typing of *M. leprae* revealed presence of predominantly type 1. SNP subtype 1D (83%) was most prevalent in all the blocks of Purulia followed by subtype 1C (15%) and subtype 1A (2%). SNP subtype 2F was noted in only one sample. SNP and VNTR combination showed presence of similar strain type in certain pockets of Purulia region which was responsible for transmission.

**Conclusion:** Presence of viable *M. leprae* in the environment, and presence of SNP Type 1 *M. leprae* in patients and environment suggests both environment and patients play a role in disease transmission.

KEYWORDS

mycobacterium leprae, environment, transmission, SNP-VNTR typing, leprosy, genotyping, clinical samples

# Introduction

Leprosy is also called Hansen's disease, a chronic infectious disease caused by *Mycobacterium leprae and Mycobacterium lepromatosis* (1, 2). It is true that the source of infection is either untreated leprosy patients or other animal reservoirs (3, 4). But in addition to this, extra-human reservoirs could be possible. Studies in Norway, India, Japan, Indonesia, Brazil, Bangladesh, England, and Suriname have shown presence of *M. leprae* in the surrounding environment of leprosy endemic regions (5–12). Hence it is important to look for the presence of *M. leprae* in the environment (soil and water) and to determine their viability status along with their genetic make-up and other factors which might help the survival of the organism in the environment.

Enormous numbers of leprosy bacilli ($2.4X10^8$) are expelled daily in the environment from the nasal discharges of lepromatous patients (13). There is also evidence to support excretion of bacilli from skin lesions (14). It was reported that *M. leprae* discharged through secretions (coughing and sneezing) from patients (15, 16) in the form of air-borne droplet may cause infection or can settle in soil (5, 17, 18) and in water (8, 19). But very limited information is available for the survival of bacilli outside the host. *M. leprae*, an obligate intracellular pathogen, have been recently shown to be associated with free living amoeba. *In vitro*, phagocytosis of *M. leprae* by amoeba was observed by florescence microscopy and *M. leprae* which remained viable for at least three days in amoebae were noted to grow in mouse foot pad (20). Further, *M. leprae* was found to survive up to 8 months within amoebic cysts (21). Hence, possibility of spreading of infection by amoebae needs to be explored in natural environmental conditions.

The new epidemiological tools developed for strain typing of *M. leprae* in the recent years will be useful in national leprosy surveillance/control efforts towards true reduction in incidence, and in epidemiological investigations. The combination of single nucleotide polymorphism (SNP) subtyping along with variable nucleotide tandem repeat (VNTR) loci determination in *M. leprae* genome have been proved to serve as a genetic marker to differentiate strains of *M. leprae* (3, 22). However, the characteristics of polymorphism vary depending on the population, and can be a reflection of that population at the national and local level.

The purpose of this study was to find out the existence of viable *M. leprae* in the surrounding environment (soil and water) of the residing areas of leprosy patients and to perform molecular genotyping using SNP typing and or VNTR analysis of *M. leprae* from patients and the environment to find out the genetic variability of the organism existing in nature which might help in tracking and understanding transmission of leprosy.

# Materials and methods

## Ethical approval

The study was approved on 22nd December 2016 by the Organization Ethical Committee of The Leprosy Mission trust India. Informed consent was obtained from all the participant enrolled in the study.

## Collection of environmental and clinical samples

Soil and water samples were collected from different blocks of Purulia district, West Bengal. Soil was dug (3-4-inch-deep) and was collected in clean plastic containers (10g each) with the help of a trowel and labelled with site code and the village name. The collected samples were transported to the laboratory at room temperature (within 2 days) and thereafter were stored at 4–8°C till further processing. One thousand and sixty soil samples and 620 water samples were collected from residing places of leprosy patients.

Multibacillary leprosy cases were diagnosed clinically based on skin lesions and impairment of nerve functions and acid-fast bacilli (AFB) positivity in slit skin smears. After taking consent, 112 slit-skin smear samples (SSS) were collected from the earlobes of active multibacillary (MB) leprosy patients. SSSs were collected during field visits in different blocks such as Joypur (n=24), Jhalda (n=13), Purulia (n=32), Arsha (n=16), Chandenkeyari (n=8), Kashipur (n=2), Para (JH) (n=10), Barabazar (n=7) of Purulia District, West Bengal. Samples

were transported in 70% ethanol in micro centrifuge tubes to the laboratory at room temperature (25°C). The tubes were kept at 4°C until further use.

## DNA extraction from environmental samples

Standard method of DNA extraction was used as described earlier (7). Briefly, pond water (50 ml) samples were centrifuged at 400 ×g for 5 min. The supernatants were collected in 50 ml sterile tubes and centrifuged again at 8000 ×g for 15 min. Pellets that contained soil and other floating matter including organisms were weighed (100 mg) in dried 1.5 ml microfuge tube and followed by the soil DNA extraction protocol. Soil samples were homogenized using bead beater followed by lysis in tube containing ethanol with zirconium beads mixed with soil. The mixture was homogenized using bead beater followed by lysis by Proteinase K in TENP buffer (50 mM Tris, 20 mM EDTA, 100 mM NaCl and 1% Polyvinylpolypyrrolidone). DNA was precipitated by adding 70% ethanol and centrifuged at 10,000 rpm for 15 mins. The pellet was air dried and dissolved in Tris EDTA (TE) buffer and stored at −20°C until further use.

## DNA extraction from slit skin smears

Proteinase K Lysis method was used for *M. leprae* DNA extraction from slit-skin smear samples (23). In brief smears collected in 1 ml 70% ethanol were centrifuged at 10,000rpm (8000xg) for 10 min. Supernatant was discarded and pellet was air dried for the removal of ethanol. After ethanol removal samples were kept for overnight lysis in lysis buffer (100 mM Tris buffer pH 8.5 with 1mg/ml proteinase K and 0.05% Tween 20) at 60°C. The Proteinase K was inactivated at 97°C for 10 minutes. This lysate preparation was further used for PCR.

## RNA extraction from environmental samples

The standardized protocol of RNA extraction was used as described earlier (7). Briefly, the samples as mentioned above were homogenised and were subjected to acid-phenol extraction followed by isopropanol precipitation and centrifugation at 12000 rpm for 10 mins at 4°C. Pellet was washed once with 70% ethanol, air dried and then dissolved in 50 µL of TE buffer.

## PCR amplification using *M. leprae* specific repetitive element (RLEP) region

PCR amplification was carried out in a total 25 µL of reaction volume that contained 2 µL of template DNA and primers at final concentration of 0.5 µM (forward and reverse) and 1X Genei Mix (Merck India) were used. We used *M. leprae* specific primers (PS1- TGCATGTCATGGCCTTGAGG; PS2 - CACCGATACCAGCGGCAGAA) as per our earlier publication (24).The amplification was carried out in a thermal cycler (Corbett) using following conditions: one cycle of denaturation at 95°C for 5 min followed by 35-45 cycles at 94°C for 30s, annealing at 58°C for 30s, extension at 72°C for 1 min and one cycle of final extension at 72°C for 10 min. PCR product (129 bp) containing amplified fragment of the target region was electrophoresed in a 2% agarose gel using Tris-Borate-EDTA buffer at 100 volts constant voltage.

## Reverse transcription-PCR of *M. leprae 16S rRNA* gene

The Reverse Transcriptase (RT) –Polymerase chain reaction was carried out by using One Step RT PCR Kit (Qiagen - 210210). Control reactions to test DNA contamination were also performed simultaneously with each experiment by carrying out PCR without prior reverse transcription. *16S rRNA* gene was amplified using *M. leprae* specific primers P2 and P3 as described earlier (25). The total volume (50 µL) of PCR amplification mixture contained 10 µL of 5X RT PCR buffer 2 µL of dNTPs, 10 µL -5X Q Solution,50ng of each primer, 2 µL of RTPCR enzyme,0.25 µL RNase inhibitor and remaining RNase free water and 10 µL of sample (template). The cycling profile for the amplification reaction was in two stages. In the first stage reverse transcription was carried out at 50°C for 30 minutes followed by inactivation step at 95°C for 15 minutes. In the second stage amplification was carried out using denaturation at 94°C for 1 min 30 seconds, annealing at 60°C for 1 min 30 seconds followed by extension at 72°C for 1 min for 37 cycles. This was followed by final extension at 72°C or 10 min. The amplification products were run on 1.5% (w/v) agarose gel, stained with ethidium bromide, and observed using Gel Documentation System (Alpha Imager).

## SNP typing and subtyping of *M. leprae*

Three SNP loci viz. 1,2 and 3 at nucleotide positions 14676, 1642875 and 2935685 in *M. leprae* genomic DNA were amplified using primers (Supplementary Table 1) and was performed using described protocols (26, 27).

Amplification of four SNP subtyping for type 1 at nucleotide positions 8453, 313361, 61425 and 1642879, *M. leprae* genomic DNA was amplified using previously reported (26, 27) primer sequences as mentioned (Supplementary Table 2).

After amplification of PCR products were run on 2% agarose gel by electrophoresis. The amplicons were outsourced for

commercial sequencing (Eurofins Genomics India Pvt. Ltd. Delhi).

## Multiplex PCR analyses using variable number of tandem repeat typing

The multiplex PCR was carried out using *M. leprae* specific primers as described earlier (28, 29) (Supplementary Table 3). The forward primers were labelled with PET, NED, VIC, and 6-FAM fluorescent dyes at the 5 termini (Invitrogen Bio-services-Applied Biosystems, India). Multiplex PCRs were performed as described earlier (30). Four sets of combination of primers were used and the reaction was carried out using multiplex PCR kit (Qiagen). Briefly, each reaction mixture (20 µL final volume) was comprised of 10 µL of 2x Qiagen master mix, 2 µL Q solution, 2 µL (each) of forward and reverse primer working stock and 2µl of template DNA, the volume was adjusted with nuclease free water. The final concentration of each primer was 0.2 µM. PCR was carried out at an initial denaturation temperature of 95 $^0$C for 15 min, followed by 40 cycles as: denaturation at 94 $^0$C for 30s, primer annealing at 60 $^0$C for 90s and primer extension at 72$^0$C for 90s, and final extension at 72 $^0$C for 10 min. 5 µL PCR products were electrophoresed in 2% Agarose gel using Tris borate-EDTA buffer (1X) at 100V constant current for 1 hour to check amplification. Amplicons were sent for commercial fragment length analysis (FLA) to Xplorigen Technologies Ltd., Delhi India.

## Data analysis

DNA fragments were visualized by Finch TV Version 1.4.0 software that was used for chromatogram analysis developed by Geospiza's research team. The chromatogram, thus generated was then compared to the standard *M. leprae* strain using nBLAST at positions mentioned in the table to track mutations and to categorize them into SNP subtypes A, B, C and D. Fragment length analysis of VNTR genotypes were analysed and copy numbers of repeat different loci determined.

Cluster analysis was done using PAST 4.03 statistical analysis software. Dendrograms were generated to see clustering if any in relation to SNP subtype and VNTR.

# Results

## PCR amplification using RLEP region of *M. leprae* from clinical and environmental samples

Clinical samples (SSS samples) were tested for presence of *M. leprae* DNA using RLEP primers. The results of *M. leprae* DNA PCR positive for SSS samples. Environmental samples were tested for presence of *M. leprae*. Out of 1060 soil samples collected from the area where patients resided, we could detect *M. leprae* in 344 samples (32.4%) (Table 1). Further, 140 (23%) water samples out of 620 samples collected from the patient residing area showed presence of *M. leprae* DNA. In control area, i.e., an area of low endemic region of Purulia from where no new case of leprosy was reported in the past ten years, we could detect *M. leprae* DNA only in 9 (3%) soil samples out of 300 samples tested. Water samples (180N) from this area, we could detect M. *leprae* DNA in only 2 (1.1%) samples.

## Detection of viable *M. leprae* from soil samples by using *16S rRNA* gene target

RT-PCR was performed by using *16S rRNA* gene target using PCR positive environmental samples. We could detect amplification in 150 (44%) soil samples out of 344 soil samples tested (Table 1). Similarly, 40% of the water samples (56 out of 140) showed RT-PCR positivity suggesting possibility of presence of viable *M. leprae* in these samples which were collected from the patient residing area. None of the environmental samples collected from the control area showed any amplification by RT-PCR (Table 1).

## PCR amplification of *M. leprae* DNA and SNP subtyping

All the *M. leprae* DNA PCR positive clinical samples and environmental soil and water samples were subjected to SNP type and subtype which were obtained from patients' area and

TABLE 1 *Mycobacterium leprae* detection in environmental samples.

| Samples type | Total number collected | *M. leprae DNA* PCR positivity (%) | RT-PCR positivity (%) *M. leprae* | Genotyping (SNP typing) of *M. leprae* DNA |
|---|---|---|---|---|
| Soil samples (Patient residing area) | 1060 | 344 (32%) | 150 (44%) | 1A=12 (15%)., 1C = 14 (18%) 1D = 50 (65%) |
| Water samples (Patient residing area) | 620 | 140(23%) | 56 (40%) | 1D = 15 (11%) |
| Control-Soil samples (No Patient area) | 300 | 9(3%) | 00 | 0 |
| Control-Water samples (No Patient area) | 180 | 2(1%) | 00 | 0 |

no patients area. Standard reference *M. leprae* DNA of NHDP63, BR 4953 and THAI 53 DNA were used as positive control (Obtained from Colorado State University, USA) and master mix without template used as negative control in PCR reaction. All the PCR positive samples were used for SNP type and SNP subtype amplicon sent for sequencing outsourcing (Eurofins Genomics India Pvt. Ltd. Delhi).

All the PCR positive SSS samples used for SNP typing and subtyping are presented in Table 2. It was observed that the almost all the samples except one belonged to SNP type 1. Further, SNP subtyping of the samples using sequencing showed 2 out of 112 samples to be of subtype 1A (2%), 16 out of 112 to be subtype 1C (15%) and 93 out of 112 belonged to subtype 1D (82%). Only one sample from Jhalda (Purulia district) was observed to be of subtype 2F (1%).

Out of 344 soil samples tested we could obtain data on SNP typing for 76 samples. All samples were of SNP Type 1 (Table 1). Of these, majority of samples (50 of 76) (65.8%) were of type 1D which is also a major SNP type noted in patients. Fourteen samples (18.4%) were of type 1C and 12 (15.8%) were of type 1A. Similarly, 140 PCR positive water samples were tested for SNP typing. We could obtain data for 15 samples and all the 15 samples showed SNP type 1D.

### VNTRs typing and fragment length analysis

Multiplex PCR was used to amplify fragments suitable for fragment length analysis from fifteen genomic VNTR loci. We found that 3 loci viz. (27 -5), (23 -3), (AC) 8b were monomorphic; four loci viz. rpoT, (AT)17, (21 -3), 18-8 were dimorphic; four loci viz. (AC)9, 12-5, (TA)10 (6, 7), were polymorphic. Four loci viz. (GGT)5, (GTA)9, (AC)8a and (TA)10 were found highly polymorphic in nature (Table 3). Interestingly, SNP subtype (1D) showed more variability in repeat number with 12 VNTR loci.

We looked at the distribution of the alleles across the samples for SNP type 1D (Table 4A). For each locus it was noted that a particular allele number was dominant e.g., for locus 18-8, there were 41 samples out of 70 which showed 8 repeats. So, we looked at the variation in allele numbers as well as the dominant allele for each locus (Table 4B). It is quite clear that some of the alleles for the loci tested are highly dominant across the samples tested.

Results of SNP type and VNTR were studied for existence of clusters. Data were analysed separately and together to plot Dendrograms for SNP type 1D and 1C (Figures 1A–C). Then, we looked at individual cases with the SNP type and VNTR allele numbers and we could easily pick up some cases showing similar *M. leprae* strain pattern from some of the blocks in Purulia district (Table 5).

## Discussion

Recent studies strongly suggest that environment could be a possible reservoir of viable *M. leprae* and might be responsible for the disease transmission as presence of viable *M. leprae* has been reported in the environment (water and soil) in Indonesia, India, Brazil, and Bangladesh, England, Surinam (6–12, 18, 25). It has been demonstrated that *M. leprae* can survive outside human body in moist soil up to 46 days (31). However, the direct proof of transmission of disease to the population has still not been clearly understood especially from environmental sources of *M. leprae* (8–10, 18).

In recent times with the advancement in molecular biological techniques a very unstable molecule like RNA can be preserved and used as a potent marker for assessment of viability of microorganisms (8, 32). *M. leprae* genome *16S rRNA* and RLEP conserved gene regions were found to be a better target for viability studies because their stability and slower degradation rate over time in comparison to superoxide dismutase gene (32).

Earlier studies have also shown the presence of viable *M. leprae* using *16S rRNA* in the environment of leprosy hospital areas which can be a possible source of infection (6, 18, 25, 27).

TABLE 2 SNP typing of *M. leprae* from slit skin smear samples.

| Block (No. of samples) | SNP Type 1 | | | | SNP Type 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H |
| Joypur (23) | 0 | 0 | 4 | 19 | 0 | 0 | 0 | 0 |
| Jhalda (10) | 1 | 0 | 0 | 8 | 0 | 1 | 0 | 0 |
| Purulia (36) | 1 | 0 | 4 | 31 | 0 | 0 | 0 | 0 |
| Arsha (16) | 0 | 0 | 5 | 11 | 0 | 0 | 0 | 0 |
| Chandankeyari (8) | 0 | 0 | 2 | 6 | 0 | 0 | 0 | 0 |
| Kashipur (2) | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Para & Pindrojara JH (10) | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| Barabazar (7) | 0 | 0 | 1 | 6 | 0 | 0 | 0 | 0 |
| Total (112) | 2 (2%) | 0 | 16 (15%) | 93 (83%) | 0 | 1(1%) | 0 | 0 |

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TABLE 3 VNTR typing of *M. leprae* from slit skin smear samples.

| VNTR loci | SNP Type1 & 2 and their subtypes | | | |
|---|---|---|---|---|
| | 1A | 1C | 1D | 2F |
| 18-8 | 7, 8 | 7, 8 | 7, 8 | 8 |
| 12-5 | 4 | 4,5 | 3,4,5,9 | 4 |
| (TA)10 | 13 | 10,11,12,13 | 10,11,12,13 | 13 |
| (AC)8a | 7 | 7,8,9,11 | 6,7,8,9,10,11 | 8 |
| (GAA)21 | 18 | 18,19, 20 | 18,19, 20 | 20 |
| (GGT)5 | 4,5 | 4,5,9,10 | 4,5,7,8,9,10,11 | 5 |
| (GTA)9 | 7,9 | 8,9,10,11 | 7,8,9,10,11,12 | 9 |
| (21 -3) | 2 | 1,2 | 1,2 | 1 |
| (6-7) | 6 | 5,6,7 | 5,6,7,11 | 6 |
| (AT)17 | 8 | 8,9 | 8,9 | 8 |
| RPOT | 3, 4 | 3, 4 | 3, 4 | 3 |
| (AC)9 | 9 | 7,9 | 5,7,9 | 9 |
| 27 -5 | 5 | 5 | 5 | 5 |
| 23 -3 | 2 | 2 | 2 | 2 |
| (AC)8b | 7 | 7 | 7 | 7 |

Existence of *M. leprae* DNA has been reported in water samples in Indonesia (12) and soil samples from high prevalence areas of North-East states of India (6, 8, 33). In some studies, it has been suggested that in endemic countries >50% of household contacts may have a history of intimate contact leprosy patients. In this study, we collected environmental samples from the residing areas of multibacillary active leprosy patients. Large proportion of environmental samples showed presence of DNA (32% for soil and 23% for water) in areas where active cases were residing suggesting that there is dynamic movement of the organism between patient and the environment. This was further supported by the fact that environmental samples from control region where there was no active case, there was rare presence of *M. leprae* in the environment. On the other hand, presence of rRNA (44% of the soil samples and 40% of the water samples with abundant presence of *M. leprae* DNA), suggests the chances of viability of *M. leprae* bacilli in these samples and their exposure to the community. Hence in the inhabitant areas of

TABLE 4A Distribution of alleles in samples showing SNP Type 1D.

| Locus | No. of samples showing the allele numbers | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| 18-8 | | | | | | | 26 | 41 | 3 | | | | | | | | | | | |
| AC8a | | | | | | 9 | 28 | 18 | 17 | 1 | 15 | | | | | | | | | |
| TA10 | | | | | | | | | 1 | 9 | 10 | 15 | 36 | | | | 1 | | | |
| 12-5 | | | 1 | 53 | 9 | | | | | | | | | | | | | | | |
| 21-3 | 40 | 26 | 2 | | | | | | | | | | | | | | | | | |
| GTA-9 | | | | | | | 2 | 21 | 26 | 9 | 7 | 4 | | | | | | | | |
| GGT-5 | | | | 23 | 41 | 1 | 1 | 1 | 3 | | 1 | 1 | | | | | | | | |
| GAA-21 | | | | | | | | | | | | | | | | | | 16 | 20 | 23 |
| AT-17 | | | | | | | | 59 | 25 | 1 | | | | | | 1 | | | | |
| rpoT | | | 70 | 19 | | | | | | | | | | | | | | | | |
| AC-9 | | | | | 2 | | 5 | 1 | 78 | | | | | | | | | | | |
| 6-7 | | | | | 5 | 69 | 13 | | | | 2 | | | | | | | | | |
| AC8b | | | | | | | 87 | | | | | | | | | | | | | |
| 23-3 | 2 | 69 | 2 | | | | | | | | | | | | | | | | | |
| 27-5 | | | | | 88 | | | | | | | | | | | | | | | |

TABLE 4B Polymorphism at different VNTR locus.

| Locus | No. of samples that showed amplification | Numbers of alleles found in tested samples | Repeat numbers in major allele | Prevalence of major allele (Percentage of total no. of samples that showed amplification) |
|---|---|---|---|---|
| 18-8 | 70 | 3 | 8 | 58.6 |
| AC8a | 88 | 6 | 7 | 31.8 |
| TA10 | 72 | 6 | 13 | 50 |
| 12-5 | 63 | 3 | 4 | 84.1 |
| 21-3 | 68 | 3 | 1 | 58.8 |
| GTA-9 | 69 | 6 | 9 | 37.7 |
| GGT-5 | 72 | 8 | 5 | 56.9 |
| GAA-21 | 59 | 3 | 20 | 39 |
| AT-17 | 86 | 4 | 8 | 68.6 |
| rpoT | 89 | 2 | 3 | 78.7 |
| AC-9 | 86 | 4 | 9 | 90.7 |
| 6-7 | 89 | 4 | 6 | 77.5 |
| AC8b | 87 | 1 | 7 | 100 |
| 23-3 | 73 | 3 | 2 | 94.5 |
| 27-5 | 88 | 1 | 5 | 100 |

leprosy cases there could be a possibility of indirect exposure to *M. leprae* to the community that may result in infection with *M. leprae* bacilli.

Further, active multibacillary leprosy patients discharge enormous numbers of leprosy bacilli from nose, mouth washes and skin to the environment which may get air borne as droplet and may cause infection or can settle in soil and water (13–16). These viable bacilli might be phagocytosed by protozoa and might survive in protozoa and can be carried to susceptible population staying in leprosy endemic area. We earlier found presence protozoa species along with viable *M. leprae* in soil and water samples (8). suggesting possible protective niche that protozoa may provide to *M. leprae* in the environment. But we are yet to find proof for the presence of *M. leprae* within protozoa in natural environmental condition. Therefore, further experiments are needed to understand and establish the mechanism of *M. leprae* viability in the environment and the factors that contribute to provide the protective niche to *M. leprae.*

Recent advancement in the molecular characterization of *M. leprae* has led to alternative and definitive methodologies that are used for identification and distribution of genotype (22, 26). Matsuoka et al. (29) reported polymorphism in *rpoT* gene of *M. leprae*. Monot et al. (26) demonstrated SNP array in *M. leprae*. In Indian population mostly SNP type-1 and rarely type-2 was observed (7, 30, 34). Several reports suggested that molecular marker for *M. leprae* were useful for distinguishing strain and epidemiological significance (3, 22, 26, 28, 35). The discovery of SNPs in *M. leprae* genome was able to distinguish four major SNP types and their distribution in different region of the world. The most common approach of SNP typing was useful and effective in molecular epidemiologic studies (26). Sixteen SNP subtypes were useful for tracking the transmission of *M. leprae* and source of infection.

The present study was based on the identification and differentiation of *M. leprae* strains from the SSS samples of index cases and their residing environmental areas from endemic region. This was to track the transmission and *M. leprae* strain prevalence in that geographical region in association with VNTR loci. Genotyping of SSS almost all of the multibacillary leprosy cases showed presence of SNP type 1 and SNP subtype 1D (82%) was most prevalent in the population. We also identified SNP type 2 and subtype 2F (1%) in one of the samples from Purulia district of West Bengal. In our earlier study we have reported SNP subtype 2E in cases from north-east Delhi and subtype 2G from West Bengal (27). All the SSS samples were obtained from different blocks of Purulia district. Genotyping of environmental samples showed SNP type 1 and subtype 1D which suggest that there is discharge of the *M. leprae* from the active cases to the environment. Similar genotype in the patient and environmental soil samples poses serious question on the source of infection for the population in the community.

SNP and VNTR genotyping studies in leprosy multi-case families have shown similar SNP type and VNTR repeat units suggesting that source of infection is common in multi-case family (30, 33, 34). We used combination of SNP and VNTR genotyping data of clinical samples to identify the pattern of transmission in different blocks of Purulia district. We observed in this study that some of the VNTR loci like (GGT)5, (GTA)9, (AC)8a and (TA)10 were highly polymorphic in nature. But every locus had at least one allele that was dominant among the samples. Similar polymorphism was reported from South Indian leprosy cases (36) and from Switzerland (26).

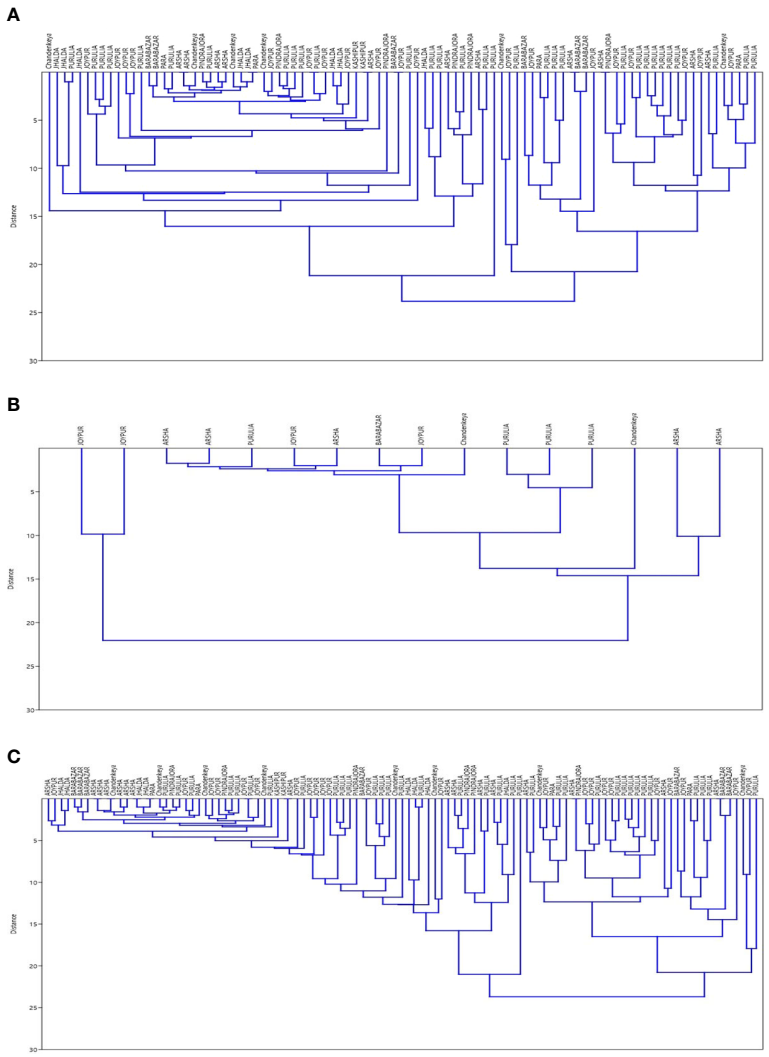


FIGURE 1
**(A)** Dendrogram for SNP Type 1D samples using data for 15 loci of VNTR 'Dendrogram was prepared using PAST 4.03 Statistical Analysis Software'. **(B)** Dendrogram for SNP Type 1C samples using data for 15 loci of VNTR'Dendrogram was prepared using PAST 4.03 Statistical Analysis Software'. **(C)** Dendrogram for SNP Type 1D & 1C samples together using data for 15 loci of VNTR 'Dendrogram was prepared using PAST 4.03 Statistical Analysis Software'.

Young et al. (36) reported 2 alleles of locus 23-3 from east and south Indian cases. Similarly, 2 alleles of loci 23-3 were also observed in China (37), Thailand, Brazil and Columbia (35). 3 copies of loci 23-3 were reported in Philippines (35). In this study we could find three alleles of 23-3 but the allele with 2 repeats was most prevalent (94.5%). We observed either 7 or 8 repeats of locus 18-8 in all the cases studied. But in Philippines, Brazil and Columbia 8 numbers of repeat were reported (35). On the other hand, 7 number of repeats were observed in China (37).

In our study we observed 3,4 and 5 repeats of VNTR locus 12-5, but 4 repeats were observed in population of Columbia (35). However, 3 repeats were reported by China (37).

Association of 5,6,7 and 11 repeats of VNTR 6-7 loci with SNP subtype 1D was noted in this study. Earlier 5 and 6 repeats were reported from India, Thailand, Columbia population (35, 38, 39). However, 7 copies were reported in Philippines (35).

We observed 8, 9 and 10 repeats for the locus (AT) 17. Monot et al. (40) reported 8 and 9 repeats from Switzerland. 3 and 4 repeats of rpoT were reported from Japan and India (18, 29, 41, 42).

With the help of cluster analysis, the SNP and VNTR combination M. *leprae* strain similarities were noted in the certain blocks of Purulia district but many variabilities in repeats

TABLE 5 Cases showing similar SNP and VNTR pattern.

| Sample code | Blocks | Subtype | Combination 1 | | | | Combination 2 | | | | Combination 3 | | | | Combination 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 18-8 | AC8a | TA10 | 12-5 | 21-3 | GTA9 | GGT5 | GAA21 | AT17 | RPOT | AC9 | 6-7 | AC8b | 23-3 | 27-5 |
| Case-55 | Barabazar | C | 8 | 9 | 13 | 4 | 1 | 8 | 5 | 19 | 8 | 4 | 9 | 6 | 7 | 2 | 5 |
| Case-57 | Barabazar | D | 8 | 9 | 13 | 4 | 1 | 8 | 5 | 19 | 8 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-62 | Barabazar | D | 8 | 9 | 13 | 4 | 2 | 8 | 4 | 19 | 8 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-36 | ARSHA | D | 7 | 8 | 13 | 4 | 2 | 9 | 5 | 20 | 8 | 4 | 9 | 6 | 7 | 2 | 5 |
| Case-42 | ARSHA | D | 7 | 8 | 13 | 4 | 2 | 9 | 5 | 20 | 8 | 4 | 9 | 6 | 7 | 2 | 5 |
| Case-43 | ARSHA | D | 8 | 8 | 13 | 4 | 2 | 9 | 5 | 20 | 9 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-46 | ARSHA | D | 8 | 8 | 13 | 4 | 2 | 9 | 5 | 20 | 8 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-48 | ARSHA | C | 7 | 8 | 13 | 4 | 2 | 9 | 4 | 20 | 8 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-32 | CHANDANKEIRY | D | 8 | 6 | 12 | 5 | 2 | 9 | 5 | 19 | 8 | 4 | 9 | 6 | 7 | 2 | 5 |
| Case-35 | CHANDANKEIRY | C | 8 | 9 | 11 | 5 | 2 | 9 | 5 | 19 | 8 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-14 | JOYPUR | C | 7 | 7 | 12 | 4 | 1 | 9 | 5 | 19 | 9 | 4 | 9 | 6 | 7 | 2 | 5 |
| Case-27 | JOYPUR | D | 7 | 9 | 12 | 4 | 1 | 11 | 5 | 19 | 9 | 3 | 7 | 6 | 7 | 2 | 5 |
| Case-38 | JOYPUR | D | 8 | 9 | 12 | 4 | 1 | 9 | 5 | 19 | 9 | 4 | 5 | 11 | 7 | 2 | 5 |
| Case-39 | JOYPUR | D | 7 | 9 | 12 | 4 | 1 | 9 | 5 | 19 | 9 | 4 | 7 | 11 | 7 | 2 | 5 |
| Case-21 | JHALDA | D | 8 | 8 | 13 | 4 | 2 | 12 | 5 | 18 | 9 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-22 | JHALDA | D | 8 | 8 | 13 | 4 | 2 | 12 | 5 | 18 | 8 | 3 | 9 | 7 | 7 | 2 | 5 |
| Case-73 | JHALDA | D | 7 | 8 | 13 | 4 | 1 | 9 | 5 | 20 | 8 | 3 | 9 | 7 | 7 | 2 | 5 |
| Case-11 | KASHIPUR | D | 8 | 11 | 13 | 5 | 1 | 8 | 4 | 18 | 8 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-12 | KASHIPUR | D | 8 | 7 | 13 | 5 | 1 | 8 | 9 | 20 | 9 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-84 | PARA | D | 7 | 7 | 13 | 4 | 1 | 9 | 5 | 20 | 8 | 3 | 9 | 7 | 7 | 2 | 5 |
| Case-99 | PARA | D | 7 | 7 | 13 | 4 | 1 | 8 | 4 | 20 | 8 | 4 | 9 | 6 | 7 | 2 | 5 |
| Case-78 | PINDROJORA | D | 8 | 7 | 13 | 4 | 1 | 9 | 5 | 20 | 8 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-80 | PINDROJORA | D | 7 | 7 | 13 | 4 | 1 | 8 | 5 | 20 | 8 | 3 | 9 | 5 | 7 | 2 | 5 |
| Case-81 | PINDROJORA | D | 8 | 7 | 13 | 4 | 1 | 9 | 5 | 20 | 9 | 3 | 9 | 6 | 7 | 2 | 5 |
| Case-82 | PINDROJORA | D | 8 | 7 | 11 | 4 | 1 | 9 | 5 | 20 | 9 | 3 | 9 | 6 | 7 | 2 | 5 |

in VNTR loci which might require large number of samples for analysis to show similar genotype of epidemiological importance.

In summary, this study undoubtedly found presence of viable *M. leprae* in inhabitant areas of leprosy patients. These viable bacilli might survive in the environment as well as might help in causing leprosy disease after repeated exposure to a susceptible host. Similar genotype in clinical and environmental samples indicate that environment could possibly act as a source of infection. SNP and VNTR combination showed *M. leprae* strain similarities and their differentiation in certain blocks of Purulia. Such studies with the combination of genetic markers may provide a tool to track transmission link in the community.

## 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 on 22nd December 2016 by the Organization Ethical Committee of The Leprosy Mission trust India. Informed consent was obtained from all the participant enrolled in the study. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

RT and RJ, conceived and designed the analysis. RT, VS, ML, IS collected the data with experimental work in lab, methodology, validation. RT wrote the paper, performed the analysis, and interpretation of data, and drafting of manuscript. RJ, ML, IS and US: Supervision, conceptualization, writing-reviewing, and editing. RJ: Data curation, software, and analysis. RT, RJ, ML and US contributed data or analysis tools, wrote the paper, analysis, and interpretation of data, and

drafting of manuscript. All authors contributed to the article and approved the submitted version.

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## 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



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2022.972682/full#supplementary-material

## References

1. Hansen GA. On the etiology of leprosy. *Br Foreign Med Chir Rev* (1875) 55 (110):459–89.

2. Seo 2. Han XY, Sizer YH, Schoberle KC, May T, Spencer GS, JS, et al. A new mycobacterium species causing diffuse lepromatous leprosy. *Am J Clin Pathol* (2008) 130(6):856–64. doi: 10.1309/AJCPP72FJZZRRVMM

3. Truman RW, Singh P, Sharma R, Busso P, Rougemont J, Paniz-Mondolfi A, et al. Probable zoonotic leprosy in the Southern United States. *N Engl J Med* (2011) 364(17):1626–33. doi: 10.1056/NEJMoa1010536

4. Avanzi C, Del-Pozo J, Benjak A, Stevenson K, Simpson VR, Busso P, et al. Red squirrels in the British isles are infected with leprosy bacilli. *Science* (2016) 354 (6313):744–7. doi: 10.1126/science.aah3783

5. Kazda J. Occurrence of non-cultivable acid-fast bacilli in the environment and their relationship to m. *leprae Lepr Rev* (1981) 52(Suppl 1):85–91. doi: 10.5935/0305-7518.19810061

6. Lavania M, Katoch K, Katoch VM, Gupta AK, Chauhan DS, Sharma R, et al. Detection of viable mycobacterium leprae in soil samples: insights into possible sources of transmission of leprosy. *Infect Genet Evol* (2008) 8(5):627–31. doi: 10.1016/j.meegid.2008.05.007

7. Turankar RP, Lavania M, Singh M, Siva Sai KS, Jadhav RS. Dynamics of mycobacterium leprae transmission in environmental context: Deciphering the role of environment as a potential reservoir. *Infect Genet Evol* (2012) 12(1):121–6. doi: 10.1016/j.meegid.2011.10.023

8. Turankar RP, Lavania M, Darlong J, Siva Sai KSR, Sengupta U, Jadhav RS. Survival of mycobacterium leprae and association with acanthamoeba from environmental samples in the inhabitant areas of active leprosy cases: A cross sectional study from endemic pockets of purulia, West Bengal. *Infect Genet Evol* (2019) 72:199–204. doi: 10.1016/j.meegid.2019.01.014

9. Mohanty PS, Naaz F, Katara D, Misba L, Kumar D, Dwivedi DK, et al. Viability of mycobacterium leprae in the environment and its role in leprosy dissemination. *Indian J Dermatol Venereol Leprol* (2016) 82(1):23–7. doi: 10.4103/0378-6323.168935

10. Holanda MV, Marques LEC, Macedo MLB, Pontes MAA, Sabadia JAB, Kerr LRFS, et al. Presence of mycobacterium leprae genotype 4 in environmental waters in northeast Brazil. *Rev Soc Bras Med Trop* (2017) 50(2):216–22. doi: 10.1590/0037-8682-0424-2016

11. Tió-Coma M, Sprong H, Kik M, van Dissel JT, Han XY, Pieters T, et al. Lack of evidence for the presence of leprosy bacilli in red squirrels from north-West Europe. *Transbound Emerg Dis* (2020) 67(2):1032–4. doi: 10.1111/tbed.13423

12. Matsuoka M, Izumi S, Budiawan T, Nakata N, Saeki K. Mycobacterium leprae DNA in daily using water as a possible source of leprosy infection. *Indian J Lepr* (1999) 71(1):61–7.

13. Davey TF, Rees RJ. The nasal dicharge in leprosy: clinical and bacteriological aspects. *Lepr Rev* (1974) 45(2):121–34. doi: 10.5935/0305-7518.19740014

14. Job CK, Jayakumar J, Kearney M, Gillis TP. Transmission of leprosy: A study of skin and nasal secretions of household contacts of leprosy patients using PCR. *Am J Trop Med Hyg* (2008) 78(3):518–21. doi: 10.4269/ajtmh.2008.78.518

15. Weddell G, Palmer E. The pathogenesis of leprosy. *Exp Approach Lepr Rev* (1963) 34:57–61. doi: 10.5935/0305-7518.19630010

16. Barton RP. A clinical study of the nose in lepromatous leprosy. *Lepr Rev* (1974) 45(2):135–44. doi: 10.5935/0305-7518.19740015

17. Blake LA, West BC, Lary CH, Todd JR4. Environmental nonhuman sources of leprosy. *Rev Infect Dis* (1987) 9(3):562–77. doi: 10.1093/clinids/9.3.562

18. Turankar RP, Lavania M, Singh M, Sengupta U, Siva Sai K, Jadhav RS. Presence of viable mycobacterium leprae in environmental specimens around houses of leprosy patients. *Indian J Med Microbiol* (2016) 34(3):315–21. doi: 10.4103/0255-0857.188322

19. Fukutomi Y, Maeda Y, Matsuoka M, Makino M. Temperature dependency for survival of mycobacterium leprae in macrophages. *Nihon Hansenbyo Gakkai Zasshi* (2009) 78(1):7–16. doi: 10.5025/hansen.78.7

20. Lahiri R, Krahenbuhl JL. The role of free-living pathogenic amoeba in the transmission of leprosy: A proof of principle. *Lepr Rev* (2008) 79(4):401–9. doi: 10.47276/lr.79.4.401

21. Wheat WH, Casali AL, Thomas V, Spencer JS, Lahiri R, Williams DL, et al. Long-term survival, and virulence of mycobacterium leprae in amoebal cysts. *PloS Negl Trop Dis* (2014) 8(12):e3405. doi: 10.1371/journal.pntd.0003405

22. Groathouse NA, Rivoire B, Kim H, Lee H, Cho SN, Brennan PJ, et al. Multiple polymorphic loci for molecular typing of strains of mycobacterium leprae. *J Clin Microbiol* (2004) 42(4):1666–72. doi: 10.1128/JCM.42.4.1666-1672.2004

23. Jadhav RS, Macdonald M, Bjune G, Oskam LMILEP2 Study Group. Simplified PCR detection method for nasal mycobacterium leprae. *Int J Lepr Other Mycobact Dis* (2001) 69(4):299–307.

