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A case series of co-infection in *Mycobacterium tuberculosis* and other pathogens: insights from nanopore sequencing

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Abstract

Background Tuberculosis (TB) continues to be a major global health burden, and co-infection with other pathogens further complicates the diagnosis and treatment of this infectious disease. The present retrospective study aimed to evaluate the clinical utility of nanopore sequencing in identifying co-infection caused by *Mycobacterium tuberculosis* (*M.tb*) and other pathogens.

Methods Patients with *M.tb* co-infection from December 2021 to March 2023 at the Jiangxi Provincial Chest Hospital were retrospectively studied. Data were collected including demographics, symptoms, imaging findings, pathogen diagnosis tests, and treatment history. Pathogen tests involved culture, AFB smear, Xpert MTB/RIF, and nanopore sequencing.

Results The enrolled patients included 20 M.*tb* cases and three nontuberculous mycobacteria (NTM) cases co-infected with other pathogens. Common clinical symptoms included cough (47.83%), expectoration (34.78%), and asthma (17.39%). Radiological examinations showed typical features of pulmonary tuberculosis, including nodules (73.91%), cord-like shadows (34.78%), cavities (34.78%), and destroyed lung manifestations (17.39%). Nanopore sequencing identified *M.tb* in a significant majority of the cases (86.96%), outperforming traditional culture tests (39.13%), acid-fast bacilli (AFB) tests (27.27%), and Xpert MTB/RIF (53.84%) tests. Notably, nanopore sequencing revealed that *M.tb* was frequently co-infected with *Candida albicans*, *Klebsiella pneumoniae*, and *Mycobacterium abscessus*. Three specific cases of co-infection with distinct diagnosis and treatment characteristics were presented in detail. They illustrated the complexity of TB co-infection management and the potential of nanopore sequencing for accurate diagnosis and informing the tailored therapeutic approaches.

Conclusion Nanopore sequencing-based metagenomics method can help clinicians to identify TB co-infection patterns and formulate a rational drug regimen in time.

Keywords *Mycobacterium tuberculosis*, Pulmonary aspergillosis, Nanopore sequencing, Case series

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Introduction

Mixed pulmonary infections are common in clinical settings, especially bacterial and fungal infections of the lower respiratory tract [1, 2]. Due to overlapping imaging features and lack of specificity in clinical manifestations, diagnosis of mixed pulmonary infection is challenging. Furthermore, prolonged empirical treatment with broad-spectrum antibiotics has contributed to the rise of drug resistance in certain pathogens, and the prevalence of drug resistance will increase accordingly [2].

Tuberculosis (TB) is the leading cause of death from a single infectious agent, which was caused by *Mycobacterium tuberculosis* (*M.tb*). There were an estimated 10.6 million people who fell ill with TB and 1.6 million deaths from TB in 2021 [3]. Co-infection of *M.tb* with other bacterial pathogens, such as *Klebsiella* and *Pseudomonas*, can worsen the severity of TB and increase the risk of complications [4]. Moreover, mixed infections of *M.tb* with aspergillosis and nontuberculous mycobacteria (NTM) are increasingly reported in pulmonary infectious disease [5, 6]. These co-infections may lead to more severe lung damage and prolonged treatment duration. *M.tb* co-infection presents unique diagnostic and therapeutic challenges that affect the accuracy of TB diagnosis and treatment outcomes. The epidemiology and optimal diagnostic approaches for co-infection are still being elucidated.

Nanopore sequencing is a promising method to personalize treatment by detecting etiologic agents and drug resistance-associated mutations in clinical applications [7]. This tool has enabled the detection of overall pathogens that may contribute to the disease burden, facilitating the identification of co-infections in TB patients [4]. There is limited available data reporting the co-infection of TB and other pathogens in regions of high TB prevalence, where TB treatment is frequently reliant on presumptive diagnosis [8, 9]. By examining a series of cases, the objective of this study is to assess the clinical significance of nanopore sequencing in the identification of *M.tb* co-infection with other pathogens.

