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Research article

Laboratory tests and analysis of drug resistance in non-tuberculous mycobacteria

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ABSTRACT

Background: This study analyzed the laboratory diagnosis results and drug resistance of patients infected with *non-tuberculous mycobacterium* (NTM).

Methods: We collected information on patients with positive indicators of NTM infection at the Henan Provincial Chest Hospital from 2020 to 2022. Acid-fast smear, mycobacterium culture, QB-SPOT assay, GeneXpert MTB/RIF assay, immunoglobulin E test, tuberculosis antibody test, and microplate method for drug sensitivity test were analyzed using strain identification as the gold standard.

Results: The 242 cases of NTM infection were predominantly detected with slow-growing *mycobacteria* (a detection rate of 87.19%), among which *Mycobacterium intracellulare* (66.53%), *Mycobacterium avium* (15.70%), and *Mycobacterium chelonei/abscessus* complex (11.16%) ranked the top three in terms of the isolation rate. Males patients accounted for a higher proportion (58.26%) than females (41.74%), and the majority of them were over 60 years (50.83%). Among laboratory tests for patients with NTM infection, mycobacterium culture showed a highest detected rate (87.20%) among laboratory tests. The results of the drug sensitivity test demonstrated that the resistance rate of NTM was generally high. Moreover, the *Mycobacterium avium* complex with the highest isolation rate showed 100% resistant to doxycycline and minocycline, but exhibited relatively high sensitivity to moxifloxacin (a resistance rate of 7.89%) and rifabutin (a resistance rate of 13.16%). The *Mycobacterium chelonei/abscessus* complex was 100% resistant to doxycycline and relatively sensitive to cefoxitin (29.17%) and clarithromycin (37.50%).

Conclusion: The NTM species isolated by the Henan Provincial Chest Hospital is dominated by *Mycobacterium intracellulare* and the highest positive rate is detected by mycobacterium culture among laboratory tests. NTM infection generally exhibits a high rate of drug resistance. Accordingly, the accurate diagnosis of NTM diseases requires enhanced drug sensitivity testing to provide patients with targeted combination drug treatment.

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1. Background

Non-tuberculous mycobacteria (NTM) is a collective name given to a large group of mycobacterium excluding *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*. With the development of strain identification technology, NTM is now found to be consisted of more than 190 species and 14 subspecies, among which most are parasites and only a few are pathogenic to humans and belong to conditional pathogenic bacteria [1–4]. NTM can commonly be found in water, soil, dust, and other natural environmental resources and can infect both humans and animals. Infections primarily affects lungs, lymph nodes, and skin. Although NTM infection has similar imaging, clinical, and pathological manifestations to tuberculosis without specificity, it has a high rate of resistance against the anti-tuberculosis drugs. Therefore, the clinical treatment options for NTM infection are significantly different from those of tuberculosis [5,6]. Besides, although there have been several scientific researches investigating the drug-resistance of NTM in other countries, such as in Tanzania and Russia, the similar exploration is lacked in China, which has become urgent for better treatments for NTM patients in China [7,8]. As a result, laboratory tests, including pathogenic diagnosis drug sensitivity tests, are critical for the clinical differential diagnosis of NTM patients [9]. Herein, this study analyzed data from patients infected with NTM strains from 2020 to 2022 identified at the Henan Provincial Chest Hospital, and assessed data from laboratory tests and results of mycobacterial drug sensitivity test by the microplate methods to provide a scientific foundation for the prevention and treatment of NTM infection.

2. Methods

2.1. Participants

Our study included 242 NTM-positive patients who underwent laboratory tests and strain identification at the Henan Provincial Chest Hospital, a tertiary care center, from 2020 to 2022. Ethical approval of this study was granted by the Ethics Committee of Henan Provincial Chest Hospital (Number: 20211212).

2.2. Methods

2.2.1. Strain identification

2.2.1.1. Microarray technology. Microarray technology (Capitalbio Technology Co., Ltd., Chengdu, China) was used for strain identification. Gene microarray microdrop technology was used to detect fluorescently labeled DNA fragments, followed by the analysis of Mycobacterium species by comparing the arrangement of specific positions on the microarray.

2.2.1.2. Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). The suspected NTM strain was identified by MALDI-TOF-MS using a time-of-flight mass spectrometer (Chongqing Zhongyuan Biological Technology Co., Ltd., Chongqing, China).

2.2.1.3. Melting curve technology. Different strains of mycobacterium were identified with a fluorescent polymerase chain reaction (PCR) melting curve method, where specific fluorescent channels and melting points were utilized to detect mycobacterium-specific nucleic acid fragments. In this study, the Mycobacterium Identification Kit by Zeesan Biotech (Xiamen, China) was used.