24. Turankar RP, Pandey S, Lavania M, Singh I, Nigam A, Darlong J, et al. Comparative evaluation of PCR amplification of RLEP, 16S rRNA, rpoT and sod a gene targets for detection of m. leprae DNA from clinical and environmental samples. *Int J Mycobacteriol* (2015) 4(1):54–9. doi: 10.1016/j.ijmyco.2014.11.062

25. Jadhav RS, Kamble RR, Shinde VS, Edward S, Edward VK. Use of reverse transcription polymerase chain reaction for the detection of mycobacterium leprae in the slit-skin smears of leprosy patients. *Indian J Lepr* (2005) 77 (2):116–27.

26. Monot M, Honoré N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A, et al. Comparative genomic and phylogeographic analysis of mycobacterium leprae. *Nat Genet* (2010) 42(4):361. doi: 10.1038/ng.477

27. Lavania M, Jadhav RS, Turankar RP, Chaitanya VS, Singh M, Sengupta U. Single nucleotide polymorphisms typing of mycobacterium leprae reveals focal transmission of leprosy in high endemic regions of India. *Clin Microbiol Infect* (2013) 19(11):1058–62. doi: 10.1111/1469-0691.12125

28. Gillis T, Vissa V, Matsuoka M, Young S, Richardus JH, Truman R, et al. Characterisation of short tandem repeats for genotyping mycobacterium leprae. *Lepr Rev* (2009) 80(3):250–60. doi: 10.47276/lr.80.3.250

29. Matsuoka M, Zhang L, Morris MF, Legua P, Wiens C. Polymorphism in the rpoT gene in mycobacterium leprae isolates obtained from Latin American countries and its possible correlation with the spread of leprosy. *FEMS Microbiol Lett* (2005) 243(2):311–5. doi: 10.1016/j.femsle.2004.12.031

30. Lavania M, Jadhav R, Turankar RP, Singh I, Nigam A, Sengupta U. Genotyping of mycobacterium leprae strains from a region of high endemic leprosy prevalence in India. *Infect Genet Evol* (2015) 36:256–61. doi: 10.1016/j.meegid.2015.10.001

31. Desikan KV, Sreevatsa. Extended studies on the viability of mycobacterium leprae outside the human body. *Lepr Rev* (1995) 66(4):287–95.

32. Martinez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, et al. Molecular determination of mycobacterium leprae viability by use of real-time PCR. *J Clin Microbiol* (2009) 47(7):2124–30. doi: 10.1128/JCM.00512-09

33. Singh V, Turankar RP, Goel A. Real-time PCR-based quantitation of viable mycobacterium leprae strain from clinical samples and environmental sources and its genotype in multi-case leprosy families of India. *Eur J Clin Microbiol Infect Dis* (2020) 39(11):2045–55. doi: 10.1007/s10096-020-03958-w

34. Turankar RP, Lavania M, Chaitanya VS, Sengupta U, Darlong J, Darlong F, et al. Single nucleotide polymorphism-based molecular typing of m. leprae from multicase families of leprosy patients and their surroundings to understand the transmission of leprosy. *Clin Microbiol Infect* (2014) 20(3):O142–9. doi: 10.1111/1469-0691.12365

35. Sakamuri RM, Harrison J, Gelber R, Saunderson P, Brennan PJ, Balagon M, et al. A continuation: study and characterisation of mycobacterium leprae short tandem repeat genotypes and transmission of leprosy in cebu, Philippines. *Lepr Rev* (2009) 80(3):272–9.

36. Young SK, Ponnighaus JM, Jain S, Lucas S, Suneetha S, Lockwood DN, et al. Use of short tandem repeat sequences to study mycobacterium leprae in leprosy patients in Malawi and India. *PloS Negl Trop Dis* (2008) 2(4):e214. doi: 10.1371/journal.pntd.0000214

37. Xing Y, Liu J, Sakamuri RM, Wang Z, Wen Y, Vissa V, et al. VNTR typing studies of mycobacterium leprae in China: assessment of methods and stability of markers during treatment. *Lepr Rev* (2009) 80(3):261–71. doi: 10.47276/lr.80.3.261

38. Lavania M, Katoch K, Sharma R, Sharma P, Das R, Gupta AK, et al. Molecular typing of mycobacterium leprae strains from northern India using short tandem repeats. *Indian J Med Res* (2011) 133(6):618–26.

39. Fontes AN, Sakamuri RM, Baptista IM, Ura S, Moraes MO, Martínez AN, et al. Genetic diversity of mycobacterium leprae isolates from Brazilian leprosy patients. *Lepr Rev* (2009) 80(3):302–15. doi: 10.47276/lr.80.3.302

40. Monot M, Honoré N, Balière C, Ji B, Sow S, Brennan PJ, et al. Are variable-number tandem repeats appropriate for genotyping mycobacterium leprae? *J Clin Microbiol* (2008) 46(7):2291–7. doi: 10.1128/JCM.00239-08

41. Matsuoka M, Maeda S, Kai M, Nakata N, Chae GT, Gillis TP, et al. Mycobacterium leprae typing by genomic diversity and global distribution of genotypes. *Int J Lepr Other Mycobact Dis* (2000) 68(2):121–8.

42. Lavania M, Katoch K, Singh H, Das R, Gupta AK, Sharma R, et al. Predominance of three copies of tandem repeats in rpoT gene of mycobacterium leprae from northern India. *Infect Genet Evol* (2007) 7(5):627–31. doi: 10.1016/j.meegid.2007.05.011

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# Do private health providers adhere to National Tuberculosis Guideline while assigning treatment outcome? Findings from a lower middle-income country

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**Background:** Treatment success rate is an important indicator to measure the performance of the National Tuberculosis Program (NTP). There are concerns about the quality of outcome data from private facilities engaged by NTP. Adherence of private providers of tuberculosis care to NTP guideline while assigning treatment outcomes to patients is rarely investigated. We aimed to determine whether Lagos private for-profit (PFP) and private not-for-profit (PNFP) facilities adhere to domestic TB guideline while assigning treatment outcome and the availability of periodic sputum acid-fast bacilli (AFB) results.

**Method:** A retrospective review of facility treatment register and treatment cards of TB patients managed between January and December 2016 across 10 private directly observed treatment short-course (DOTS) facilities involved in the public–private mix (PPM) in Lagos, Nigeria. The study took place between January and June 2019.

**Results:** Of the 1,566 patients, majority (60.7%) were male, >30 years (50.2%), HIV-negative (88.4%), and attended PNFP (78.5%). The reported treatment success rate (TSR) was 84.2% while the actual TSR was 53.8%. In total, 91.1, 77.6, and 70.3% of patients had sputum acid-fast bacilli (AFB) at 2/3, month 5, and month 6, respectively, while 68.6% had all the three sputum AFB in the register. Healthcare workers (HCWs) were adherent in assigning treatment outcome for 65.6% of TB patients while 34.4% of patients were assigned incorrect treatment outcomes. Most variations between reported and actual treatment outcomes were found with cured (17%) and completed (13.4%). Successful and unsuccessful outcomes were overreported by 30.4% and 4.1%,

respectively. DOTS providers in private facilities with available TB guideline (OR 8.33, CI 3.56–19.49, $p < 0.0001$) and PNFP facility (OR 4.42, CI 1.91–10.3, $p = 0.001$) were more likely to adhere to National TB Guideline while assigning TB treatment outcome.

**Conclusion:** Frontline TB providers in Lagos private hospitals struggled with assigning correct treatment outcome for TB patients based on NTBLCP guideline. Increased access to all the periodic follow-up AFB tests for TB patients on treatment and availability of National TB Guideline for referencing could potentially improve the adherence of private TB service providers while assigning TB treatment outcomes.

KEYWORDS

**tuberculosis, adherence, guideline, sputum, acid fast bacilli, treatment success rate, NTP**

## Background

Nigeria is one of the 30 high-burden countries for tuberculosis (TB), tuberculosis/HIV co-infection, and multidrug-resistant tuberculosis (MDR-TB). Annually, Nigeria accounts for 9% of TB cases missed globally either because they were not diagnosed, or they were diagnosed but not reported to the National TB, Buruli Ulcer and Leprosy Control Program (NTBLCP) (1). Only 104,000 of the estimated 434,000 TB cases in Nigeria were notified in 2018 (2). In contrast, Nigeria has consistently reported treatment success rate (TSR) that is close to the 90% global target over the years (2). Successful treatment outcome is defined as the sum of patients cured and those who have completed treatment (1). The international target is to successfully treat at least 90% of new sputum smear-positive TB cases (1). In 2015, the overall TB treatment success rate in Nigeria was 87% (with a cure rate of 78% and completion rate of 8.8%) while TSR was 84% in 2016 (3–5). At the subnational level, the tuberculosis cure rate in Lagos grew from 64% in 2003 to 76% in 2014 (6). Also, 77% of a cohort of multidrug-resistant TB patients initiated in 2013 was reported to be successfully treated in 2015 compared with the 56% recorded globally (7, 8). TSR for tuberculosis provides a useful indicator to measure the quality of health services and the performance of the NTBLCP. Suboptimal TSR suggests that infectious patients may not be receiving adequate treatment. TSR has been benchmarked at 90%, and underperforming National TB Programs (NTPs) are usually placed on closer monitoring to improve the performance. Evaluation of successful treatment outcomes of new smear-positive pulmonary TB patients could also be used to determine the effectiveness of DOTS implementation.

The World Health Organization (WHO) estimated that the prevalence of MDR-TB was 4.3% among new TB cases and 25% among retreatment cases, respectively (5). Although treatment coverage is still low in Nigeria, a successful TB control program should match high treatment success rate with low figures of reported MDR-TB cases and vice versa. In addition to the poor treatment coverage, there were concerns about the accuracy of TSR figures reported by health facilities and adherence to NTBLCP guidelines while assigning those treatment outcomes. Nonadherence to guideline often manifests as incomplete documentation, underreporting and overreporting of treatment outcomes.

A study by Measure Evaluation identified nine overarching barriers to Health Management Information Systems (HMIS) in low- and middle-income countries (LMICs). These include insufficient skills in data use core competencies; poor data quality (completeness, validity, reliability, and timeliness of reporting); insufficient expertise in data synthesis and visualization; lack of systems thinking in HMIS design and development; lack of leadership and culture of data use, and, finally, poor knowledge and motivation of healthcare workers on data use (9). In Nigeria, variances between TB data reported at facility and LGA levels, the lack of data for planning, incomplete and delayed quarterly reporting,

Abbreviations: AFB, acid-fast bacilli; CQI, continuous quality improvement; CPT, cotrimoxazole therapy; DOTS, directly observed therapy, short-course; DS-TB-drug, susceptible tuberculosis; ETR, electronic tuberculosis reporting; HCWs, healthcare workers; LGATBLS, Local Government Area Tuberculosis, Buruli Ulcer and Leprosy Control Supervisor; LGA, Local Government Area; LTFU, lost to follow-up; LMICs, low- and middle-income countries; LSTBLCP, Lagos State Tuberculosis and Leprosy Control Program; STBLCO, State TB, Buruli Ulcer and Leprosy Control Officer; MDR-TB, multidrug-resistant tuberculosis; NTP, National Tuberculosis Program; NTBLCP, National Tuberculosis, Buruli Ulcer and Leprosy Control Program; PPM, public–private mix; TSR, treatment success rate; TB, tuberculosis; WHO, World Health Organization.

enormous missing records in health facilities, poor storage of surveillance data, and weak workforce capacity in data management have also been reported (10, 11). Custodians and managers of TB data oftentimes are not provided with the logistics needed for tracking patient lost to follow-up, conduct contact tracing, and supervise and collate data with far-reaching implications on quality and completeness of TB data (12).

A previous comparative evaluation of the performance of the public and private healthcare system in low- and middle-income countries suggested that private sector providers of TB services frequently failed to adhere to guidelines and medical standards of care leading to poor patient outcomes. The authors argued that the efficiency of the data reporting system is worse in the private sector (13). A World Health Organization (WHO) survey in Mexico showed that private practitioners managed one-third of patients who died from TB (14). Also, 85% of DR-TB patients were reported to have been previously managed for TB in the private sector (15). Despite the relatively high reported overall national TSR over the years, DR-TB case notification in Nigeria has increased from 21 in 2010 to 1,686 in 2016 and a further increase of 35% from 1,686 in 2016 to 2,286 in 2017 (2). Similarly, from 665 MDR-TB cases notified in 2013, the number of cases increased to 29,469 in 2020. Disaggregation of TSR data into the private sector component is often lacking in the annual national TB report. There is also a lack of data to confirm previous concerns that many TB patients managed in the private facilities were poorly managed and that private facilities contributed to poor TSR. This raises concerns about the reported treatment outcome data from the private sector. Analysis of adherence of staff in assigning patient outcome can help focus supervision and mentorship program to enhance the quality of care in the private sector. Therefore, we aimed to investigate adherence to the National TB Guideline in assigning treatment outcome to bacteriologically diagnosed TB patients in private facilities, Lagos, Nigeria. The specific objectives of the study include: (1) to investigate the availability of sputum AFB follow-up test needed to assign treatment outcome at various stages of TB treatment, (2) to determine the level of adherence of healthcare workers (HCWs) to NTBLCP guideline while assigning TB treatment outcome and adherence predictive factors, and (3) to highlight variations between reported and actual treatment outcomes based on standard NTBLCP case definitions for treatment outcomes in Nigeria.

# Method

## Study design

A retrospective study among all bacteriologically positive TB patients as recorded in the facility TB treatment register between January and December 2016 in Lagos, Nigeria.

## Study setting

Lagos state has a population of 24 million people and is divided into 20 Local Government Areas (LGAs) (16).

Health care in Nigeria is organized into primary, secondary, and tertiary care, and the governance of the National TB Program (embedded within the Federal Ministry of Health) is also organized in alignment with the federal system of government. The NTBLCP was established in 1989 and saddled with policy development, tertiary care, mobilization and development of human and material resource, coordination and provision of technical support to state programs. TB control activity is coordinated at the national level by the NTBLCP coordinator, at the state level by the State Tuberculosis, Buruli Ulcer and Leprosy Control Officer (STBLCO), and at the LGA level by the LGA TB, Buruli Ulcer and Leprosy supervisor (LGATBLS) assisted by the DOTS officer in the facilities (17). The health facility focal person known as the directly observed treatment short-course (DOTS) officer captures each presumptive patient in the TB presumptive register, after the preliminary test has been conducted. Once the patient is confirmed TB-positive, the details of the patient are registered in the facility TB treatment register. Records of registered TB patients are collated monthly by facility DOTS provider and sent to the LGA supervisor who will now report to the STBLCO at the end of the quarter. The STBLCO subsequently reports the state data to the NTBLCP. Similarly, TB treatment outcome data are reported in cohorts on a quarterly basis, taking cohorts of patients who initiated TB treatment in the preceding year. There are 774 LGA supervisors in the country. They all report to the state TB control officers in their respective states. The state TB officers then transmit the data to the national level (18). Also, there are six zonal arrangements at the subnational level. Each zone comprises six states. Reports from the six zones are collected and aggregated at the national level for the production of quarterly report and presentations. The NTBLCP also generates annual report based on the aggregated data from the 36 states and the Federal Capital Territory. Data validation takes place monthly or quarterly depending on the availability of funds although priority is given to public facilities and high-volume private hospitals. Electronic reporting of TB data starts at the state level when transmitting state data to the NTBLCP.

Lagos harbors 11% of the Nigerian population. Each of the Lagos LGA is supervised by an LGA TB supervisor. Healthcare service in Lagos is provided at three levels: primary, secondary, and tertiary. The Lagos State TB, Buruli Ulcer and Leprosy Control Program (LSTBLCP) was inaugurated in 2003 and expanded to engage the private sector in 2008. By the end of 2016, private facilities engaged in Lagos for tuberculosis program have increased from 8 to over 150. Facilities are often engaged under four service schemes, i.e., referral of presumptive TB only, provision of directly observed therapy short-course (DOTS) treatment only, provision of microscopy

service only, and provision of both microscopy and DOTS. LSTBLCP is responsible for the training of healthcare workers and the provision of reagents, recording, and reporting tools such as the presumptive TB register, TB treatment register, and treatment cards, among others. All individuals having symptoms of TB in particular, cough of two weeks or more, are regarded as presumptive TB and documented in the presumptive TB register (17).

## Participants

A total of 10 facilities were recruited for this study. They provide a range of TB services including AFB microscopy and DOTS. Only five (50%) of the 10 centers provide AFB microscopy, and one of them also provides molecular testing services. These facilities are all private and have been engaged by TB program for at least 1 year. All the facilities recruited also provide TB/DOTS service.

In Figure 1, a total of 1,654 patients (across the 10 facilities) extracted from the facility treatment register are included in the study. A total of 4.7% (77/1,654) had missing treatment outcome and were excluded from the analysis of the initial treatment outcome. Of the remaining 1,577 patients with assigned treatment outcome in the register, 11 (0.7%) were excluded from the final analysis of adherence to NTBLCP guideline since they were transferred out before the end of treatment. A total of 1,566 patients were included in the final adherence analysis.

## Bias

Where an outcome was given but AFB results were missing in the TB treatment register, a second-level check of the treatment card was conducted. In such a scenario, any missing AFB result in treatment register now found in the TB treatment card was used to update the TB treatment register accordingly.

## Study size

A sample size of all the 1,654 patients (across 10 private facilities) was extracted from the facility treatment register and included in the study.

## Sampling technique

A four-stage sampling technique (summarized below) was used to select facilities for this study.

- Stage 1: Purposive sampling technique was used in selecting the seven high TB burden LGAs in Lagos State out of the total of 20 LGAs.
- Stage 2: The TB facilities were stratified into private and public, and convenient sampling technique was used to select the private facilities. This was because of less bureaucratic processes in getting access to data from private than public facilities.
- Stage 3: Ten (10) out of 57 facilities across the seven high TB burden LGAs were selected proportionately. This implies that the facilities were selected based on the numbers of facilities in each LGA.
- Stage 4: All pulmonary tuberculosis patients were extracted from the facility treatment register.

## Data sources and measurement

Data were collected by six trained data clerks using Microsoft Excel data collection template. Information from the TB treatment register was inputted on this template. In minimizing bias in data extraction, data clerks were trained on the data collection processes. The research was also piloted in a community outside the study setting to test the practical knowledge of data collection by the trained data clerks. The objective of the study was also masked from data clerks. Daily reviews were held with data collectors to assess collected data for any missing information or double counting. No double counting was observed during the process. Prior to analysis, collected data by data clerks were triangulated with different data collection sources such as the patient treatment card and the TB treatment register. Collected data were also randomly picked by multiple observers to ensure interrater reliability. For each patient, data were collected on the date of registration, age, sex, patient category, type of facility, regimen type, date of commencement of treatment, referral setting, site of TB, means of diagnosis of TB, follow-up AFB result and grade, treatment outcome, HIV result, and cotrimoxazole preventive therapy (CPT) status. For each health facility, data were also collected on for-profit status, availability of TB guideline, provider training, refresher training, and year of training. Using these pieces of information, assigned treatment outcomes were manually linked to their test results under the result columns and interpreted against the case definition of treatment outcome assigned to determine if the assigned outcome for the patient was correct (adhere to NTP guideline) or incorrect (did not adhere to NTP guideline). Information on follow-up AFB was either collected directly from the TB treatment register or updated from TB care card if available. Adherent or non-adherent status was registered against each patient after evaluation depending on whether the assigned outcome met the case definition or otherwise.

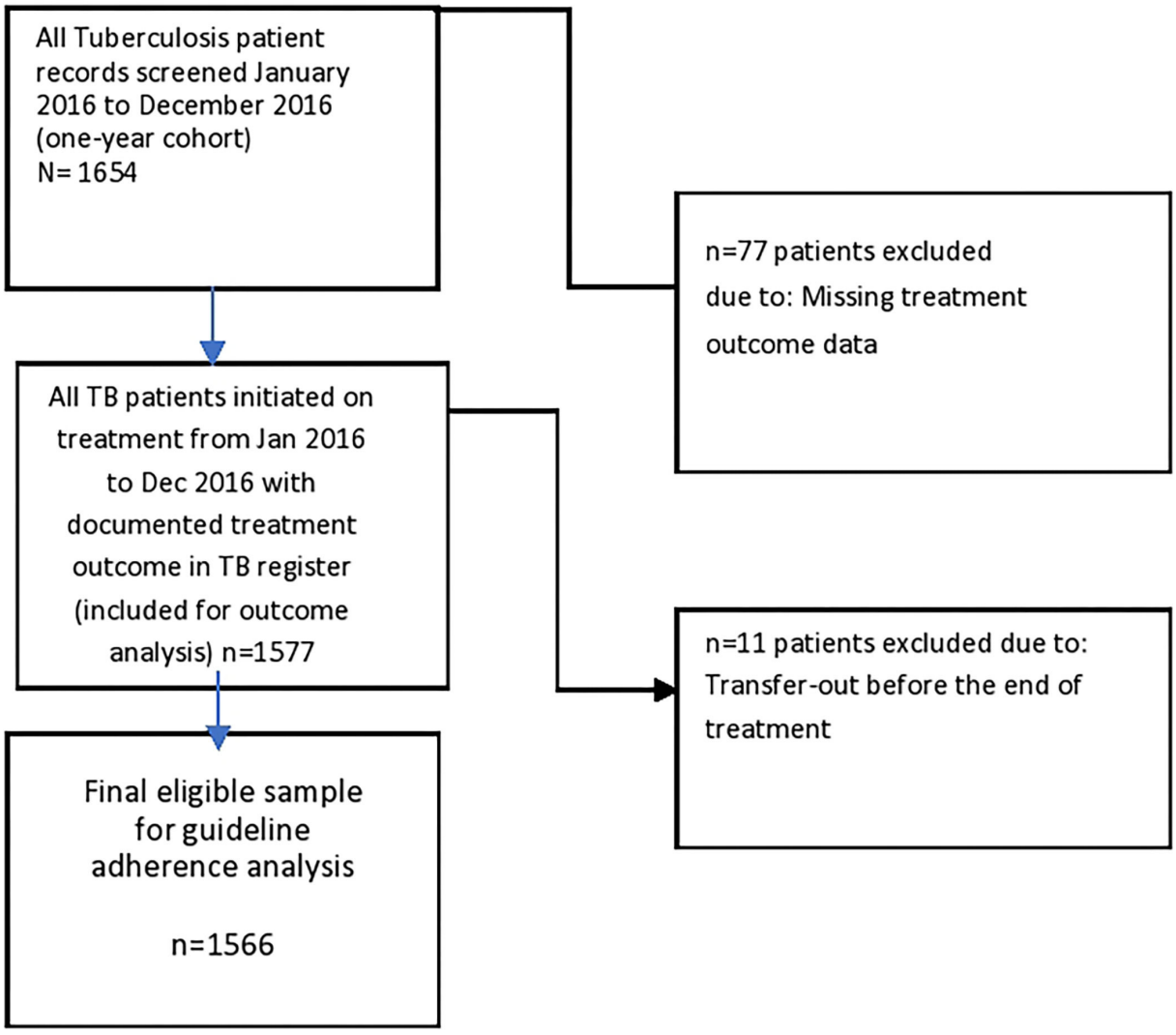


FIGURE 1
Flowchart on patient selection for analysis of adherence to National Guideline in assigning TB treatment outcome.

## Definitions of terms

### Cure

Patient who is sputum smear-negative in the last month of treatment and on at least one previous occasion (17).

### Treatment completed

A patient who has completed treatment but who does not meet the criteria to be classified as a cure or a failure (17).

### Treatment failure

Patient who is sputum smear-positive at 5 months or later during the treatment (17).

### Died

Patient who died for any reason during the treatment (17).

### Lost to follow-up

Patient whose treatment was interrupted for two consecutive months or more (17).

### Transfer out

Patient who has been transferred to another recording and reporting unit and for whom the treatment outcome is not known (17).

### Treatment success

It is defined as the sum of patients cured and those who have completed the treatment (17).

## Statistical method

The proportion of bacteriologically diagnosed TB patients in the facility tuberculosis treatment register that had follow-up sputum smear results available at month 2/3, month 5, and month 6 was calculated using frequency and percentages. Among those with available smear follow-up results, the proportions with smear conversion were also estimated and used to determine actual treatment outcomes. Among bacteriologically confirmed TB patients with end-of-treatment (month 6) smear result, the proportion of each outcome (cure, failure, treatment completed) as derived from the TB treatment register was validated for adherence against case definitions provided by NTBLCP guideline and marked as adhered (correct outcome) or non-adherent (incorrect treatment outcome). Descriptive statistics such as frequency and percentages were used to present adherence level and availability of sputum AFB follow-up result. In addition, predictors of healthcare workers' adherence to TB guideline were also determined at univariate and multivariate levels using Statistical Program for Social Sciences, version 17, at a level of significance $p < 0.001$ and confidence interval of 95%.

## Ethical approval

As data for this study were retrieved from routinely collected surveillance register of the LSTBLCP, no ethical clearance was required. Permission was received from the Lagos State Ministry of Health. Patient information was deidentified before the analysis for confidentiality.

# Results

## Descriptive data

In Table 1, of the 1,577 eligible records, majority (60.7%) of the participants were male, >30 years (50.2%), HIV-negative (88.4%), and attended private not-for-profit (78.5%). In total, 0.7% of the patients were transferred out.

## Outcome data

In Table 2, 91.1, 77.6, and 70.3% of patients were respectively offered sputum smear microscopy at months 2/3, month 5, and month 6 in that decreasing order. In total, 68.6% of patients in our study had all the three sputum follow-up results.

TABLE 1 Demographic characteristics and reported treatment outcomes for TB patients who initiated treatment across 10 private hospitals in Lagos State between January–December 2016 ($n = 1,577$).

| Variable | Number | Percentage |
|---|---|---|
| **Patient age** | | |
| 0–30 years | 785 | 49.8 |
| >30 years | 992 | 50.2 |
| **Sex** | | |
| Male | 957 | 60.7 |
| Female | 620 | 39.3 |
| **Type of hospital** | | |
| Private for profit | 339 | 21.5 |
| Private not for profit | 1,238 | 78.5 |
| **TB service scheme** | | |
| 3a | 8 | 80 |
| 3b | 2 | 20 |
| **Provider training (year)** | | |
| ≤2012 | 7 | 70 |
| >2012 | 3 | 30 |
| **TB refresher training** | | |
| Yes | 2 | 20 |
| No | 8 | 80 |
| **Guideline availability** | | |
| Yes | 4 | 40 |
| No | 6 | 60 |
| **HIV status** | | |
| Positive | 100 | 6.3 |
| Negative | 1,394 | 88.4 |
| Unknown | 83 | 5.3 |
| **Treatment outcome** | | |
| Cured | 1,064 | 67.5 |
| Completed | 255 | 16.1 |
| LTFU | 186 | 11.8 |
| Died | 47 | 3.0 |
| Treatment failure | 14 | 0.9 |
| Transfer out | 11 | 0.7 |
| **Treatment success rate (TSR)** | | |
| Successful | 1,319 | 83.6 |
| Not successful | 258 | 16.4 |

Table 3 highlights the level of adherence to NTBLCP guideline while assigning treatment outcome to TB patients. Overall, HCWs were adherent in assigning treatment outcome for 65.6% of TB patients while 34.4% of patients were assigned incorrect treatment outcome. Nonadherence was highest when assigning "treatment completion," 82% (209/255), followed by treatment failure (57.1%) and LTFU 29.6% (55/186) but least for death outcome 0% (0/14) and cure, 25.1% (267/1,064). Most variations between reported and actual treatment outcomes were found with cured (17%) and

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TABLE 2 Analysis of availability of Acid-Fast Bacilli (AFB) Sputum Follow up result at months 2/3, 5 and 6 in Tuberculosis Treatment Register ($n = 1,566$).

| Follow-up month | Number (*N*) | Percentage % |
|---|---|---|
| **Month 2/3** | | |
| 0 | 1,332 | 85.6 |
| Scanty | 10 | 0.6 |
| 1+ | 78 | 5.0 |
| Not done | 146 | 9.0 |
| Done | 1,420 | 91.0 |
| **Month 5** | | |
| 0 | 1,198 | 76.4 |
| Scanty | 2 | 0.1 |
| 1+ | 18 | 1.1 |
| Not done | 348 | 22.2 |
| Done | 1,218 | 77.6 |
| **Month 6** | | |
| 0 | 1,096 | 70.0 |
| Scanty | 1 | 0.06 |
| 1+ | 5 | 0.3 |
| Not done | 464 | 29.8 |
| Done | 1,102 | 70.3 |
| **Months 2/3,5&6** | 1,074 | 68.6 |

completed (13.34%) outcomes. Successful and unsuccessful outcomes were overreported by a difference of 30.4 and 4.1%, respectively.

In Table 4, multivariate analysis shows that DOTS providers in private facilities with available TB guideline (OR 8.33, CI 3.56–19.49, $p < 0.0001$), PNFP facility (OR 4.42, CI 1.91–10.3, $p = 0.001$) were more likely to adhere to National TB Guideline while assigning TB treatment outcome.

## Discussion

The study aimed to investigate whether private hospital TB service providers assigned correct treatment outcome to tuberculosis patient based on case definitions in NTBLCP guidelines. The study highlighted the availability of periodic sputum AFB results in treatment records as well as variations in reported and actual treatment outcome and predictive factors. In total, 4.7% of patient entries in this study had missing treatment outcome. This is lower than findings from Lagos and Kenya studies where 53.8 and 30% of records had missing treatment outcomes, respectively (19, 20). Healthcare workers might not document treatment outcome if they were not able to retrieve complete sputum AFB results from the laboratory. It is also possible that some of the laboratory results got missing before they were documented. The above findings underline the need to strengthen interventions that improve patient access to follow-up test and results, particularly month 6 result in the absence of which "cured" outcome cannot be declared.

Only 65.6% of TB patients received correct treatment outcome based on current domestic NTBLCP guideline for frontline healthcare workers. This contrasts with the Lagos study where reported treatment success was associated with full adherence (19). However, the study only mentioned that 53.8% of treatment outcome records were missing but failed to describe how adherence to treatment outcome for patients managed in the private sector was specifically measured rather than specified (19). All the 34 DOTS sites recruited in the study had microscopy services which may indicate better access; unlike in our study, where only half of the facilities had AFB services.

In the current study, reported lost to follow-up (LTFU) was 11.9%, but the validated figure was 8.4% giving a difference of 3.5%. The figure is lower than findings from Nepal in which 16.8% of patients assigned LTFU were found to have completed treatment during community tracking and validation by NTP

TABLE 3 Analysis of level of adherence to National TB Guideline in assigning various TB treatment outcomes ($n = 1,566$).

| Indicator | Not adherent (incorrect)% | Adherent (correct)% | Reported treatment outcome (%) | Actual treatment outcome (%) | % Diff (actual-reported) |
|---|---|---|---|---|---|
| Cured | 267 (25.1) | 797 (74.9) | 1,064 (67.9) | 797 (50.9) | 17.0 |
| Completed | 209 (82.0) | 46 (18.0) | 255 (16.3) | 46 (2.94) | 13.36 |
| Died | 0 (0.0) | 47 (100) | 47 (3.0) | 47 (3.0) | 0.0 |
| Failure | 8 (57.1) | 6 (42.9) | 14 (0.9) | 6 (0.38) | 0.52 |
| LTFU | 55 (29.6) | 131 (70.4) | 186 (11.9) | 131 (8.4) | 3.5 |
| Successful outcome | 476 (36.1) | 843 (63.9) | 1,319 (84.2) | 843 (53.8) | 30.4 |
| Unsuccessful outcome | 63 (25.5) | 184 (74.5) | 247 (15.8) | 184 (11.7) | 4.1 |
| Overall adherence | 539 (34.4) | 1,027 (65.6) | | | |

% Adherence, # of correct outcome/# same category of outcome; % Non-adherent: # of incorrect outcome/# same category of outcome; % Reported, Treatment outcome Total # of specific outcome category/Total # of patients (1,577); % Actual treatment outcome, # of correct outcome out of same reported outcome category/Total # of patients (1,577); %Diff in outcome, Diff in Reported and Actual patient/Total # of patients (1,577).

TABLE 4 Facility and DOTS provider correlates of adherence to National TB Guideline.

| Variable | Non-adherent | Adherent | cOR, 95% CI | aOR, 95% CI | *p*-Value |
|---|---|---|---|---|---|
| **Facility** | | | | | |
| PFP | 98 (28.9) | 241 (71.4) | | Ref | |
| PNFP | 452 (36.5) | 786 (23.5) | 0.71 (0.5–0.9) | 4.42 (1.91–10.3) | 0.001 |
| **Refresher training** | | | | | |
| No | 26 (32.5) | 30 (41.7) | 1.09 (1.07–1.11) | – | 1.000 |
| Yes | 54 (67.5) | 42 (58.3) | | | |
| **TB guideline** | | | | | |
| No | 9 (11.3) | 4 (5.6) | | | |
| Yes | 71 (88.8) | 68 (94.4) | 0.99 (0.8–1.3) | 8.33 (3.56–19.49) | <0.0001 |
| **TB service scheme** | | | | | |
| 3a | 27 (33.8) | 24 (34.7) | 0.53 (0.3–0.9) | 1.62 (0.82–3.19) | 0.162 |
| 3b | 53 (66.3) | 47 (65.3) | | | |

cOR, crude odd ratio; aOR, adjusted odd ratio; 3a, TB treatment services only; 3b, TB diagnosis and treatment.

(21, 22). It is possible that providers erroneously assigned LTFU for TB patients yet to fulfill LTFU case definition or were unable to update TB registers for TB patients who later presented at health facilities after the initial missed appointments. In Botswana, for instance, 39% of sputum smears were declared as not done instead of a lower figure of 16%. This gap was due to the failure of documentation by HCWs. This finding highlights why emphasis should be placed on knowledge of case definitions of TB treatment outcomes by private providers during supervisory visits, the need for home tracking, and prompt recording (23).

In this study, the percentage difference in the reported (documentation in register) and validated/actual (NTBLCP case definitions) treatment completed and cure rates were 13.4% and 17%, respectively. The investigators noted that as against the reported cure rate of 67.9%, the validated figure was 50.9%, while the completion rate was validated as 2.94% as against the reported completion rate of 16.3%. Overall, treatment success in this study was overreported by a difference of 30.4%. This figure is higher than findings from South African study where TSR was overreported by 12% (24) but lower than findings from KwaZulu natal study where only 34.8% of the reported cure rate was validated (25). It was obvious in our study that HCWs poorly understood how 'treatment completed' differed from 'cured'. Many patients who had negative month 6 AFB result with at least one previous negative AFB result were still labeled as completed in the treatment records, while several others without month 6 follow-up test were assigned cured. This highlights the need to structure supervisory and training program to specific needs of private providers with the view to bridging gaps in knowledge and health systems performance. Overreporting of cure rate can give a false impression of TB control. If patients are managed according to their smear results and their follow-up smear is inaccurately interpreted, healthcare workers may be inappropriately and unknowingly treating drug-resistant TB patients with first-line anti-TB drugs.

We also found that PNFPs (compared to PFPs) were four times more likely to adhere to national guideline while assigning treatment outcome while DOTS providers in facilities with National TB Guidelines (compared to those with no guideline) were eight times more likely to adhere to guideline when assigning TB treatment outcomes. These findings have implications for policy and practices. A study from Northern Nigeria found that the poor quality of TB services in the private sector is impacting TB treatment outcome (26). These differential findings in the documentation among private providers by profit status could mean that PFPs fear that adhering to the TB guidelines would reduce the monetary profit they will make from treating TB patients. These findings are pertinent because they are critical to the use of the guidelines by the practitioners and subsequent good outcomes for patients treated in private health facilities.

Guidelines traditionally serve as reference guide and means of refresher for providers across different knowledge areas, for example, clinical management, monitoring and evaluation, documentation and reporting. Prioritization of public sector facility while disseminating such guidelines with private sector left out is not uncommon. Studies have shown that 60% of guideline available at the central level were not available at service delivery point and over 50% of nurses did not document nursing care provided (27, 28). Making these guidelines available at lower level of care could help providers to reference and clarify technical issues toward improving on documentation and reporting practices. Such guidelines need to be precise, less bulky for busy private sector providers who oftentimes backstop for multiple disease areas including TB services. There is a need for NTBLCP to intensify structured training and supervision/mentorship visits targeted at private facilities with emphasis on case definitions of treatment outcomes. The approach could change the narrative in the private sector where data issues may be a big challenge due to the limited human

resources needed to provide quality TB services. Innovative, real-time digitization of reportable case-based TB indicators could help to reduce the volume of paper-based documentation, improve the quality and fidelity of reported TB data, and help in real-time decision-making toward the realization of TB control and elimination goals (29).

## Limitation

One of the limitations of this study is that follow-up results were not traced to the laboratory since there was no digital linkage of the laboratory with facility TB surveillance system. However, the objective was not to validate results with laboratory records but to check for the adherence of HCWs and the correctness of the assigned treatment outcome based on the case definitions of the NTBLCP guidelines. We did a second-level check for missing AFB results in the TB treatment card which reduced the possibility of underestimation of treatment outcome. In rare instances, it was possible that treatment outcomes of some patients were validated during data collation by the LGA TB supervisors before reporting to NTBLCP, although we expect such cases to have been updated in the facility TB register or patient treatment card. The study also reviewed TB surveillance data in 2017. Therefore, it will be relevant if follow-up study is done to evaluate any changes or improvement between 2017 and till date. This study is a retrospective review of facility TB register and treatment card and did not consider other factors that could have influenced the quality of TB data management.

# Conclusion

A huge percentage of patients managed in private facilities in a lower middle-income country were not assigned the correct treatment outcome based on the NTBLCP guideline. Only 68% of patients had all the three periodic sputum follow-up results needed to assign correct treatment outcomes. The huge proportion of TB patients with missing end-of-treatment, month 6 follow-up results is of great concern. The result was a huge discrepancy between the reported and actual cure and completion rate data. Giving the importance of sputum AFB results in the management of TB patients and assigning the correct treatment outcome, measures should be put in place to improve patient access to laboratory follow-up tests where TB drugs were collected. There is a need to establish systems for complete logging and tracking of all sputum samples received in the laboratory, timely retrieval, and accurate recording of results at the facility level. Linking laboratory directly with the NTBLCP surveillance system is recommended since it has the potential to minimize missing data and discrepancies and more accurately influence the quality of TB program outcome data. National TB Programs in low- and middle-income countries (LMICs) need to invest more on regular supervision, training and retraining of staff, provision of updated guidelines, and exchange mentorship from highly adherent private and public facilities to poorly adherent private facilities. In addition, we call for a business model that links the quality of data reported by the private facility providers with annual government accreditation programs. Future research should investigate the impact of removal of fees for laboratory follow-up test through health insurance programs and the impact that digitalization of TB Health Information Management Systems will have on documentation practices and the integrity of the TB treatment outcome data in the private sector.

# 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.

# Author contributions

VA: conceptualization, formal analysis, methodology, validation, and writing-original draft. OAA, MA, and VA: visualization. OAA, MA, AA, and VA: writing-review and editing. OEA and IN: writing methodology. MA: writing result. VE and VO: rewriting introduction and project administration. VA and AA: overall project administration and supervision. IN, OAA, and VA: data analysis. JOI: wrote the introduction and part of the discussion. OO: wrote part of the discussion, revised the manuscript based on feedback from the reviewers. All authors contributed to the article and approved the submitted version.

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# 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



## References

1. World Health Organization. *Global tuberculosis Report*. (2018). Available online at: https://apps.who.int/iris/handle/10665/274453 (accessed March 18, 2022).

2. Federal Ministry of Health. *National TB and leprosy control program annual report*. (2018). Available online at: www.ntblcp.or.ng/resources (accessed March 28, 2022).

3. USAID Nigeria. *MDR-TB Country profile*. (2016). Available online at: https://www.usaid.gov/sites/default/files/documents/1864/Nigeria_MDR-TB_508_ck.pdf (accessed March 28, 2022).

4. World Health Organization. *Nigeria Tuberculosis Profile*. (2016). Available online at: https://extranet.who.int/sree/Reports?op=Replet&name=/WHO_HQ_Reports/G2/PROD/EXT/TBCountryProfile&ISO2=NG&outtype=pdf (accessed March 18, 2022).

5. Federal Ministry of Health. *National TB and leprosy control program annual report*. (2017). Available online at: www.ntblcp.or.ng/resources (accessed March 14, 2022).

6. Lagos State. *Tuberculosis Control Program*. (2014). Available online at: https://health.lagosstate.gov.ng/2017/04/03/tuberculosis-control-program/ (accessed February 21, 2022).

7. Federal Ministry of Health. *National TB and leprosy control program annual report*. (2016). Available online at: www.ntblcp.or.ng/resources (accessed November 20, 2021).

8. World Health Organization. *Global Tuberculosis Report*. (2016). Available online at: https://apps.who.int/iris/handle/10665/250441 (accessed April 11, 2022).

9. Michelle L, Isabel B, Eric G. *Barriers to Use of Health Data in Low- and Middle Income Countries: A Review of the Literature*. (2018). Available online at: https://www.measureevaluation.org/resources/publications/wp-18-211/at_download/document (accessed May 26, 2022).

10. Onyeonoro UU, Chukwu JN, Nwafor CC, Meka AO, Omotowo BI, Ogbudebe C, et al. Assessment of the quality of tuberculosis surveillance data in six selected states in Southern Nigeria. *Ann Trop Med Public Health*. (2015) 8:94–100. doi: 10.4103/1755-6783.162318

11. Ogbuabor DC. Onwujekwe, OE. Governance of tuberculosis control programme in Nigeria. *Infect Dis Poverty*. (2019) 8:45. doi: 10.1186/s40249-019-0556-2

12. Adejumo OA, Daniel OJ, Adepoju VA, Femi-Adebayo T, Adebayo BI, Airauhi AO. Challenges of tuberculosis control in Lagos State, Nigeria: a qualitative study of health-care providers' perspectives. *Niger Med J*. (2020) 61:37–41. doi: 10.4103/nmj.NMJ_108_19

13. Basu S, Andrews J, Kishore S, Panjabi R, Stuckler D. Comparative performance of private and public healthcare systems in low- and middle-income countries: a systematic review. *PLoS Med*. (2012) 9:1. doi: 10.1371/journal.pmed.1001244

14. World Health Organization. *Involving Private Practitioners in Tuberculosis Control: Issues, Interventions, and Emerging Policy Framework*. (2001). Available online at: http://apps.who.int/iris/bitstream/handle/10665/66733/WHO_CDS_TB_2001.285.pdf?sequence=1 (accessed July 7, 2022).