Material and methods

Study design and procedures

Patients infected with *M.tb* and other pathogens were enrolled from December 2021 to March 2023 at the Jiangxi Chest Hospital, China. Epidemiological and clinical data was retrospectively analyzed to assess the clinical availability of nanopore sequencing for the identification of *M.tb* and other pathogens. Comprehensive clinical data were collected from the electronic medical records. Clinical data included patient demographics, symptoms of tuberculosis, radiographic features, underlying conditions, smoking history, history of previous tuberculosis disease, and the clinical course of anti-TB drugs. To confirm the presence of pulmonary tuberculosis (PTB), all individuals with suspected clinical symptoms and abnormal imaging findings were initially screened by acid-fast bacilli (AFB) smear analysis. Confirmation was obtained by mycobacterial culture, nanopore sequencing for etiologic microbes, or the Xpert MTB/RIF assay. Culture test and nanopore sequencing have been performed in all the samples. Suspicious chest radiographic findings included patchy opacities, possibly with or without cavities, miliary shadows, or pleural effusions [10].

Mycobacterial culture and Xpert MTB/RIF testing were performed in the hospital microbiology laboratory. All samples for mycobacterial culture, identification, and drug susceptibility testing were performed using the BD BACTEC MGIT 960 Mycobacteria Culture System (Becton Dickinson, USA) as per the manufacturer's instructions. The Xpert MTB/RIF assay procedure consisted of mixing either centrifuged BALF or a 1-mL sputum sample with 2-mL sample processing solution according to the manufacturer's instructions. After an incubation period of 15 min, 2.0 mL of the mixture was pipetted into the reaction cassette (Cepheid, USA). The cassette was then inserted into the test module to initiate the automated test. The system automatically interpreted the MTB test results within 2 h. The sequencing was processed on the nanopore sequencing device GridION (Oxford Nanopore Technologies) at Zhejiang ShengTing Biotech Co., Ltd. (Hangzhou, China) following standard operating procedures as previously described [11]. *Mycobacterial* species identification is performed by targeting the 16S rDNA and hsp65 gene regions. *M.tb* was considered as positive when at least one read was mapped to either the species or genus level.

Data analysis

Categorical data are presented as numbers and proportions. Continuous data are presented as the median and interquartile range. IBM SPSS Statistics 24 software was used for data analysis. Heatmap was plotted by https://www.bioinformatics.com.cn (last accessed on 10 July 2023), an online platform for data analysis and visualization.

Results

The result of nanopore sequencing showed that 20 MTB cases and 3 NTM cases were co-infected with other etiological organism: 11 (47.83%) were male, and 12 (52.17%) were female patients. The median age was 58 years (IQR 36, 65). Thirteen (47.83%) patients presented with cough, and 8 (34.78%) patients had expectoration. The laboratory data are listed as median values in Table 1. There were also six patients

Table 1 Patient characteristics

Characteristics	Data
Median age (IQR)	58 (35, 65)
Male, *n* (%)	11 (47.83%)
Symptoms	
Cough, *n* (%)	13 (56.52%)
Asthma, *n* (%)	4 (17.39%)
Fever, *n* (%)	3 (13.04%)
Expectoration, *n* (%)	8 (34.78%)
Hemoptysis, *n* (%)	3 (13.04%)
Laboratory tests	
ESR (mm/h), (IQR)	26.50 (11.75, 84.25)
CRP (mg/dL), (IQR)	18.51 (1.03, 69.87)
GM test (ODI), (IQR)	0.24 (0.15, 0.27)
BG test (pg/mL), (IQR)	37.50 (3.63, 136.05)
Lesion location	
Lung, *n* (%)	17 (73.91%)
Bronchus, *n* (%)	6 (26.09%)
Underlying conditions	
Severe pulmonary infection, *n* (%)	7 (30.43%)
Anemia, *n* (%)	5 (21.74%)
Malnutrition, *n* (%)	4 (17.39%)
Hypohepatia, *n* (%)	3 (13.04%)
Hepatitis B surface antigen carriers, *n* (%)	3 (13.04%)
Type 2 diabetes, *n* (%)	4 (17.39%)

Abbreviations: *ESR* Erythrocyte sedimentation rate, *CRP* C-reactive protein, *GM test* Galactomannan test. BG test, beta-(1,3)-D-glucan test

with complicated tracheobronchial tuberculosis. Extrapulmonary underlying conditions included anemia (21.74%, 5/23), hypohepatia (13.04%, 3/23), diabetes (17.39%, 4/23), hypertension (4.35%, 1/23), infarct of the brain (4.35%, 1/23), and rheumatoid arthritis (4.35%, 1/23). Iron deficiency anemia is associated with a higher incidence of TB [12]. All the patients had typical imaging features of pulmonary tuberculosis, except for two NTM cases. The prevalent radiological finding was the presence of nodules (73.91%, 17/23), followed by cord-like shadows (34.78%, 8/23), cavities (34.78%, 8/23), and destroyed lung with consolidation and increased density manifestations (17.39%, 4/23). Destroyed manifestations were found in patients who suffered secondary pulmonary tuberculosis. According to the results of nanopore sequencing, we speculated that the radiographic finding of these four destroyed lungs was related to the coexisting infection caused by *Candida*, *Aspergillus*, or *Klebsiella*.