15. Sidharta SD, Yin JD, Yoong JS, Mishal SK. High use of private providers for first healthcare seeking by drug-resistant tuberculosis patients: a cross-sectional study in Yangon, Myanmar. *BMC Health Serv Res*. (2018) 18:276. doi: 10.1186/s12913-018-3077-y

16. Lagos State Government Nigeria. *Tuberculosis Control Program*. Available online at: https://health.lagosstate.gov.ng/tuberculosis-control-program/ (accessed July 6, 2022).

17. Federal Ministry of Health, Nigeria. *National Tuberculosis and Leprosy Control Program, Workers Manual Revised*, 6th ed. Lagos: BamPrint Ventures (2015), p. 95.

18. National Tuberculosis and Leprosy Control Programme, Department of Public Health, Federal Ministry of Health, Nigeria. *The National Strategic Plan for Tuberculosis Control (NSP-TB 2015 - 2020): Towards Universal Access to Prevention, Diagnosis and Treatment*. (2014). Available online at: https://www.health.gov.ng/doc/NFM_Nigeria_NSP-for-TB-Control2015-2020_anx5.pdf (accessed July 6, 2022).

19. Adejumo O, Daniel O, Gidado M. Otesanya, AF, Esther N, Adejumo E, et al. Are tuberculosis patients managed according to the national guidelines in Lagos State Nigeria? *Int J Phys Med Rehabil*. (2016) 7:16–24. doi: 10.4236/ijcm.2016.71003

20. Sharma A, Ndisha M, Ngari F, Kipruto H, Kevin PC, Joseph S, et al. Review of data quality of an electronic tuberculosis surveillance system for case-based reporting in Kenya. *Eur J Public Health*. (2015) 25:1095–7. doi: 10.1093/eurpub/ckv092

21. Shakya TM, Shrestha KB. Compliance among tuberculosis patients with short course chemotherapy in the national tuberculosis Centre, Thimi, Bhaktapur, Nepal. In: *Proceedings of the Conference on Globar Lung Health and the 1995 Annual Meeting of the IUATLD/UICTMR; 1995 Oct 82*. Amsterdam: Elsevier Limited (2002), p. 82. doi: 10.1016/0962-8479(95)90316-X

22. Wares DF, Singh S, Acharya AK, Dangi R. Non-adherence to tuberculosis treatment in the Eastern Tarai of Nepal. *Int J Tuberc Lung Dis*. (2003) 7:327–35.

23. Alpers L, Chrouser K, Halabi S, Moeti T. Reingold A, Binkin N, et al. Validation of the surveillance system for tuberculosis in Botswana. *Int J Tuberc Lung Dis*. (2000) 4:737–43.

24. Dreyer AW, Mbambo D, Machaba M, Oliphant CEM, Claasens MM. Tuberculosis cure rates and the ETRNet: investigating the quality of reporting treatment outcomes from primary healthcare facilities in Mpumalanga province, South Africa. *Health Serv Res*. (2017) 17:190. doi: 10.1186/s12913-017-2128-0

25. Dilraj A, Bristow CC, Connolly C, Margot B, Dlamini S, Podewils LJ. Validation of sputum smear results in the Electronic TB Register for the management of tuberculosis, South Africa. *Int J Tuberc Lung Dis*. (2013) 17:1317–21. doi: 10.5588/ijtld.12.0904

26. Ibrahim LM, Oleribe OO, Nguku P, Tongwong GC, Mato LG, Longkyer MI, et al. Evaluation of quality of TB control services by private healthcare providers in Plateu state, Nigeria; 2012. *Pan Afr Med J*. (2014) 17:77. doi: 10.11604/pamj.2014.17.77.3412

27. Nabyonga O J, Bataringaya W J, Bakeera SK, Bart C. Do guidelines influence the implementation of health programs? — Uganda's experience. *Implementation Sci*. (2012) 7:98. doi: 10.1186/1748-5908-7-98

28. Tasew H, Mariye T, Teklay G. Nursing documentation practice and associated factors among nurses in public hospitals, Tigray, Ethiopia. *BMC Res Notes*. (2019) 12:612. doi: 10.1186/s13104-019-4661-x

29. Blaya JA, Shin SS, Yale G. Electronic laboratory system reduces errors in National Tuberculosis Program: a cluster randomized controlled trial. *Int J Tuberc Lung Dis*. (2010) 14:1009–15.

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# Modified aluminosilicates display antibacterial activity against nontuberculous mycobacteria and adsorb mycolactone and *Mycobacterium ulcerans in vitro*

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*Mycobacterium ulcerans* (MU) infection of skin and soft tissue leads to chronic skin ulceration known as Buruli ulcer. MU releases a lipid-like toxin, mycolactone, that diffuses into the tissue, effecting disease through localized tissue necrosis and immunosuppression. Cutaneous Buruli ulcer wounds slowly advance from a painless pre-ulcerative stage to an ulcerative lesion, leading to disparities in the timing of medical intervention and treatment outcomes. Novel Buruli ulcer wound management solutions could complement and supplement systemically administered antimicrobials and reduce time to healing. Capitalizing on nanopore structure, adsorption, and exchange capacities, aluminosilicate nanozeolites (nZeos) and geopolymers (GPs) were developed and investigated in the context of therapeutics for mycobacterial disease ulcerative wound care. nZeos were ion exchanged with copper or silver to assess the antimicrobial activity against MU and *Mycobacterium marinum*, a rapid growing, genetic ancestor of MU that also causes skin and soft tissue infections. Silver- and copper-exchanged nZeos were bactericidal against MU, while only silver-exchanged nZeos killed *M. marinum*. To mediate adsorption at a biological scale, GPs with different pore sizes and altered surface modifications were generated and assessed for the ability to adsorb MU and mycolactone. Macroporous GPs with and without stearic acid modification equivalently adsorbed MU cells, while mesoporous GPs with stearic acid adsorbed mycolactone toxin significantly better than mesoporous GPs or GPs modified with phenyltriethoxysilane (PTES). In cytotoxicity assays, Cu-nZeos lacked toxicity against Detroit 551, U-937, and WM-115 cells. GPs demonstrated limited cytotoxicity in Detroit 551 and WM-115, but produced time-dependent toxicity in U-937 cells. With their large surface area and adsorptive capacities, aluminosilicates nZeos and GPs may be modified and developed to support conventional BU wound care. Topical application of nZeos and GPs could kill MU within the cutaneous wound

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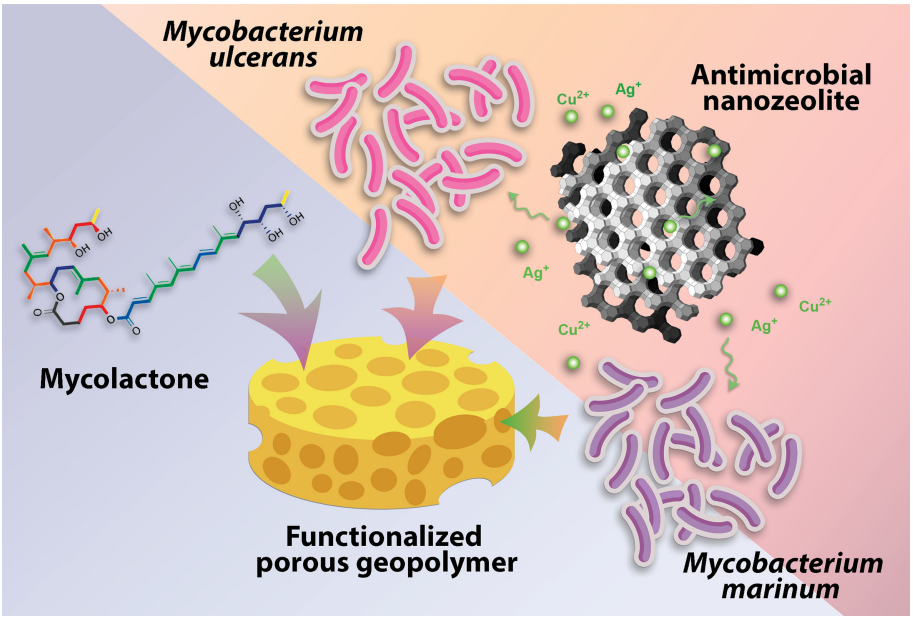
environment and physically remove MU and mycolactone with wound dressing changes, thereby improving wound healing and overall patient outcomes.

KEYWORDS

mycobacteria, *Mycobacterium ulcerans*, *Mycobacterium marinum*, aluminosilicates, antimicrobial ions, silver, copper, adsorption




GRAPHICAL ABSTRACT

## Introduction

Buruli ulcer is a painless, progressive skin and soft tissue infection (SSTI) caused by *Mycobacterium ulcerans* (MU) (1). Cases of this neglected tropical disease are observed in equatorial countries, wherein disease occurrence is associated with proximity to wetlands and bodies of water (2, 3). Infection manifests as a diminutive nodule, wherein the lengthy MU replicative cycle yields protracted ulceration (3, 4). MU accumulates in the subcutaneous extracellular space (5) and secretes the polyketide toxin mycolactone (6, 7), generating cytopathic analgesia (8). The resulting painlessness prompts benign negligence of the wound, condoning bacterial penetration of underlying tissue (9). As in the case of latent *Mycobacterium tuberculosis*, deferred treatment of Buruli ulcer compromises therapeutic efforts (10, 11).

A fully oral regimen of rifampicin and clarithromycin by the World Health Organization has greatly advanced treatment of Buruli ulcer (12). However, success of this regimen is confined to early cases of the disease (13, 14). Despite indications of antibiotic susceptibility *in vitro*, parenteral antibiotic therapies must account for penetration of the infection site (12). Localized treatment, as utilized in the care of other SSTIs, supports greater therapeutic access to the wound (15). Evidence of the antimicrobial activity of clay materials against MU (16) has garnered interest as an alternative to conventional small molecule compounds (1, 17). Aluminosilicate materials, a group of modulated clay derivatives, provide a versatile framework for topical therapeutic delivery (18).

Our research team created two synthetic, customized aluminosilicates – nanozeolites (nZeos) and geopolymers (GPs) – to mimic clay's predominant antimicrobial features: ion content (19) and adsorption (20), respectively (21–23), and to ensure consistency, safety, and efficacy of developed products for treating SSTIs (24). Ion-exchanged nanozeolites (IE-nZeos)

and GPs strategically exploit ion release and adsorptive capacities for leveraging topical therapeutic applications with site-directed antimicrobial delivery and bacterial and toxin adsorption, respectively, as we previously demonstrated (21–23). Metal ions with previously reported antimicrobial properties, such as copper and silver (16, 25), can be interchangeably attached to the extensive nZeos surface, enabling subsequent ion exchange with water or moisture and dispersion of metal ions from the scaffold into the environment (22, 23, 26).

*Mycobacterium marinum* (Mmar), the closest genetic relative of MU and a fast-growing nontuberculous mycobacterium SSTI pathogen (27–30), causes non-ulcerative granulomatous skin lesions that are often linked with fish aquaria (31) or fishing-related injuries (32). As a close relative of both MU and *Mycobacterium tuberculosis* (33), Mmar provides context to decades of research focused on ion-dependent antimicrobial activity against mycobacteria (25, 34, 35). Silver nanoparticles exhibit antimicrobial activity against Mmar (35), whereas silver enriched dressings (36, 37) and copper-rich clay materials (16) kill MU. Jointly, these findings suggest the pertinence of IE-nZeos as intermediates for mycobacterial wound care.

Geopolymers (GPs), amorphous counterparts to zeolites, are aluminosilicate materials that can be customized to control porosity, physicochemical properties, and functionality (18, 38, 39). The porous structure of these compounds adsorbs heavy metals and industrial manufacturing waste (18). In a biomedical context, we recently demonstrated that porous GPs adsorb whole bacterial cells, toxins, and other secreted products (21, 22). Given that Buruli ulcer pathology is largely ascribable to mycolactone secretion and activity (6, 40), sequestration of either (a) mycolactone alone or (b) whole MU cells is expected to vastly improve disease prognosis (41). GPs have significant technological potential due to ease and low cost of production (38), making GPs exceptional candidates for use in endemic MU areas with minimal healthcare infrastructure (3). With high mechanical and chemical stabilities, GPs can be functionalized by coating the surface with operative agents and/or moieties that impart specific physicochemical properties (42).

To assess the antimicrobial activity of nZeos against SSTI-associated mycobacteria, we investigated *in vitro* antimicrobial activity of copper and silver nZeos against MU and Mmar (43). Based on pore size capacities and our previous GP adsorption studies with bacterial cells and toxins (21), macroGPs were tested for the adsorption of MU cells and mesoGPs were assessed for mycolactone adsorption. To impart GPs with heightened hydrophobicity, the surface of GP materials was modified with stearic acid (saturated fatty acid) or phenyltriethoxysilane (PTES) (a silane coupling agent with hydrophobic characteristics). These steric acid-coated GPs (SA-GPs) or PTES-coated GPs (PTES-GPs) were predicted to display greater lipophilic or hydrophobic behavior than their uncoated counterparts (44). As a result, we expected that these surface-modified GPs would bind apolar mycolactone (45) and lipid-rich MU (46) with increased affinities.

# Materials and methods

## Synthesis and characterization of Ag-nZeos and Cu-nZeos

The synthesis and characterization of Ag-nZeos and Cu-nZeos were reported previously (22, 23). In short, nZeos were prepared by heating a Na-based geopolymer resin with the nominal composition of $3.0Na_2O$: $1.0Al_2O_3$: $4.0SiO_2$: $32.4H_2O$, which was mixed homogeneously with canola oil. The precursors for the geopolymer resin were NaOH pellets (Sigma Aldrich), water glass (Sigma Aldrich), metakaolin (MetaMax® from BASF), and deionized (DI) water. The oil reduces the alkalinity of the reaction mixture during heating, thereby improving the zeolitic crystallinity of the final product. The zeolite product was washed repetitively with DI water until the pH of the filtrate reached about 8. Ag-nZeos and Cu-nZeos were prepared by exchanging $Na^+$ ions in nZeos with $Ag^+$ and $Cu^{2+}$ ions, respectively. To exclude the nZeos as a source of antimicrobial activity, nZeo carriers were enriched with sodium (Na-nZeos) (22). For characterization of chemical structures and morphologies, powder X-ray diffraction (PXRD) patterns, scanning electron microscopy (SEM) images, and transmission electron microscopy (TEM) of dried samples were collected on Siemens D5000 powder X-ray diffractometer, XL30 environmental FEG microscope and Titan 80-300 FEG-TEM (FEI Co., Hillsboro, OR), respectively.

Brunauer-Emmett-Teller (BET) surface areas were estimated with a Micrometrics ASAP 2020 volumetric adsorption analyzer with nitrogen as the adsorbate at 77 K. Prior to the analysis, samples (approximately 300 mg) were degassed at 300°C for at least 6 h under a vacuum until a residual pressure of ≤10 µm Hg was reached. Specific surface areas were determined from the BET equation. The *t*-plot method was used to distinguish the contributions from micropores (pore size: < 2 nm) and from the mesopores (pore size: from 2 nm to 50 nm) to the pore volume (the combined volume of micropores and mesopores) and surface area. The mesopore volumes were calculated after subtracting the micropore volume from the total pore volume. Pore size distributions were obtained using the Barrett-Joyner-Halenda (BJH) method assuming a cylindrical pore model from the desorption branch of sorption isotherms (47). Elemental compositions of the zeolite samples were determined by combining Rutherford backscattering and particle induced X-ray emission data. The hydrodynamic

particle size of the nZeos sample was measured on Malvern Nano-ZS instrument with a helium neon laser.

## Synthesis and characterization of macroporous and mesoporous GPs

The synthesis and characterization of macroporous and mesoporous GPs were reported previously (21). For macroporous GPs, the synthetic process resembles nZeos preparation. Fumed silica (4.15 g) (Cabot, CA-BO-SIL® EH-5; aggregate particle size: 0.2 – 0.3 μm; primary size: 5 -25 nm) was added to 12.0 mL of KOH (11.6 *M*) solution and mechanically mixed (IKA® RW 60 digital mixer) at 800 rpm for 30 min to dissolve the silica. Metakaolin [7.64 g (Metamax, BASF; average particle size: 1.3 μm)] was added to the solution and the mixture was stirred again at the same speed for 40 min to form a homogenous fluidic liquid. Paraffin oil (Alfa Aesar, Haverhill, MA) was then added to the resin at a 1:1 oil-to-water volume ratio and stirred under the same mixing condition for 15 min, resulting in a homogeneous, viscous emulsion. After heating at 60°C for 24 h, the cured monolithic product was crushed into small pieces (1 – 2 mm) and subjected to Soxhlet extraction with hexane as the solvent. The product was washed extensively with DI water until a neutral pH was achieved (pH ~7) and dried in a lab oven overnight at 90°C.

For mesoporous GPs, an aluminosilicate precursor mixture with a composition of $3.1K_2O$: $Al_2O_3$: $5.5SiO_2$: $66H_2O$ was prepared by first adding 6.05 g of KOH pellets and 18.03 g of potassium silicate solution (PQ corporation, $H_2O$: 60.8 wt%, $K_2O$: 12.65 wt%, $SiO_2$: 26.55 wt%) in 14.55 mL of DI water in a cold bath. Once the KOH pellets were dissolved, the solution was brought to room temperature and mixed with 5.09 g of the metakaolin. Stirring with the mechanical mixer at 800 rpm for 40 min produced a visually homogeneous and free-flowing geopolymer resin. The resin was transformed into a paste by heating the resin at 90°C for 6 h in a sealed 50 ml polypropylene tube. To isolate the solid component, the paste was subjected to repetitive washings with DI water, followed by centrifugation at 6000 rpm (g-force: ~2500 $m/s^2$) for 15 min, until the supernatant was pH ~8. After drying at 90°C overnight, the resulting powder product was stored in sealed glass vials at room temperature.

The chemical structures and morphological features of all the samples were examined by collecting PXRD patterns and SEM/TEM images. Brunauer-Emmett-Teller (BET) surface area analysis was performed using a Micrometrics ASAP 2020 volumetric adsorption analyzer with nitrogen as the adsorbate at 77 K. The *t*-plot method was used to distinguish the micropores from the mesopores in the samples and to calculate the external surface areas. By subtracting the micropore volume from the total pore volume, mesopore volumes were calculated. Mesopore size distributions were obtained using the Barrett-Joyner-Halenda (BJH) method assuming a cylindrical pore model and calculating the distance between opposite walls of the pore (47).

## GP surface modifications to enhance hydrophobicity

As previously described (21), surface modification of the GP products with stearic acid was carried out by an esterification reaction between silanol groups on the surfaces of geopolymers. Stearic acid (0.2 g) was dissolved in 25 mL of 0.6 M NaOH solution in a 90°C water bath. After adding 1.0 g of macroGP or mesoGP to the hot solution, the mixture was magnetically stirred for 10 min at 90°C and slowly cooled to room temperature while agitating. Precipitate products were filtered and washed extensively with DI water to reduce the pH to ~8.0. The products were dried in a lab oven at 60°C overnight and then placed in another oven at 160°C for 4 h to induce crosslinking of carboxylic acid groups to the surface of the geopolymer. Finally, the dried products were washed with hot toluene multiple times to remove unbound stearic acid and then dried again at 60°C overnight. After producing stearic acid-modified macroGPs (SA-macroGPs) and stearic acid-modified mesoGPs (SA-mesoGPs), Fourier transform infrared (FT-IR) spectra were recorded using a Bruker IFS66 V/S attenuated total reflection FT-IR spectrometer. Carbon–hydrogen–nitrogen (CHN) elemental analyses were performed by employing Perkin-Elmer 2400 Series II CHNS/O Analyzer (Waltham, MA) with a thermal conductivity detector.

Phenyltriethoxysilane (PTES)-GPs were prepared with PTES as a surface modifier and motor oil as a pore-forming agent with a 1:1 oil: resin volume ratio. First, 0.96 g of DI water, 0.74 g of NaOH, 12.23 g of water glass (Sigma-Aldrich; $SiO_2$ 27 wt %, NaOH 14 wt %, $H_2O$ 59 wt %), and 1.01 g of PTES (Gelest, Inc., Morrisville, PA) were mixed and stirred with a high-shear mixer at 500 RPM for 10 min. To the mixture solution, 6.17 g of metakaolin was added and stirred at 100 RPM for 30 min. The resulting GP resin was then mixed with 10 mL of motor oil and the liquid mixture was stirred at 800 RPM for 30 min until becoming homogeneous, after which the mixture was heated at 70°C for 24 h in a tightly sealed container. After heating, the resulting hard monolithic solid was crushed into a coarse powder and washed with hexane and then ethanol repetitively to remove the oil from the sample, yielding the PTES-GP sample.

## Mycobacterial strains and growth conditions

Cultures of Mmar ATCC 927 and MU 1615 (graciously provided by Dr. Pamela Small, University of Tennessee, Knoxville, TN, USA) were grown in Middlebrook 7H9

supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% oleic acid-dextrose-catalase (OADC), hereafter referred to as M7H9. All liquid cultures were maintained at 30°C with gentle rotation. To produce homogenous bacterial suspensions, clumps were dispersed with 23-gauge needles. Samples were adjusted to an $OD_{600}$ of ~0.3 (~2 x $10^7$ cells/ml), then diluted 1:100 prior to experimental use. Middlebrook 7H10 agar plates, designated M7H10, were supplemented with 0.5% glycerol and 10% OADC. To maintain a microaerophilic environment and prevent desiccation, M7H10 plates were sealed and incubated at 30°C. Mmar and MU colony forming units (CFU) were counted after incubation for 1 week or 6 weeks, respectively.

## *In vitro* Mmar IE-nZeos susceptibility testing

A five-day M7H9 broth microdilution susceptibility assay determined sensitivity to metal ions released by IE-nZeos (48). The antimicrobial activity of streptomycin (STR) (4, 8 μg/ml) was analyzed in parallel (48). At 24 h intervals, all experimental wells were sampled. Samples were subsequently diluted and plated on M7H10 and incubated at 30°C for one week. The minimum bactericidal concentration required to reduce the bacterial population by 99% ($MBC_{99}$) was determined with resultant CFU counts.

## *In vitro* MU IE-nZeos susceptibility testing

To assess the susceptibility of MU to IE-nZeos, two-fold serial dilutions of IE-nZeos (4-128 μg/ml) were incubated with MU for 21 d in M7H9 (48). STR (4 μg/ml) was used as a control for *in vitro* antimicrobial activity. Aliquots were collected at one-week intervals, serially diluted ten-fold in PBS-Tween 80 (0.1% v/v, pH 7), and plated on M7H10 agar. Pursuant to a six-week incubation at 30°C, $MBC_{99}$ was quantified.

## *In vitro* cytotoxicity assays

Representative skin, soft tissue, and immune cells were used to assess the cytotoxicity of Cu-nZeos and GPs, as previously demonstrated with Ag-nZeos (23) Detroit 551 human skin fibroblasts (ATCC CCL-110), WM-115 human skin epithelial cells (ATCC CRL-1675), and U-937 human monocytes (ATCC CRL-1593.2) were propagated according to manufacturer instructions. In 96-well microtiter plates, each cell type was seeded at a density of 3-6 x $10^3$ cells per well. Following an overnight attachment period, cells were exposed to two-fold dilutions of Cu-nZeos (128–8 μg/ml) or GPs (10 mg/ml) in 180 μL. Untreated cells served as controls for all independent experimental replicates. After a 24-hour incubation, supernatant was transferred to a black-walled, clear bottom, 96-well microtiter plate, and CellTox Green cytotoxicity reagent (Promega Corporation, Madison, WI) was added to experimental and control wells. Excitation and emission fluorescence was measured in relative fluorescent units (RFUs) with wavelengths of 485 nm and 520 nm, respectively. Cytotoxicity was determined by comparing RFU measurements of control, untreated cells and Cu-nZeo- and GP-treated cells (23).

## MU bacterial cell adsorption assays

MacroGPs or SA-macroGPs (10 mg) were added to MU saline suspensions (1 ml). MU-GP mixtures were incubated for 1 h at 30°C with gentle orbital agitation (100 rpm) to prevent sedimentation. MU saline suspensions without the addition of macroGPs were included as untreated controls. To remove GPs and GP-bound MU from solution, all samples (including MU untreated controls), were filtered using a sterile 5-μm cellulose syringe filter. Filtered supernatants containing unbound MU cells were subjected to serial dilutions in PBS-Tween 80 (0.1% v/v) and plated in duplicate on M7H10 agar. After the M7H10 plates were incubated at 30°C for 6 weeks, CFU/ml for each MU-GP co-incubation was determined.

## Mycolactone extraction

TLC isolation of mycolactone was performed as previously described from four- to six-week-old MU cultures incubated on M7H10 agar (7, 49). A 2:1 (v/v) chloroform: methanol mixture extracted total lipids from whole cells. The chloroform-soluble fraction was dried and resuspended in ice-chilled acetone. Acetone-soluble lipids were separated on a TLC plate in a solution of 90:10:1 (v/v/v) chloroform: methanol: water. Upon exposure to long wave UV light (365 nm), the yellow band observed at $R_f$ = 0.23 was collected and solubilized in absolute ethanol. Following silica removal with a 0.2 μm PTFE filter, mycolactone was isolated *via* HPLC (50).

## Mycolactone toxin adsorption assays

Purified mycolactone (two independent isolates) was adjusted to an $OD_{362}$ of 0.5 in 100% ethanol (51). MesoGPs, SA-mesoGPs, or PTES-GPs (10 mg) were added to mycolactone suspensions (~25 mg per 1 ml). Untreated mycolactone solutions were analyzed in parallel. To minimize photodegradation (51) and evaporation of the mycolactone suspension, samples were incubated in amber vials for 1 h at

room temperature. Thereafter, samples were briefly centrifuged (100 RPM) to sediment GP particles. To determine the level of remaining mycolactone, we analyzed the absorbance ($\Lambda_{max}$ = 362 nm) of each sample in a quartz cuvette (Hellma, Müllheim, Germany). GP-treated, 100% ethanol-only samples were measured to detract background signal. The final mycolactone concentration was subtracted from the starting concentration to determine the amount adsorbed by GPs.

## Statistical analyses

We assessed statistical significance using Welsh's t-tests for normally distributed data, Mann-Whitney U tests for data reflecting non-normal distributions, and ANOVA with Brown-Forsythe test and Welsh's correction. All statistical analyses were conducted using GraphPad Prism 9 software (San Diego, CA, United States). Samples with a difference of $p < 0.05$ were regarded significant.

# Results

## Characteristics and properties of modified aluminosilicate nZeo materials

Synthesis and detailed characteristics of nZeos, Ag-nZeos, Cu-nZeos, macroGPs, mesoGPs, and SA-mesoGPs were described previously (21–23, 26). In summary, nZeos, Ag-nZeos, and Cu-nZeos are 100 to 700 nm-sized aggregates composed of intergrown zeolite nanocrystals with lateral dimensions of 20 – 40 nm. Textual pores between the aggregated nanocrystals revealed a hierarchical pore structure with both zeolitic micropores and mesopores to the aggregates. Their PXRD patterns indicated formation of faujasite-type structures. From the elemental analysis, the silver and copper loadings for Ag-nZeos and Cu-nZeos were estimated to be 24 and 8.3 wt% (2.2 and 1.3 mmol/g), respectively (23, 26).

## Characteristics and properties of modified aluminosilicate GP materials

The PXRD patterns of macroGPs and mesoGPs (21) showed that both are non-crystalline geopolymeric materials (52). After modification with SA, the PXRD patterns of SA-macroGPs and SA-mesoGPs resembled their parent materials, indicating that geopolymer structure was not affected by the surface modification experiments. The macroGPs exhibited discrete spherical pores with pore diameters in the range of 50 – 200 µm, with additional smaller pores ranging from 200 – 500 nm on the pore walls (21). The smaller pores are due to the nanoscopic biphase formation between the inorganic geopolymer component and the organic paraffin oil component. The much larger spherical pores coexist after the biphase formation due to excess paraffin oil remaining in the mixture as large oil droplets. After curing the GP component, oil extraction leaves the two types of pore structures in the product. As expected, the SA-macroGPs and SA-mesoGPs exhibit the same morphology as the parent macroGPs and mesoGPs, respectively, indicating that surface modification does not change the morphology of the inorganic geopolymer (21). Meanwhile, the mesoGPs form aggregates composed of strongly interconnected primary nanoparticles of 20 – 30 nm in diameter with apparent textural porosity (21). BJH analyses revealed that the average pore size of mesoGPs was 31 nm due to the presence of textural porosity observed in the TEM studies (23). Meanwhile, macroGPs exhibit large pore sizes in the range of 50 – 200 µm and 200 – 500 nm with a small number of mesopores with an average pore size of 26 nm (21).

Characterization of the PTES-GPs using SEM imaging and $N_2$ gas sorption studies revealed both spherical macropores and mesopores walls (Figure 1), indicating similarity with macroGPs (21, 26). SEM imaging revealed PTES-GPs with seemingly discrete spherical pores with pore diameters in the range of in the 2 – 50 µm (Figures 1A, B). From $N_2$ gas sorption analysis (Figure 1C), the total surface area was 44.1 $m^2/g$, while the BJH analysis revealed a 12 nm of average pore size and estimated 0.13 $cm^3/g$ of mesopore volume (Figure 1D). Colorimetric titration with methylene blue indicated that only 0.7% of the total surface area could absorb the cationic dye molecules, proving a strong hydrophobic surface for PTES-GPs (data not shown).

## *In vitro* nZeos activity against Mmar is ion specific

Since Mmar is susceptible to silver nanoparticles [$Ag^+$ equivalency of 1 µg/ml (35)], we subjected Mmar to *in vitro* antimicrobial susceptibility assays with Ag-nZeos. At the 5-day experimental endpoint, 64 µg/ml Ag-nZeos ($Ag^+$ equivalency of 15.36 µg/ml (23); Table 1) reduced Mmar ≥100 fold, indicating $MBC_{99}$ bactericidal activity similar to the streptomycin antibiotic controls (Figure 2A). In the presence of Ag-nZeos (32-256 µg/ml), Mmar viability decreased from 0 to 3 days with CFU levels subsequently remaining static through day 5 (Figure 2A). Conversely, Cu-nZeos (4-128 µg/ml) did not affect Mmar viability (Figure 2B). All tested concentrations of Na-nZeos (16-128 µg/ml) grew synchronously with the Mmar growth control (Figure 2C), indicating the innocuous nature of nZeos carriers. Cumulatively, these results indicate that silver released from Ag-nZeos is responsible for the Mmar antimicrobial activity observed.

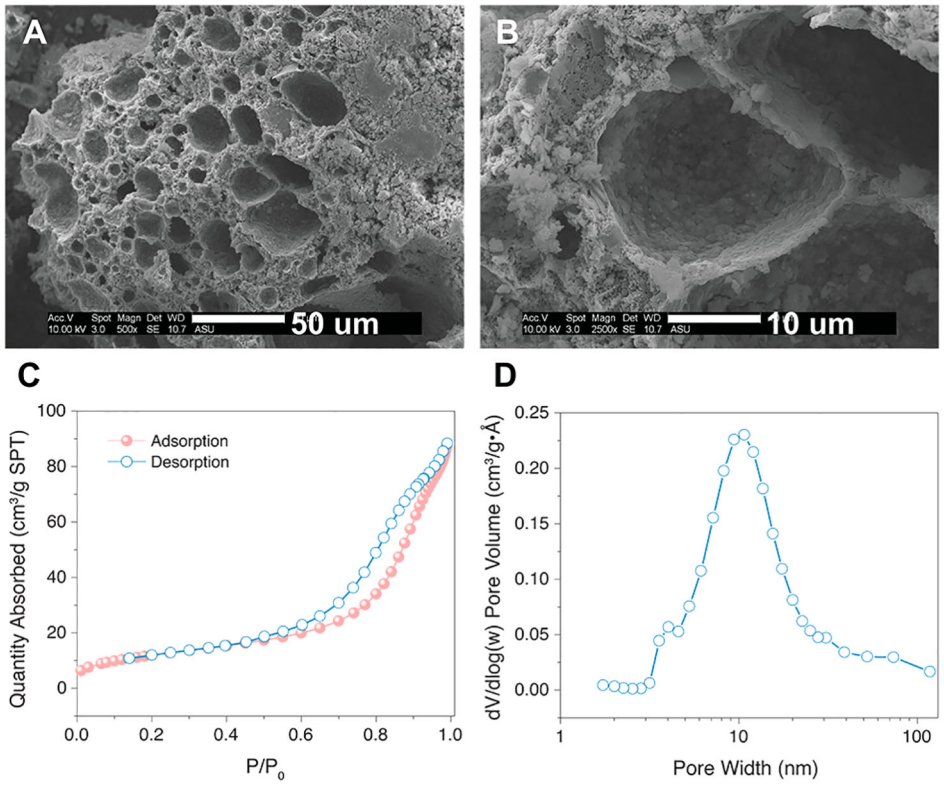



FIGURE 1
**(A)** Low-magnification and **(B)** high-magnification SEM images of PTES-GPs. Scale bar = 50 µm **(A)**, 10 µm **(B)**. **(C)** $N_2$ gas sorption isotherm and **(D)** BJH pore size distribution of PTES-GPs.

TABLE 1 nZeos antimycobacterial activity and ion equivalency.

| nZeos | Mmar $MBC_{99}$ (µg/ml) | Mmar $MBC_{99}$ Ion equivalence (µg/ml) | MU $MBC_{99}$ (µg/ml) | MU $MBC_{99}$ Ion equivalence (µg/ml) |
|---|---|---|---|---|
| Ag-nZeos | 64 | 15.36 $Ag^+$ | 16 | 3.84 $Ag^+$ |
| Cu-nZeos | N/A | N/A | 32 | 2.69 $Cu^{2+}$ |

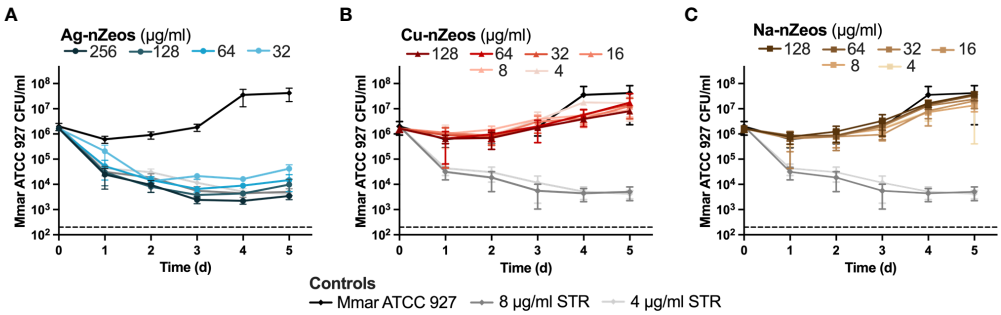



FIGURE 2
IE-nZeos antimicrobial activity against Mmar is ion specific. Mmar was exposed to two-fold dilutions of **(A)** Ag-nZeos (256 – 32 µg/ml), **(B)** Cu-nZeos (128 – 4 µg/ml), **(C)** Na-nZeos (128 – 4 µg/ml), and **(A, B, C)** STR (8 – 4 µg/ml) for 5 d. Viability was assessed by plating samples at 1 d intervals. Each point represents the triplicate sample mean (SD). The assay limit of detection is demarcated by the hatched line at 200 CFU/ml.

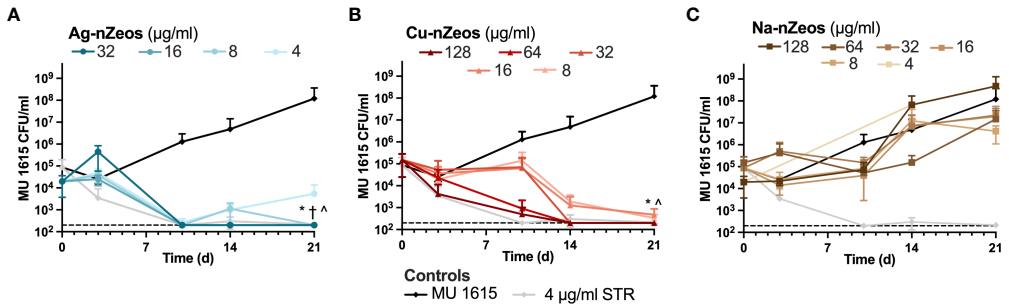



FIGURE 3
Ag-nZeos and Cu-nZeos kill MU. MU 1615 was exposed to two-fold dilutions of **(A)** Ag-nZeos (32 – 4 µg/ml), **(B)** Cu-nZeos (128 – 8 µg/ml), **(C)** Na-nZeos (128 – 4 µg/ml), and **(A, B, C)** streptomycin (STR; 4 µg/ml) with CFU viability assessed at 3, 10, 14 and 21 d. Each point represents the triplicate sample mean (SD). The assay limit of detection is demarcated by the hatched line at 200 CFU/ml. *32 µg/ml, †16 µg/ml; ^8 µg/ml with $p < 0.05$; Ag- and Cu-nZeos were compared to corresponding concentrations of Na-nZeos.

## Antimicrobial activity of nZeos against MU

Having demonstrated ion specificity and the ability of Ag-nZeos to kill Mmar, we sought to examine the relative ion species-specific antimicrobial activity of nZeos against MU. Similar to STR, ≥4 µg/ml Ag-nZeos ($Ag^+$ equivalency of 0.96 µg/ml (23); Table 1) reduced MU to the limit of detection, revealing the $MBC_{99}$ by the 10 d time point (Figure 3A). Cu-nZeos enacted antimicrobial activity in a protracted manner, whereby 32 µg/ml Cu-nZeos ($Cu^{2+}$ equivalency of 2.69 µg/ml (22); Table 1) was observed as the $MBC_{99}$ at 14 d (Figure 3B). MU co-incubated with Na-nZeos (4-128 µg/ml) demonstrated no significant difference ($p > 0.05$) from the untreated control (Figure 3B), corroborating the innocuous character of nZeo carriers. The concentration- and time-dependent antimicrobial activity of Ag- and Cu-nZeos against MU reflects ion-specific mechanisms of action. IE-nZeos indicate proximity-based bactericidal activity which reflects the potential of aluminosilicates as topical therapeutic agents.

## Cytotoxicity assessment of Cu-nZeos

We previously determined that nZeo carriers lack toxicity against eukaryotic cells (23). Furthermore, Chen et al. (23) demonstrated an Ag-nZeos $LC_{50}$ of 64 µg/ml against Detroit 551 and WM-115 cells, whereas U-937 cell viability was not

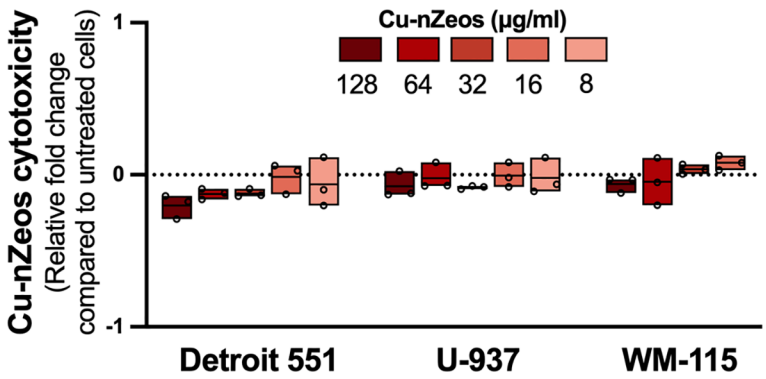



FIGURE 4
Cu-nZeos lack mammalian cell cytotoxicity. Detroit 551, U-937, and WM-115 mammalian cells were exposed to Cu-nZeos (8 – 128 µg/ml) for 24 h, followed by cell viability measurements, as outlined in materials and methods. Data are represented as the relative fold change in excitation/emission fluorescence values between Cu-nZeo experimental samples and untreated cells. Values for individual replicates are flanked by floating box plots, wherein the mean of each experimental group is represented as a line. The dotted line represents the normalized value for untreated cells and the fold-change threshold for Cu-nZeo experimental replicates.