We were able to identify *M.tb* by nanopore sequencing in 20 patients (86.96%, 20 out of 23), while culture tests yielded positive results in 9 samples (39.13%, 9 out of 23). The positive rate for acid-fast bacilli (AFB) was 27.27% (6 out of 22), and for Xpert MTB/RIF, it was 53.84% (7 out of 13). Additionally, co-infection with *Candida albicans*, *Klebsiella pneumoniae*, and *Mycobacterium abscessus* was commonly observed alongside *Mycobacterium tuberculosis* (Fig. 1).

We presented three cases of PTB patients that peaked our interest. One of them was a 20-year-old woman (case 1) with extensively drug-resistant tuberculosis (XDR-TB). The second patient has recurrent and multidrug-resistant tuberculosis (case 21). The third patient had a double negative culture and AFB staining but with symptoms worsening (case 23).

Case 1

A 21-year-old female presented with a 10-month history of cough and sputum production. She complained of coughing up a small amount of yellow, purulent sputum, which gradually worsened over time. She also developed hoarseness 4 months before admission. On January 19, 2022, she was admitted to our hospital. A laryngoscopy performed at another hospital suggested laryngeal tuberculosis. Previously, she had been diagnosed with tuberculosis-associated erythema nodosum in January 2020 and had received anti-TB therapy with isoniazid, rifampicin, pyrazinamide, and ethambutol for 1 year. On admission, she was given symptomatic treatment with amoxicillin and clavulanate potassium, as well as pasiniazid, rifampicin, pyrazinamide, ethambutol, and levofloxacin for anti-TB treatment.

On January 27, 2022, the results of nanopore sequencing indicated that the patient was infected with *M.tb* (28,333 reads). Variants in the *rrs*, *rpoB*, *katG*, *gyrA*, *pncA*, and *rpsL* genes suggested resistance to streptomycin, rifampicin, isoniazid, and fluoroquinolones. These findings were consistent with the culture of *M.tb* and the drug susceptibility testing of the bronchoalveolar lavage fluid. The drug susceptibility testing performed on February 07, 2022, confirmed resistance to streptomycin, isoniazid, rifampicin, and levofloxacin. As a result, the antituberculosis therapy regimen was adjusted to include bedaquiline, linezolid, cycloserine, protionamide, and ethambutol.

During the course of antituberculosis treatment, the patient experienced recurrent episodes of prolonged cough and expectoration, accompanied by yellow pus expectoration. Piperacillin and tazobactam were used empirically. Subsequent drug susceptibility testing revealed resistance to ethambutol and susceptibility to amikacin. Therefore, ethambutol was discontinued, and amikacin was added to the antituberculosis regimen. However, even after 3 months of treatment with bedaquiline, linezolid, cycloserine, and protionamide, the patient's cough and sputum production did not improve. Chest CT showed multiple patchy infiltrates, nodules,