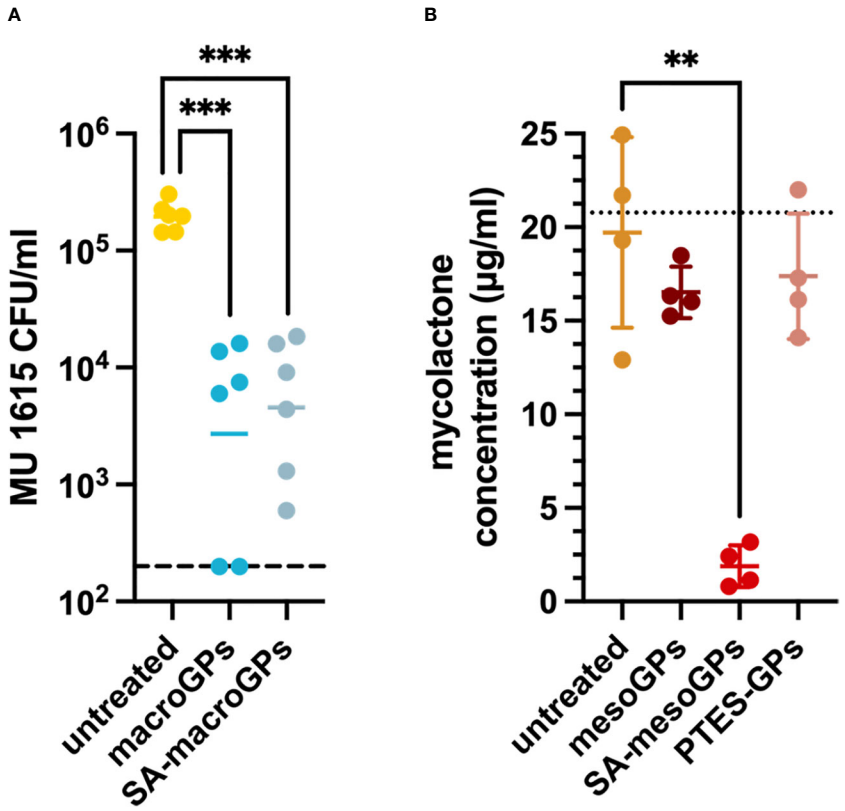



FIGURE 5
Geopolymers adsorb MU bacteria and mycolactone toxin. **(A)** MU 1615 cells were incubated with macroGPs (10 mg/ml) or SA-macroGPs (10 mg/ml) for 1 hour at 30°C. Each point represents a single technical replicate collected from triplicate samples with geometric mean. Experimental limit of detection **(A)** is indicated by a hatched line at 200 CFU/ml. **(B)** Mycolactone (~20 mg) was incubated with mesoGPs (10 mg/ml), SA-mesoGPs (10 mg/ml), or PTES-GPs (10 mg/ml) for 1 hour at room temperature. Each point represents a single technical replicate collected from duplicate samples with mean (SD). The dotted line corresponds to mycolactone concentration prior to experimental incubation. ***$p < 0.001$; ANOVA test with Brown-Forsythe test and Welsh's correction to compare GP-treated and untreated MU samples. **$p < 0.01$; Welsh's t-test comparing GP-treated samples to untreated mycolactone samples.

affected by Ag-nZeos at any tested concentration (2 - 128 µg/ml). To establish the potential host toxicity, Cu-nZeos-associated mammalian cell lysis was measured in the same three eukaryotic cells: Detroit 551 human skin fibroblasts, U-937 human monocytes, and WM-115 dermal epithelial cells. Compared to untreated cells, Cu-nZeos lacked cytotoxicity against Detroit 551, WM-115, and U-937 cells at or above the biologically relevant concentration (32 µg/ml) that killed MU (Figures 4, 3B). These results indicate that nZeos effect differential toxicity in bacterial and mammalian cells.

## MacroGPs and SA-macroGPs sequester MU cells in suspension

Since macroGPs and SA-macroGPs adsorb MRSA (21), we sought to determine if these GPs could physically adsorb and remove MU cells in suspension. MU bacterial suspensions were incubated with macroGPs or SA-macroGPs for 1 h, and nonadherent MU cells were quantified. A single application of macroGPs or SA-macroGPs (10 mg/ml) adsorbed ≥95% of MU cells (Figure 5A). Surface-modification with SA, a saturated fatty acid, to heighten hydrophobicity did not enhance binding to lipid-rich MU (Figure 5A). Nevertheless, these findings further support the role of macroGPs as immuring and adsorptive agents of bacteria that cause cutaneous infections.

## SA-mesoGPs effectively bind mycolactone

Throughout the course of infection, MU secretes the polyketide toxin mycolactone. Much of the pathology observed in Buruli ulcer has been ascribed to the broad range of mycolactone cytotoxicity, suggesting that toxin removal

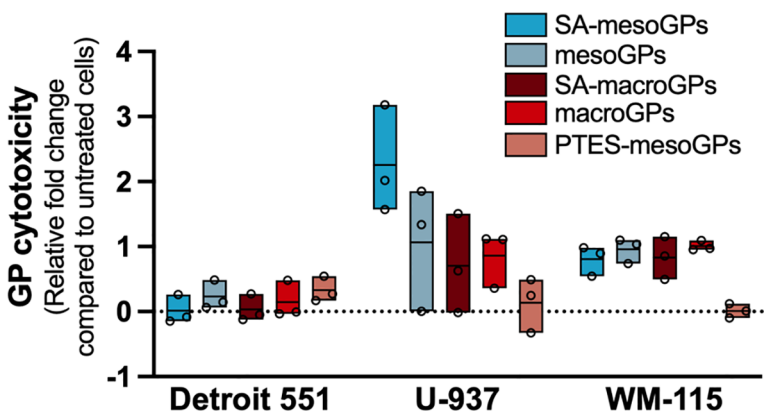



FIGURE 6
Cytotoxicity assessment of geopolymers. Detroit 551, U-937, and WM-115 mammalian cells were exposed to GPs (10 mg/ml) for 24 h, followed by cell viability measurements, as outlined in materials and methods. Data are represented as the relative fold change in excitation/emission fluorescence values between GP experimental samples and untreated cells. Values for individual replicates are flanked by floating box plots, wherein the mean of each experimental group is represented as a line. The dotted line represents the normalized value for untreated cells and the fold-change threshold for GP experimental replicates.

restores immune function at the infection site (40). In our previous studies, GPs adsorb diverse bacterial toxins, including α-hemolysin and streptolysin O (21). Due to greater hydrophobicity, we hypothesized that SA-mesoGPs and PTES-GPs would adsorb greater quantities of mycolactone than their uncoated counterpart. Mycolactone remaining in solution was regarded as unbound. SA-mesoGPs (10 mg/ml) neared total adsorption (92.8%) of the initial amount of mycolactone (~20 mg) (Figure 5B). In contrast, mesoGPs adsorbed ~35% of total mycolactone, whereas PTES-GPs adsorbed less than 10% mycolactone (Figure 5B). SA-mesoGPs displayed significant affinity for mycolactone sequestration, indicating that the SA saturated fatty acid plays an important role in hydrophobic interactions with the polyketide mycolactone toxin.

## Cytotoxicity assessment of macroGPs and mesoGPs

Cytotoxicity of the GP materials was assessed using Detroit 551, WM-115, and U-937 mammalian cells incubated with GPs (10 mg/ml) for 24 h. The five GP products only minimally decreased viability (0.01 - 0.35-fold) of Detroit 551 human skin fibroblasts compared to untreated cells (Figure 6). Except for PTES-modified mesoGPs, other tested GP materials increased cytotoxicity 0.8 - 1-fold in WM-115 dermal epithelial cells (Figure 6). Compared to untreated samples, U-937 cells exposed to macroGPs and SA-macroGPs for 24 h also exhibited 0.7 – 0.9-fold increases in cytotoxicity (Figure 6). While PTES-mesoGPs had minimal effects on U-937 human monocytes, mesoGPs and SA-mesoGPs exhibited 1- to 2.3-fold increases in cytotoxicity compared to untreated cells (Figure 6). Since macroGPs and SA-mesoGPs significantly adsorbed MU cells (Figure 5A) and mycolactone toxin (Figure 5B), respectively, with 1 h incubations and exhibited minimal cytotoxic effects on skin fibroblasts and epithelial cells with 24 h incubations, these results further support potential wound-adjacent applications for mycobacterial skin and soft tissue infections (53) (Figure 6).

# Discussion

Despite an improved standard of care in early Buruli ulcer cases (13, 14), the prognosis for advanced disease remains grim with surgical procedures, such as invasive debridement, frequently necessitated and often delayed (31). Non-small molecule compounds may arbitrate the treatment of advanced cases (13). Modified and customized aluminosilicate materials offer many benefits as potential therapeutics, including low production cost, long-term stability, and ease of application (21–23, 26).

IE-nZeos demonstrated divergent antimicrobial activity against the tested mycobacterial species. While Ag-nZeos killed both Mmar and MU, only MU was susceptible to Cu-nZeos (Figures 2B, 3B). The antimycobacterial activity profile of each nZeos species (Figures 2A, 3A) may stem from physiological differences between species. Though undefined, silver ion antibacterial activity is speculated to result from disruption of the membrane structure, cellular ion gradient (54), and electron transport, whereas copper ions are speculated to metabolic disturbance (55, 56).

The differential identity of Mmar and MU as fast- and slow-growing species, respectively, likely influences susceptibility to ion-based metabolic interference (57). Efflux pump activity imbues mycobacteria with broad-spectrum chemotherapeutic resistance (58). Because of their permissive nature, mycobacterial efflux pumps also hinder intracellular metal ion accumulation and toxicity (59, 60). Greater active transporter activity in Mmar, the fast-growing species, may contribute to the species' copper resistance (61). Host macrophages appear to exploit the copper susceptibility of slow-growing intracellular pathogens, as in the case of *Mycobacterium tuberculosis* (56, 62). The reductive MU genome may also lack genes associated with copper toxicity recovery in Mmar, its evolutionary derivative (63). As a result of synergistic activity between antibiotics and metal ions (56, 62, 64), IE-nZeos may support the adaptation of lower dosage multidrug therapy.

Although we hypothesized that SA-macroGPs would adsorb MU better than their uncoated macroGP counterparts, both macroGPs and SA-macroGPs significantly adsorbed MU (Figure 5). MacroGPs and SA-macroGPs also adsorbed MRSA in significant quantities (21), implying potential broad-spectrum adsorptive activity that may benefit treatment of secondary infections of the Buruli ulcer (65, 66). These results may indicate either consistency in GP-binding motifs and/or GP pore sizes (Figure 1) between bacteria or curtailed performance of SA-enriched GPs resulting from side group dissociation (21).

As a highly hydrophobic molecule, mycolactone was also anticipated to demonstrate greater adherence to PTES-GPs and SA-mesoGPs. Our mycolactone – mesoGP adsorption results parallel GP adhesion of MRSA α-hemolysin toxin (21). Though both SA-mesoGPs and non-coated mesoGPs adsorbed α-hemolysin, SA-coated GPs demonstrated a modestly enhanced binding compared to uncoated GPs (21). Despite the greater hydrophobicity of PTES-GPs, the aromatic ring structure of this coating may have produced greater steric hindrance (67) than the fatty acid chain of the SA-GPs coating. Future studies may seek to establish longitudinal surface group stability and identify target-specific GP binding domains.

One of the greatest obstacles to effective MU treatment is the discordance between *in vitro* and *in vivo* susceptibility to small molecule compounds. Because IE-nZeos and GPs demonstrate antimicrobial and adsorptive activities *in vitro*, their unconventional antimicrobial mechanisms warrant further investigation for activity *in vivo*. As topical treatments, aluminosilicates offer the potential to physically protect mycobacterial skin and soft tissue wounds and provide antimicrobial and mycolactone adsorptive therapy for individuals with Buruli ulcer or Mmar infections.

## 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.

## Author contributions

D-KS and SH conceived and supervised the research. RD, JP, SC, D-KS, and SH designed the experiments. RD, FA, JP, and SC performed the experiments. All authors analyzed the experimental results and participated in writing and editing the manuscript. All authors contributed to the article and approved the submitted version.

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## 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

# References


1. Portaels F, Johnson P, Meyers WM. *Diagnosis of Mycobacterium ulcerans disease: a manual for health care providers*. Geneva (CH): World Health Organization (2001).

2. Wallace JR, Mangas KM, Porter JL, Marcsisin R, Pidot SJ, Howden B, et al. *Mycobacterium ulcerans* low infectious dose and mechanical transmission support insect bites and puncturing injuries in the spread of buruli ulcer. *PloS Negl Trop Diseases.* (2017) 11(4):e0005553. doi: 10.1371/journal.pntd.0005553

3. Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, de Rijk P, et al. First cultivation and characterization of *Mycobacterium ulcerans* from the environment. *PloS Negl Trop Dis* (2008) 2(3):e178. doi: 10.1371/journal.pntd.0000178

4. Zingue D, Panda A, Drancourt M. A protocol for culturing environmental strains of the buruli ulcer agent, *Mycobacterium ulcerans*. *Sci Rep* (2018) 8(1):6778. doi: 10.1038/s41598-018-25278-y

5. Zingue D, Bouam A, Tian RBD, Drancourt M. Buruli ulcer, a prototype for ecosystem-related infection, caused by *Mycobacterium ulcerans*. *Clin Microbiol Rev* (2018) 31(1):e00045–17. doi: 10.1128/CMR.00045-17

6. Sarfo FS, Phillips RO, Rangers B, Mahrous EA, Lee RE, Tarelli E, et al. Detection of mycolactone A/B in *Mycobacterium ulcerans*–infected human tissue. *PloS Negl Trop Diseases.* (2010) 4(1):e577. doi: 10.1371/journal.pntd.0000577

7. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, et al. Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. *Science* (1999) 283(5403):854–7. doi: 10.1126/science.283.5403.854

8. En J, Goto M, Nakanaga K, Higashi M, Ishii N, Saito H, et al. Mycolactone is responsible for the painlessness of *Mycobacterium ulcerans* infection (Buruli ulcer) in a murine study. *Infect Immun* (2007) 76(5):2002-2007. doi: 10.1128/IAI.01588-07

9. Houngbédji GM, Bouchard P, Frenette J. *Mycobacterium ulcerans* infections cause progressive muscle atrophy and dysfunction, and mycolactone impairs satellite cell proliferation. *Am J Physiol Regul Integr Comp Physiol* (2011) 300(3): R724–32. doi: 10.1152/ajpregu.00393.2010

10. Gomez JE, Mckinney JD. *M. tuberculosis* persistence, latency, and drug tolerance. *Tuberculosis* (2004) 84(1-2):29–44. doi: 10.1016/j.tube.2003.08.003

11. Kibadi K, Tsakala M, Mputu-Yamba JB, Muyembe T, Kashongwe M, Imposso B, et al. [Buruli ulcer in angolese refugees in the Kimpese area, lower Congo, D.R. Congo]. *Sante* (2003) 13(1):39–41.

12. Van Der Werf TS, Barogui YT, Converse PJ, Phillips RO, Stienstra Y. Pharmacologic management of *Mycobacterium ulcerans* infection. *Expert Rev Clin Pharmacol* (2020) 13(4):391–401. doi: 10.1080/17512433.2020.1752663

13. Klis S, Ranchor A, Phillips RO, Abass KM, Tuah W, Loth S, et al. Good quality of life in former buruli ulcer patients with small lesions: long-term follow-up of the BURULICO trial. *PloS Negl Trop Diseases.* (2014) 8(7):e2964. doi: 10.1371/journal.pntd.0002964

14. Klis S, Stienstra Y, Phillips RO, Abass KM, Tuah W, van der Werf TS. Long term streptomycin toxicity in the treatment of buruli ulcer: follow-up of participants in the BURULICO drug trial. *PloS Negl Trop Diseases.* (2014) 8(3): e2739. doi: 10.1371/journal.pntd.0002739

15. Stevens DL, Bisno AL, Chambers HF, Dellinger EP, Goldstein EJC, Gorbach SL, et al. Practice guidelines for the diagnosis and management of skin and soft tissue infections: 2014 update by the Infectious Diseases Society of America. *Clin Infect Diseases.* (2014) 59(2):e10–52. doi: 10.1093/cid/ciu296

16. Adusumilli S, Haydel SE. *In vitro* antibacterial activity and *in vivo* efficacy of hydrated clays on *Mycobacterium ulcerans* growth. *BMC Complementary Altern Med* (2015) 16(1). doi: 10.1186/s12906-016-1020-5

17. WHO Advisory Group on Buruli Ulcer. *Report of the 6th WHO advisory group meeting on buruli ulcer*. WHO headquarters, Geneva, Switzerland. Geneva (CH): World Health Organization (2003).

18. Provis JL. Geopolymers and other alkali activated materials: why, how, and what? *Materials Structures* (2014) 47(1-2):11–25. doi: 10.1617/s11527-013-0211-5

19. Otto CC, Haydel SE. Exchangeable ions are responsible for the *in vitro* antibacterial properties of natural clay mixtures. *PloS One* (2013) 8(5):e64068. doi: 10.1371/journal.pone.0064068

20. Morrison KD, Misra R, Williams LB. Unearthing the antibacterial mechanism of medicinal clay: a geochemical approach to combating antibiotic resistance. *Sci Rep* (2016) 6(1):19043. doi: 10.1038/srep19043

21. Popovich J, Chen S, Iannuzo N, Ganser C, Seo D-K, Haydel SE. Synthesized geopolymers adsorb bacterial proteins, toxins, and cells. *Front Bioengineering Biotechnol* (2020) 8. doi: 10.3389/fbioe.2020.00527

22. Chen S, Popovich J, Zhang W, Ganser C, Haydel SE, Seo D-K. Superior ion release properties and antibacterial efficacy of nanostructured zeolites ion-exchanged with zinc, copper, and iron. *RSC Advances.* (2018) 8(66):37949–57. doi: 10.1039/C8RA06556J

23. Chen S, Popovich J, Iannuzo N, Haydel SE, Seo D-K. Silver-ion-exchanged nanostructured zeolite X as antibacterial agent with superior ion release kinetics and efficacy against methicillin-resistant *Staphylococcus aureus*. *ACS Appl Materials Interfaces* (2017) 9(45):39271–82. doi: 10.1021/acsami.7b15001

24. Otto C, Haydel S. Microbicidal clays: composition, activity, mechanism of action, and therapeutic applications. In: Méndez-Vilas A, editor. *Microbial pathogens and strategies for combating them: science, technology and education*, vol. 2 . Badajoz, Spain: Formatex Research Center (2013). p. 1169–80.

25. Coelho TS, Halicki PCB, Silva L, Menezes Vicenti JR, Gonçalves BL, Almeida Da Silva PE, et al. Metal-based antimicrobial strategies against intramacrophage *Mycobacterium tuberculosis*. *Lett Appl Microbiol* (2020) 71 (2):146–53. doi: 10.1111/lam.13298

26. Chen S, Zhang W, Sorge LP, Seo D-K. Exploratory synthesis of low-silica nanozeolites through geopolymer chemistry. *Crystal Growth Design.* (2019) 19 (2):1167–71. doi: 10.1021/acs.cgd.8b01636

27. Hashish E, Merwad A, Elgaml S, Amer A, Kamal H, Elsadek A, et al. *Mycobacterium marinum* infection in fish and man: epidemiology, pathophysiology and management; a review. *Veterinary Quarterly.* (2018) 38 (1):35–46. doi: 10.1080/01652176.2018.1447171

28. Pidot SJ, Asiedu K, Käser M, Fyfe JAM, Stinear TP. *Mycobacterium ulcerans* and other mycolactone-producing mycobacteria should be considered a single species. *PloS Negl Trop Diseases*. (2010) 4(7):e663. doi: 10.1371/journal.pntd.0000663

29. Haydel SE, Remenih CM, Williams LB. Broad-spectrum *in vitro* antibacterial activities of clay minerals against antibiotic-susceptible and antibiotic-resistant bacterial pathogens. *J Antimicrobial Chemotherapy.* (2007) 61 (2):353–61. doi: 10.1093/jac/dkm468

30. Jernigan JA, Farr BM. Incubation period and sources of exposure for cutaneous *Mycobacterium marinum* infection: case report and review of the literature. *Clin Infect Diseases.* (2000) 31(2):439–43. doi: 10.1086/313972

31. Franco-Paredes C, Marcos LA, Henao-Martínez AF, Rodríguez-Morales AJ, Villamil-Gómez WE, Gotuzzo E, et al. Cutaneous mycobacterial infections. *Clin Microbiol Rev* (2018) 32(1):e00069–18. doi: 10.1128/CMR.00069-18

32. Johnson MG, Stout JE. Twenty-eight cases of *Mycobacterium marinum* infection: retrospective case series and literature review. *Infection* (2015) 43(6):655–62. doi: 10.1007/s15010-015-0776-8

33. Prasanna AN, Mehra S. Comparative phylogenomics of pathogenic and non-pathogenic mycobacterium. *PloS One* (2013) 8(8):e71248. doi: 10.1371/journal.pone.0071248

34. Tăbăran A-F, Matea CT, Mocan T, Tăbăran A, Mihaiu M, Iancu C, et al. Silver nanoparticles for the therapy of tuberculosis. *Int J Nanomedicine* (2020) 15:2231–58. doi: 10.2147/IJN.S241183

35. Mohanty S, Jena P, Mehta R, Pati R, Banerjee B, Patil S, et al. Cationic antimicrobial peptides and biogenic silver nanoparticles kill mycobacteria without eliciting DNA damage and cytotoxicity in mouse macrophages. *Antimicrobial Agents Chemotherapy.* (2013) 57(8):3688–98. doi: 10.1128/AAC.02475-12

36. Geroult S, Phillips RO, Demangel C. Adhesion of the ulcerative pathogen *Mycobacterium ulcerans* to DACC-coated dressings. *J Wound Care* (2014) 23 (8):417–24. doi: 10.12968/jowc.2014.23.8.417

37. Bowler PG, Welsby S, Towers V. *In vitro* antimicrobial efficacy of a silver-containing wound dressing against mycobacteria associated with atypical skin ulcers. *Wounds* (2013) 25(8):225–30.

38. Medpelli D, Seo J-M, Seo D-K. Geopolymer with hierarchically meso-/macroporous structures from reactive emulsion templating. *J Am Ceramic Society.* (2014) 97(1):70–3. doi: 10.1111/jace.12724

39. Kriven WM, Bell J, Gordon M. Geopolymer refractories for the glass manufacturing industry. In: *64th Conference on Glass Problems: Ceramic Engineering and Science Proceedings*. (2004) Wiley Online Library. 25 (1):57–79. doi: 10.1002/9780470294857.ch5

40. Adusumilli S, Mve-Obiang A, Sparer T, Meyers W, Hayman J, Small PLC. *Mycobacterium ulcerans* toxic macrolide, mycolactone modulates the host immune response and cellular location of m. ulcerans in vitro and *in vivo*. *Cell Microbiol* (2005) 7(9):1295–304. doi: 10.1111/j.1462-5822.2005.00557.x

41. Demangel C, Stinear TP, Cole ST. Buruli ulcer: reductive evolution enhances pathogenicity of *Mycobacterium ulcerans*. *Nat Rev Microbiol* (2009) 7(1):50–60. doi: 10.1038/nrmicro2077

42. Duxson P, Provis JL, Lukey GC, Mallicoat SW, Kriven WM, Van Deventer JSJ. Understanding the relationship between geopolymer composition, microstructure and mechanical properties. *Colloids Surfaces A: Physicochemical Eng Aspects* (2005) 269(1-3):47–58. doi: 10.1016/j.colsurfa.2005.06.060

43. Scherr N, Pluschke G, Thompson CJ, Ramón-García S. Selamectin is the avermectin with the best potential for buruli ulcer treatment. *PloS Negl Trop diseases*. (2015) 9(8):e0003996. doi: 10.1371/journal.pntd.0003996

44. Al-Ani A, Freitas C, Zholobenko V. Nanostructured large-pore zeolite: The enhanced accessibility of active sites and its effect on the catalytic performance. *Microporous Mesoporous Materials*. (2020) 293:109805. doi: 10.1016/j.micromeso.2019.109805

45. Scherr N, Gersbach P, Dangy J-P, Bomio C, Li J, Altmann K-H, et al. Structure-activity relationship studies on the macrolide exotoxin mycolactone of *Mycobacterium ulcerans*. *PloS Negl Trop Dis* (2013) 7(3):e2143. doi: 10.1371/journal.pntd.0002143

46. Daffé M, Lanéelle MA, Lacave C. Structure and stereochemistry of mycolic acids of *Mycobacterium marinum* and *Mycobacterium ulcerans*. *Res Microbiol* (1991) 142(4):397–403. doi: 10.1016/0923-2508(91)90109-N

47. Barrett EP, Joyner LG, Halenda PP. The determination of pore volume and area distributions in porous substances. i. computations from nitrogen isotherms. *J Am Chem Society*. (1951) 73(1):373–80. doi: 10.1021/ja01145a126

48. Woods GL, Brown-Elliott BA, Conville PS, Desmond EP, Hall GS, Lin G, et al *M24-A2: Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes*. Wayne (PA): Clinical Laboratory Standard Institute (2011).

49. Gunawardana G, Chatterjee D, George KM, Brennan P, Whittern D, Small PLC, et al. Characterization of novel macrolide toxins, mycolactones A and B, from a human pathogen, *Mycobacterium ulcerans*. *J Am Chem Soc* (1999) 121(25):6092–3. doi: 10.1021/ja990017l

50. Cadapan LD, Arslanian RL, Carney JR, Zavala SM, Small PL, Licari P. Suspension cultivation of *Mycobacterium ulcerans* for the production of mycolactones. *FEMS Microbiol Letters*. (2001) 205(2):385–9. doi: 10.1111/j.1574-6968.2001.tb10977.x

51. Marion E, Prado S, Cano C, Babonneau J, Ghamrawi S, Marsollier L. Photodegradation of the *Mycobacterium ulcerans* toxin, mycolactones: considerations for handling and storage. *PloS One* (2012) 7(4):e33600. doi: 10.1371/journal.pone.0033600

52. Davidovits J. Geopolymers. *J thermal analysis*. (1991) 37(8):1633–56. doi: 10.1007/BF01912193

53. Lu Z-H, Mu Y-M, Wang B-A, Li X-L, Lu J-M, Li J-Y, et al. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular leydig cell. *Biochem Biophys Res Commun* (2003) 303(4):1002–7. doi: 10.1016/S0006-291X(03)00449-2

54. Swathy JR, Sankar MU, Chaudhary A, Aigal S, Anshup, Pradeep T. Antimicrobial silver: An unprecedented anion effect. *Sci Rep* (2015) 4(1):7161. doi: 10.1038/srep07161

55. Macomber L, Imlay JA. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc Natl Acad Sci* (2009) 106(20):8344–9. doi: 10.1073/pnas.0812808106

56. Shah S, Dalecki AG, Malalasekera AP, Crawford CL, Michalek SM, Kutsch O, et al. 8-hydroxyquinolines are boosting agents of copper-related toxicity in mycobacterium tuberculosis. *Antimicrobial Agents Chemotherapy* (2016) 60 (10):5765–76. doi: 10.1128/AAC.00325-16

57. Rowland JL, Niederweis M. A multicopper oxidase is required for copper resistance in *Mycobacterium* tuberculosis. *J Bacteriol* (2013) 195(16):3724–33. doi: 10.1101/2021.03.11.434961

58. Viveiros M, Martins M, Rodrigues L, Machado D, Couto I, Ainsa J, et al. Inhibitors of mycobacterial efflux pumps as potential boosters for anti-tubercular drugs. *Expert Rev anti-infective Ther* (2012) 10(9):983–98. doi: 10.1586/eri.12.89

59. Neyrolles O, Wolschendorf F, Mitra A, Niederweis M. Mycobacteria, metals, and the macrophage. *Immunol Rev* (2015) 264(1):249–63. doi: 10.1111/imr.12265

60. Adams KN, Takaki K, Connolly LE, Wiedenhoft H, Winglee K, Humbert O, et al. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* (2011) 145(1):39–53. doi: 10.1016/j.cell.2011.02.022

61. Black PA, Warren RM, Louw GE, van Helden PD, Victor TC, Kana BD. Energy metabolism and drug efflux in *Mycobacterium tuberculosis*. *Antimicrobial Agents Chemotherapy* (2014) 58(5):2491–503. doi: 10.1128/AAC.02293-13

62. Dalecki AG, Haeili M, Shah S, Speer A, Niederweis M, Kutsch O, et al. Disulfiram and copper ions kill *Mycobacterium tuberculosis* in a synergistic manner. *Antimicrobial Agents Chemotherapy*. (2015) 59(8):4835–44. doi: 10.1128/AAC.00692-15

63. Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, Johnson PDR, et al. Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of mycobacterium tuberculosis. *Genome Res* (2008) 18(5):729–41. doi: 10.1101/gr.075069.107

64. Agertt VA, Bonez PC, Rossi GG, Flores VDC, Siqueira FDS, Mizdal CR, et al. Identification of antimicrobial activity among new sulfonamide metal complexes for combating rapidly growing mycobacteria. *BioMetals* (2016) 29 (5):807–16. doi: 10.1007/s10534-016-9951-3

65. Amissah NA, Glasner C, Ablordey A, Tetteh CS, Kotey NK, Prah I, et al. Genetic diversity of *Staphylococcus aureus* in buruli ulcer. *PloS Negl Trop Diseases*. (2015) 9(2):e0003421. doi: 10.1371/journal.pntd.0003421

66. Ruf M-T, Chauty A, Adeye A, Ardant M-F, Koussemou H, Johnson RC, et al. Secondary buruli ulcer skin lesions emerging several months after completion of chemotherapy: paradoxical reaction or evidence for immune protection? *PloS Negl Trop Dis* (2011) 5(8):e1252. doi: 10.1371/journal.pntd.0001252

67. Lin W, Zheng J, Zhuo J, Chen H, Zhang X. Characterization of sol-gel ORMOSIL antireflective coatings from phenyltriethoxysilane and tetraethoxysilane: Microstructure control and application. *Surface Coatings Technology*. (2018) 345:177–82. doi: 10.1016/j.surfcoat.2018.01.059

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# Systematic assessment of clinical and bacteriological markers for tuberculosis reveals discordance and inaccuracy of symptom-based diagnosis for treatment response monitoring

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**Background:** Clinical symptoms are the benchmark of tuberculosis (TB) diagnosis and monitoring of treatment response but are not clear how they relate to TB bacteriology, particularly the novel tuberculosis-molecular bacterial load assay (TB-MBLA).

**Methods:** Presumptive cases were bacteriologically confirmed for TB and assessed for symptoms and bacteriological resolution using smear microscopy (SM), culture, and TB-MBLA over 6-month treatment course. Kaplan–Meier and Kappa statistics were used to test the relationship between symptoms and bacteriological positivity.

**Results:** A cohort of 46 bacteriologically confirmed TB cases were analyzed for treatment response over a 6-month treatment course. Pre-treatment symptoms and bacteriological positivity concurred in over 70% of the cases. This agreement was lost in over 50% of cases whose chest pain, night sweat, and loss of appetite had resolved by week 2 of treatment. Cough resolved at a 3.2% rate weekly and was 0.3% slower than the combined bacteriological (average of MGIT and TB-MBLA positivity) resolution rate, 3.5% per week. A decrease in TB-MBLA positivity reflected a fall in bacillary load, 5.7 ± 1.3- at baseline to 0.30 ± 1.0- $\log_{10}$ eCFU/ml at month 6, and closer to cough resolution than other bacteriological measures, accounting for the only one bacteriologically positive case out of seven still coughing at month 6. Low baseline bacillary load patients were more likely to be bacteriologically negative, HR 5.6, $p$ = 0.003 and HR 3.2, $p$ = 0.014 by months 2 and 6 of treatment, respectively.

**Conclusion:** The probability of clinical symptoms reflecting bacteriological positivity weakens as the patient progresses on anti-TB therapy, making the symptom-based diagnosis a less reliable marker of treatment response.

KEYWORDS

TB-MBLA, diagnosis, monitoring, TB symptoms, bacteriological tests

## Introduction

Tuberculosis (TB) is the leading cause of death attributed to a single microbial pathogen worldwide, ranking above HIV/AIDS until the recent coronavirus pandemic (1). In 2020, around 10 million people developed TB disease and 1.4 million died (2). The current 6-months TB treatment with Isoniazid (H), Rifampicin (R), Pyrazinamide (P), and Ethambutol (E) regimens is effective in healthcare settings but can be hampered by low adherence and long treatment duration that can be challenging to finish (3–5). Accurate and rapid tests are required to help clinicians to identify patients at risk of treatment failure including those who may require an extended treatment or treatment change (6). The availability of such tools would reduce treatment costs and expedite the evaluation of new TB medicines (7).

Currently, diagnosis and treatment monitoring relies on the initial assessment of TB symptoms followed by bacteriological confirmation in clinical specimens (8, 9). Presence of TB symptoms such as cough, fever, night sweats, hemoptysis, sputum production, and weight loss are associated with active TB disease (9, 10). Despite the technical difficulties to screen TB symptoms and its low sensitivity and specificity (11), a combination of three symptoms (cough, fever, and sweats) demonstrated a 93% sensitivity and 36% specificity for active TB in people living with Human Immune Deficiency Virus (PLHIV) (12). Previous clinical trials showed the association of cough, fever, and night sweats with TB bacillary burden at diagnosis and relapse (13, 14). Thus, careful assessment of TB symptoms may be an alternative diagnostic and monitoring tool in healthcare settings with limited capacity to perform bacteriological tests.

Bacteriological tests are the reference standard for the diagnosis of active TB and monitoring. Tests detecting DNA of *Mycobacterium tuberculosis* (*M.tb*) such as the Xpert MTB/RIF Assay are rapid and sensitive for diagnosis but are not useful for monitoring (15) as DNA can be detected from both live and dead cells (16, 17). Reliance on standard smear microscopy (SM) and culture methods have many limitations for monitoring (18–22). Sputum smear is cheap and easy to perform but has low sensitivity and specificity (23, 24). Therefore, in the absence of the WHO-recommended tools, many clinicians use clinical symptoms as the means for monitoring treatment response (24, 25).

Tuberculosis-molecular bacterial load assay (TB-MBLA) is a promising test for treatment response monitoring. It quantifies 16S rRNA of *M.tb* by reverse transcriptase polymerase chain reaction as the marker viable *M.tb* (26–28). Early studies have consistently retrieved *M.tb* 16S rRNA in patient samples reflecting viable bacilli during treatment with a strong correction with standard MGIT culture (29–32). In this study, we prospectively assessed clinical measures of patient improvement compared to standard-of-care bacteriological tests and with the novel TB-MBLA before and throughout 6 months of standard TB therapy.

## Materials and methods

### Ethics

The study was approved by the Mbeya Medical Research and Ethics Committee (MRH/R.10/18VOLL.VII/12) and the University of St Andrews Teaching and Research Ethics Committee (MD12678). National approval was obtained from the National Institute for Medical Research (NIMR/HQ/R.8a/Vol.IX/2400) in Tanzania. All participants provided written consent or witnessed verbal consent for those who could not write or read.

### Study design

This was a longitudinal prospective study assessing the relationship of TB symptoms with bacteriological measures before and during standard 6 months of TB therapy in routine healthcare settings.

### Study sites

The study was conducted in Mbeya, Tanzania from January 2017 to March 2018. Patients were enrolled from

the Mbeya Zonal Referral Hospital (MZRH), Mbeya Regional Referral Hospital (MRRH), and Mbeya Rural District Hospital (MRDH). Mbeya is a high TB burden border region and major transport gateway to Malawi, Zambia, and the Democratic Republic of Congo.

## Patient recruitment and selection criteria

Patients with presumed symptoms of pulmonary TB (PTB) and those who had at least one of the following symptoms; cough, night sweats, chest pain, loss of appetite, and weight loss aged between 18 and 65 years were selected to provide sputum specimens for initial diagnosis with Xpert MTB/RIF Assay or sputum SM at the health facility. Xpert MTB/RIF Assay positive who tested negative to RIF resistance were given permission to participate in the study, which included providing follow-up samples for the bacteriological assessment of treatment response. Patients who were severely ill, unable to produce sputum, and those who were more than 50 km from a health facility, making it difficult for them to attend all follow-up visits, were excluded from the study.

## Bacteriological confirmation

Prior to the initiation of standard TB therapy based on diagnostic results at the health facility, all consented patients provided a second pre-treatment sputum sample for bacteriological confirmation at NIMR-Mbeya Medical Research Centre (NIMR-MMRC) using sputum SM and Xpert MTB/RIF Assay, Culture and novel TB-MBLA. Patients who were bacteriologically negative or with the discordant confirmatory result at NIMR-MMRC including those who were treated based on the clinician's judgment were excluded in the study (**Figure 1**). All patients received standard TB therapy consisting of H-Z-R-E for 2 months followed by 4 months with H-R.

## Treatment response monitoring and study outcomes

Participants were monitored for symptom and bacteriological positivity at weeks 2, 8, 22, and 26 of standard TB therapy. TB symptoms such as cough, chest pain, sputum production, loss of appetite, and night sweats were assessed at diagnosis, and all treatment follow-up visits by a clinician or a TB and leprosy coordinator at the healthcare facility prior to the collection of sputum specimens for bacteriological tests. Bacteriological response during treatment was measured using standard ZN microscopy, culture, and the novel TB-MBLA.

The study outcomes were the resolution of TB clinical symptoms and bacteriological conversion from positive to negative to inform the treatment continuation phase at week 8 and to establish final outcomes at the end of treatment weeks 22 and 26. Assessment of the final study outcomes was performed using standard SM, culture and results compared to the novel TB-MBLA. TB cured patients were defined as PTB patients with bacteriologically confirmed at diagnosis who converted to smear or culture negative in the last month of treatment and on at least one previous occasion (33).

## Bacteriological tests

Sputum samples were homogenized using a sterile magnetic stirrer for 30 min and 1 ml of aliquot was mixed with 4 ml of guanidine thiocyanate (GTC) and used for TB-MBLA. Total RNA extraction for TB-MBLA was performed using the FastRNA Pro Kit (MP Biomedicals) and removal of genomic DNA was done using Turbo DNA free kit (Ambion). Reverse transcriptase-quantitative polymerase chain reactions (RT-qPCR) were performed on the Rotor-Gene Q machine (Qiagen) using the QuantiTect PCR NoROX mix (Qiagen) and TaqMan dual labeled probes (Eurofins Genomics). Using a pre-developed standard curve, the RT-PCR quantification cycle (Cq) values were converted to bacterial load (BL) and reported as estimated colony-forming units per/ml of sputum (eCFU/ml) as previously described (27, 34). The standard curve was developed using RNA extracted from mycobacterial cells with known colony-forming units per ml (CFU/ml).

A total of more than 2 ml of sputum was decontaminated with 1% of *N*-acetyl-L-cysteine combined with 2% of sodium hydroxide for 20 min. Concentrated sputum pellets were used for ZN microscopy, MGIT liquid culture (mycobacterial growth indicator tubes), and solid culture (LJ) (Beckton and Dickson Company, MD, USA) following the manufacturer's instructions. The growth of *M.tb* in culture was confirmed by ZN stain, lateral flow antigen test (MPT64, Beckton and Dickson Company, MD, USA), and blood agar plate (BAP) to confirm the purity of culture (35). Antimicrobial drug susceptibility tests for standard regimens (H-Z-R-E) were performed in the BACTEC MGIT 960 System following manufacturer instructions (36).

Treatment outcome was assessed at weeks 22 and 26 of treatment using the culture as the gold standard compared to TB-MBLA positivity at this stage of treatment. To assess the effect of baseline BL on the resolution of TB symptoms during treatment, we computed the median BL at baseline and stratified the patients into "High BL" for those with BL greater than the median value and "Low BL" for those with BL below the median value. Additionally, we stratified patients based on HIV status to determine if HIV has an impact on the resolution of TB symptoms. The average percent positivity of the most sensitive bacteriological tests, MGIT culture, and TB-MBLA were used to calculate the bacteriological positivity score.

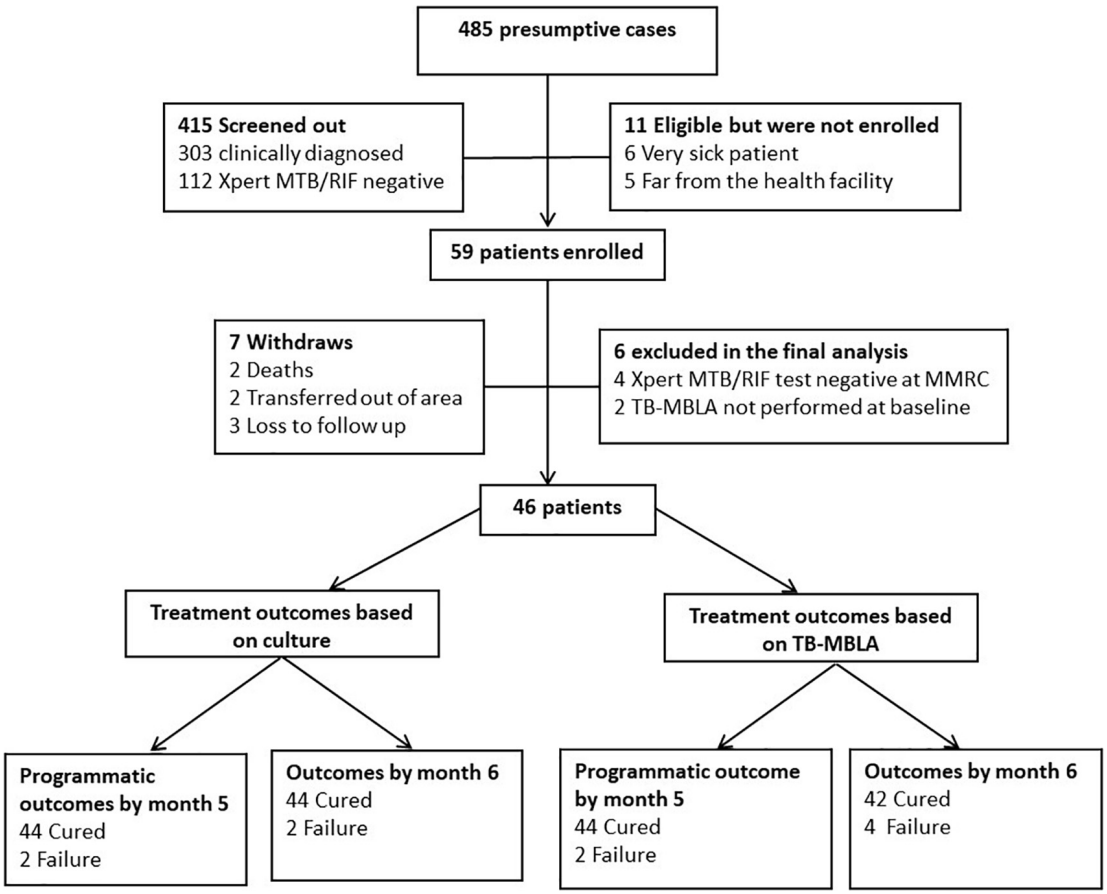


FIGURE 1
Screening workflow, enrollment, follow-up, and final outcomes. Three hundred and three patients were screened out because they were bacteriological test negative at diagnosis and were treated clinically. Of the 59 enrolled patients, seven patients withdrew from the study (two died, three transferred out of the area, and three were lost to follow-up). Six patients were excluded from the final analysis (four due to inconsistent Xpert MTB/RIF results at the health facility and confirmatory Xpert MTB/RIF test at the NIMR-MMRC testing laboratory and two patients had low sputum volume for TB-MBLA at baseline). Three patients were lost to follow-up and two patients who were HIV positive died.

## Statistical analyses

Data were analyzed using STATA software (version 14) and GraphPad Prism version 9.3.1 (GraphPad Software, La Jolla, CA, USA). Multivariate hazard ratio was used to estimate the association between baseline BL and time to the resolution of TB symptoms from the start of TB therapy. Cox proportion hazard ratio was used to determine the association between the median time to the resolution of TB symptoms stratified by baseline BL or HIV status. Fischer exact test was used to assess the association of TB symptoms with bacteriological tests at week 8, exploring whether TB symptoms may be used to inform treatment continuation phase as for standard culture and SM. Kaplan–Meier curves were used to calculate the probability of TB bacillary load clearance at weeks 8 and 26 of treatment and Kappa statistics to assess percentage agreement between TB symptoms and bacteriological tests. All statistical analyses were considered significant at a $p$-value of less than 0.05.

# Results

## Patients' characteristics

Figure 1 depicts the flow of screened patients, enrollment, and follow-up. Of the 485 presumptive patients screened, 59 (12.8%) were enrolled in the study and 13 (22.03%) were excluded from the analysis because they either did not complete follow-up or their initial Xpert MTB/RIF Assay result at the health facility was discordant with the second confirmatory MTB/RIF Assay test performed NIMR-MMRC. As a result, 46 patients with a total of 230 serial samples collected longitudinally during the 6 months of standard treatment period were analyzed. Median age (range) was 37 years (18–65), 60.9% (28/46) were male subjects, 39.1% (18/46) were HIV positive, and 13% (6/46) were re-treatment cases (Table 1). Using the WHO definition of cure, 4.3% (2/46) each at months 5 and 6 were MGIT culture positive and considered as treatment failure.

TB-MBLA concurred with MGIT at month 5 detecting 4.3% (2/46) patients but two more at month 6, 8.6% (4/46). All 46 (100%) patients were phenotypically susceptible to the first-line H-Z-R-E regimen determined by the BD BACTEC MGIT 960 Culture Systems.

## Resolution of tuberculosis symptoms compared to bacteriological tests positivity throughout treatment

The overall patients with detectable TB symptoms at diagnosis were 97.8, 80.4, 86.9, 76, and 100% for cough, chest pain, sweats, loss of appetite, and sputum production, respectively, and matched with bacteriological positivity of 84.8, 84.7, 93.5, and 100% by SM, LJ culture, MGIT culture, and TB-MBLA, respectively. The proportion of patients with three clinical symptoms, such as night sweats, loss of appetite, and chest pain declined sharply to 9.1, 6.8, and 30.8%, respectively, by week 8 of treatment compared to bacteriological test positivity of 8.6, 17.4, 28.3, and 60.9% by sputum SM, LJ culture, MGIT culture, and TB-MBLA, respectively. No patients reported sweats at week 26 of treatment and only 13.3 and 4.4% had chest pain and loss of appetite, respectively. In contrast, cough resolution was slower than both MGIT culture and TB-MBLA (Figure 2) or average bacteriological positivity throughout treatment (Figure 3), except at week 8 with 50% positivity cough compared to 60.9% of TB-MBLA. Similar to cough, sputum production resolved more slowly than all bacteriological tests except TB-MBLA clearance at weeks 2 and 8 with a positivity of 89.1 and 60.9% compared to 82.6 and 56.8% for sputum production, respectively. A total of seven (15.2%) and 10 patients (21.7%) were coughing and producing sputum at week 26 of treatment, respectively, compared to four (8.6%) patients with TB-MBLA and two (4.3%) patients with MGIT positive. Only two out of the seven coughing patients at week 26 were among the six patients who were treatment failures.

TABLE 1 Baseline characteristics of the patients ($N$ = 46).

| **Age in years, median (range)** | **37 (18–65)** |
|---|---|
| **Sex** | |
| Male, *n* (%) | 28 (60.9) |
| Re-treatment, *n* (%) | 6 (13.0) |
| **HIV status** | |
| HIV positive, *n* (%) | 18 (39.1) |
| HIV negative, *n* (%) | 25 (54.3) |
| Unknown HIV status, *n* (%) | 3 (6.5) |
| **Bacteriological tests** | |
| Xpert MTB/RIF Assay, *n* (%) | 46 (100) |
| Sputum smear microscopy, *n* (%) | 39 (84.8) |
| Liquid culture in MGIT, *n* (%) | 43 (93.5) |
| Solid culture in LJ, *n* (%) | 39 (84.7) |
| TB-MBLA, *n* (%) | 46 (100) |
| **TB clinical symptoms** | |
| Cough, *n* (%) | 45 (97.8) |
| Chest pain, *n* (%) | 37 (80.3) |
| Sputum production, *n* (%) | 46 (100) |
| Sweats, *n* (%) | 40 (86.9) |
| Loss of appetite, *n* (%) | 35 (76.1) |

HIV, human immunodeficiency virus; LJ, Lowenstein–Jensen media; MGIT, mycobacterium growth indicator tubes; TB, tuberculosis; TB-MBLA; tuberculosis molecular bacterial load assay.

## Agreements between clinical symptoms and bacteriological measures

At the time of diagnosis, there was more than 70% agreement between TB-MBLA, other bacteriological measures, and clinical symptoms. Cough, loss of appetite, and sputum production were 100% in agreement with TB-MBLA. Apart from cough and sputum production that was over 70% in agreement with TB-MBLA by week 2 of treatment, chest pain, sweats, and loss of appetite had significantly resolved, which increased discordance with TB-MBLA by over 60% (Table 2). Resolution of cough was consistent with bacteriological positivity score with a SLOPE of −3.2 and −3.5%, respectively, per week. The lowest agreement of TB-MBLA with clinical symptoms and other bacteriological tests was at week 8 between 32–44 and 45–59%, respectively, and increased to 71–87 and 82–89%, respectively, by the end of treatment week 26 (Table 2). Bacteriological positivity was not associated with the presence of TB symptoms at week 8 of treatment, $p > 0.05$ (Supplementary Table 1).

## Effect of bacillary load on tuberculosis symptoms and bacteriological test positivity

The median BL at baseline was 7.46E+05 eCFU/ml. A total of 23 patients (50%) had high BL above the median value (Table 3). Chest pain, night sweats, and loss of appetite resolved rapidly in response to treatment irrespective of baseline BL, $p > 0.05$. Cough resolved slowly and independently of the pre-treatment BL, $p = 0.95$. In contrast to cough, sputum production resolved more slowly in patients with low BL than those with high BL, $p = 0.04$. In a multivariate analysis, there was no association between low or high BL with the resolution of cough [AHR, 0.85 (0.42–1.73); $p = 0.65$], sputum production [AHR, 1.77 (0.90–3.47); $p = 0.09$], and chest pain [AHR, 1.25 (0.60–2.59); $p = 0.55$] after adjustment for HIV, age, and sex (Supplementary Table 2).

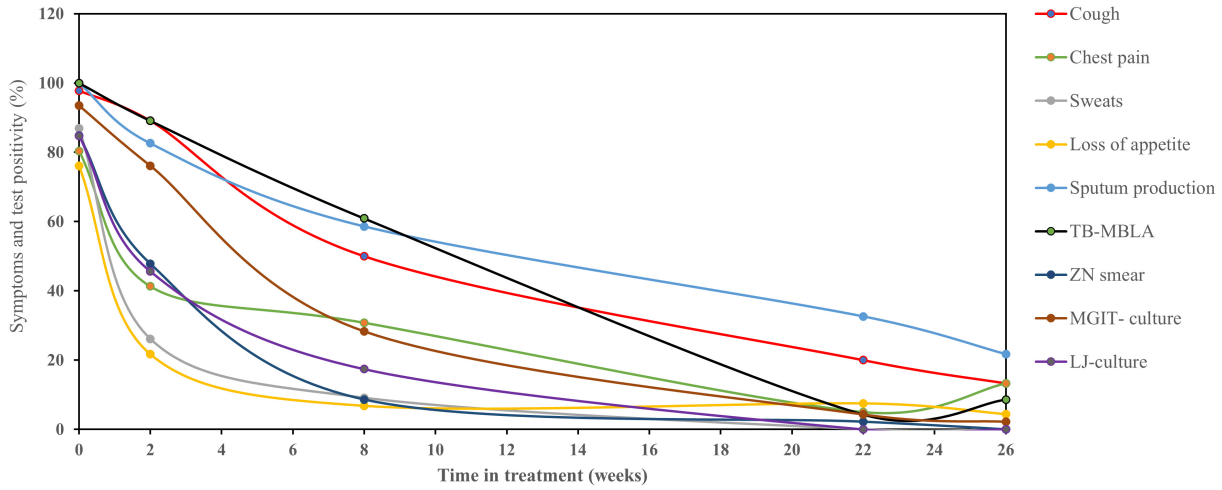


FIGURE 2
Relationship of TB clinical symptoms clearance with bacteriological tests. Clinical symptoms resolved rapidly during TB therapy with the exception of cough and sputum production which resolved slowly than culture, microscopy, and TB-MBLA. Data are presented as the percentage (%) of patients with clinical symptoms and bacteriological positivity over the time in treatments (weeks).

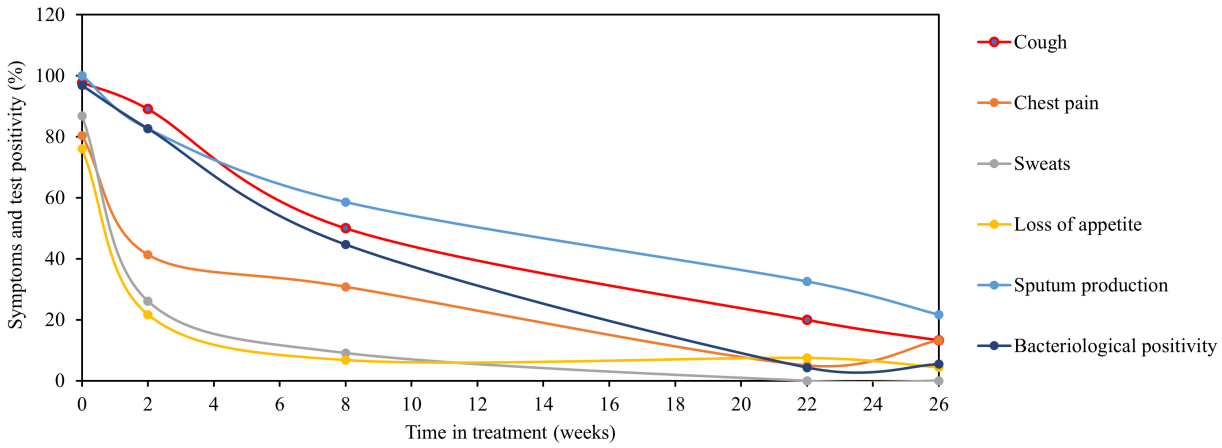


FIGURE 3
Average bacteriological positivity compared to clinical symptoms clearance. Relationship of clinical symptoms clearance compared to average bacteriological positivity of TB-MBLA and culture. Clinical symptoms (sweats, loss of appetite, and chest pain) resolved more rapidly than the average bacteriological positivity. Resolution of cough was slow matching the resolution of average bacteriological positivity, while sputum production resolved more slowly than average bacteriological positivity. Data are presented as the percentage (%) of patients with clinical symptoms and average bacteriological positivity over the time in treatments (weeks). The average bacteriological positivity (±SD) was 96.7 ± 4.6, 82.6 ± 9.2, 44.6 ± 23.1, 4.3 ± 0, and 6.5 ± 3.04 at baseline (week 0), weeks 2, 8, 22, and 26, respectively.

The median time to sputum smear and LJ culture conversion-to-negative was shorter than the time for MGIT culture negative, while TB-MBLA had the highest median time to a negative result for patients with both low and high BL (Table 3). Unlike SM and MGIT culture, patients with low BL had a significantly shorter median time for conversion-to-negative, 2 weeks versus 8 weeks in patients with high BL, $p = 0.03$. Patients with low BL were more likely to clear TB bacillary load at weeks 8 and 26 of treatment, HR 5.6, $p = 0.003$ and HR 3.2, $p = 0.014$, respectively (Figure 4).

## Effect of HIV status on tuberculosis symptoms and bacteriological test positivity

The prevalence of HIV was 39.1%. Chest pain, night sweats, and loss of appetite resolved rapidly in response to treatment and there was no difference in the median time to the resolution of TB symptoms between patients with HIV positive and negative, $p > 0.05$ (Table 4). Overall, the median time to the resolution of cough and sputum production was slightly higher

in patients with HIV positive than HIV negative, $p > 0.05$. For bacteriological tests, with the exception to SM, the median time to MGIT and LJ culture conversion from positive to negative was not statistically different among patients with HIV positive and negative, $p > 0.05$. Overall, TB-MBLA had the highest time to BL clearance among patients with HIV positive and negative compared to smear and culture tests, $p > 0.05$ (Table 4).

TABLE 2 Percentage (%) agreement of clinical symptoms with bacteriological tests.

| | | Agreement (%) throughout treatment | | | | |
|---|---|---|---|---|---|---|
| | **Variable** | **Baseline** | **Week 2** | **Week 8** | **Week 22** | **Week 26** |
| Smear | Cough | 86.96 | 41.30 | 53.49 | 68.29 | 86.67 |
| | Chest pain | 80.43 | 50.00 | 69.77 | 90.00 | 86.67 |
| | Sweats | 76.09 | 56.52 | 90.70 | 95.00 | 97.78 |
| | Loss of appetite | 73.91 | 63.04 | 86.05 | 87.50 | 97.78 |
| | Sputum production | 86.96 | 52.17 | 40.00 | 60.00 | 77.78 |
| LJ culture | Cough | 86.96 | 43.48 | 41.86 | 68.29 | 82.22 |
| | Chest pain | 80.43 | 43.48 | 53.49 | 90.00 | 82.22 |
| | Sweats | 80.43 | 58.70 | 72.09 | 95.00 | 93.33 |
| | Loss of appetite | 80.43 | 54.35 | 67.44 | 87.50 | 93.33 |
| | Sputum production | 86.96 | 36.96 | 33.33 | 60.00 | 73.33 |
| MGIT culture | Cough | 93.48 | 86.96 | 73.26 | 70.73 | 88.89 |
| | Chest pain | 78.26 | 34.78 | 55.81 | 87.50 | 84.44 |
| | Sweats | 82.61 | 34.78 | 72.09 | 92.50 | 91.11 |
| | Loss of appetite | 76.09 | 28.26 | 62.79 | 85.00 | 91.11 |
| | Sputum production | 93.48 | 67.39 | 62.79 | 62.50 | 75.56 |
| TB-MBLA | Cough | 100 | 82.61 | 44.19 | 65.85 | 75.56 |
| | Chest pain | 84.78 | 39.13 | 32.56 | 82.50 | 80.00 |
| | Sweats | 89.13 | 32.61 | 39.53 | 87.50 | 86.67 |
| | Loss of appetite | 100 | 30.43 | 34.88 | 80.00 | 86.67 |
| | Sputum production | 100 | 71.74 | 37.78 | 62.50 | 71.11 |
| | Smear | 86.96 | 54.35 | 45.65 | 88.37 | 88.89 |
| | LJ culture | 86.96 | 56.52 | 54.35 | 88.37 | 86.67 |
| | MGIT culture | 93.48 | 82.61 | 58.70 | 90.70 | 82.22 |

The percentage agreement between TB-MBLA with other bacteriological measures and clinical symptoms was over 80% at the time of diagnosis. Cough, loss of appetite, and sputum production were 100% in agreement with TB-MBLA. The percentage agreement decreased between treatment weeks 2 and 8, then increased between treatment weeks 22 and 26. The lowest agreement of TB-MBLA with all clinical symptoms and other bacteriological measures was at week 8 of treatment.

TABLE 3 Time to the resolution of clinical and bacteriological measures stratified by bacterial loads.

| | Median time to resolution of clinical symptoms (weeks) | | | |
|---|---|---|---|---|
| **Clinical symptoms** | **All patients ($n$ = 46)** | **High-bacterial load ($n$ = 23)** | **Low-bacterial load ($n$ = 23)** | ***P*-value** |
| Cough, *n* (IQR) | 17.9 (8.0–23.9) | 17.9 (8.0–20.5) | 19.8 (8.0–23.9) | 0.95 |
| Sputum production, *n* (IQR) | 19.4 (8.0–21.8) | 8.1 (2.0–19.9) | 19.9 (8.0–23.9) | 0.04 |
| Chest pain, *n* (IQR) | 2.1 (2.0–19.8) | 2.0 (2.0–11.5) | 2.4 (2.0–19.9) | 0.39 |
| Sweats, *n* (IQR) | 2.0 (2.0–8.0) | 2.0 (2.0–8.0) | 2.0 (2.0–6.0) | 0.33 |
| Loss of appetite, *n* (IQR) | 2.0 (2.0–6.0) | 2.0 (2.0–8.0) | 2.3(2.0–6.0) | 0.58 |
| Smear microscopy, *n* (IQR) | 7.8 (2.0–8.0) | 2.1 (2.0–8.0) | 8.0(2.0–8.0) | 0.67 |
| LJ, *n* (IQR) | 7.8 (2.0–8.1) | 8.0 (2.8–18.4) | 2.0 (2.0–8.0) | 0.03 |
| MGIT, *n* (IQR) | 8.0 (7.8–18.0) | 8.0 (7.8–18.4) | 8.0 (7.8–8.0) | 0.83 |
| TB-MBLA, *n* (IQR) | 19.8 (8.0–19.9) | 19.9 (11.7–19.9) | 8.1 (7.8–19.9) | 0.39 |

Cox proportion hazard ratio for the association between median time to the resolution (in weeks) of TB clinical symptoms stratified by the baseline bacterial load. IQR, interquartile range; LJ, Lowenstein–Jensen media; MGIT, mycobacterium growth indicator tubes; TB-MBLA; tuberculosis-molecular bacterial load assay.

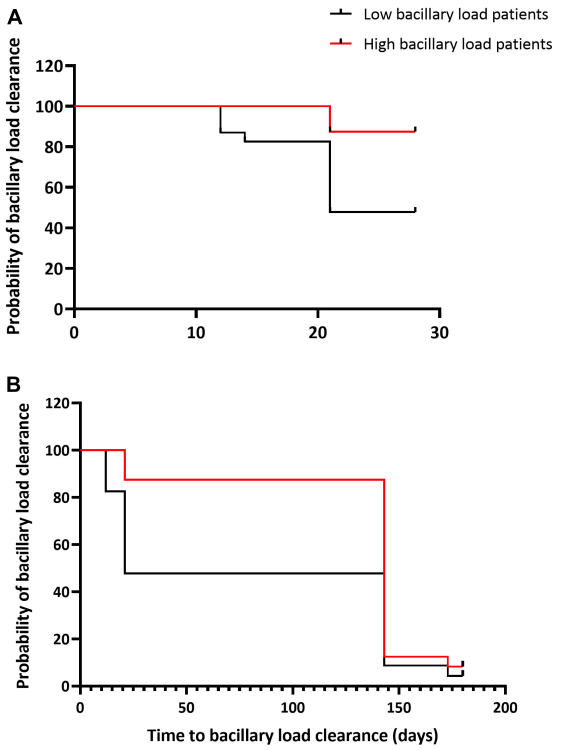


FIGURE 4
Bacillary load clearance during treatment in relation to baseline bacterial loads. Kaplan–Meier curves show TB-MBLA bacillary load clearance by week 8 of treatment **(A)** and by the end of treatment week 26 **(B)** among patients with high and low baseline bacterial load. Low baseline bacterial load patients were more likely to clear bacillary load at week 8, HR 5.6, $p = 0.003$ and at week 26 of treatment, HR 3.2, $p = 0.014$ than high baseline bacterial load patients.

## Discussion

Using the data from this clinical study, we have demonstrated that the concordance of symptoms and bacteriological tests is high before and low after treatment initiation. The strong pre-treatment agreement is in line with a recent report from South Africa (11). While other symptoms improved rapidly in the first 2 weeks of treatment, cough and sputum production improved slowly than bacteriological tests, as previously reported (13, 14). The rapid resolution of symptoms such as chest pain, night sweats, and loss of appetite seems to reflect the early bactericidal activity of anti-TB therapy. Studies have shown that most actively growing bacilli are killed within the first 2 weeks of treatment leaving the dormant (hard to clear) bacilli to persist longer on treatment course (23, 37).

Cough resolution followed closely that of bacillary load measured by TB-MBLA. The cough-TB-MBLA resolution relationship observed was more pronounced than the relationship with standard culture and sputum SM. Early clinical trials have demonstrated an association of TB symptoms with bacteriological tests during treatment (14, 38). Most importantly, analysis of the data from four clinical trials showed that TB symptoms (fever and sweats) resolved rapidly in the first 8 weeks of treatment whereas cough resolved slowly and was present in 20% of patients at the end of treatment (14). This finding is consistent with our study in which 20 and 15% of patients were coughing at weeks 22 and 26 of treatment, respectively, of whom four out of seven patients (57%) were HIV positive. A total of 14.2% of the coughing patients at week 26 were TB-MBLA positive. The presence of residual inflammation or other ailments arising as TB bacteria are cleared may explain the high positivity of cough at the end of treatment.

Interestingly, patients with low baseline BL took longer time to resolve sputum production than those with high BL. Further analysis of these patients revealed that 44% were HIV positive, an indication that the persistent sputum production could be driven by other opportunistic ailments and not underlying TB bacillary load. It is long established that HIV infection increases the risk of infection with other respiratory pathogens that may exacerbate and prolong symptoms such as cough and sputum production (39, 40). Thus, this interpretation and the overall relationship of clinical symptom clearance in TB/HIV-coinfected patients including those with low bacillary load warrants further investigation in a large cohort of HIV/TB-coinfected patients.

We further compared our results with a clinical trial for shortening the treatment of patients with drug-sensitive HIV-negative PTB conducted in Uganda, Brazil, and the Philippines. The study revealed that clinical symptoms declined rapidly by week 8 of treatment and that symptoms including fever, cough, and chest pain were more common among patients who had culture positive during follow-up (13). In contrast, the presence of TB symptoms at week 8 of treatment was not associated with bacteriological positivity in our study. Most patients with TB symptoms such as cough and sputum production at week 8 were negative by culture and sputum smear tests. Moreover, bacteriological tests were positive in some patients who had no TB symptoms at week 8 of treatment. At week 26, two patients who had no TB symptoms would have been declared clinically well and discharged from treatment in the absence of culture as the case in resource-poor countries. The novel TB-MBLA demonstrated highest positivity rate among patients with and without TB symptoms including those who were culture and smear negative. This discrepancy may be explained by the low sensitivity of SM, which is limited to 10,000 CFU/ml (6, 17), the effect of chemical decontamination on viable TB bacilli, and the presence of non-culturable bacteria, which do not grow in routine culture media (17, 21).

It is important to note that both clinical and bacteriological measures responded to treatment, falling to less than 20% positivity except for sputum production which was slightly above 20% positivity. The discordance could be due to the limitation of sputum-based tests as they only capture a

TABLE 4 Time to the resolution of TB symptoms and bacteriological tests stratified by HIV status.

| | Median time to resolution of clinical symptoms (weeks) | | | |
|---|---|---|---|---|
| **Clinical symptoms** | **All patients (*n* = 46)** | **HIV positive (*n* = 23)** | **HIV negative (*n* = 23)** | ***P*-value** |
| Cough, *n* (IQR) | 17.9 (8.0–23.9) | 19.9 (9.8–23.9) | 8.0 (7.8–23.9) | 0.33 |
| Sputum production, *n* (IQR) | 19.9 (8.0–23.9) | 19.9 (8.1–23.9) | 8.0 (8.0–21.8) | 0.67 |
| Chest pain, *n* (IQR) | 2.0 (2.0–11.5) | 8.0 (2.0–19.9) | 2.0 (2.0–8.0) | 0.09 |
| Sweats, *n* (IQR) | 2.0 (2.0–8.0) | 2.0 (2.0–11.5) | 2.0 (2.0–2.8) | 0.07 |
| Loss of appetite, *n* (IQR) | 2.0 (2.0–8.0) | 2.0 (2.0–6.0) | 2.0(2.0–8.0) | 0.52 |
| Smear microscopy, *n* (IQR) | 8.0 (2.0–8.0) | 2.1 (2.0–8.0) | 8.0(2.0–8.0) | 0.60 |
| LJ, *n* (IQR) | 6.0 (2.0–8.1) | 2.0 (2.0–17.9) | 8.0 (2.0–8.0) | 0.60 |
| MGIT, *n* (IQR) | 8.0 (7.8–18.0) | 8.0 (7.8–17.9) | 8.0 (7.8–8.0) | 0.84 |
| MBLA, *n* (IQR) | 19.8 (8.0–19.9) | 18.4 (8.0–19.9) | 19.8 (8.0–19.9) | 0.86 |

Cox proportion hazard ratio shows the association between median time to resolution (in weeks) of TB clinical symptoms stratified by HIV status. *n* (IQR), number of weeks (interquartile range); LJ, Lowenstein–Jensen media; MGIT, mycobacterium growth indicator tubes; TB-MBLA; tuberculosis-molecular bacterial load assay.

proportion of bacilli in the lungs and only those that can growth in culture (41). This may explain why TB-MBLA which measures total RNA reflecting both actively growing and dormant bacilli is more sensitive and appears more consistent with the resolution of cough. Moreover, clinical symptoms are not specific and can be caused by any other respiratory disease. For example, cough and chest pain are some of the most common symptoms of COVID-19 (42).

Our study has several limitations. Screening and enrollment were based on the presence of TB symptoms at diagnosis and a positive Xpert MTB/RIF Assay result. This approach selectively included only patients who had symptoms of active PTB and increased the chance of those patients being positive by bacteriological tests at diagnosis and a higher percentage agreement than in treatment follow-up visits. Secondly, we enrolled a low number of patients with 6-month follow-up visits and we were not able to establish if treatment failure was due to relapse or re-infections. Because of the small number of patients, we were unable to find a difference in time to the resolution of TB symptoms or bacteriological positivity in relation to baseline BL and HIV status. Clinical studies involving larger number of patients and longer follow-up past the current 6 months of TB therapy in healthcare settings are underway and will shed more light on the long-term relationship of clinical symptoms particularly cough with bacteriological tests and final outcomes. Such studies will further help to understand the clinical relevance of TB positive results by TB-MBLA when routine standard bacteriological tests, culture, and microscopy are negative and provide additional data about the causes of recurrent tuberculosis after successful treatment.

## Conclusion

We showed a high percentage agreement of clinical symptoms with bacteriological positivity for TB at diagnosis which weakens rapidly during the early weeks of anti-TB therapy. Our findings provide new evidence that relying solely on TB symptoms for diagnosis or monitoring may mislead treatment decisions of some patients and final treatment outcomes. Our findings advocate for more investments in bacteriological tests to improve the accuracy of TB diagnosis and treatment monitoring in routine healthcare settings.

## Data availability statement

The data supporting the conclusions of this article will be available by authors upon request and without undue reservation.

## Ethics statement

The studies involving human participants were reviewed and approved by the Mbeya Medical Research and Ethics Committee (MRH/R.10/18VOLL.VII/12) and University of St Andrews Teaching and Research Ethics Committee (MD12678). National approval was obtained from National Institute for Medical Research (NIMR/HQ/R.8a/Vol.IX/2400) in Tanzania. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

BM, WS, NN, IS, and SG conceived and designed the study. BM, WS, IS, and NN developed the research tools. BM, ES, and JJ collected the data. BM, WS, and WO led database cleaning, data analysis, and created figures and tables. BM drafted the manuscript. SG, WS, and NN proofread the manuscript. All authors reviewed the manuscript before submission.

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## Conflict of interest

Life Arc, which is developing TB-MBLA for clinical use, receives pro bono advice from SG and WS.

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.

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## Supplementary material

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## References

1. Maclean, E, Mckenna L, Ruhwald M. *Pipeline Report 2021*. New York, NY: Treatment Action Group (2021).

2. World Health Organization. *Global Tuberculosis Report*. Geneva: World Health Organization (2021).

3. Tiberi S, du Plessis N, Walzl G, Vjecha MJ, Rao M, Ntoumi F, et al. Tuberculosis: progress and advances in development of new drugs, treatment regimens, and host-directed therapies. *Lancet Infect Dis.* (2018) 18:e183–98.

4. Cadosch D, Abel zur Wiesch P, Kouyos R, Bonhoeffer S. The role of adherence and retreatment in de novo emergence of MDR-TB. *PLoS Comput Biol.* (2016) 12:e1004749. doi: 10.1371/journal.pcbi.1004749

5. Gillespie SH, Crook AM, McHugh TD, Mendel CM, Meredith SK, Murray SR, et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *N Engl J Med.* (2014) 371:1577–87. doi: 10.1056/NEJMoa1407426

6. Horne DJ, Royce SE, Gooze L, Narita M, Hopewell PC, Nahid P, et al. Sputum monitoring during tuberculosis treatment for predicting outcome: systematic review and meta-analysis. *Lancet Infect Dis.* (2010) 10:387–94. doi: 10.1016/S1473-3099(10)70071-2

7. Goletti D, Lindestam Arlehamn CS, Scriba TJ, Anthony R, Cirillo DM, Alonzi T, et al. Can we predict tuberculosis cure? What tools are available? *Eur Respir J.* (2018) 52:1801089.

8. Diktanas S, Vasiliauskiene E, Polubenko K, Danila E, Celedinaite I, Boreikaite E, et al. Factors associated with persistent sputum positivity at the end of the second month of tuberculosis treatment in Lithuania. *Tuberc Respir Dis (Seoul).* (2018) 81:233–40. doi: 10.4046/trd.2017.0096

9. Loddenkemper R, Lipman M, Zumla A. Clinical aspects of adult tuberculosis. *Cold Spring Harb Perspect Med.* (2016) 6:1–25.

10. Cudahy P, Haven N, Shenoi S. Diagnostic for pulmonary tuberculosis. *Postgr Med J.* (2016) 92:187–93.

11. Govender I, Karat AS, Olivier S, Baisley K, Beckwith P, Dayi N, et al. Prevalence of Mycobacterium tuberculosis in sputum and reported symptoms among clinic attendees compared with a community survey in rural South Africa. *Clin Infect Dis.* (2022) 75:314–22.

12. Cain KP, McCarthy KD, Heilig CM, Monkongdee P, Tasaneeyapan T, Kanara N, et al. An algorithm for tuberculosis screening and diagnosis in people with HIV. *N Engl J Med.* (2010) 362:707–16.

13. Bark CM, Dietze R, Okwera A, Quelapio MI, Thiel BA, Johnson JL. Clinical symptoms and microbiological outcomes in tuberculosis treatment trials. *Tuberculosis.* (2011) 91:601–4.

14. Hales CM, Heilig CM, Chaisson R, Leung CC, Chang KC, Goldberg SV, et al. The association between symptoms and microbiologically defined response to tuberculosis treatment. *Ann Am Thorac Soc.* (2013) 10:18–25.

15. Friedrich SO, Rachow A, Saathoff E, Singh K, Mangu CD, Dawson R, et al. Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. *Lancet Respir Med.* (2013) 1:462–70.

16. Kayigire XA, Friedrich SO, Karinja MN, van der Merwe L, Martinson NA, Diacon AH. Propidium monoazide and Xpert MTB / RIF to quantify *Mycobacterium tuberculosis* cells. *Tuberculosis.* (2016) 101:79–84. doi: 10.1016/j.tube.2016.08.006

17. van Zyl-Smit RN, Binder A, Meldau R, Mishra H, Semple PL, Theron G, et al. Comparison of quantitative techniques including Xpert MTB/RIF to evaluate mycobacterial burden. *PLoS One.* (2011) 6:e28815. doi: 10.1371/journal.pone.0028815

18. Phillips PPJ, Mendel CM, Burger DA, Crook AM, Nunn AJ, Dawson R, et al. Limited role of culture conversion for decision-making in individual patient care and for advancing novel regimens to confirmatory clinical trials. *BMC Med.* (2016) 14:19. doi: 10.1186/s12916-016-0565-y

19. de Knegt GJ, Dickinson L, Pertinez H, Evangelopoulos D, McHugh TD, Bakker-Woudenberg IAJM, et al. Assessment of treatment response by colony forming units, time to culture positivity and the molecular bacterial load assay compared in a mouse tuberculosis model. *Tuberculosis.* (2017) 105:113–8. doi: 10.1016/j.tube.2017.05.002

20. Mtafya B, Sabi I, Ntinginya NE, Sabiiti W, Msuya Y, et al. *Clinical Evaluation of the Novel Molecular Bacterial Load Assay for Monitoring of Tuberculosis Treatment Response NIMR-Mbeya Medical Research Centre*. Mbeya: Tanzania

National TB and Leprosy Control Programme-Mbeya Regional TB and Leprosy Control (2019). p. 1–24.

21. Pheiffer C, Carroll NM, Beyers N, Donald P, Duncan K, Uys P, et al. Time to detection of *Mycobacterium tuberculosis* in BACTEC systems as a viable alternative to colony counting. *Int J Tuberc Lung Dis.* (2008) 12:792–8.

22. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor Countries: challenges and opportunities. *Clin Microbiol Rev.* (2011) 24:314–50.

23. Kayigire XA, Friedrich SO, Van Der Merwe L, Donald PR, Diacon AH. Simultaneous staining of sputum smears for acid-fast and lipid-containing *Myobacterium tuberculosis* can enhance the clinical evaluation of antituberculosis treatments. *Tuberculosis.* (2015) 95:770–9. doi: 10.1016/j.tube.2015.08.001

24. Rockwood N, du Bruyn E, Morris T, Wilkinson RJ. Assessment of treatment response in tuberculosis. *Expert Rev Respir Med.* (2016) 10:643–54.

25. Global Tuberculosis Report. *Global Tuberculosis Report*. Geneva: Global Tuberculosis Report (2021).

26. Sabiiti W, Azam K, Farmer ECW, Kuchaka D, Mtafya M, Bowness R, et al. Tuberculosis bacillary load, an early marker of disease severity: the utility of tuberculosis molecular bacterial load assay. *Thorax.* (2020) 75:606–8. doi: 10.1136/thoraxjnl-2019-214238

27. Honeyborne I, Mtafya B, Phillips PPJ, Hoelscher M, Ntinginya EN, Kohlenberg A, et al. The molecular bacterial load assay replaces solid culture for measuring early bactericidal response to antituberculosis treatment. *J Clin Microbiol.* (2014) 52:3064–7. doi: 10.1128/JCM.01128-14

28. Moore DF, Curry JI, Knott CA, Jonas V. Amplification of rRNA for assessment of treatment response of pulmonary tuberculosis patients during antimicrobial therapy. *J Clin Microbiol.* (1996) 34:1745–9. doi: 10.1128/JCM.34.7.1745-1749.1996

29. Honeyborne I, McHugh TD, Phillips PPJ, Bannoo S, Bateson A, Carroll N, et al. Molecular bacterial load assay, a culture-free biomarker for rapid and accurate quantification of sputum *Mycobacterium tuberculosis* bacillary load during treatment. *J Clin Microbiol.* (2011) 49:3905–11. doi: 10.1128/JCM.00547-11

30. Deutscher MP. Degradation of RNA in bacteria: comparison of mRNA and stable RNA. *Nucleic Acids Res.* (2006) 34:659–66.

31. Desjardin LE, Perkins MD, Wolski K, Haun S, Teixeira L, Chen Y, et al. Measurement of sputum mycobacterium tuberculosis messenger RNA as a surrogate for response to chemotherapy. *Am J Respir Crit Care Med.* (1999) 160:203–10. doi: 10.1164/ajrccm.160.1.9811006

32. Rustad TR, Minch KJ, Brabant W, Winkler JK, Reiss DJ, Baliga NS, et al. Global analysis of mRNA stability in *Mycobacterium tuberculosis*. *Nucleic Acids Res.* (2013) 41:509–17.

33. World Health Organization. *Definitions and Reporting Framework for Tuberculosis: 2013 Revision (WHO/HTM/TB/2013.2)*. Geneva: World Health Organization (2014).

34. Gillespie SH, Sabiiti W, Oravcova K. Mycobacterial load assay. In: Bishop-Lilly K editor. *Diagnostic Bacteriology: Methods and Protocols, Methods in Molecular Biology*. New York, NY: Humana Press (2017). p. 89–105. doi: 10.1007/978-1-4939-7037-7_5

35. Stinson KW, Eisenach K, Kayes S, Matsumoto M, Siddiqi S, Nakashima S. *Mycobacteriology Laboratory Manual:Global Laboratory Initiatives Advancing TB diagnosis*. 1st ed. Geneva: Global Laboratory Initiative (2014).

36. Siddiqi SH, Rüsch-Gerdes S. *MGIT Procedure Manual: For Bactectm MGIT 960TM TB System*. Geneva: Foundation for Innovative New Diagnostics (2006).

37. Bowness R, Boeree MJ, Aarnoutse R, Dawson R, Diacon A, Mangu C, et al. The relationship between *Mycobacterium tuberculosis* mgit time to positivity and cfu in sputum samples demonstrates changing bacterial phenotypes potentially reflecting the impact of chemotherapy on critical sub-populations. *J Antimicrob Chemother.* (2015) 70:448–55. doi: 10.1093/jac/dku415

38. Woimo TT, Yimer WK, Bati T, Gesesew HA. The prevalence and factors associated for anti-tuberculosis treatment non-adherence among pulmonary tuberculosis patients in public health care facilities in South Ethiopia: a cross-sectional study. *BMC Public Health.* (2017) 17:269. doi: 10.1186/s12889-017-4188-9

39. Crothers K, Thompson BW, Burkhardt K, Morris A, Flores SC, Diaz PT, et al. HIV-Associated lung infections and complications in the Era of combination antiretroviral therapy. *Proc Am Thorac Soc.* (2011) 8:275–81.

40. Benito N, Moreno A, Miro JM, Torres A. Pulmonary infections in HIV-infected patients: an update in the 21st century. *Eur Respir J.* (2012) 39:730–45. doi: 10.1183/09031936.00200210

41. Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR. Resuscitation-promoting factors reveal an occult population of tubercle *Bacilli* in sputum. *Am J Respir Crit Care Med.* (2010) 181:174–80. doi: 10.1164/rccm.200905-0661OC

42. Viscaa D, Ong CWM, Tiberi S, Centisg R, D'Ambrosio L, Chen B, et al. Tuberculosis and COVID-19 interaction: a review of biological, clinical and public health effects. *Pulmonary.* (2021) 27:151–65.

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# Exploring modulations in T-cell receptor-mediated T-cell signaling events in systemic circulation and at local disease site of patients with tubercular pleural effusion: An attempt to understand tuberculosis pathogenesis at the local disease site

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**Introduction:** T cells are crucial for pathogenesis as well as control for tuberculosis (TB). Although much is known about the signaling pathways which are required for the activation of T cells during acute infection but the way these cells respond during persistent of infection still remained elusive. Therefore, it is rationale to understand T cell activation during tuberculous pleural effusion (TPE), which is similar to bacterial persistency system.

**Methods:** Herein, we will employ T cell receptor (TCR) based approaches for studying events of T cell activation pathways in cells of blood and pleural fluid among patients with TPE. We performed spectrofluorimetric analysis to study effect of M. tuberculosis antigens, ESAT-6 and Ag85A stimulation on intracellular calcium levels, Phosphorylation levels of ZAP-70 (Zeta-chain-associated protein kinase 70), PKC-θ (Protein kinase C theta), Erk1/2 (Extracellular signal-regulated kinase 1 and 2) and p-38 two important members of MAPKs (Mitogen activated Protein kinases) in CD3 and CD28 induced cells of blood and pleural fluid of same patients with TPE by western blotting. Patients with non-TPE were also included as matching disease controls in this study.

**Results:** We observed significantly higher intracellular calcium levels, Phosphorylation levels of ZAP-70, Erk1/2 and p-38 in CD3 and CD28 induced cells of pleural fluid as compared to the blood cells of same patients with TPE. Alteration in the activation of these events has also been noted after stimulation of ESAT-6 and Ag85A.

**Discussion:** Present study demonstrated up-regulated activation of TCR mediated T cell signaling events at local disease site (Pleural fluid) as compared to the blood sample of TB pleurisy patients which could be involved in T-cell dysfunctioning during the progression of the disease and also could be responsible for Th 1 dominance at local disease site in patients with TPE.