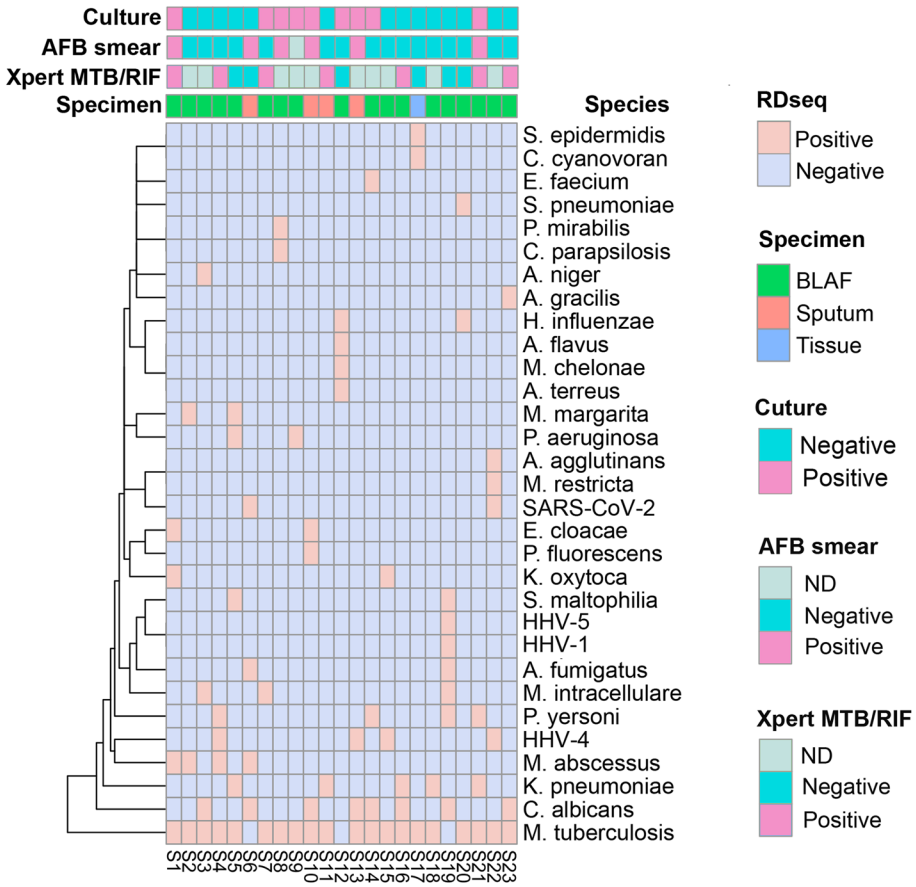

Fig. 1 Heatmap of the test results by different methods. The diagnostic results of culture, AFB smear, Xpert MTB/RIF, and specimen type are shown as in the top of the heatmap. Species identified by nanopore sequencing directly from clinical samples are shown as in the bottom of the heatmap. Each column represents a sample from a patient with tuberculosis or NTM. Abbreviations: BALF, bronchoalveolar lavage fluid; HHV, human herpes virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ND, not done

segmental consolidations, and a miliary shadows in the left lower lobe. Diffuse miliary changes were observed in both lungs. Meanwhile, bronchoscopy showed left main bronchus mucosal hyperemia, lumen stenosis, left upper lobe and lower lobe openings narrowed, and mucosal erosion with little necrosis (Fig. 2). Nanopore sequencing of the bronchoalveolar lavage fluid indicated the presence of *Mycobacterium abscessus* (3 reads), *Mycobacterium tuberculosis* (2 reads), *Klebsiella oxytoca* (2195 reads), and *Enterobacter cloacae* (603 reads). To address this,

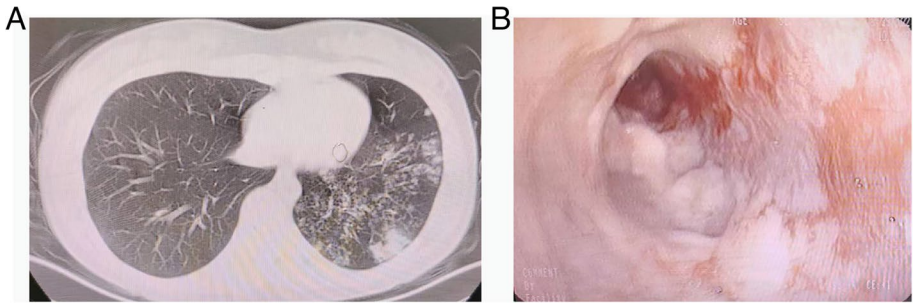

Fig. 2 Chest CT images of case 1. Multiple patchy infiltrations, nodules, segmental solid shadows in the left upper lobe of the lung. Diffuse distribution of spots and chestnut grain shadows in both lungs (**A**). Left main bronchus mucosal hyperemia, lumen stenosis, left upper lobe and lower lobe openings narrow, mucosal erosion a little necrosis (**B**)

imipenem and cilastatin were added for anti-infection treatment, and clarithromycin was included for anti-NTM treatment. Bedaquiline, linezolid, cycloserine, protionamide, and clarithromycin were continued for anti-TB and anti-NTM treatment after the patient's discharge. Currently, the patient does not experience any symptoms.