KEYWORDS

**tuberculous pleural effusion, T-cell receptor, cytokines, activation, T-cell signaling**

## Introduction

Globally, an estimated 10 million people fall ill with tuberculosis (TB) worldwide and a total of 1.5 million people die from TB (1). TB remains the leading cause of death globally triggered from a single pathogen, and this TB-related problem is even exacerbated by the human immunodeficiency virus (HIV) infection. *Mycobacterium tuberculosis* (*M. tuberculosis*) elicits an immune response in the host, and the immune cells are activated against the bacteria. Despite activation, the immune system is not capable enough to eliminate bacteria from the body. This goes to prove that *M. tuberculosis* is proficient in altering the immune response for its own survival, leading to disease or latent infection. The molecules and mechanisms utilized to accomplish the survival of bacteria inside the host are not fully understood. The host–pathogen interactions in TB should be analyzed at the disease site because *M. tuberculosis* is predominantly contained in the local tissue lesions. Active TB is characterized by the expansion of *M. tuberculosis*-specific T cells at the site of infection. A very low proportion of *M. tuberculosis*-specific effector T cells are found in the blood compared with the infected tissue, and thus, considerable differences in the cellular immune response and regulatory mechanisms are induced in these diverse compartments. Therefore, it is important to study the immune responses at the local site of infection to improve the understanding of the immunological mechanisms that are involved in the containment and progression of TB. Tuberculous pleural effusion (TPE) is the second most common type of extrapulmonary TB, which is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of *M. tuberculosis* infection (2). An accumulation of lymphocytes, in TPE, has been well documented (3–7), which is involved in the pathogenesis of TB pleurisy. The profusion and easy accessibility of pleural fluid mononuclear cells (PFMCs) in the TPE make it a good model to study the *M. tuberculosis*-specific T-cell responses at the local disease site. The signaling pathways triggered by *M. tuberculosis* in human T cells have not yet been studied in a relevant physiological system, hence requiring further investigation. The modulations in T-cell receptor (TCR)-mediated cell signaling mechanism are not completely elucidated to date. It was previously observed that T cells from human TB patients showed a decreased expression of CD3-ζ, a key signaling domain of the TCR/CD3 complex (8). Wang et al. showed that the potent T-cell antigen ESAT-6 can directly suppress interferon-gamma (IFN-γ) production in CD4+ T cells (9). Mahon et al. (10) reported that *M. tuberculosis* cell wall glycolipids directly inhibit polyclonal murine CD4+ T-cell activation by blocking ZAP-70 phosphorylation. Later, they extended their study by reporting on mannose-capped lipoarabinomannan (ManLAM)-induced inhibition of the TCR signaling by interfering with ZAP-70 (zeta-chain-associated protein kinase 70), Lck (lymphocyte-specific protein tyrosine kinase), and LAT (linker for activation of T cells) phosphorylation in antigen-specific murine CD4+ T cells and primary human T cells (11). Palma-Nicholas (12) reported T-cell downmodulation of the mitogen-activated protein kinases–extracellular signal-regulated kinase 1 and 2 (MAPK–ERK1/2) pathway in total spleen cells from naive BALB/c mice by the cell-surface lipid di-O-acyl-trehalose (DAT). Recently, the regulation of interferon-gamma (IFN-γ) production by the ERK and p38 MAPK signaling pathway and through CD150 signaling lymphocytic activation molecule (SLAM) co-stimulation has been suggested to take place in TB (13). The inhibition of IFN-γ production through the p38 MAPK pathway by ESAT-6 has been reported in T cells from healthy individuals (14). We also reported modulations in T-cell signaling events in Jurkat T cells and TB patients (15, 16) in earlier studies. The signaling pathways triggered by *M. tuberculosis* in human T cells have not yet been studied at a local disease site, but there is a previous study that showed that, among pleural fluid lymphocytes, natural killer (NK) cells are a major source of IFN-γ production in a mechanism enhanced by interleukin 12 (IL-12), dependent on calcineurin, p38, and the ERK pathways and these cells can directly recognize *M. tuberculosis* antigens (17). One more study by Chen et al. showed that Toll-like receptor 2 (TLR2) ligand activity was also significantly higher in the tuberculous pleural fluid (TPF) than in the serum. They also observed that *M. tuberculosis* TLR2 ligands, 19-kDa lipoprotein, and live Bacillus Calmette Guerin (BCG) all modulated cytokine production by CD4+ T cells isolated from pleural fluid and activated with anti CD3 and anti CD28 (18). However, there is yet no study on TCR-mediated modulation in

T-cell activation in pleural fluid (PF) and peripheral blood (PB) of patients with TB pleurisy. Although very few reports on the alterations of signaling molecules of T-cell activation in patients with TB are available in India. Previously, our study found that the phosphorylation of MAPKs, ERK1/2, and p38 was curtailed by *M. tuberculosis* antigens in patients with TB, whereas, in purified protein derivative (PPD)+ve healthy individuals, only ERK1/2 phosphorylation was inhibited. In addition to this development, we also observed that the binding of transcription factors, such as the nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF-κB), was also altered by *M. tuberculosis* antigens (15). The effect of the secretory protein ESAT-6 of *M. tuberculosis* on the modulation of macrophage signaling pathways has been studied (19). Still, there is yet no study to demonstrate whether the mechanisms and molecules were involved in the production of IFN-γ and interleukin 2 (IL-2) (two crucial cytokines for immune responses against TB) by activated T lymphocytes on *M. tuberculosis* stimulation, using any relevant biological model. At the site of the active *M. tuberculosis* infection, as opposed to other forms of TB, PFMCs are readily accessible and provide an opportunity to study the aspects of TB pathogenesis on cells from the local disease site. Thus, it is important to evaluate the immune responses at the local site of infection and in peripheral blood, to improve the understanding of the immunological mechanisms involved in the containment and progression of TB.

# Methodology

## Patient selection

The study protocol was approved by the Institutional Human Ethics Committee of ICMR–National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra (India). Informed written consent was obtained from all study subjects. Blood samples and pleural fluid samples were collected from pleurisy patients ($n = 22$) who were aged between 18 and 60 years. Pleural effusions were classified into tuberculous pleural effusion (TPE) ($n = 15$) and non-tuberculous pleural effusion (non-TPE) ($n = 7$) groups. Matching disease controls/patients with non-tuberculous pleural effusion (non-TPE), such as parapneumonic effusion, empyema, and malignancies, were included in the non-TPE group. All patients involved in the study were those who attended the Outpatient Department (OPD) of the Department of *Tuberculosis* and Chest Diseases, Sarojni Naidu Medical College (S.N.M.C), Agra. We also noted their BCG vaccination status and their PPD status, with data shown in Table 1. The effusions were classified as exudates according to at least one of the criteria of Light et al. (20). All patients provided a detailed medical history and underwent a detailed physical examination. The diagnosis of TPE was confirmed on the basis of: medical history, chest X-ray, physical examination, and isolation of *M.tuberculosis* in a positive mycobacterial culture, a smear positive for acid-fast bacilli from the pleural fluid. None of the subjects received anti-tuberculous or steroid therapy at the time of the study. The exclusion criteria included a positive test for human immunodeficiency virus, a pregnant woman, and the presence of concurrent infectious diseases. The diagnosis of tuberculosis was confirmed in all cases by a microscopic examination and culture of *M. tuberculosis* from pleural fluid specimens.

TABLE 1 Demographic data of the study population.

| Characteristics | TPE | Non-TPE |
|---|---|---|
| Patients (n)=22 | 15 (68.18%) | 7 (31.82%) |
| **Age** | | |
| Median | 43 | 40 |
| Range (Lower-Upper) | (32.84–47.16) | (28.54–52.89) |
| **Sex** | | |
| Men | 11 (73.33%) | 4 (57.14%) |
| Women | 4 (26.66%) | 3 (42.86%) |
| **BCG** | | |
| Vaccinated | 11 (73.33%) | 5 (71.43%) |
| Non-vaccinated | 4(26.66%) | 2 (28.57%) |
| **PPD status** | | |
| Positive | 7 (46.67%) | 3 (42.86%) |
| Negative | 8 (53.33%) | 4 (57.14%) |

TPE, Tuberculous pleural effusion; Non-TPE, Non-tuberculous pleural effusion.

## Thoracentesis and mononuclear cells

Blood samples and pleural fluid samples were collected at the time of therapeutic thoracentesis. Briefly, pleural fluid (PF) (∼50 ml) and peripheral blood (10 ml) were obtained from the patients during diagnostic thoracentesis before the initiation of chemotherapy. As much as >50 ml of PF was aspirated under sterile conditions, using 18-gauge needles, collected in heparinized vials and kept in ice during transportation. Blood samples were also collected in heparinized vials and kept at room temperature. Pleural fluid (PF) and blood were obtained simultaneously. Peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) were isolated from heparinized blood and pleural fluid (PF) using the standard Ficoll-Hypaque density gradient centrifugation method and suspended in RPMI (Roswell Park Memorial Institute) 1,640 tissue culture medium (Sigma, USA) supplemented with 2 mM L-glutamine, antibiotic–antimycotic solution (Sigma, USA), and 10% heat-inactivated human AB serum (MP Biomedicals, India). Cell cultures were maintained in a humidified 5% CO2 incubator at 37°C. Cell viability ≥95% was determined by the trypan blue exclusion test.

## Chemicals and antigens

Mouse immunoglobulin G (IgG) anti-human pure CD3 (clone UCHT1), ionomycin, goat anti-mouse-IgG (GAM), sodium fluoride (NaF), sodium orthovanadate, anti-protease cocktail and Bradford reagent were purchased from Sigma, USA. Fura-2-acetoxymethyl ester (Fura-2/AM) was procured from Calbiochem, USA. Cell lysis buffer was purchased from Invitrogen, USA. Anti-human CD3 (clone OKT-3) and anti-human CD28 (clone CD28.2) were procured from eBioscience, USA. Phosphorylated ZAP-70, phosphorylated ERK1/2, phosphorylated p38 MAPK, phosphorylated protein kinase C theta (PKC-θ), β-Actin and goat polyclonal IgG anti-mouse peroxidase-conjugated antibodies were procured from Cell Signaling Technology (CST), USA. Enhanced chemiluminescence (ECL) reagents were procured from Millipore, USA. Lyophilized *M. tuberculosis* antigens (ESAT-6 and Ag85A) were procured from the BEI Research Resources Repository funded by the National Institute of Allergy and Infectious Diseases and managed by ATCC, USA. PPD RT-49 (Research Tuberculin) was procured from Statens Serum Institut, Denmark. All antigens were dissolved in filtered phosphate-buffered saline (PBS) at 7.4 pH to make a 1 mg/ml concentration.

## Quantification of transmembrane $Ca^{2+}$ mobilization

Peripheral blood mononuclear cells (PBMCs) and PFMCs ($5 \times 10^6$/ml) were rested at least for 2 h in a 37°C $CO_2$ incubator before stimulation with appropriate doses of *M. tuberculosis* antigens. Cells were stimulated with 5μg/ml Ag85A and 10μg/ml of ESAT-6 for 4 h in a 37°C $CO_2$ incubator. After incubation, the cells were washed with PBS, pH 7.4. Cells were incubated with Fura-2/AM at 1 μM for 30 min at 37°C in loading buffer [(in mM): NaCl, 110; KCl, 5.4; $NaHCO_3$, 25; $MgCl_2$, 0.8; $KH_2PO4$, 0.4; HEPES, 20; $Na_2HPO4$, 0.33; $CaCl_2$, 1.2. pH 7.4]. After loading, cells were washed three times (500×g for 5 min) and remained suspended in an identical buffer. $[Ca^{2+}]i$ was measured, as reported elsewhere (21, 22). Fluorescence intensities were measured in ratio mode using a Varian ECLIPSE spectrofluorometer equipped with fast filter accessory (Varian Incorporation, St. Helens, Australia) at 340 and 380 nm (excitation filters) and 510 nm (emission filter). Cells were stirred continuously throughout the experiment. For anti-CD3-stimulated calcium studies: after stabilization of the basal levels of cytosolic calcium, 10 μg/ml of pure anti-CD3 (Clone UCHT1) was added to the cuvette. For the measurement of Fmax, ionomycin 5μM was added to the cuvette and for Fmin, $MnCl_2$ 2mM was added to the cuvette.

The intracellular concentrations of free $Ca^{2+}$ $[Ca^{2+}]i$ were calculated by using the following equation: $[Ca^{2+}]i = Kd \times (R - Rmin)/(Rmax - R) \times (Sf2/Sb2)$. A value of 224 nM for Kd was added to the calculations. Rmax and Rmin values were obtained by the addition of ionomycin (5 μM) and $MnCl_2$ (2 mM), respectively. All experiments were performed at 37°C.

## Treatment and activation of cells

Peripheral blood mononuclear cells (PBMCs) and PFMCs ($5 \times 10^6$/ml) were stimulated with appropriate doses of *M. tuberculosis* antigens: 5 μg/ml of Ag85A and 10 μg/ml of ESAT-6 for overnight hours in a 37°C $CO_2$ incubator, and few cells were left unstimulated. After overnight stimulation with antigens, the cells were activated with plate-bound anti-CD3 and anti-CD28 (clones OKT3 and CD28.2 at 2 μg/ml each procured from eBiosciences). The antibody-coated plates were prepared by coating the wells with goat anti-mouse IgG for 1 h at 37°C; then, the plates were washed and then coated with both anti-CD3 and anti-CD28 also for 1 h at 37°C in a humidified atmosphere of 5% $CO_2$. The antigen-stimulated cells were then added to the antibody-coated wells and incubated overnight at 37°C in a humidified atmosphere of 5% $CO_2$. Few cells were left unstimulated with antigens and also untreated with CD3 and CD28 antibodies.

## Western blot analysis of MAPK activation

Antigen-stimulated and anti-CD3 and CD28-activated cells were treated with chilled PBS and then lysed with 50 μl of cell lysis buffer (with freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM anti-protease cocktail). After centrifugation (13,000×g for 5 min), cell lysates were used immediately or stored at −80°C. The protein contents were determined with the Bradford reagent. Denatured proteins (35 μg) were separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) (10%) and transferred to polyvinylidene difluoride (PVDF) membrane. Immunodetection of phosphorylated forms of ZAP-70, PKC-θ, Erk1/2, and p38MAPK was performed using 1:1,000 dilution of phospho-specific antibodies for ZAP-70, PKC-θ, Erk1/2, and p38 MAPK in 5% bovine serum albumin Tris-buffered saline (BSA TBS) for overnight incubation at 4°C. After washing with TBST (TBS with 0.05% Tween 20), the PVDF membranes were treated with peroxidase-conjugated secondary antibodies, and the peroxidase activity was detected with ECL reagents. Equal loading of the proteins was confirmed after stripping the blot and reprobing for total forms of β-Actin. Densitometric analysis of bands was performed using Quantity OneTM software (Bio-Rad, Hercules, USA).

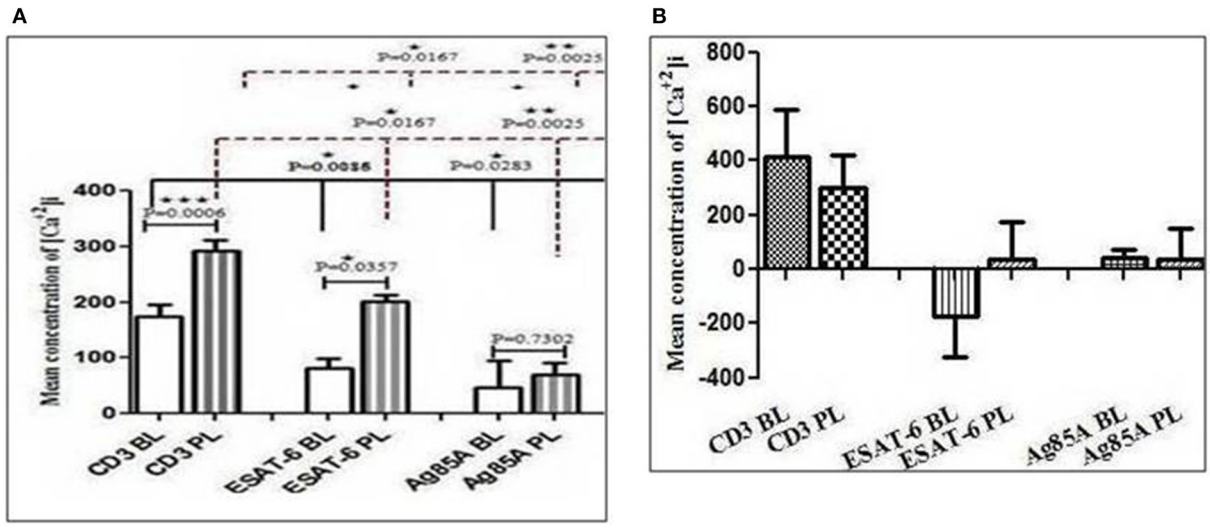


FIGURE 1
Modification in CD3 (cluster of differentiation 3)-induced free intracellular calcium concentration in blood and pleural fluid after *M. tuberculosis* antigens (ESAT-6 and Ag86A) stimulation: The Fura-2-acetoxymethyl ester (Fura-2/AM)-loaded cells peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) were used to study intracellular calcium levels and fluorescence intensities were measured in ratio mode using a Varian ECLIPSE spectrofluorometer, as described in materials and methods section. Bar diagrams show changes in intracellular calcium levels in CD3-treated cells. The effect of *M. tuberculosis* antigens on CD3-stimulated calcium influx is shown in graphs in blood and pleural fluid of patients with tuberculous pleural effusion (TPE) **(A)**, while graph **(B)** is showing the effect of *M. tuberculosis* antigens on blood and pleural fluid of non-TPE patients. The bar is showing the mean ± SEM. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$.

## Statistical analysis

Data were presented as mean ± SEM and comparisons of paired PB and PF samples and different treatments of the same sample were performed using the non-parametric Wilcoxon paired *t*-test. Analysis was done with Prism 5.0 software (GraphPad, La Jolla, USA) and *p*-values <0.05 were considered to indicate statistical significance.

# Results

## TCR triggered intracellular calcium mobilization in pleural fluid and blood samples of TPE patients and matching disease controls and *M. tuberculosis* antigens differentially altered intracellular calcium mobilization

To find out the difference between intracellular calcium mobilization in the blood and the pleural fluid and the effect of *M. tuberculosis* antigens on intracellular calcium mobilization, we measured the intracellular calcium concentration with a spectrofluorometer. We assessed the TCR-triggered calcium mobilization by adding anti-CD3 antibodies on cells from the blood and the pleural fluid pretreated with optimum doses of *M. tuberculosis* antigens (Ag85A and ESAT-6). We noticed a significantly higher intracellular level of calcium in cells from the pleural fluid of patients with TPE, as compared to the intracellular calcium levels in their blood after the addition of anti-CD3. We also observed significantly reduced intracellular levels of calcium in ESAT-6 and Ag85A pretreated cells after the addition of anti-CD3 in the blood and the pleural fluid. Interestingly, the reduction was more in blood as compared to the pleural fluid of patients with TPE (Figure 1A). Diminished levels of intracellular calcium were also noted in blood and pleural fluid cells of non-TPE patients, but they were not significant and there was no significant difference between the intracellular calcium levels of blood and pleural fluid of non-TPE patients (Figure 1B).

## TCR-induced ζ-chain-associated 70-KDa tyrosine phosphoprotein (ZAP-70) activation in pleural fluid and blood samples of TPE patients

To determine whether there is any difference in TCR/CD28-induced ZAP-70 activation in *the* blood and the pleural fluid of patients with TPE and whether *M. tuberculosis* antigens

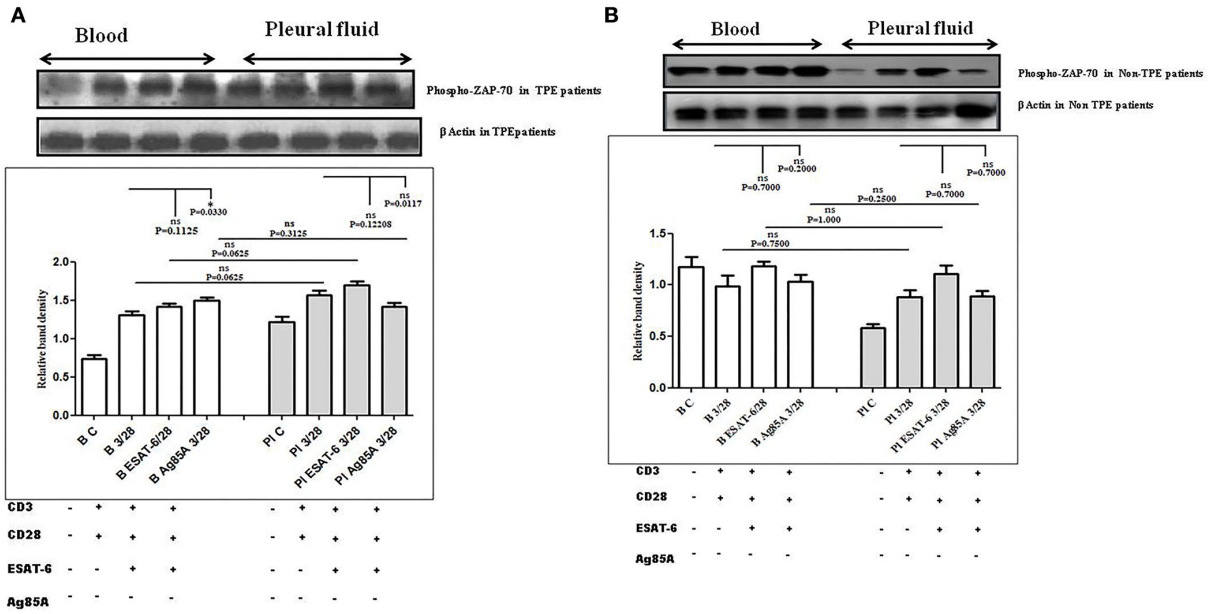


FIGURE 2
TCR/CD28 (T-cell receptor/cluster of differentiation 28)-induced phosphorylation of zeta-chain-associated protein kinase 70 (ZAP-70) before and after *Mycobacterium tuberculosis* antigen stimulation in blood and pleural fluid of patients with tuberculous pleural effusion (TPE) **(A,B)** non-tuberculosis (non-TB) pleurisy patients. Peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) from blood and pleural fluid were activated with CD3 (cluster of differentiation 3) with CD28 antibodies after pretreatment with *M. tuberculosis* antigens, and few cells were left unstimulated and untreated with antibodies as a negative control, and few cells were only activated without any antigen stimulation, and western blotting was done as mentioned in materials and methods. β-Actin antibody was used to conform to equal loading. **(A)** Densitometric analysis of the phosphorylation of ZAP-70 in blood and pleural fluid of patients with TPE. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated Zap-70 and β-Actin is shown, where Lane 1 is showing Control in blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. **(B)** Densitometric analysis of phosphorylation of zeta-chain-associated protein kinase 70 (ZAP-70) in blood and pleural fluid of non-TPE (tuberculous pleural effusion) patients. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated Zap-70 and β-Actin is shown, where Lane 1 is showing Control in blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. Densitometric analysis was done and the ratios of phosphorylated ZAP-70 to β-Actin protein expression were expressed as arbitrary units. Statistical significance was determined using Mann–Whitney $*P < 0.05$.

modulate TCR and TCR/CD28-induced ZAP-70 activation, we measured the phosphorylation of ZAP-70 by Western blot. Phosphorylation of ZAP-70 was studied in Ag85A and ESAT-6-stimulated PBMCs and PFMCs of blood and pleural fluid of patients with TPE. We observed significantly higher levels of phosphorylation of ZAP-70 in pleural fluid, as compared to the blood samples of patients with TPE ($n = 10$). After stimulation with *M. tuberculosis* antigens, altered activation of ZAP-70 was observed both in the blood and the pleural fluid (Figure 2A), whereas a significantly increased level of phosphorylated ZAP-70 was observed only in blood after Ag85A stimulation. However, a lower degree of phosphorylation of ZAP-70 was observed in the blood of non-TPE patients ($n = 5$), but not significantly (Figure 2B).

## TCR-induced protein kinase C-theta (PKC-Θ) activation in the pleural fluid and the blood samples of patients with TPE and *M. tuberculosis* antigens-induced alterations

To determine the differences in TCR/CD28-induced PKC-θ activation in the blood and the pleural fluid of patients with TPE and to study the effect of *M. tuberculosis* on TCR/CD28-induced PKC-θ activation, we measured the phosphorylation of PKC-θ by Western blot. We observed significantly higher levels of phosphorylated PKC θ in pleural fluid, as compared to the blood samples of TPE patients ($n = 10$). After stimulation with *M. tuberculosis* antigens, a significantly altered activation was observed both in the blood and the pleural fluid. ESAT-6

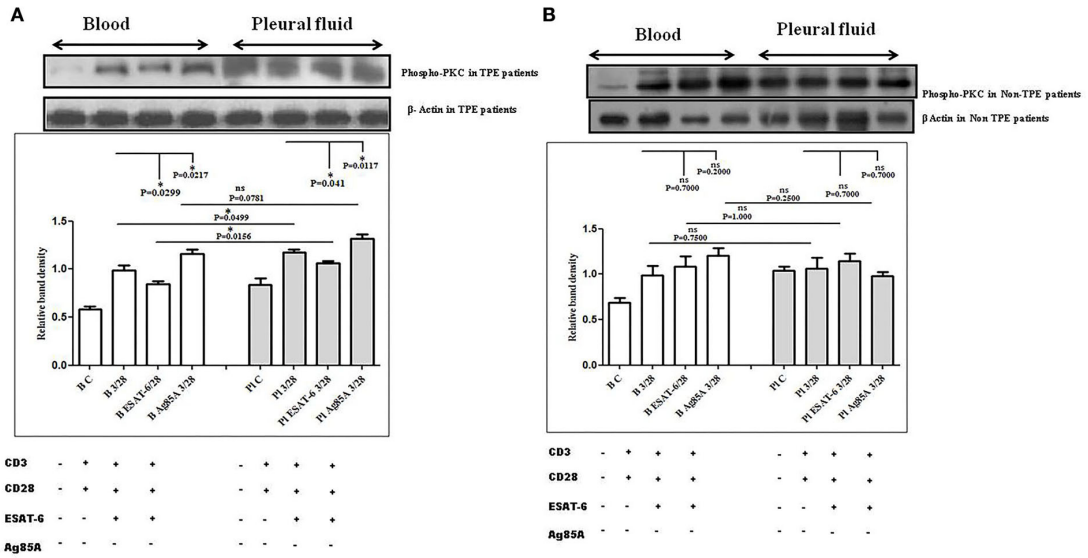


FIGURE 3
TCR/CD28 (T-cell receptor/cluster of differentiation 28)-induced phosphorylation of protein kinase C theta (PKC-θ) before and after *M. tuberculosis* antigen stimulation in blood and pleural fluid of patients with tuberculous pleural effusion (TPE) **(A,B)** non-TB (non-tuberculosis) pleurisy patients. Peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) from blood and pleural fluid were activated with CD3 (cluster of differentiation 3) with CD28 antibodies after pretreatment with *M. tuberculosis* antigens, and few cells were left unstimulated and untreated with antibodies as the negative control, and few cells were only activated without any antigen stimulation, and western blotting was done as mentioned in materials and methods. β-Actin antibody was used to conform to equal loading. **(A)** Densitometric analysis of phosphorylation of PKC-θ in blood and pleural fluid of patients with TPE. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated PKC-θ and β-Actin is shown, where Lane 1 is showing Control in blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. **(B)** Densitometric analysis of phosphorylation of PKC-θ in blood and pleural fluid of non-TPE patients. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated PKC-θ and β-Actin is shown, where Lane 1 is showing Control in blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. Densitometric analysis was done and the ratios of phosphorylated PKC-θ to β-Actin protein expression were expressed as arbitrary units. Statistical significance was determined using Mann–Whitney $^*P < 0.05$.

significantly reduced the phosphorylation of PKC θ and Ag85A significantly increased phosphorylated PKC θ levels both in the blood and the pleural fluid. The activation of PKC θ was more in pleural fluid as compared to the blood before and after stimulation with *M. tuberculosis* antigen stimulation (Figure 3A). A higher activation of PKC θ was also observed in non-TPE patients ($n = 5$) but not significantly (Figure 3B).

## Differences in TCR-induced mitogen-activated protein kinases (MAPKs) activation in pleural fluid and blood samples of patients with TPE and *M. tuberculosis* antigens-induced alterations

To determine the differences in TCR/CD28-induced MAPK activation in blood and pleural fluid of patients with TPE and to study the effect of *M. tuberculosis* on TCR/CD28-induced MAPKs activation, we measured the phosphorylation of Erk ½ and p38 by Western blot. The phosphorylation of MAPKs was studied in Ag85A and ESAT-6-stimulated PBMCs and PFMCs. We observed significantly higher levels of phosphorylation of Erk ½ in pleural fluid as compared to the blood of patients with TPE ($n = 12$), and after stimulation with *M. tuberculosis* antigens, altered activation of Erk ½ was observed both in th4 blood and pleural fluid. Increased phosphorylation of Erk ½ was observed in blood after *M. tuberculosis* antigen stimulation Ag85A significantly increased, while it was not significant after ESAT-6 stimulation in blood. On the other hand, in pleural fluid, significantly increased phosphorylation was observed after ESAT-6 stimulation but not significantly with Ag85A stimulation (Figure 4A). Altered levels of phosphorylated Erk½ were also observed in the blood and the pleural fluid of non-TPE patients ($n = 5$), but not significantly (Figure 4B). We observed significantly increased phosphorylation of p38 in pleural fluid and blood of patients with TPE ($n = 12$), and after stimulation

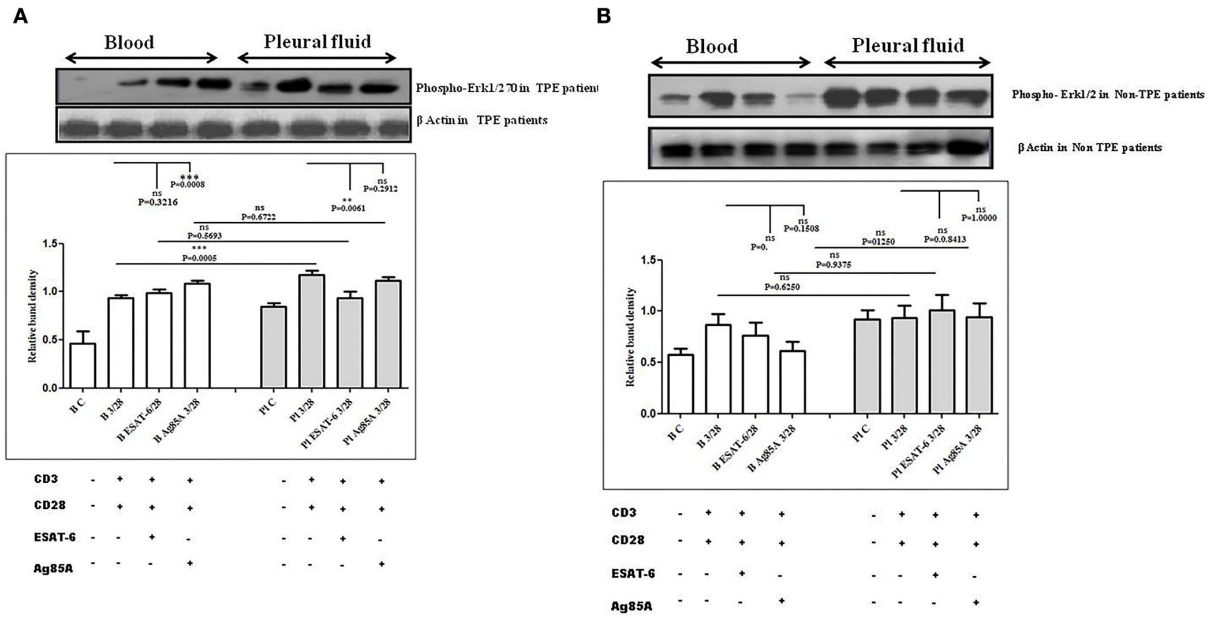


FIGURE 4
TCR/CD28 (T-cell receptor/cluster of differentiation 28)-induced phosphorylation of Erk1/2 before and after *M. tuberculosis* antigen stimulation in blood and pleural fluid of patients with tuberculous pleural effusion (TPE) **(A,B)** non-TPE patients. Peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) from blood and pleural fluid were activated with CD3 with CD28 antibodies after pretreatment with *M. tuberculosis* antigens, few cells were left unstimulated and untreated with antibodies as the negative control, and few cells were only activated without any antigen stimulation, and western blotting was done as mentioned in materials and methods. β-Actin antibody was used to conform to equal loading. **(A)** Densitometric analysis of phosphorylation of Erk1/2 in blood and pleural fluid of patients with tuberculosis (TB) pleurisy. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated Erk1/2 and β-Actin is shown, where Lane 1 is showing Control in blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. **(B)** Densitometric analysis of the phosphorylation of Erk1/2 in blood and pleural fluid of non-TB (non-tuberculosis) patients with TB pleurisy. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated Erk1/2 and β-Actin is shown, where Lane 1 is showing Control in blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. Densitometric analysis was done and the ratios of phosphorylated Erk1/2 to β-Actin protein expression were expressed as arbitrary units. Statistical significance was determined using Mann–Whitney ** $P < 0.005$; *** $P < 0.005$.

with *M. tuberculosis* antigens, altered activation was observed both in the blood and the pleural fluid, but not significantly. p38 phosphorylation was more significantly increased in the pleural fluid than in the blood after stimulation with Ag85A; on the other hand, decreased phosphorylation of p38 was observed after ESAT-6 stimulation but it was not significant (Figure 5A). We also observed reduced p38 phosphorylation in the pleural fluid of non-TPE patients as compared to blood ($n = 5$) but not significantly (Figure 5B).

## Discussion

TB pathogenesis is driven by a complex interplay between the host immune system and the survival strategies of *M. tuberculosis*. The inflammatory response to *M. tuberculosis* infection is tightly regulated by both the host and the pathogen and the protection against TB is based on cell-mediated immune responses. A consistent feature in TB patients has been the *in vitro* dysfunction of circulating T lymphocytes and mononuclear cells, especially at chronic stages of the disease (23). The real progress requires more detailed knowledge of the host immune responses. Mycobacterial species are well adapted to the hostile environment of phagocytic cells, and they use several strategies for survival within the host cells that are not seen in other bacteria. Our understanding of the mechanisms of interaction between mycobacteria and host cells, and of the consequent changes that are induced by mycobacteria in the host signaling machinery, is still incomplete.

T-cell activation and proliferation require the binding of the TCR complex and CD4 or CD8 (cluster of differentiation

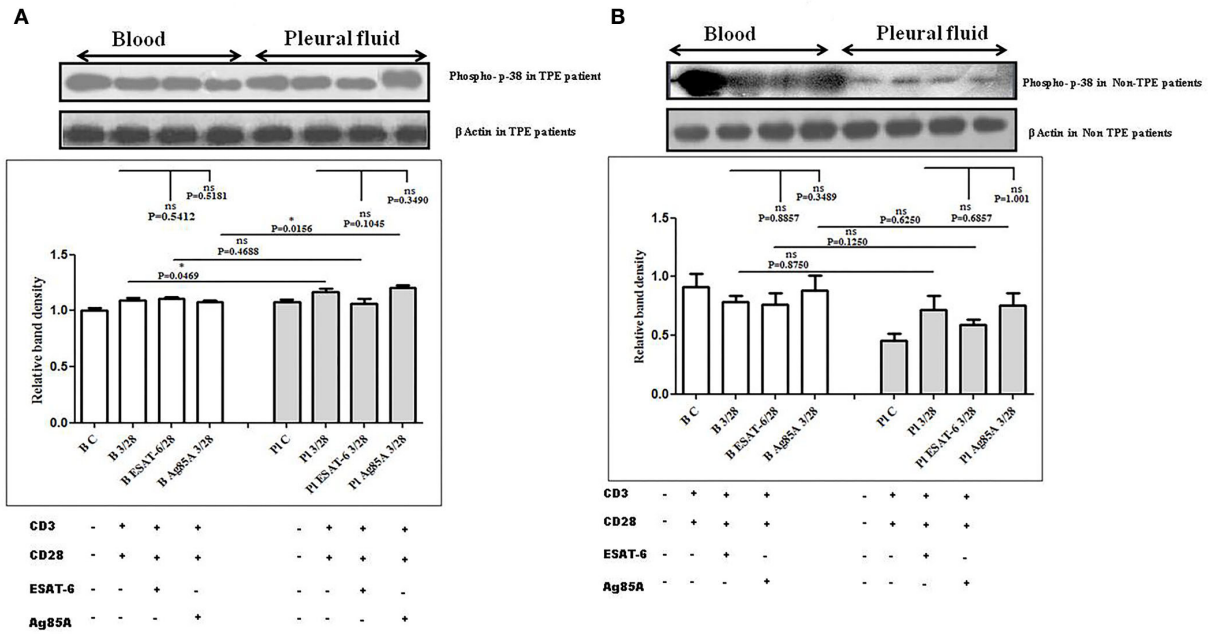


FIGURE 5
TCR/CD28 (T-cell receptor/cluster of differentiation 28)-induced phosphorylation of p38 before and after *M. tuberculosis* antigen stimulation in blood and pleural fluid of patients with tuberculous pleural effusion (TPE) **(A,B)** non-TPE patients. Peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) from blood and pleural fluid were activated with CD3 (cluster of differentiation 3) with CD28 antibodies after pretreatment with *M. tuberculosis* antigens, and few cells were left unstimulated and untreated with antibodies as a negative control, and few cells were only activated without any antigen stimulation, and western blotting was done as mentioned in materials and methods. β-Actin antibody was used to conform to equal loading. **(A)** Densitometric analysis of phosphorylation of p38 in blood and pleural fluid of patients with TPE. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated p38 and β-Actin is shown, where Lane 1 is showing Control in the blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. **(B)** Densitometric analysis of phosphorylation of p38 in blood and pleural fluid of non-TPE patients. Relative band intensity values are expressed as mean±SEM in bar diagrams. A representative blot of one experiment with phosphorylated p38 and β-Actin is shown, where Lane 1 is showing Control in the blood, Lane 2 is showing anti-CD3 + anti-CD28 activated cells in the blood, Lane 3 is showing anti-CD3 + anti-CD28 activated cells of blood with pretreatment with ESAT-6, Lane 4 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A, Lane 5 is showing Control in pleural fluid, Lane 6 is showing anti-CD3 + anti-CD28 activated cells in pleural fluid, Lane 7 is showing anti-CD3 + anti-CD28 activated cells of pleural fluid with pretreatment with ESAT-6, and Lane 8 is showing anti-CD3 + anti-CD28 activated cells of blood pretreated with Ag85A. Densitometric analysis was done and the ratios of phosphorylated p38 to β-Actin protein expression were expressed as arbitrary units. Statistical significance was determined using Mann–Whitney $*P < 0.05$.

8) receptor to specific major histocompatibility complex (MHC) bound antigens. Co-stimulatory signals delivered by the interaction of T-cell membrane molecules and antigen-presenting cells (APCs) also play an important role in the complete activation of a T cell. After the activation, the intracellular calcium level rises and cytoplasmic kinases also get activated, which further leads to the translocation of nuclear transcription factors. The Lck binding acts as a docking site for ZAP-70, which converts the TCR to an active protein tyrosine kinase that is able to phosphorylate, leading to the generation of downstream signals, and the resting T cell enters the cell cycle, inducing various genes to produce cytokines and further go for differentiation and apoptosis (24). Any impairment in the T-cell signaling cascade leads to defects in cellular responses. There are many stages in the T-cell activation pathway, which have not been thoroughly explored in the case of TB. The effect of *M. tuberculosis* infection leads to a modulation in the T- cell activation pathway, which should be investigated to understand the pathogenesis of *M. tuberculosis* completely.

We proposed that, to explore pathogenesis of TB with the aim to develop new strategies for prevention and treatment, host–pathogen interactions should be studied at the local site of *M. tuberculosis* infection. TPE is of particular immunological interest and the study of T-cell activation mechanism in TPE provides an opportunity to evaluate a component of the human immune response at the infection site that is probably involved in mediating the containment of *M. tuberculosis in vivo*. The signaling pathways triggered by *M. tuberculosis* in human T cells

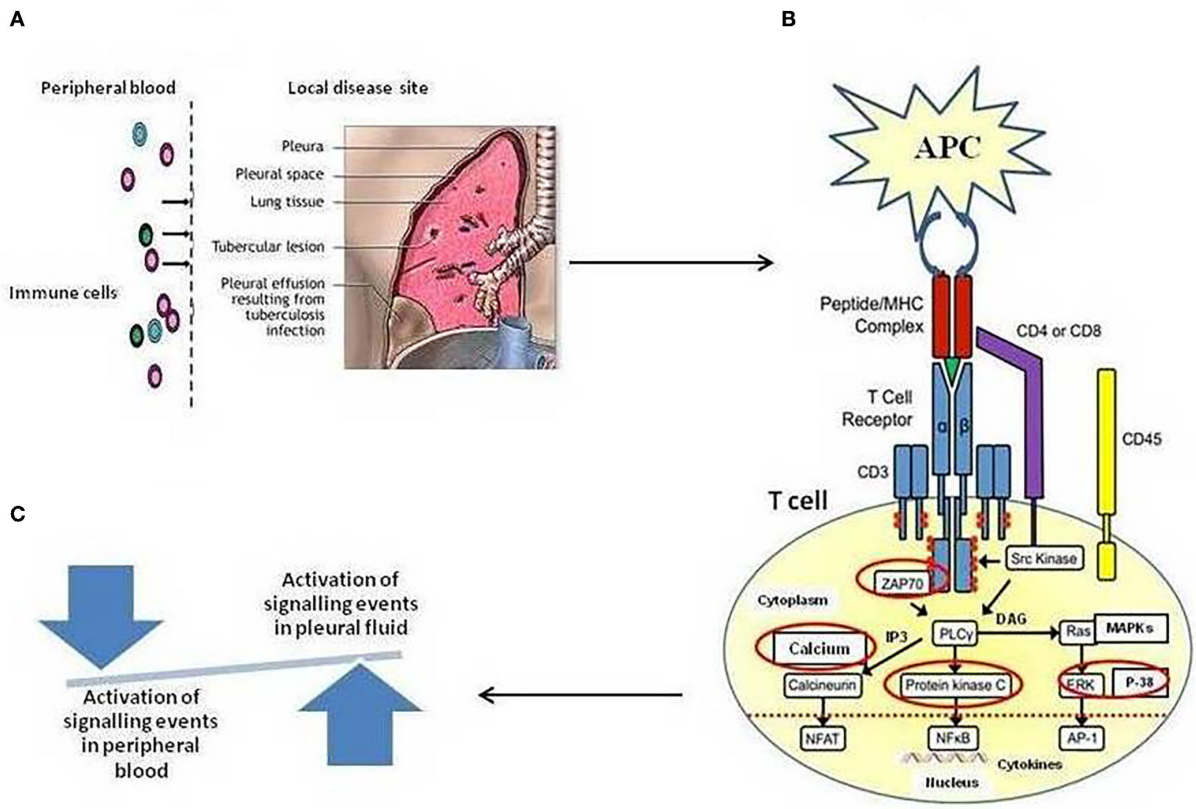


FIGURE 6
**(A)** Schematic illustration of Compartmentalization of different immune cell subsets at the local site/tuberculous pleural effusion (TPE) of *Mycobacterium tuberculosis* infection. High frequencies of *in vivo*-activated *M. tuberculosis*-specific T cells at local disease site as compared to peripheral blood, showing a snapshot of a specific temporal window of tuberculosis disease **(B)**. Regulation of T-cell receptor (TCR)-mediated T-cell activation mechanism. The figure depicts the activation of various enzymes and adaptor molecules upon engagement of TCR with the major histocompatibility complex (MHC) antigenic peptide complex. The red-circled molecules are the molecules whose activation is being studied. **(C)** A shift in the local immune response not only tips the balance toward suboptimal immunity and impaired control of tuberculosis disease but also results in excessive cellular immunity and tissue destruction.

have not yet been studied in a relevant physiological system. The presence of mycobacterial antigens in the pleural space elicits an intense cellular immune response, resulting in lymphocyte-predominant exudative effusions (3, 25) making this biological sample a physiologically relevant model of human tuberculosis infection. We used the pleural fluid of the patient with TPE as a sample from the local disease site and a blood sample as the sample from systemic circulation.

The signaling pathways triggered by *M. tuberculosis* in human T cells have not yet been studied in a relevant physiological system like TPE. One previous study showed that, among pleural fluid lymphocytes, natural killer (NK) cells are a major source of IFN-γ production in a mechanism enhanced by IL-12, dependent on calcineurin, p38, and Erk1/2 pathways, and these cells can directly recognize *M. tuberculosis* antigens (17). One more study by Chen et al. showed that TLR2 ligand activity was also significantly higher in the tuberculous pleural fluid than in the serum. They also observed that *M. tuberculosis* TLR2 ligands, 19-kDa lipoprotein, and live BCG all modulated cytokine production by CD4+ T cells isolated from pleural fluid and activated with anti-CD3 and anti-CD28 (18). However, no study is available for TCR-mediated modulation in T-cell activation in the pleural fluid and the peripheral blood of patients with TPE.

Herein, we employed TCR-based approaches for studying events pertaining to T-cell activation pathways in cells of blood and tuberculous pleural fluid among patients with TPE. In the present study, we hypothesized that the T cells in tuberculous pleural effusion can directly recognize *M. tuberculosis* antigens and can be activated more rapidly in comparison to the cells from peripheral blood in a mechanism dependent on TCR-mediated activation pathway involving TCR-induced intracellular $Ca^{+2}$ mobilization, ZAP-70 activation, PKC-θ activation, and MAPKs' activation that regulates the transcription of IFN-γ and IL-2. As shown in Figure 6, a pictorial representation of the hypothesis. Host

defense against TB infection involves T-lymphocyte-mediated cellular immune responses, and to understand TB pathogenesis, signaling pathways induced by mycobacteria have long been a subject of interest.

The intracellular $Ca^{2+}$ signaling is critical to T-cell activation as a means of rapidly activating and integrating numerous signaling pathways to generate widespread changes in T-cell gene expression and function. $Ca^{2+}$ is a second messenger that functions by binding to and altering the function of key proteins leading to pleiotropic changes in cell function. Binding of antigen to the TCR triggers the mobilization of $Ca^{2+}$ and is needed for effective T-cell activation, anergy, gene expression, motility, synapse formation, cytotoxicity, development, and differentiation of T cells (26). ZAP-70 is a cytoplasmic protein tyrosine kinase that plays a critical role in the events involved in initiating T-cell responses by the antigen receptor. Protein kinase C theta (PKC-θ) is a key kinase in mediating TCR signals. PKC-θ activated by TCR engagement translocates to immunological synapses (ISs) and regulates the activation of transcriptional factors NF-κB, AP-1, and NFAT. These transcription factors then activate target genes such as IL-2 and IFN-γ. Activation of mitogen-activated protein kinases (MAPKs) further downstream to calcium/PKC is an important event for both cytokine production and cell activation. Serine/threonine protein kinases-MAPKs comprise a family of protein kinases, including extracellular signal-regulated kinases 1 and 2 (Erk1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK), which have been implicated as important cellular signaling molecules activated by mycobacteria (26). CD4+ T cells play a central role in the containment of *M. tuberculosis* infection by secreting IFN-γ and IL-2 (27). The modulations in the TCR-mediated cell signaling mechanism are not completely elucidated to date. Although very few reports of alterations in signaling molecules are available so far, this study used Jurkat T cells, peripheral blood either from humans or from mice (10–12, 15, 16).

In the present study, significantly higher CD3-induced $Ca^{2+}$ levels were observed in pleural fluid as compared to blood and we also noted modulation in the $Ca^{2+}$ level after *M. tuberculosis* antigen stimulation in blood and pleural fluid of patients with TPE. In a previous study, we observed that ESAT-6 significantly downregulated intracellular $Ca^{2+}$ in the blood of pulmonary TB patients (15). We also noted the same observation in this study and our finding showed that ESAT-6 reduced intracellular $Ca^{2+}$ in blood and pleural fluid of TPE patients. Direct inhibitory mechanisms of T-cell activation has until now not been extensively studied. It was previously observed that T cells from human TB patients had decreased expression of CD3-ζ, a key signaling domain of the TCR/CD3 complex (8). Mahon et al. found that *M. tuberculosis* bacilli directly inhibited CD4+ T-cell activation. Mahon et al. (10) reported that *M. tuberculosis* cell wall glycolipids directly inhibit polyclonal murine CD4+ T-cell activation by blocking ZAP-70 phosphorylation and later they extended their study by reporting that ManLAM induced inhibition of TCR signaling by interfering with ZAP-70, Lck, and LAT phosphorylation in antigen-specific murine CD4+ T cells and primary human T cells (11). In the present study, we studied ZAP-70 activation in blood and pleural fluid of anti-CD3 and CD28-activated cells after being pretreated with *M. tuberculosis* antigen. We observed the increased activation of ZAP-70 in pleural fluid, as compared to blood in TPE patients. We observed significantly higher levels of phosphorylation of ZAP-70 in pleural fluid, as compared to blood samples of TPE patients. After stimulation with *M. tuberculosis* antigens, altered activation was observed both in blood and pleural fluid, whereas the significantly increased level of phosphorylated ZAP-70 was observed only in the blood after Ag85A stimulation. On the other hand, lesser phosphorylation of ZAP-70 was observed in non-TPE patients but not significantly. The ZAP-70 activation regulates further activation of other downstream signaling molecules, so we further studied the activation of PKC-θ. PKC-θ is the first PKC family member described to be recruited to the immunological synapse (IS) (28). It plays an integral role in activating a range of signaling cascades that ultimately result in a transcriptional network in T cells. PKC includes a large family of homologous serine/threonine protein kinases that are widely conserved in eukaryotes (29). We observed significantly higher levels of phosphorylated PKC θ in pleural fluid, as compared to blood samples of TPE patients. After stimulation with *M. tuberculosis* antigens, significantly altered activation was observed in blood and pleural fluid both. Higher activation of PKC θ was also observed in non-TPE patients but not significantly. We also observed the activation of MAPKs and higher activation of Erk1/2 and p38 in pleural fluid as compared to blood samples and alteration in the activation of these MAK molecules was also noted after stimulation with ESAT-6 and Ag85A. We did not find any significant change in the activation of MAPKs in blood and pleural fluid samples of non-TPE patients. The earlier studies of the T-cell activation mechanism in TB have investigated various events of T-cell signaling. Wang et al. showed that the potent T-cell antigen ESAT-6 can directly suppress IFN-γ production in CD4+ T cells (9), Palma-Nicholas (12) reported T-cell downmodulation of the MAPK–ERK1/2 pathway in total spleen cells from naive BALB/c mice by the cell-surface lipid di-O-acyl-trehalose (DAT). The regulation of IFN-γ production by the ERK and p38 MAPK signaling pathway, through SLAM co-stimulation, had been studied in TB (13). Inhibition of IFN-γ production through the p38 MAPK pathway by ESAT-6 was reported in T cells from healthy individuals (14). The effect of the secretory protein ESAT-6 of *M. tuberculosis* on the modulation of macrophage signaling pathways was also studied earlier (19). Our previous study showed that the phosphorylation of MAPKs–Erk1/2 and p38 was curtailed by *M. tuberculosis* antigens in patients with TB, whereas in PPD+ve healthy individuals only Erk1/2 phosphorylation was inhibited, the

inhibitory effect of secretory antigens of *M. tuberculosis* on the modulation of T-cell signaling pathways was also observed in this study (15).

*M. tuberculosis* is capable of establishing infection in the host by altering the different cell machineries. It modulates the cell processes according to itself for the survival inside the host. The modulation in T-cell signaling is not explored enough, but *M. tuberculosis* is strongly thought of to cause changes in the signal transduction pathway. This study explored the mechanisms in *M. tuberculosis* infection, which are used to destabilize the host's T-cell response, besides what are already established. Although previous studies of cell signaling pathways in TB have contributed to marked advances in our knowledge about their role in host protective immune responses, a number of critical questions are still unstated. Research into the development of TB vaccines and immunodiagnostics has focused on the proteins released by *M. tuberculosis*, because these antigens are thought to induce protective cell-mediated immunity and immune responses of diagnostic value. Ag85A and ESAT-6 are widely studied for their potential to trigger effective host immune responses against TB, but our knowledge regarding their role in the T-cell signaling mechanisms underlying proinflammatory cytokine secretion by T cells is not well established. Presently, follow-up studies are also needed to determine whether such alterations in the activation mechanism revert after successful treatment and also whether these can be modulated by immunotherapy. It is also of interest to know the variations in different forms of TB and to determine the relationship with the activation of different cytokines. These observations of molecular and functional characteristics in TB may provide new tools to analyze and monitor patients, to reveal how these characteristics affect the development of immune dysfunction, and to study new pathways to block suppressor mechanisms. This endeavor enhances our knowledge of disease pathogenesis, contributing to a better understanding of the immune response to *M. tuberculosis*. It provides insight into the specific immune responses to *M. tuberculosis* at the site of infection, which may differ from those in blood. Hence, a study of these T-cell activation pathways in pleural fluid and blood from the same patient with TPE would be able to reveal the role of these events in the dominance of the TH1 profile in TPE. The understanding of human local immune responses to *M. tuberculosis* may facilitate the evaluation of the efficacy of new anti-TB vaccines. Further investigations may unravel the critical targets for therapeutic intervention in chronic inflammatory diseases.

## Data availability statement

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

## Ethics statement

The studies involving human participants were reviewed and approved by National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra Human Ethics Committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## Author contributions

BS conceived, designed the study, performed the experiments, analyzed, interpreted the data, and drafted the manuscript. DR and SU performed the experiments. BJ contributed to designing the study. DC contributed reagents and materials. SK carried out the clinical evaluation of patients with and without tuberculous pleural effusion. BJ, DC, and SK critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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## 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

# References


1. World Health Organization (WHO) (2021). *Global Tuberculosis Report*. Geneva: WHO/HTM/TB.

2. Light RW. Update on tuberculous pleural effusion. *Respirology.* (2010) 15:451–8. doi: 10.1111/j.1440-1843.2010.01723.x

3. Mitra DK, Sharma SK, Dinda AK, Bindra MS, Madan B, Ghosh B, et al. Polarized helper T cells in tubercular pleural effusion: phenotypic identity and selective recruitment. *Eur J Immunol.* (2005) 35:2367–75. doi: 10.1002/eji.200525977

4. Yokobori N, Schierloh P, Geffner L, Balboa L, Romero M, Musella R„ et al. CD3 expression distinguishes two γδ cell receptor subsets with different phenotype and effector function in tuberculous pleurisy. *Clin Exp Immunol.* (2009) 157:385–94. doi: 10.1111/j.1365-2249.2009.03974.x

5. Wu C, Zhou Q, Qin XJ, Qin SM, Shi HZ. CCL22 is involved in the recruitment of CD4+ CD25high T cells into tuberculous pleural effusions. *Respirology.* (2010) 15:522–9. doi: 10.1111/j.1440-1843.2010.01719.x

6. Saha PK, Sharma PK, Singh BK, Verma C. Role of CXCR3+CCR5+Th1 cells in pulmonary tuberculosis patients: At pathogenic site. *Biomed Biotechnol Res J.* (2019) 3:80–6. doi: 10.4103/bbrj.bbrj_78_19

7. Roofchayee ND, Adcock IM, Marjani M, Dezfuli NK, Varahram M, Garssen J, et al. Helper Cell Subsets in the Pleural Fluid of Tuberculous Patients differentiate patients with non-tuberculous Pleural Effusions. *Front Immunol.* (2021) 12:780453. doi: 10.3389/fimmu.2021.780453

8. Seitzer U, Kayser K, Hohn H, Entzian P, Wacker HH, Ploetz S, et al. Reduced T-cell receptor CD3ξ-chain protein and sustained CD3ε expression at the site of mycobacterial infections. *Immunology.* (2001) 104:269–77. doi: 10.1046/j.1365-2567.2001.01323.x

9. Wang X, Barnes PF, Dobos-Elder KM, Townsend JC, Chung YT, Shams H, et al. ESAT-6 inhibits production of IFN-gamma by Mycobacterium tuberculosis-responsive human T cells. *J Immunol.* (2009) 15:3668–77.

10. Mahon RN, Rojas RE, Fulton SA, Franko JL, Harding CV, Boom WH, et al. Mycobacterium tuberculosis cell wall glycolipids directly inhibit CD4+ T cell activation by interfering with proximal T-cell receptor signalling. *Infect Immun.* (2009) 10:4574–83. doi: 10.1128/IAI.00222-09

11. Mahon RN, Sande OJ, Rojas RE, Levine AD, Harding CV, Boom WH, et al. Mycobacterium tuberculosis ManLAM inhibits T-cell-receptor signalling by interference with ZAP-70, Lck and LAT phosphorylation. *Cell Immunol.* (2012) 275:98–105. doi: 10.1016/j.cellimm.2012.02.009

12. Palma-Nicolás JP, Hernández-Pando R, Segura E, Ibarra-Sánchez MJ, Estrada-García I. Mycobacterial di-O-acyl trehalose inhibits Th-1cytokine gene expression in murine cells by down-modulation of MAPK signalling. *Immunobiology.* (2010) 215:143–52. doi: 10.1016/j.imbio.2009.03.010

13. Pasquinelli V, Rovetta AI, Alvarez IB, Jurado JO, Musella RM, et al. Phosphorylation of mitogen-activated protein kinases contributes to interferon γ production in response to mycobacterium tuberculosis. *J Infect Dis.* (2013) 207:340–50. doi: 10.1093/infdis/jis672

14. Peng H, Wang X, Barnes PF, Tang H, Townsend JC, Samten B, et al. The Mycobacterium tuberculosis Early Secreted Antigenic Target of 6 kDa Inhibits T Cell Interferon-γ production through the p38 Mitogen-activated Protein Kinase Pathway. *J Bio Chem.* (2011) 27:24508–18. doi: 10.1074/jbc.M111.234062

15. Sharma B, Upadhyay R, Dua B, Khan NA, Katoch VM, Bajaj B, et al. Mycobacterium tuberculosis secretory proteins downregulate T cell activation by interfering with proximal and downstream T cell signalling events. *BMC Immunol.* (2015) 16:67. doi: 10.1186/s12865-015-0128-6

16. Sharma B, Dua B, Joshi B. Investigating role of Mycobacterium tuberculosis secretory antigens in altering activation of T cell signaling events in Jurkat T cells. *Int J Mycobacteriol.* (2020) 9:405–10.

17. Schierloh, P., Yokobori, N., Aleman M, Landoni V, Geffner L, Musella RM„ et al. (2007). Mycobacterium tuberculosis-Induced gamma interferon production by natural killer cells Requires cross talk with antigen-presenting cells involving Toll-Like Receptors 2 and 4 and the Mannose Receptor in Tuberculous Pleurisy. *Infect Immun* 11, 5325–5337. doi: 10.1128/IAI.00381-07

18. Chen X, Zhang M, Zhu X, Deng Q, Liu H, Larmonier N, et al. Engagement of toll-like receptor 2 on CD4+ T cells facilitates local immune responses in patients with tuberculous pleurisy. *J Infect Dis.* (2009) 200:399–408. doi: 10.1086/600075

19. Ganguly N, Giang PH, Basu SK, Mir FA, Siddiqui I, Sharma P. *Mycobaterium tuberculosis* 6-kDa Early Secreted Antigenic Target (ESAT-6) protein downregulates Lipopolysaccharide induced c-myc expression by modulating the Extracellular Signal Regulated Kinases ½. *BMC Immunol.* (2007) 8:24–35. doi: 10.1186/1471-2172-8-24

20. Light RW, Macgregor MI, Luchsingar PC, Ball WC. Pleural effusions: the diagnostic separation of transudatesand exudates. *Ann Intern Med.* (1972) 77:507–13. doi: 10.7326/0003-4819-77-4-507

21. Grynkiewicz G, Poenie M, Tsien RYA. new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem.* (1985) 260:3440–50. doi: 10.1016/S0021-9258(19)83641-4

22. Joshi B, Kedouchi S, Dagur PK, Hichami A, Sengupta U. On cell signalling mechanism of *Mycobaterium leprae* soluble antigen (MLSA) in Jurkat T-cells. *Mol Cell Biochem.* (2006) 287:157–64. doi: 10.1007/s11010-006-9132-8

23. Delgado JC, Tsai EY, Thim S, Baena A, Boussiotis VA, Reynes JM, et al. Antigen- specific and persistent tuberculin anergy in a cohort of pulmonary tuberculosis patients from rural Cambodia. *Proc Natl Acad Sci.* (2002) 99:7576–81. doi: 10.1073/pnas.062056099

24. Smith-Garvin JE, Koretzky GA, Jordan MST. Cell activation. *Annu Rev Immunol.* (2009) 27:591–519. doi: 10.1146/annurev.immunol.021908.132706

25. Nemeth J, Winkler HM, Zwick RH, Rumetshofer R, Schenk P, Burghuber OC, et al. Recruitment of Mycobacterium tuberculosis specific CD4+ T cells to the site of infection for diagnosis of active tuberculosis. *J Intern Med.* (2009) 265:163–8. doi: 10.1111/j.1365-2796.2008.02012.x

26. Hwang JR, Byeon Y, Kim D, et al. Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. *Exp Mol Med.* (2020) 52:750–61. doi: 10.1038/s12276-020-0435-8

27. Millington KA, Innes JA, Hackforth S, Hinks TS, Deeks JJ, Dosanjh DP, et al. Dynamic relationship between IFN-gamma and IL-2 profile of Mycobacterium tuberculosis-specific T cells and antigen load. *J Immunol.* (2007) 178:5217–26. doi: 10.4049/jimmunol.178.8.5217

28. Arendt CW, Albrecht B, Soos TJ, Littman DR. Protein kinase C-theta: signaling from the center of the T-cell synapse. *Curr Opin Immunol.* (2002) 14:323–30. doi: 10.1016/S0952-7915(02)00346-1

29. Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J.* (1998) 332:281–92. doi: 10.1042/bj3320281

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# Transmission of tuberculosis in an incarcerated population during the subclinical period: A cross-sectional study in Qingdao, China

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**Objectives:** As a closed gathering place, prison is the cradle of tuberculosis (TB) outbreak. Therefore, the analysis of the prevalence rate and risk factors of latent tuberculosis infection (LTBI) in prison will be a necessary measure to intervene in the spread of tuberculosis.

**Methods:** In this study, we consecutively recruited 506 adult prisoners in Qingdao to carry out this cross-sectional study. TB and LTBI were screened by IGRA, X-ray, X-pert, sputum smear and culture.

**Results:** A total of 17 TB, 101 LTBI and 388 HC were identified, with an infection rate of 23.32% (118/506) and a TB incidence rate of 3282/100,000 population. Age, malnutrition and inmates living with TB prisoners were risk factors for LTBI. Additionally, most TB cases (70.59%, 12/17) were subclinical tuberculosis (STB), contributing significantly to TB transmission.

**Conclusion:** Our results demonstrate that the transmission efficiency of asymptomatic patients is not essentially different from that of symptomatic patients, indicating that TB transmission occurs during the subclinical period. Our findings highlight the need to strengthen active case-finding strategies to increase TB case detection in this population.

KEYWORDS

**tuberculosis, incarcerated population, transmission, subclinical period, cross-sectional study**

## Introduction

*Mycobacterium tuberculosis* (MTB) is one of the leading causes of death due to a single infectious agent, accounting for an estimated 1.6 million death in 2021 (1). As an airborne-transmitted disease, MTB is majorly transmitted by inhaling droplet nuclei carrying viable bacilli. Although early clearance of MTB by innate immunity in the majority of cases, infection occurs in a substantial proportion of susceptible individuals, either leading to a dormant state of the bacilli or developing active tuberculosis (ATB) disease (2). It is estimated that approximately one-quarter of the world's population has been infected with tubercle bacilli (1). The LTBI is the major contributor to the pool of active tuberculosis cases, thereby constituting a pivotal barrier to TB elimination (3).

Prisons have consistently been recognized as high-risk environments for TB transmission due to overcrowded locations with poor ventilation (4). The estimated prevalence of infection

in prisons is reported to be much higher than that in the general population (5). In a survey on TB control in Europe, it was estimated that European prions counted on average 17 times higher incidence than the general population (6). This is also true for low-income, high-burden countries, with more than 40 times higher TB prevalence rates in prions than in the general population (5). Given that infectious TB in prison inmates can be transmitted to the community after prisoners are released, prions have emerged as a threat to TB control; prisoners should be chosen as a priority population for intervention (7).

China has the second-highest TB burden in the world. Notably, the prevalence of bacteriologically positive TB exhibits significant geographic diversity across this country, varying from high in western China (212/100,000 population) to fewer than 66/100,000 population in eastern China (8). Qingdao is an eastern coastal city in China, with an incidence rate of 26.82/100,000 population, which is remarkably lower than the national average level (9). Despite significant achievements during past decades, prisons are neglected reservoirs of TB control in this region, majorly attributed to inadequate provision of health services (10). More importantly, international guidelines and national tuberculosis programmes focus on case detection and preventive interventions for certain high-risk groups (such as people living with HIV and family contacts with diagnosed TB patients), but relatively little attention is paid to incarcerated populations, thus hampering the formulation and implementation of effective policies for these individuals living in the risk environment (11). To address this concern, we undertook a cross-sectional study to determine the prevalence of active TB and risk factors of LTBI in a prison population.

# Methods

## Study design and participants

In 2022, we conducted cross-sectional screening for tuberculosis in prison in Qingdao, China. HIV/AIDS, TB and LTBI screening were carried out at the beginning of everyone's admission; HIV-positive, TB and LTBI prisoners were held incommunicado in different wards and excluded from this study. Finally, a total of 506 prisoners aged ≥18 were included. Demographic information was collected through a questionnaire, and preliminary TB screening was carried out. Further laboratory tests will be carried out on the screened TB patients (Figure 1).

## Ethical approval

This study was approved by the Ethics Commission of the Municipal Center of Disease Control and Prevention of Qingdao, and written informed consent was obtained from each participant before enrollment.

## TB screening

We collected the clinical symptoms of all participants. All participants underwent chest radiographs to diagnose tuberculosis in accordance with international standards and guidelines (8). Professional medical workers used BD Vacutainer Venous Blood Collection Tubes (Becton Dickinson, Sunnyvale, CA, USA) to collect venous blood while checked Mycobacterium bovis BCG vaccine scars.

The collected blood was centrifuged within 2 h at 2,500 rpm for 5 min. Then collect the upper serum, and next detected for IGRA through QuantiFERON-TB Gold Kit (QIAGEN, https://www.qiagen.com). Suspected TB (IGRA seropositive with X-ray positive) sputum samples were collected for smear, bacterial culture and X-pert detection ① Sputum smear was examined under a microscope after acid-fast staining. ≥1 acid-fast bacilli/100 visual fields were found in both examinations, which was defined as positive. ② The bacteria are cultured on Löwenstein–Jensen (L-J) medium, incubated at 37°C with daily examinations for 8 weeks until the sample isolates at most minuscule one *Mycobacterium tuberculosis* complex colony, which is defined as positive. ③ After adding the corresponding reagent of GeneXpert MTB/RIF (Xpert, Cepheid, Sunnyvale, CA, USA), the sample was processed according to the manufacturer's instructions, and finally put into the machine to read the results (12).

## Definitions

We defined the population of IGRA (–, negative) with X-ray (–) as Health control (HC); IGRA (–) with X-ray (+, positive) participants as LTBI. And performed a series of laboratory tests, including sputum smear, culture, and X-pert, on IGRA (+) with X-ray (+) participants. People who were positive in any of the laboratory tests were immediately defined as having TB, and the chest X-ray of all negative samples were re-read by medical imaging expert panel to determine whether it was TB (8). Patients with negative chest X-rays after re-read were included in the LTBI cohort. The positive population and the previous TB were defined as ATB and STB (Table 3), according to clinical symptoms (cough, expectoration, hemoptysis, etc.,).

## Statistical analysis

This study, the $\chi^2$ test was used to analyze the transition relationship between LTBI and HC. The risk factors from HC to LTBI were counted by multivariate logistic regression, and the corrected OR and 95% CI were calculated. The difference was considered statistically significant when $P < 0.05$. All calculations were performed using SPSS 22.0 software for Windows (SPSS Inc., Chicago, IL, United States).

# Result

## Study population

A total of 506 participants included 17 TB, 101 LTBI and 388 HC of which 17 TB were screened from 25 IGRA seropositive with X-ray positive participants using smear, culture, GeneXpert

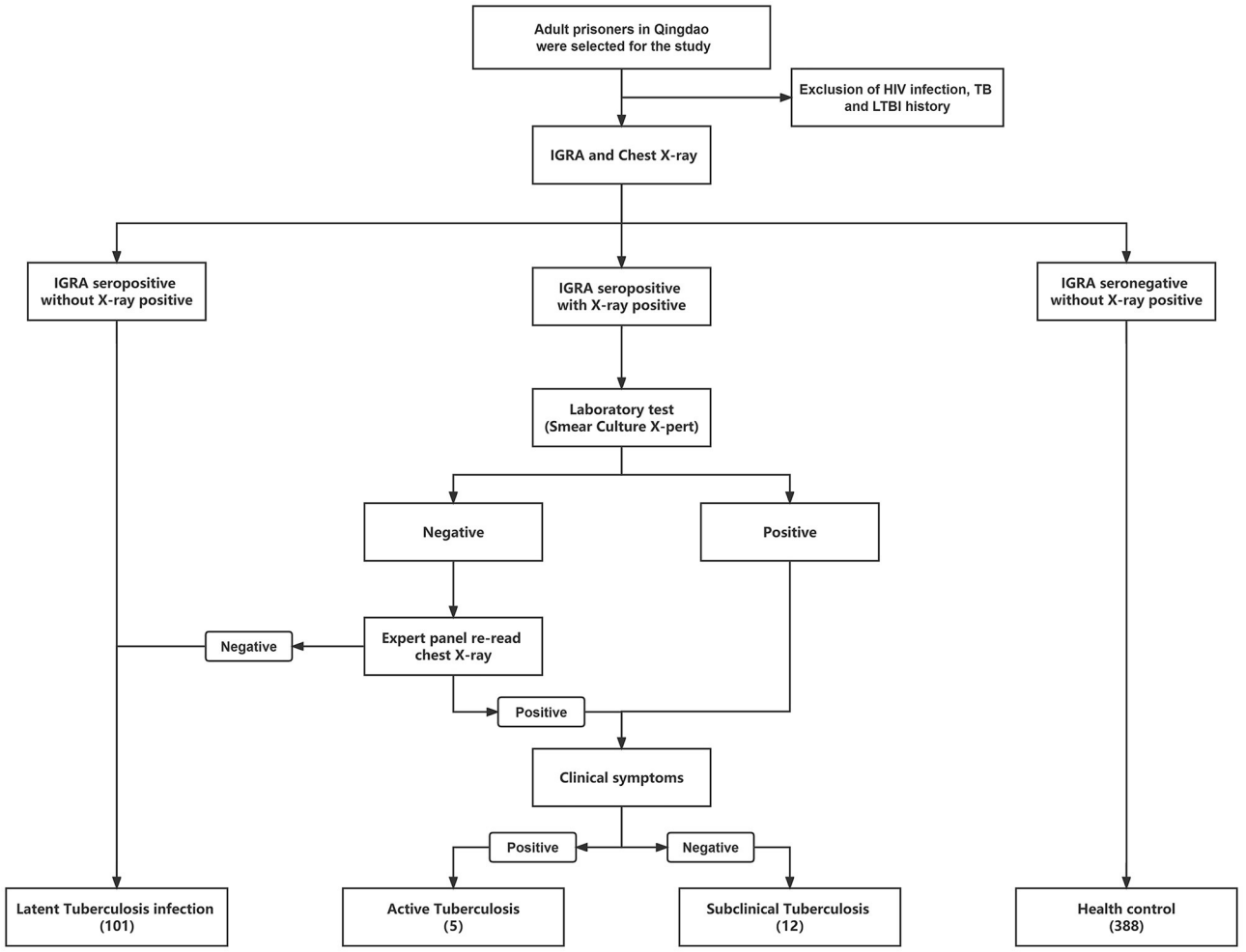


FIGURE 1
The workflow of this study is to classify different groups of prisoners with different health status. HIV, human immunodeficiency virus; TB, tuberculosis; LTBI, Latent Tuberculosis infection.

and rechecked chest X-ray, and a pulmonary TB incidence of 3,373 cases/100,000 persons (Table 1). Almost all of them were male (504/506, 99.60%), and only two females were LTBI and HC, respectively. The average age of the participants was 40 years old (range 18–78). Junior high school education accounted for 40.12% (203/506), followed by 38.34% (194/506) with high school education or above, and 21.54% (109/506) with primary school education. Most of the participants were non-local population, and only 15.42% (78/506) of the participants were local registered residents. 84.58% (428/506) of the participants were employed before being jailed. As of the time of information collection, the duration of incarceration was from 1 to 214 months (median term 2 months), and nearly half of the participants exceeded 2 months in jail (262/506, 51.78%).

According to the WHO standard, 13.04% (66/506) of people were malnutrition (BMI <18.5) (13, 14). 15.61% (79/506) of the participants had a history of close contact with TB (family member with a TB history and history of close contact with a TB patient). Surprisingly, only 10.67% (54/506) of people with the BCG vaccine (*Mycobacterium bovis* BCG scar). 19.37% (98/506) of participants had complications. After screening, 30.63% (155/506) of them were found to share a room with TB inmates.

## Characteristics between LTBI and HC

Preliminary tests showed that 19.96% (101/506) of the prisoners changed from HC to LTBI during their incarceration. Therefore, we compared the characteristic frequencies of LTBI and HC (Table 2). In terms of age, most people with LTBI were ≥45 years old (45.54%, 46/101). The IGRA-seropositive was proportional to age, and the risk of becoming LTBI increases ($P < 0.05$). In addition, malnourished prisoners were at greater risk and had a higher incidence ($P = 0.045$, 95% CI: 1.024–7.091). Remarkably, there was a significant relationship between the high incidence of LTBI and the cohabitation of the TB population we had identified ($P < 0.001$, up to 50–60 people per cell), and it was an inextricable risk factor ($P < 0.001$, 95% CI: 0.010–0.042).

In addition, the sentence length and place of origin were not related to the incidence of LTBI in this study ($P = 0.652$, 0.107). Participants with a history of close contact with TB had a higher incidence ($P = 0.003$), but did not affect the conversion of HC to LTBI ($P = 0.892$, 95% CI: 0.467–1.939). However, the incidence of LTBI was not statistically significant in terms of education level, pre-prison work, excess nutrition, complications, and BCG vaccination ($P > 0.05$).

TABLE 1 Comparison of characteristics in prisoners, Qingdao, China.

| Characteristics | Total $n = 506$ (100.0%) | TB $n = 17$ (3.36%) | LTBI $n = 101$ (19.96%) | HC $n = 388$ (76.68%) | *P*-value by $\chi^2$ test |
|---|---|---|---|---|---|
| **Sex** | | | | | |
| Male | 504 (99.60%) | 17 (100.00%) | 100 (99.01%) | 387 (99.74%) | 0.304 |
| Female | 2 (0.40%) | 0 (0.00%) | 1 (0.99%) | 1 (0.26%) | |
| **Age (years)** | | | | | |
| <25 | 39 (7.71%) | 4 (23.53%) | 4 (3.96%) | 31 (7.99%) | 0.049 |
| 25–34 | 154 (30.43%) | 6 (35.29%) | 26 (25.74%) | 122 (31.44%) | |
| 35–44 | 141 (27.87%) | 3 (17.65%) | 25 (24.75%) | 113 (29.12%) | |
| ≥45 | 172 (33.99%) | 4 (23.53%) | 46 (45.54%) | 122 (31.44%) | |
| **Education level** | | | | | |
| Primary school | 109 (22.33%) | 4 (23.53%) | 28 (27.72%) | 77 (19.85%) | 0.229 |
| Middle school | 203 (41.11%) | 4 (23.53%) | 38 (37.62%) | 161 (41.49%) | |
| High school | 194 (38.74%) | 9 (52.94%) | 35 (34.65%) | 150 (38.66%) | |
| **Residence** | | | | | |
| Local | 364 (71.94%) | 13 (76.47%) | 66 (65.35%) | 285 (73.45%) | 0.107 |
| Migrant | 142 (28.06%) | 4 (23.53%) | 35 (34.65%) | 103 (26.55%) | |
| **Employment** | | | | | |
| No | 78 (15.42%) | 3 (17.65%) | 21 (20.79%) | 54 (13.92%) | 0.088 |
| Yes | 428 (84.58%) | 14 (82.35%) | 80 (79.21%) | 334 (86.08%) | |
| **Malnutrition (BMI<18.5)** | | | | | |
| No | 440 (86.96%) | 14 (82.35%) | 94 (93.07%) | 332 (85.57%) | 0.045 |
| Yes | 66 (13.04%) | 3 (17.65%) | 7 (6.93%) | 56 (14.43%) | |
| **Contact history** | | | | | |
| No | 431 (85.18%) | 13 (76.47%) | 77 (76.24%) | 341 (87.89%) | 0.003 |
| Yes | 75 (14.82%) | 4 (23.53%) | 24 (23.76%) | 47 (12.11%) | |
| **Concurrent condition** | | | | | |
| No | 408 (80.63%) | 14 (82.35%) | 76 (75.25%) | 318 (81.96%) | 0.430 |
| Yes | 98 (19.37%) | 3 (17.65%) | 25 (24.75%) | 70 (18.04%) | |
| ***Mycobacterium bovis* BCG scar** | | | | | |
| No | 452 (89.33%) | 15 (88.24%) | 89 (88.12%) | 348 (89.69%) | 0.648 |
| Yes | 54 (10.67%) | 2 (11.76%) | 12 (11.88%) | 40 (10.31%) | |
| **Current duration of incarceration, month** | | | | | |
| <2 | 244 (48.22%) | 2 (11.76%) | 52 (51.49%) | 190 (48.97%) | 0.652 |
| ≥2 | 262 (51.78%) | 15 (88.24%) | 49 (48.51%) | 198 (51.03%) | |
| **Living in the same room as TB inmates** | | | | | |
| No | 351 (69.37%) | 0 (0.00%) | 18 (17.82%) | 333 (85.82%) | <0.001 |
| Yes | 155 (30.63%) | 17 (100.00%) | 83 (82.18%) | 55 (14.18%) | |

TB, tuberculosis; LTBI, Latent tuberculosis infection; HC, Health control; BCG, Bacillus Calmette Guerin; BMI, body mass index; OR, odds ratio; CI, confidence interval.

## Subclinical tuberculosis transmission ability

We further improved the demographic, clinical symptoms and laboratory testing information of 17 TB (Table 3). It was found that this TB was mainly concentrated in four cells. And 70.59% (12/17) of TB were in the subclinical tuberculosis (STB) state; there were no clinical symptoms of tuberculosis such as cough and expectoration. Even half of the cells had only STB prisoners, but it still caused HC in the same room to be infected with LTBI (Figure 2). This indicated that STB still has a solid ability to spread tuberculosis.

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TABLE 2 Factors associated with LTBI among prisoners, Qingdao, China.

| Factor | OR (95% CI) | *P*-value by multivariable regression |
|---|---|---|
| Age (years) | | |
| <25 | Referent | 0.044 |
| 25–34 | 4.809 (1.309–17.665) | |
| 35–44 | 1.759 (0.869–3.563) | |
| ≥45 | 2.110 (1.031–4.318) | |
| Malnutrition (BMI<18.5) | | |
| No | Referent | 0.045 |
| Yes | 2.694 (1.024–7.091) | |
| Contact history | | |
| No | Referent | 0.892 |
| Yes | 0.952 (0.467–1.939) | |
| Living in the same room as TB inmates | | |
| No | Referent | <0.001 |
| Yes | 0.952 (0.467–1.939) | |

TB, tuberculosis; LTBI, Latent tuberculosis infection; OR, odds ratio; CI, confidence interval.

## Discussion

Prisons are regarded as a hotspot for TB disease and transmission, emphasizing that prevention strategy must focus on this hotspot to achieve the global target of ending TB (11). In the present study, we confirm the prediction of a significant burden of TB in an incarcerated population in Qingdao, where 3.36% of inmates had pulmonary TB. The prevalence of TB found in our study was six times higher than the estimated prevalence for the general population (0.5% in 2010) (8). This prevalence corroborated that in Cameroon (3.3%) (5) and Zambia (4.0%) (15), although it is lower than that in Ethiopia (10.4%) (16), and South Africa (7.5%) (17), and higher than that in Malawi (0.7%) (18). The cause for this discrepancy is majorly due to different sampling strategies, screening methodologies, and case definitions across studies (17). Indeed, several of the studies mentioned above registered only prisoners with typical symptoms and chest radiographs as having smear-positive pulmonary TB, which leads to a possible underestimation of the rate of pulmonary TB. The high prevalence of active TB in incarcerated populations calls for urgent strategies to address this concern, considering that it is not only a risk to other prisoners and prison staff; it also has the potential to put the communities in danger of TB disease after their release from prison.

By employing the active case-finding strategy, 17 cases of active pulmonary TB were detected in our cohort, of which 5 had clinical symptoms suggestive of active TB and 12 were declared free of symptoms, demonstrating that about two-thirds of patients were subclinical diseases. By reviewing data from TB prevalence population surveys, Frascella and colleagues found that the median percentage of subclinical TB cases was 50.4 and 56.4% in African and Asian countries, respectively (19). Existing data are increasingly clear that subclinical TB accounts for a substantial fraction of prevalent disease in the general population, as well as in the incarcerated population, suggesting the heterogeneity in the natural history of TB. In the subclinical period, the absence of symptoms indicating active TB challenges the passive case-finding strategy for diagnosing TB in most low-income countries, where chest radiography is conducted only for cough of 2 or more weeks' duration, undoubtedly resulting in subclinical TB patients without clinical symptoms are missed. This could be intensified by the fact that prisons are often underfunded and without mandate to provide comprehensive healthcare services (7). Thus, our findings highlight the need to strengthen active case-finding strategies to increase TB case detection in this population.

Our finding of a great proportion of subclinical TB inmates raised the very interesting question of whether TB transmission occurred during the subclinical period. Traditionally, it is well known that TB transmission is caused by the aerosolization of infectious droplet nuclei, which are majorly generated by coughing (20). A previous household study in Uganda demonstrated that cough-expelled aerosols with tubercle bacilli were a much stronger predictor of recent infection (21). However, our results supported the opposite notion, that the transmission efficiency of asymptomatic patients was not essentially different from that of symptomatic patients. One possible explanation for the infectivity of asymptomatic patients is that respiratory droplets can produce during routine activities such as talking and breathing (22). In line with our hypothesis, multiple recent studies by analyzing MTB DNA from face masks worn by individuals with active TB found no association between the quantity of exhaled MTB and cough frequency or sputum positivity (23). Despite exhibiting no disease symptoms, people can still have varying bacterial loads contributing to further transmission. Similar results were reported in COVID-19-infected patients that symptom expression was not always positively correlated with virus load. This phenomenon may reflect that clinical symptoms are determined by an intricate balance between the immune system of the host and the virulence of tubercle bacilli. It also underlines the fact that the majority of individuals are highly susceptible to TB because of the limited protective efficacy of the BCG vaccine; thus infection with MTB can occur even after exposure to small numbers of viable bacilli (23).