Case 21

A 26-year-old male, who had been treated for tuberculosis for 9 months and had been found enlarged lung cavity for more than 1 day, was admitted to our hospital on February 14, 2023. Initially, the patient presented with a persistent cough and night sweats in May 2022, resulting in a diagnosis of infiltrative pulmonary tuberculosis and concurrent lung infection. Treatment with ampicillin sodium sulbactam for the infection and isoniazid, rifampicin, pyrazinamide, and ethambutol for tuberculosis led to the improvement of symptoms and subsequent discharge from the hospital. Nevertheless, shortly after leaving the hospital, the patient developed pruritus, skin rashes, nausea, and vomiting, which was indication for readmission due to liver function impairment. The antituberculosis medication was stopped and started anti-allergic and hepatoprotective treatment. After improvement in liver function, the patient's treatment plan underwent multiple changes due to adverse drug reactions, including a change in the antituberculosis drug regimen to levofloxacin, isoniazid, ethambutol, and rifapentine.

Imaging revealed an enlargement of a lung cavity, pleural thickening, and pleural effusion on the right, and cord-like shadows in both lungs (Fig. 3) on February 13, 2023, which prompted additional hospitalization for diagnosis and treatment. AFB smear tests yielded negative results on both day 2 and day 3 after admission, and common bacterial cultures were negative on day 4. However, the results of nanopore sequencing detected *Mycobacterium tuberculosis* (4263 reads), *Klebsiella pneumoniae* (3302 reads), and *Pneumocystis yersoni* (1 read) on day 2, along with resistance to β-lactams, fluoroquinolones, rifampicin, isoniazide, and ethambutol, identified by *blaTEM*, *blaSHV*, *gyrA*, *rpoB*, *katG*, and *embB*. On day 3 of hospitalization, Xpert MTB/RIF results indicated resistance to rifampicin. Based on the molecular findings from nanopore sequencing and Xpert RIF/RIF, the antituberculosis treatment was changed from pasiniazid, rifapentine, levofloxacin, and ethambutol to bedaquiline, linezolid, cycloserine, clofazimine, and protionamide. Netilmicin was also administered for other bacterial infection. After 25 days of hospitalization, precise diagnosis and treatment led to the improvement of the patient's symptoms, and no adverse drug reactions were observed.

Case 23

A 58-year-old male patient was diagnosed with tuberculosis about a year ago and presented with symptoms of cough and white sputum. He was initially treated with antituberculosis drugs for 2–3 months, which relieved the cough, and he stopped the treatment on his own. On January 25, 2023, the patient was admitted to another hospital for chest tightness, asthma, and hemoptysis. The detection results of *Mycobacterium tuberculosis* antibody and AFB test were positive after the hospitalization. Isoniazide, rifapentine, pyrazinamide, ethambutol, linezolid, and moxifloxacin were used as antituberculosis treatment. After his discharge, there were no further episodes of hemoptysis, and he was diagnosed with cavitary pulmonary tuberculosis, with positive sputum microscopy for tuberculosis retreatment, as well as chronic obstructive pulmonary disease.

On February 24, 2023, he was admitted to our hospital with a history of coughing and sputum production for 1 year and chest tightness and asthma for over 2 months. Chest CT showed pulmonary tuberculosis with multiple cavity formation, multiple spots, nodules, and cord-like shadows in both lungs (Fig. 4). Laboratory test abnormalities on the first day of admission included

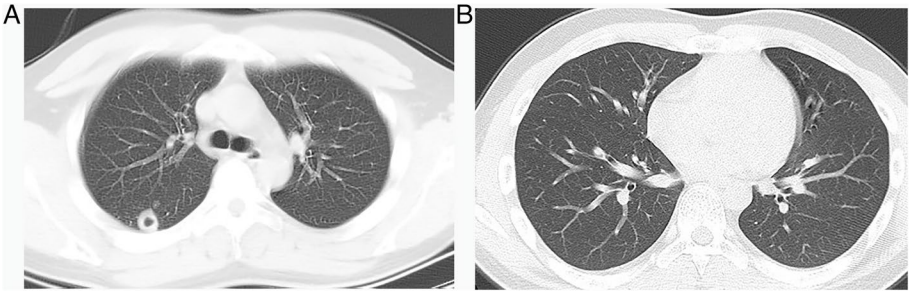

Fig. 3 Computed tomography scan of the chest in case 21. Cavitation, pleural thickening, and pleural effusion in the right lung (**A**). Nodules, spots, and cord shadows were observed in the right lower lobe and right upper lobe of the lung (**A**, **B**)

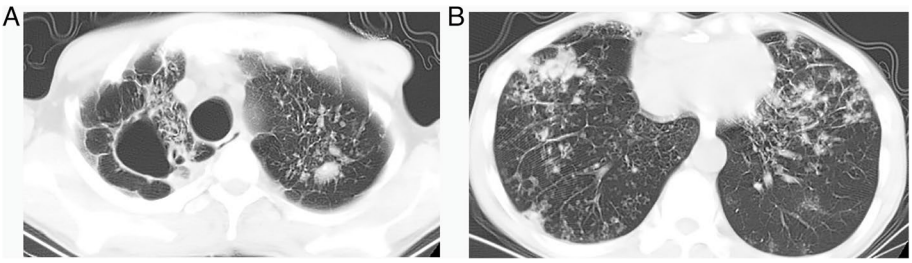

Fig. 4 Computed tomography scan of the thorax in case 23. There is a cavity in the right upper lobe (**A**), and multiple spots, nodules, and cords were observed in both lungs (**A**, **B**). Dilated bronchi were shown in some segments of the bronchus

WBC 16.9×10^9/L, CRP 132 mg/L (reference range: 0–10), ESR 45 mm/h (reference range: 0–15), and IL-6 20.85 pg/mL (reference range: < 7 pg/mL). The results of the five indicators test for hepatitis B indicated that he was a hepatitis B surface antigen carrier. Sputum AFB was negative on the first day of admission. The common bacterial culture and cryptococcal polysaccharide antigen were negative, and *M.tb* was detected by Xpert MTB/RIF without rifampicin resistance on the third day of admission. Five days after antituberculosis treatment, the inflammatory indicators were slightly elevated with CRP 117.99 mg/L, IL-6 67.2 pg/mL, and WBC 18.6×10^9/L. Based on the isoniazide, levofloxacin, pyrazinamide, and ethambutol antituberculosis treatment, piperacillin, tazobactam, imipenem, and etimicin were added to the anti-infection regimen.

However, 3 days after antituberculosis and anti-infection, the inflammatory indicators remained high with CRP 120.19 mg/L, ESR 80 mm/h, IL-6 102.0 pg/mL, WBC 15.5×10^9/L, and BNP 497.7 ng/L. Bronchoscopy was performed on March 06, 2023, to further search for evidence of an epidemic factor. The cell morphology of bronchoalveolar lavage fluid was consistent with infection, and no malignant tumor cells were found. In addition, AFB staining was negative. Finally, *Mycobacterium tuberculosis* (22,194 reads), *Aspergillus gracilis* (45 reads), and *Candida albicans* (49 reads) were identified by BLAF using nanopore sequencing. Voriconazole was used for anti-fungal therapy and entecavir for anti-HBV therapy. Inflammatory indicators gradually decreased with CRP 52.26 mg/L, ESR 69 mm/h, IL-6 32.2 pg/mL, and WBC 12.0×10^9/L. After 19 days admission, the patient was discharged with improved cough, reduced chest tightness and asthma, and no other symptoms or adverse drug reactions.

Discussion

In this study, infection with more than one species of pathogen is described as a mixed infection or co-infection. The differentiation of mixed infection plays an important role in the accurate diagnosis, appropriate treatment, and control of TB [13], particularly when the patient's diagnostic or treatment outcomes are not as expected. This series documented the availability of nanopore sequencing for differentiating the co-infection from a single infection. We report 20 cases of PTB and 3 patients with NTM identified by nanopore sequencing. Moreover, these cases revealed the challenges in diagnosing and treating tuberculosis, particularly in the context of drug resistance and co-infection.