We also explored potential risk factors for TB infection in an incarcerated population. As expected, the inmates exposed to hospital roommates with active TB disease were the most significant factor for TB infection. This could be explained by the nature of the cells shared by the inmates, including high-density crowds and poor ventilation. In addition, we found a trend toward low BMI being associated with an increased risk of TB infection. An observational study concerning the influence of BMI on immune response indicated that low BMI was related to the impairment of cell-mediated immunity functions (24), which is essential for MTB clearance in host. Moreover, Han and colleagues found that white adipose tissue provided a niche for the long-term maintenance of pathogen-specific memory T cells (25). Therefore, impaired T cell immunity is a potential major contributor to an increased risk of TB infection in individuals with low BMI.

This study had several limitations. First, the major limitation of the present study was the small number of inmates, which may limit the overall significance of our conclusion. Second, due to the cross-sectional nature of this study, we did not conduct intensive

TABLE 3 Information of prisoners with tuberculosis.

| Patient ID | Room number | Demographic information | | | | Clinical symptoms | | | | | | | | Laboratory testing | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Age | Sex | Employment | Resistance | Cough | Cough up phlegm | Hemoptysis | Chest pain | Night sweat | Weakness | Smoking history | History of tuberculosis | Smear | Culture | Xpert |
| 1 | 1 | 41 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ |
| 2 | | 23 | M | ● | ○ | ○ | ○ | ○ | ○ | ○ | ● | ○ | ○ | ○ | ○ | ○ |
| 3 | | 28 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ |
| 4 | | 32 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ● | ○ | ○ | ○ | ○ | ● |
| 5 | 2 | 25 | M | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ● | ● | ○ | ○ | ○ |
| 6 | | 31 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ● |
| 7 | 3 | 60 | M | ● | ○ | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ● |
| 8 | | 33 | M | ● | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 9 | | 18 | M | ● | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ● |
| 10 | | 43 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ● | ○ | ● | ○ | ○ | ○ |
| 11 | | 42 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ | ● | ○ | ○ | ● | ● |
| 12 | 4 | 53 | M | ○ | ● | ○ | ○ | ○ | ○ | ○ | ○ | ● | ○ | ○ | ○ | ● |
| 13 | | 23 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ● | ● |
| 14 | | 21 | M | ○ | ● | ● | ○ | ○ | ○ | ○ | ● | ○ | ○ | ○ | ● | ● |
| 15 | | 56 | M | ● | ● | ● | ○ | ○ | ○ | ○ | ○ | ● | ● | ○ | ○ | ○ |
| 16 | | 45 | M | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| 17 | | 28 | M | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |

○ Indicate no/negative; ● Indicate yes/positive; M, male.





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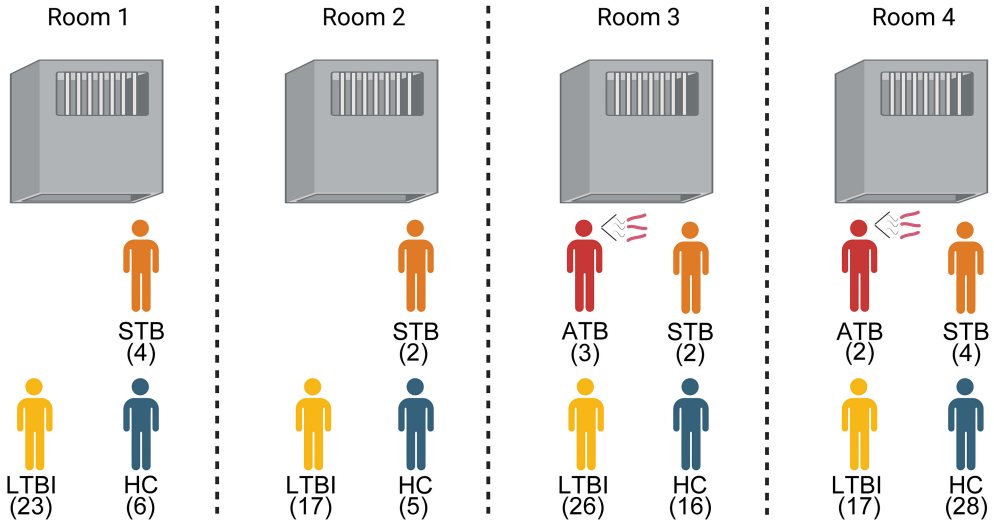


FIGURE 2
Composition of the health status of prisoners living with tuberculosis. ATB, Active tuberculosis; STB, Subclinical tuberculosis; LTBI, Latent Tuberculosis infection; HC, Healthy control.

follow-ups on individuals with evidence of recent infection. Third, a recent modeling study demonstrated that the sensitivity of chest X-rays for asymptomatic individuals might be lower than 88% (23), indicating that the burden of subclinical TB in our cohort may be underestimated. In addition, further laboratory testing of the cases with IGRA or CXR negative cohorts may help to find hidden TB or LTBI. Finally, only a small number of patients had positive cultures, hampering us to perform molecular genotyping analysis, and identifying the timing and directionality of disease transmission.

To conclude, we confirm the prediction of a large burden of TB in an incarcerated population in Qingdao, where 3.3% of inmates had pulmonary TB. About two-thirds of patients are subclinical diseases without symptoms indicating active TB. In addition, our results demonstrate that the transmission efficiency of asymptomatic patients is not essentially different from that of symptomatic patients, indicating that TB transmission occurs during the subclinical period. The inmates exposed to hospital roommates with active TB disease and low BMI are great risk factors for TB infection. Our findings highlight the need to strengthen active case-finding strategies to increase TB case detection in this population.

## Data availability statement

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

## Ethics statement

This study was approved by the Ethics Commission of Municipal Center of Disease Control and Prevention of Qingdao and written informed consent was obtained from each participant prior to enrollment. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

YP and ZW: conceptualization and methodology. ZW, HL, MC, and SS: formal analysis and investigation. XD, SS, MC, and HS: data curation. ZW and HL: writing—original draft preparation. YP and HZ: writing—review and editing. YP: funding acquisition. All authors contributed to the article and approved the submitted version.

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## 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.

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# References


1. Organisation WH. *Global Tuberculosis Report*. Geneva: World Health Organization (2022).

2. Migliori GB, Ong CWM, Petrone L, D'Ambrosio L, Centis R, Goletti D. The definition of tuberculosis infection based on the spectrum of tuberculosis disease. *Breathe*. (2021) 17:210079. doi: 10.1183/20734735.0079-2021

3. Zellweger JP, Sotgiu G, Corradi M, Durando P. The diagnosis of latent tuberculosis infection (Ltbi): currently available tests, future developments, and perspectives to eliminate tuberculosis (Tb). *Med Lav*. (2020) 111:170–83. doi: 10.23749/mdl.v111i3.9983

4. Altice FL, Azbel L, Stone J, Brooks-Pollock E, Smyrnov P, Dvoriak S, et al. The perfect storm: incarceration and the high-risk environment perpetuating transmission of Hiv, hepatitis C virus, and tuberculosis in Eastern Europe and Central Asia. *Lancet*. (2016) 388:1228–48. doi: 10.1016/S0140-6736(16)30856-X

5. Noeske J, Ndi N, Mbondi S. Controlling tuberculosis in prisons against confinement conditions: a lost case? Experience from cameroon. *Int J Tuberc Lung Dis*. (2011) 15:223–7.

6. Aerts A, Hauer B, Wanlin M, Veen J. Tuberculosis and tuberculosis control in European prisons. *Int J Tuberc Lung Dis*. (2006) 10:1215–23.

7. Kamarulzaman A, Reid SE, Schwitters A, Wiessing L, El-Bassel N, Dolan K, et al. Prevention of transmission of Hiv, Hepatitis B virus, Hepatitis C virus, and tuberculosis in prisoners. *Lancet*. (2016) 388:1115–26. doi: 10.1016/S0140-6736(16)30769-3

8. Wang L, Zhang H, Ruan Y, Chin DP, Xia Y, Cheng S, et al. Tuberculosis prevalence in China, 1990–2010: a longitudinal analysis of national survey data. *Lancet*. (2014) 383:2057–64. doi: 10.1016/S0140-6736(13)62639-2

9. Hai-yan SU, Shuo LI, Zhong-dong WA, Zhi-sheng RE, Song SO, Bai XU, et al. Characteristics of the tuberculosis epidemic in schools in Qingdao in 2010–2019. *Chinese J Antituberculosis*. (2020) 42:994–7. doi: 10.3969/j.issn.1000-6621.2020.09.021

10. MA Guangren CJ, LIN Mei, CHU Yan, LI Tongxia. Drug resistance of mycobacterium tuberculosis in tuberculosis patients in Qingdao of China in 2016–2021. *J Precision Med*. (2022) 37:418–21. doi: 10.13362/j.jpmed.202205010

11. Cords O, Martinez L, Warren JL, O'Marr JM, Walter KS, Cohen T, et al. Incidence and prevalence of tuberculosis in incarcerated populations: a systematic review and meta-analysis. *Lancet Public Health*. (2021) 6:e300–e8. doi: 10.1016/S2468-2667(21)00025-6

12. Huang S, Qin M, Shang Y, Fu Y, Liu Z, Dong Y, et al. Performance of Xpert Mtb/Rif in diagnosis of lymphatic tuberculosis from fresh and formaldehyde-fixed and paraffin embedded lymph nodes. *Tuberculosis*. (2020) 124:101967. doi: 10.1016/j.tube.2020.101967

13. Weir CB, Jan A. *BMI Classification Percentile and Cut Off Points*. Treasure Island (FL): StatPearls Publishing (2022).

14. Obesity: Preventing and Managing the Global Epidemic. Report of a who consultation. *World Health Organ Tech Rep Ser*. (2000) 894:i-xii.

15. Habeenzu C, Mitarai S, Lubasi D, Mudenda V, Kantenga T, Mwansa J, et al. Tuberculosis and multidrug resistance in Zambian prisons, 2000–2001. *Int J Tuberc Lung Dis*. (2007) 11:1216–20.

16. Moges B, Amare B, Asfaw F, Tesfaye W, Tiruneh M, Belyhun Y, et al. Prevalence of smear positive pulmonary tuberculosis among prisoners in North Gondar Zone Prison, Northwest Ethiopia. *BMC Infect Dis*. (2012) 12:352. doi: 10.1186/1471-2334-12-352

17. Telisinghe L, Fielding KL, Malden JL, Hanifa Y, Churchyard GJ, Grant AD, et al. High tuberculosis prevalence in a South African Prison: the need for routine tuberculosis screening. *PLoS ONE*. (2014) 9:e87262. doi: 10.1371/journal.pone.0087262

18. Banda HT, Gausi F, Harries AD, Salaniponi FM. Prevalence of smear-positive pulmonary tuberculosis among prisoners in Malawi: a national survey. *Int J Tuberc Lung Dis*. (2009) 13:1557–9.

19. Frascella B, Richards AS, Sossen B, Emery JC, Odone A, Law I, et al. Subclinical tuberculosis disease-a review and analysis of prevalence surveys to inform definitions, burden, associations, and screening methodology. *Clin Infect Dis*. (2021) 73:e830–e41. doi: 10.1093/cid/ciaa1402

20. Cardona PJ. Pathogenesis of tuberculosis and other mycobacteriosis. *Enferm Infecc Microbiol Clin*. (2018) 36:38–46. doi: 10.1016/j.eimce.2017.10.009

21. Jones-López EC, White LF, Kirenga B, Mumbowa F, Ssebidandi M, Moine S, et al. Cough aerosol cultures of mycobacterium tuberculosis: insights on Tst/Igra discordance and transmission dynamics. *PLoS ONE*. (2015) 10:e0138358. doi: 10.1371/journal.pone.0138358

22. Smither SJ, Eastaugh LS, Findlay JS, Lever MS. Experimental aerosol survival of SARS-CoV-2 in artificial saliva and tissue culture media at medium and high humidity. *Emerg Microbes Infect*. (2020) 9:1415–7. doi: 10.1080/22221751.2020.1777906

23. Kendall EA, Shrestha S, Dowdy DW. The epidemiological importance of subclinical tuberculosis. A critical reappraisal. *Am J Respir Crit Care Med*. (2021) 203:168–74. doi: 10.1164/rccm.202006-2394PP

24. Chandra RK. Nutrition and the immune system: an introduction. *Am J Clin Nutr*. (1997) 66:460s–3s. doi: 10.1093/ajcn/66.2.460S

25. Kornfeld H, Sahukar SB, Procter-Gray E, Kumar NP, West K, Kane K, et al. Impact of diabetes and low body mass index on tuberculosis treatment outcomes. *Clin Infect Dis*. (2020) 71:e392–e8. doi: 10.1093/cid/ciaa054

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# Characteristics analysis of 157 cases of central airway stenosis due to tracheobronchial tuberculosis: A descriptive study

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**Background:** Tracheobronchial stenosis, particularly central airway stenosis, which frequently results in severe complications such as lung damage, occurs in patients with tracheobronchial tuberculosis (TBTB).

**Objectives:** To analyze the clinical characteristics of patients with central airway stenosis due to tuberculosis (CASTB).

**Methods:** Retrospective analysis was performed on the clinical features, radiological features, bronchoscopic features and treatment of 157 patients who were diagnosed with CASTB in two tertiary hospitals in Chongqing, China, from May 2020 to May 2022.

**Results:** CASTB mostly occurs in young patients and females. Patients with CASTB exhibited different symptoms repeatedly during the disease, especially varying degrees of dyspnea, prompting many patients to undergo bronchoscopic intervention and even surgery. Patients with cicatricial strictures constituted the highest proportion of the TBTB subtype with CASTB and 35.7% of the patients with CASTB were found to have tracheobronchomalacia (TBM) under bronchoscopy. CASTB and TBM mainly involved the left main bronchus. Patients with lower levels of education had higher rates of TBM. Patients with TBM manifested shortness of breath more frequently than patients without TBM. Patients with TBTB who had undergone bronchoscopic interventions have a higher rate of TBM.

**Conclusions:** Despite mostly adequate anti-tuberculosis chemotherapy, patients with TBTB can present with CASTB involving severe scarring stenosis, bronchial occlusion, tracheobronchomalacia and even destroyed lung.

KEYWORDS

**tracheobronchial tuberculosis, central airway stenosis, bronchoscopic findings, tracheobronchomalacia, clinical characteristics**

## 1. Introduction

The global tuberculosis report 2022 points out that tuberculosis (TB) is still ranked in the top 10 causes of death worldwide. According to the latest World Health Organization statistics, there are approximately 10.6 million people with TB disease, and 1.6 million deaths were due to TB in 2021 (1).

Tracheobronchial tuberculosis (TBTB) is a special type of TB infecting the tracheobronchial tree that could affect any portion or layer of the tracheobronchial wall (2). Published

evidence suggests that TBTB may be present in as many as 40% of patients with pulmonary tuberculosis (PTB) (3–5). The incidence of airway stenosis associated with TBTB can be as high as 68% during the first 4–6 months of illness (6). However, due to the lack of specific symptoms and the lack of attention from patients and medical staff, some cases of early reversible airway stenosis may progress gradually and eventually result in permanent cicatricial stenosis. If the stenosis occurs in the central airway, it is called central airway stenosis due to tuberculosis (CASTB), which is the most severe type because it can lead to varying degrees of respiratory distress, recurrent respiratory infections, and even death from asphyxia (7–9).

However, the clinical characteristics of CASTB are relatively rarely reported in the literature. Consequently, we aimed to retrospectively analyze the clinical characteristics of patients with CASTB to increase physician awareness of the clinical features and harmfulness of the condition.

# 2. Materials and methods

## 2.1. Law and ethics

This study is a retrospective case study approved by the Medical Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (No. 20188501) without the need for participants' explicit consent. The institutional review board of each hospital approved the analysis of patients' clinical, radiological, and bronchoscopic data.

## 2.2. Study design

We performed a retrospective analysis of patients with CASTB treated at the First Affiliated Hospital of Chongqing and the Public Health Medical Center (PHMC) from May 2020 to May 2022. The inclusion criteria were as follows: (1) patients diagnosed with TBTB based on positive acid-fast bacilli smear or culture of *Mycobacterium tuberculosis* (Mtb) or histopathologically and with endoscopic findings suggestive of TBTB (9); (2) patients with proven TBTB with endoscopic findings of central airway stenosis (including trachea, right and left main bronchi and right middle segment bronchi) (10, 11). The exclusion criteria were as follows: (1) patients with tracheobronchial stenosis secondary to etiologies other than TB; (2) patients with incomplete clinical data; (3) patients diagnosed with life-threatening cardiovascular, hepatic, hematopoietic, or other serious diseases.

According to the Chinese guidelines for the classification of TBTB (9), bronchoscopic subtypes of TBTB may be classified as inflammatory infiltration, ulceration necrosis, granulation hyperplasia, cicatricial stricture, tracheobronchomalacia and lymphatic fistula. Endobronchial stenosis was evaluated by comparing the decrease in cross-sectional area and grade under bronchoscopy as follows: Grade I (<50%); Grade II (51–70%); Grade III (>70%) (12). Clinical case notes and electronic medical records of all patients were reviewed. Demographic information, symptoms, radiological features, bronchoscopic findings, and treatment were recorded.

## 2.3. Statistical analysis

Statistical analyses were performed using SPSS version 26.0 (SPSS Inc, Chicago, IL, USA), with $P < 0.05$ being statistically significant (all *P*-values are from two-sided tests). Normality was tested using the Shapiro-Wilk test separate parametric and non-parametric variables. The continuous variables of normally distributed data are expressed as means + standard deviation and differences between any two groups were assessed by an unpaired *t*-test. Categorical data are expressed as numbers (percentage), and comparisons for testing statistically significant differences were made using the $\chi^2$-test (minimum expected values ≥5) or Fisher's exact test (minimum expected values <5).

# 3. Results

## 3.1. Characteristics of patients with CASTB

The study included 157 patients. Of the patients diagnosed with CASTB (mean age 32.5 years, range 17–64 years), 132 were female (84.1%, median age 32.4 years) and 25 were male (15.9%, mean age 33.4 years). The proportion of patients aged 20–29 years diagnosed with CASTB was significantly higher than that of other age groups, followed by the 30–39 age group of patients. Thirty-three patients diagnosed with CASTB (21.0%) were underweight (BMI < 18.5 kg/m$^2$), 14 (8.9%) were the national minority, 36 (22.9%) lived in rural areas, 99 (63.1%) had high school or lower education level, 104 (66.2%) were married, 7 (4.5%) had smoking history, 3 (1.9%) had diabetes, 40 (25.5%) had a history of PTB, 14 (8.9%) had drug resistance and 18 (11.5%) had irregular anti-TB treatment. The common symptoms of CASTB included cough (132/157, 84.1%), expectoration (118/157, 75.2%), shortness of breath (91/157, 58.0%), hemoptysis (29/157, 18.5%) and weight loss (40/157, 25.5%). One hundred and twenty-three patients (78.3%) underwent bronchoscopic intervention (including cryotherapy, electric coagulation, balloon dilatation, and stent insertion) and 2 of them underwent surgery. The remaining 34 patients were conservatively managed (Table 1).

## 3.2. Chest CT and bronchoscopy

The results of chest CT and bronchoscopy are presented in Table 2. Based on chest CT, 51 (32.5%) patients showed tracheobronchial wall thickening, 136 (86.6%) showed tracheobronchial stenosis, 33 (21.0%) patients showed mediastinal nodes increase, 34 (21.7%) patients showed mediastinal nodes calcification and 19 (12.1%) patients showed cavities. Of all the study patients, 5 (3.2%) had destroyed lung and two of them underwent a surgical operation. The results of bronchoscopy indicated 101 (64.3%) cases of cicatricial strictures, 7 (4.5%) cases of TBM, and 49 (31.2%) mixed cicatricial strictures and TBM. The lesions were mainly located in the left main bronchus (58.0%), followed by the right bronchus (30.6%), the left upper lobe bronchus (26.8%), and the right upper lobe bronchus (23.6%). Patients with multiple lesions under bronchoscope constituted 77.1%. A total of 56 (35.7%) of the 157 patients with CASTB manifested TBM and the highest proportion of TBM lesions was the left main bronchus

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TABLE 1 Demographic and clinical characteristics of 157 patients diagnosed with CASTB.

| | Patients ($n = 157$) |
|---|---|
| Age [mean (SD)] | 32.5 (11.41) |
| **Age categories (y)** | |
| <20 | 10 (6.4%) |
| 20–29 | 73 (46.5%) |
| 30–39 | 32 (20.4%) |
| 40–49 | 24 (15.3%) |
| 50–59 | 15 (9.6%) |
| ≥60 | 3 (1.9%) |
| BMI [mean (SD)] | 20.93 (3.12) |
| **BMI (kg/m²)** | |
| <18.5 (underweight) | 33 (21.0%) |
| 18.5–24.9 (normal weight) | 113 (72.0%) |
| 25.0–29.9 (overweight) | 9 (5.7%) |
| ≥30.0 (obese) | 2 (1.3%) |
| **Gender** | |
| Male | 25 (15.9%) |
| Female | 132 (84.1%) |
| **National minority** | |
| Yes | 14 (8.9%) |
| No | 143 (91.9%) |
| **Residence** | |
| Urban | 121 (77.1%) |
| Rural | 36 (22.9%) |
| **Education** | |
| College or above | 58 (36.9%) |
| High school or less | 99 (63.1%) |
| **Marital status** | |
| Married | 104 (66.2%) |
| Spinsterhood | 53 (33.8%) |
| **Smoking history** | |
| Never-smoker | 150 (95.5%) |
| Former or current smoker | 7 (4.5%) |
| **Diabetes** | |
| Yes | 3 (1.9%) |
| No | 154 (98.1%) |
| **History of PTB** | |
| Yes | 40 (25.5%) |
| No | 117 (74.5%) |
| **Drug resistance** | |
| Yes | 14 (8.9%) |
| No | 85 (54.1%) |
| **Irregular anti-tuberculosis treatment** | |
| Yes | 18 (11.5%) |
| No | 139 (88.5%) |
| **Symptoms at presentation** | |
| **Cough** | |
| Yes | 132 (84.1%) |
| No | 25 (15.9%) |
| **Expectoration** | |
| Yes | 118 (75.2%) |
| No | 39 (24.8%) |
| **Shortness of breath** | |
| Yes | 91 (58.0%) |
| No | 66 (42.0%) |
| **Hemoptysis** | |
| Yes | 29 (18.5%) |
| No | 128 (81.5%) |
| **Weight loss** | |
| Yes | 40 (25.5%) |
| No | 117 (74.5%) |
| **Treatment methods** | |
| Bronchoscopic intervention | 123 (78.3%) |
| Cryotherapy | 120 (76.4%) |
| Electric coagulation | 94 (59.9%) |
| Balloon dilatation | 104 (66.2%) |
| Stent insertion | 13 (8.3%) |
| Surgical operation | 2 (1.3%) |

Data are expressed as numbers (percentages). CASTB, central airway stenosis due to tracheobronchial tuberculosis; BMI, body mass index.

(37/56, 66.1%). Of these patients including all lesions detected bronchoscopically, 20 (12.7%) were mild stenosis, 56 (35.7%) were intermediate stenosis, and 81 (51.6%) were severe stenosis.

## 3.3. Comparison of TBM group vs. non-TBM group

Patients with lower levels of education exhibited a higher incidence of TBM compared with patients with higher levels of education ($p = 0.008$; Table 3). A greater percentage of patients with TBM experienced shortness of breath (73.2 vs. 49.5%, $p = 0.004$) than patients without TBM. Not only that, a greater percentage of patients with TBM had modified Medical Research Council (mMRC) dyspnea scores ≥ 2 (30.4 vs. 12.9%, $p = 0.008$) than patients without TBM. A larger proportion of patients with TBM had ever undergone bronchoscopic interventions (92.3 vs. 70.3%, $p = 0.001$) than patients without TBM. No statistically significant difference was found in

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TABLE 2 Chest CT and bronchoscopy features of 157 patients with CASTB.

| | Patients ($n$ = 157) |
|---|---|
| **Chest CT features** | |
| Tracheobronchial wall thickening | 51 (32.5%) |
| Tracheobronchial stenosis | 136 (86.6%) |
| Mediastinal nodes increase | 33 (21.0%) |
| Mediastinal nodes calcification | 34 (21.7%) |
| Cavity | 19 (12.1%) |
| Destroyed lung | 5 (3.2%) |
| **Bronchoscopic features** | |
| **Type of TBTB** | |
| Cicatricial stricture | 101 (64.3%) |
| Tracheobronchomalacia | 7 (4.5%) |
| Cicatricial stricture + Tracheobronchomalacia | 49 (31.2%) |
| **Site of lesions** | |
| Trachea | 29 (18.5%) |
| LMB | 91 (58.0%) |
| RMB | 48 (30.6%) |
| RBI | 33 (21.0%) |
| LULB | 42 (26.8%) |
| LLLB | 23 (14.6%) |
| RULB | 37 (23.6%) |
| RMLB | 15 (9.6%) |
| RLLLB | 15 (9.6%) |
| **Number of lesions involved** | |
| Single | 36 (22.9%) |
| Multiple | 121 (77.1%) |
| **Site of TBM lesions** | |
| Trachea | 5 (3.2%) |
| LMB | 37 (23.6%) |
| RMB | 10 (6.4%) |
| RMI | 8 (5.1%) |
| **Grade of tracheobronchial** | |
| **Stenosis (including all bronchi)** | |
| Grade 1 | 20 (12.7%) |
| Grade 2 | 56 (35.7%) |
| Grade 3 | 81 (51.6%) |

Data are expressed as numbers (percentages). CASTB, central airway stenosis due to tracheobronchial tuberculosis; CT, computed tomography; LMB, left main bronchus; RMB, right main bronchus; RBI, right bronchus intermedius; LULB, left upper lobe bronchus; LLLB, left lower lobe bronchus; RULB, right upper lobe bronchus; RMLB, right middle lobe bronchus; RLLB, right upper lobe bronchus; TBM, tracheobronchomalacia.

the number of lesions and degree of stenosis under bronchoscope between the two groups ($P > 0.05$; Table 3).

TABLE 3 Comparison of TBM group ($n$ = 56) vs. non-TBM group ($n$ = 101).

| | TBM ($n$ = 56) | Non-TBM ($n$ = 101) | $P$-value |
|---|---|---|---|
| Age[mean (SD)] | 32.52 (1.56) | 32.63 (1.13) | 0.952 |
| BMI [mean (SD)] | 21.18 (0.43) | 20.79 (0.31) | 0.456 |
| **Gender** | | | 0.160 |
| Male | 12 (21.4%) | 1,312.9 (%) | |
| Female | 44 (78.6%) | 88 (87.1%) | |
| **Residence** | | | 0.950 |
| Urban | 43 (76.8%) | 78 (77.2%) | |
| Rural | 13 (23.2%) | 23 (22.8%) | |
| **Education** | | | 0.008 |
| College or above | 13 (23.2%) | 45 (44.6%) | |
| High school or less | 43 (76.8%) | 56 (55.4%) | |
| **Symptoms** | | | |
| **Cough** | | | 0.970 |
| Yes | 47 (83.9%) | 85 (84.2%) | |
| No | 9 (16.1%) | 16 (15.8%) | 0.973 |
| **Expectoration** | | | |
| Yes | 42 (75.0%) | 76 (75.2%) | |
| No | 14 (25.0%) | 25 (24.8%) | |
| **Hemoptysis** | | | 0.254 |
| Yes | 13 (23.2%) | 16 (15.8%) | |
| No | 43 (76.8%) | 85 (84.2%) | |
| **Shortness of breath** | | | |
| Yes | 41 (73.2%) | 50 (49.5%) | 0.004 |
| No | 15 (26.8%) | 51 (50.5%) | |
| **Weight loss** | | | 0.296 |
| Yes | 17 (30.4%) | 23 (22.8%) | |
| No | 39 (69.6%) | 78 (77.2%) | |
| **mMRC dyspnea scores ≥2** | | | 0.008 |
| Yes | 17 (30.4%) | 13 (12.9%) | |
| No | 39 (69.6%) | 88 (87.1%) | |
| **Treatment methods** | | | |
| Bronchoscopic intervention | 52 (92.3%) | 71 (70.3%) | 0.001 |
| Cryotherapy | 51 (91.1%) | 69 (68.3%) | 0.001 |
| Electric coagulation | 39 (69.6%) | 55 (54.5%) | 0.063 |
| Balloon dilatation | 45 (80.4%) | 59 (58.4%) | 0.005 |
| Stent insertion | 5 (8.9%) | 8 (7.9%) | 0.826 |
| Surgical operation | 1 (1.8%) | 1 (1.0%) | 0.670 |
| **Number of lesions involved under bronchoscope** | | | 0.211 |
| Single | 16 (28.6%) | 20 (19.8%) | |
| Multiple | 40 (71.4%) | 81 (80.2%) | |

*(Continued)*

TABLE 3 (Continued)

| | TBM ($n = 56$) | Non-TBM ($n = 101$) | *P*-value |
|---|---|---|---|
| Grade of tracheobronchial stenosis (including all bronchi) | | | 0.118 |
| Grade 1 | 3 (5.4%) | 17 (16.8%) | |
| Grade 2 | 22 (39.3%) | 34 (33.7%) | |
| Grade 3 | 31 (55.4%) | 50 (49.5%) | |

Continuous data are expressed as means ± standard. Differences between the 2 groups were tested by the unpaired t-test for normally distributed continuous variables. Categorical data are expressed as numbers (percentage), and comparisons for testing statistically significant differences were made using the $\chi^2$ test (minimum expected values ≥5) or Fisher's exact test (minimum expected values <5). TBM, tracheobronchomalacia; BMI, body mass index; mMRC, Medical Research Council; LMB, left main bronchus; RMB, right main bronchus; RBI, right bronchus intermedius.

## 4. Discussion

This study was the first dual-center, retrospective report analyzing the clinical characteristics of CASTB. This study found that CASTB mostly occurs in young patients and females. Patients with CASTB manifested different symptoms repeatedly during the disease, especially varying degrees of dyspnea, prompting many patients to undergo bronchoscopic intervention and even surgery. The TBTB subtypes of patients with CASTB which accounted for the highest proportion were cicatricial strictures and 35.7% of the patients with CASTB were found to have TBM under bronchoscope. The left main bronchus was more prone to be involved in patients with CASTB and TBM. Patients with lower levels of education had higher rates of TBM. Patients with TBM experienced shortness of breath more frequently than patients without TBM. Patients with TBTB who had undergone bronchoscopic interventions had a higher rate of TBM. Of the 157 patients with CASTB, 68.8% had cicatricial stricture, 35.7% had tracheobronchomalacia and 3.2% had destroyed lung.

CASTB is seldom reported. In 2018, a large-scale prospective study (13) enrolled 392 patients with scarring airway stenosis from 18 tertiary hospitals. It reported that TBTB was the most common cause of scarring airway stenosis in Chinese adults with a high rate of incidence in young women. Similar sex- and age-related preponderance was observed in our study of CASTB. In the present study, nearly half of the patients were aged between 20 and 29 years probably due to the stronger immunity and inflammatory response to the disease, which may increase the risk of airway injury, even leading to permanent scar stenosis. The study found a higher proportion of females in patients with CASTB, which was consistent with previous reports (4, 14, 15). The bronchi were smaller and thinner in adult women than in men, and sputum retention promoted chronic infection of the bronchial lumen with Mtb (15). In addition, Gan et al. (16) suggested that estradiol may play an important role in the pathogenesis of TBTB by binding to estrogen receptor α (ERα) and affecting Mtb proliferation in bronchial epithelial cells. Estrogen is known to increase the levels of transforming growth factor β1 (TGF-β1) and fibronectin, which affects the wound healing response (17, 18). This may also explain the occurrence of severe cicatricial strictures in TBTB mostly in younger women.

In 2018, a large prospective study in southern China found that the proportion of retreated patients was 13.2% of the 1,442 patients with PTB, while this proportion was 10.7% among patients with TBTB (4). However, our study indicated that 25.5% of patients with CASTB had a history of PTB probably due to the significantly reduced cellular immune function in patients with retreated PTB than in newly treated patients (19).

During the disease, 21% of patients with CASTB were underweight and 25.5% of patients manifested symptoms of weight loss. TB itself is a wasting disease. However, changes in weight which may reflect disease activity, are not often the focus of outpatient consultations. Patients with CASTB manifested different symptoms during the disease, especially varying degrees of dyspnea, which was the main reason why patients underwent bronchoscopic interventions and even surgery. Most patients' symptoms were relieved after active treatment. However, patients with severe scarring stenosis, bronchial occlusion, malacia, and even lung damage, were still detected.

Our findings showed that the highest proportion of TBTB subtypes was cicatricial strictures (64.3%). The proportion of patients who had multiple lesions under bronchoscopy was as high as 77.1%, suggesting that the study patients had severe and extensive tracheobronchial lesions during the disease. The higher frequency of left bronchial involvement with CASTB and TBM is also consistent with previous studies (4, 13, 20). This finding may be explained by the location of the left main bronchus anatomically adjacent to the aortic arch and vulnerability to compression, increasing the risk of endobronchial infection of the left main bronchus (2).

This study found that 35.7% of the patients with CASTB had TBM and patients with lower levels of education had higher rates of TBM, which might be related to the fact that patients with lower levels of education had poorer compliance with standardized anti-tuberculosis treatments. Compared with patients without TBM, those with TBM were more likely to experience dyspnea because of the weakening of the airway wall and dynamic collapse of the airway lumen during respiration (21). Our analysis also found that patients with previous bronchoscopic interventions had a higher rate of TBM. There are two possibilities to be considered. One explanation is that this is an accidental phenomenon. Another possible explanation is that bronchoscopic intervention as a type of surgical procedure may damage cartilage or normal structures further contributing to the development of TBM. The impact of bronchoscopic interventions in the longer term is less predictable although it is a logical approach to relieve symptoms. Additional studies are needed to investigate the outcomes of bronchoscopic interventions, both immediate and in the longer term.

Some limitations of this study should be noted. First, this study was a retrospective study with a relatively small sample size, suggesting the possibility of bias. Second, lesions were graded based on the results of bronchoscopic examinations. It was possible that lesions were over or underestimated due to the subjective judgment of the physicians performing bronchoscopy despite similar evaluation criteria. Third, this study could not identify the predictors of TBM in patients with CASTB definitively. Further prospective, multicenter, and large-scale studies are needed to establish the incidence and predictors of CASTB and TBM in patients with TBTB.

## 5. Conclusion

Despite mostly adequate anti-tuberculosis chemotherapy, patients with TBTB can present with CASTB involving severe

scarring stenosis, bronchial occlusion, tracheobronchomalacia, and even destroyed lung. Future studies should focus on the pathogenesis of bronchial fibrosis and bronchomalacia to prevent tracheobronchial stenosis at an early stage.

## Data availability statement

The datasets presented in this article are not readily available because the raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Requests to access the datasets should be directed to RZ, raphaela720@163.com.

## Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (No. 20188501). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

## Author contributions

The data collection of the project was done by RZ, YinL, YC, TH, and XN. The idea for the paper, the data cleaning, the data analysis, and the writing was done by RZ and MY. The review of the paper and suggested ideas were done by LX, YisL, XY, XK, and SY. The review and final edits of the paper were done by RZ and SG. All authors contributed to the article and approved the submitted version.

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## 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



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1115177/full#supplementary-material

## References

1. World Health Organization. *Global Tuberculosis Report 2022*. Geneva: World Health Organization (2022).

2. Shahzad T, Irfan M. Endobronchial tuberculosis-a review. *J Thorac Dis.* (2016) 8:3797–802. doi: 10.21037/jtd.2016.12.73

3. Chung HS, Lee JH. Bronchoscopic assessment of the evolution of endobronchial tuberculosis. *Chest.* (2000) 117:385–92. doi: 10.1378/chest.117.2.385

4. Su Z, Cheng Y, Wu Z, Zhang P, Chen W, Zhou Z, et al. Incidence and predictors of tracheobronchial tuberculosis in pulmonary tuberculosis: a multicentre, large-scale and prospective study in Southern China. *Respiration.* (2019) 97:153–9. doi: 10.1159/000492335

5. Jung SS, Park HS, Kim JO, Kim SY. Incidence and clinical predictors of endobronchial tuberculosis in patients with pulmonary tuberculosis. *Respirology.* (2015) 20:488–95. doi: 10.1111/resp.12474

6. Kashyap S, Solanki A. Challenges in endobronchial tuberculosis: from diagnosis to management. *Pulm Med.* (2014) 2014:594806. doi: 10.1155/2014/594806

7. Li YQ, Li Q. Etiological analysis of 386 cases of benign central airway stenosis. *J Nav Med.* (2007) 307–309. doi: 10.3969/j.issn.1009-0754.2007.04.010

8. Um S-W, Yoon YS, Lee S-M, Yim J-J, Yoo C-G, Chung HS, et al. Predictors of persistent airway stenosis in patients with endobronchial tuberculosis. *Int J Tuberc Lung Dis.* (2008) 12:57–62.

9. Chinese Medical Association. Diagnosis and treatment guideline for tracheobronchial tuberculosis. *Chin J Tuberc Respir Dis.* (2012) 35:581–7. doi: 10.3760/cma.j.issn.1001-0939.2012.08.007

10. Murgu SD, Egressy K, Laxmanan B, Doblare G, Ortiz-Comino R, Hogarth DK. Central airway obstruction: benign strictures, tracheobronchomalacia, and malignancy-related obstruction. *Chest.* (2016) 150:426–41. doi: 10.1016/j.chest.2016.02.001

11. Guedes F, Mauricio AC, Bugalho A. Central airway stenosis: opening the path. *Arch Bronconeumol.* (2020) 56:695–6. doi: 10.1016/j.arbres.2019.12.011

12. Freitag L, Ernst A, Unger M, Kovitz K, Marquette CH. A proposed classification system of central airway stenosis. *Euro Respir J.* (2007) 30:7–12. doi: 10.1183/09031936.00132804

13. Wang T, Zhang J, Qiu XJ, Wang J, Pei YH, Wang YL. Scarring airway stenosis in Chinese adults: characteristics and interventional bronchoscopy treatment. *Chin Med J.* (2018) 131:276–81. doi: 10.4103/0366-6999.223850

14. Peng AZ, Yang A, Li SJ, Qiu Q, Yang S, Chen Y. Incidence, laboratory diagnosis and predictors of tracheobronchial tuberculosis in patients with pulmonary tuberculosis in Chongqing, China. *Exp Ther Med.* (2020) 20:174. doi: 10.3892/etm.2020.9304

15. Lee JY, Yi CA, Kim TS, Kim H, Kim J, Han J, et al. CT scan features as predictors of patient outcome after bronchial intervention in endobronchial TB. *Chest.* (2010) 138:380–5. doi: 10.1378/chest.09-1846

16. Gan Y, Hu Q, Li A, Gu L, Guo S. Estradiol inhibits autophagy of *Mycobacterium tuberculosis*-infected 16HBE cells and controls the proliferation of intracellular *Mycobacterium tuberculosis*. *Mol Med Rep.* (2022) 25:196. doi: 10.3892/mmr.2022.12712

17. Ashcroft GS, Dodsworth J, van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, et al. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta1 levels. *Nature medicine.* (1997) 3:1209–15. doi: 10.1038/nm1197-1209

18. Smith LC, Moreno S, Robertson L, Robinson S, Gant K, Bryant AJ, et al. Transforming growth factor beta1 targets estrogen receptor signaling in bronchial epithelial cells. *Respir Res.* (2018) 19:160. doi: 10.1186/s12931-018-0861-5

19. Zhang SJ, Xiao HP. Changes and significance of T cell subsets in peripheral blood of patients with retreated and newly treated tuberculosis. *Chin J Tuberc Respir Dis.* (2011) 34:884–7. doi: 10.3760/cma.j.issn.1001-0939.2011.12.002

20. Wu Y, Li Y, Bai Y, Jiang J, Wang X, Guo S. Left main bronchus stenosis lesion, neutrophil count, and platelet count are predictors of post-tuberculosis bronchomalacia. *Med Sci Monit.* (2021) 27:e931779. doi: 10.12659/MSM.931779

21. Hysinger EB, Bates AJ, Higano NS, Benscoter D, Fleck RJ, Hart CK, et al. Ultrashort echo-time MRI for the assessment of tracheomalacia in neonates. *Chest.* (2020) 157:595–602. doi: 10.1016/j.chest.2019.11.034

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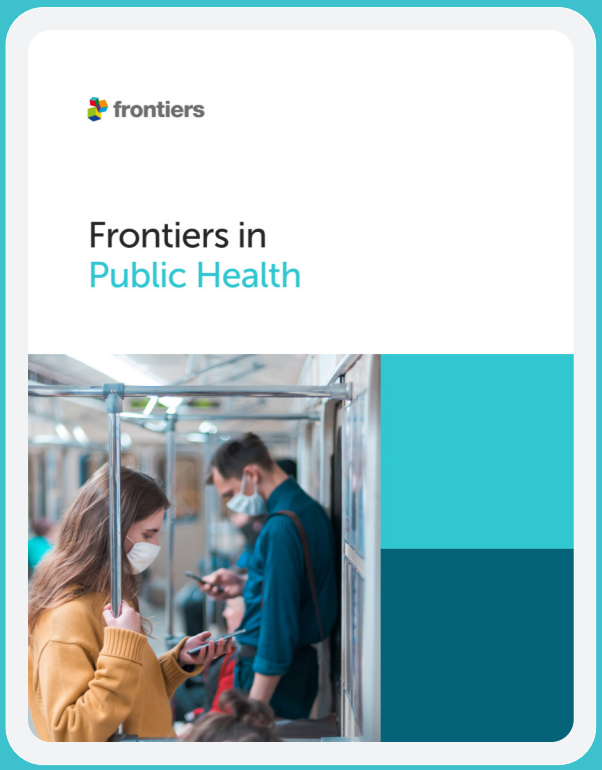


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