Timely detection of mixed infection was available by nanopore sequencing, and these three cases were accurately diagnosed and effectively treated. The patient of case 1 was repeatedly hospitalized and treated for recurrent symptoms and imaging evidence (CT and tracheobronchoscopy) of pulmonary tuberculosis (PTB) complicated with extrapulmonary tuberculosis (EPTB). Early diagnosis and management of EPTB are challenging due to the low detection rate [14]. Additionally, our study results indicate that nanopore sequencing can be used to accurately characterize the microbe. The patient was concurrently infected with multiple microorganisms, including *Mycobacterium abscessus*, *Mycobacterium tuberculosis*, *oxytoca*, and *Enterobacter cloacae*. Besides resistance to multiple first-line and second-line anti-TB drugs, the patient exhibited antimicrobial resistance to β-lactam antibiotics. Pneumonia caused by *Klebsiella oxytoca* has a similar presentation to PTB [15]. Physicians should be aware that pneumonia caused by *Klebsiella* may occur in patients with PTB, especially TB patients with chronic lung disease [16]. Empiric antibiotic treatment with β-lactams is not appropriate, because carbapenem-resistant *Klebsiella oxytoca* complexes are increasingly detected [17]. The delay in diagnosis of XDR-TB and mixed infection may have contributed to the deterioration of the patient's condition and the need for multiple treatment adjustments.

Lung cavitation is a hallmark of tuberculosis and is associated with antibiotic resistance [18]. Previous study suggests that TB and bacterial co-infection may

be more likely among patients with cavitary lesions on chest radiography. *Klebsiella* and *Pseudomonas* were the predominant causes of respiratory infections with and without TB co-infection [19]. The patient in case 21 initially responded to treatment but later relapsed, demonstrating the persistence of TB even after apparent recovery. AFB smear and common bacterial culture were negative, but *Mycobacterium tuberculosis* (resistance gene mutations were detected in *gyrA*, *rpoB*, *katG*, and *embB* conferring resistance to fluoroquinolones, rifampicin, isoniazide, and ethambutol, respectively) and *Klebsiella pneumoniae* (resistance gene mutations were detected in *blaTEM* and *blaSHV* conferring resistance to β-lactam antibiotics) were identified by nanopore sequencing, 2 days after hospital admission. Xpert MTB/RIF was also positive. *M.tb* was finally confirmed by mycobacterial culture and AFB smear tests 24 days after hospitalization. The discovery of nanopore sequencing prompted a critical shift in the patient's treatment plan. A certain combination of drugs was tailored to combat the drug resistance identified. The patient's symptoms improved after receiving an appropriate drug regimen. It is important to tailor treatment for drug-resistant TB according to the timely diagnosis of nanopore sequencing.

Previous studies have shown that pulmonary tuberculosis and chronic pulmonary aspergillosis (CPA) may coexist, making diagnosis difficult due to similar clinical and radiological features [20, 21]. Case 23 was a pulmonary tuberculosis patient with CPA, who carried hepatitis B surface antigen. Culture and AFB smear results were negative. *Mycobacterium tuberculosis*, *Aspergillus gracilis*, and *Candida albicans* were identified by nanopore sequencing. For this patient, we speculated that the delayed diagnosis of co-infection may have contributed to the patient's prolonged inflammatory response. Previous studies have also suggested that pulmonary fungal colonization or infection in bacteriologically negative patients presenting with clinical symptoms has been associated with poor treatment outcomes in PTB patients [22]. The patient's symptoms finally improved after confirmation of the pathogens by nanopore sequencing and a combination of anti-TB and antifungal therapies. TB and other bacterial or fungal mixed infections are very common in clinical experience. Missed diagnosis of the pathogen spectrum may lead to poorer outcomes [23]. A complex pathogen spectrum of infection requires comprehensive diagnosis and treatment measures.

Conclusion

In conclusion, these cases highlight the challenges of diagnosing and treating tuberculosis, especially in cases of drug resistance and co-infection. Nanopore sequencing is the effective tool to clinically detect mixed infections and guide treatment decisions. Tailoring treatment regimens to individual patient profiles is crucial for achieving successful outcomes in complex TB cases.

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Authors' contributions
WS and WC contributed to the study conception and design. Material preparation, data collection, and analysis were performed by WS, LY, MS, ZW, WX, YL, and TW. The first draft of the manuscript was written by WS, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was performed in accordance with the principles stated in the Declaration of Helsinki and approved by Ethics Committee of the Jiangxi Provincial Chest Hospital. Written informed consent was obtained from all the participants before the study began.

Consent for publication
Written informed consent for publication of their details was obtained from the patients.

Competing interests
The authors declare that they have no competing interests.

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