2.2.2. Acid-fast staining

The acid-fast staining solution (cold staining method) was provided by Baso (Zhuhai, China). The modified basic fuchsin method was used for the chemical staining of acid-fast bacteria such as mycobacterium.

2.2.3. Mycobacterium culture

Mycobacterium culture was performed with the BACTEC MGIT 960 Automatic Mycobacterium Detection System. After the instrument automatically reported positive results, the bacterial suspension was collected for acid-fast staining, and then seeded on blood agar plates to exclude common bacterial contamination. The strain was identified as NTM.

2.2.4. Drug sensitivity test of mycobacterium

The NTM bacterial suspension of 1.0 Mack turbidity was prepared. The microplate method for the drug sensitivity test (Encode Medical Engineering Co., Ltd., Zhuhai, China) was utilized for analyzing the resistance of NTM to the Clinical and Laboratory Standards Institute susceptibility testing standard M24-recommended 14 drugs for NTM, including ethambutol (EMB), clarithromycin (CLR), linezolid (LZD), rifabutin (RFB), rifampicin (RFP), gatifloxacin (GAT), doxycycline (DOX), cefoxitin (FOX), tobramycin (TOB), sulfamethoxazole (SMZ), minocycline (MIN), moxifloxacin (MFX), azithromycin (AZM), and amikacin (AK).

2.2.5. Immunoglobulin E (IgE) test

The IgE turbidity in the serum of NTM-infected patients was detected by a Chemiluminescence immunosandwich assay using the human IgE assay kit (chemiluminescence method; Snibe, Shenzhen, China).

2.2.6. QB-SPOT assay

An enzyme-linked immunospot assay was performed to detect tuberculosis-specific antigen-stimulated effector T cells in lymphocytes obtained from freshly drawn anticoagulated peripheral venous blood of patients. The detection reagents used in this study were purchased from Beijing Kinghawk Pharmaceutical Co., Ltd. (Beijing, China).

2.2.7. Tuberculosis antibody test

Mycobacterium tuberculosis IgG antibodies in the serum of patients were qualitatively detected with the detection reagents of three tuberculosis antibodies (Beijing Beier Medical Equipment Company, Beijing, China; Shanghai UPPER Biotech Pharma Co., Ltd., Shanghai, China; Shandong Kanghua Biotechnology Co., Ltd., Shandong, China). Positive results for any of the antibodies were considered positive results.

2.2.8. GeneXpert MTB/RIF assay

The 81-bp RFP resistance core region of rpoB of *Mycobacterium tuberculosis* in the samples was detected by semi-nested real-time fluorescence quantitative PCR technology using the fully automated medical PCR system (Cepheid, Shanghai, China) to identify whether the bacteria were *Mycobacterium tuberculosis* and the RFP resistance status.

2.3. Statistical analysis

The distribution of 242 strains of NTM infection was first investigated, and the specific gender and age distribution of these cases were analyzed. Then the corresponding laboratory test results were listed to explore the pathological features of NTM. We also represented the identification approaches for 114 NTM strains to assess the drug susceptibility. All the data are shown in Tables 1–3 and Figs. 1–2 with the case/strain number and percentage. ANOVA was used to determine differences between research results from three or more groups.

2.4. Flow chart of methodology

The representative chart showing the methodology of our study is presented as Supplementary Fig. 1.

3. Results

3.1. Strain identification results

3.1.1. NTM isolation rates

Based on clinical features, imaging, and laboratory tests, from 2020 to 2022, a total of 1316 cases with *Mycobacterium tuberculosis* complex and 242 cases with NTM infection was identified at the Henan Provincial Chest Hospital, with an isolation rate for NTM of 15.53%. Among the 242 cases of nontuberculous mycobacteria infection, 139 strains were obtained from sputum samples, 92 strains were from alveolar lavage fluid samples, and 7 strains were isolated from pus samples, 3 strains from pleural fluid and 1 strain from wound secretions. Through strain identification The 242 cases with NTM infection comprised 211 strains of slow-growing mycobacterium (87.19%), including 161 strains of *Mycobacterium intracellulare* (66.53%), 38 strains of *Mycobacterium avium* (15.7%), 11 strains of *Mycobacterium kansasii* (4.55%), and 1 strains of *Mycobacterium smegmatis* (0.41%). There were 31 strains of fast-growing mycobacteria, accounting for 12.81%, including 4 strains of *Mycobacterium fortuitum* (1.65%), 27 strains of *Mycobacterium chelonei/abscessus* (11.16%) (Table 1). Collectively, slow-growing mycobacterium, especially *Mycobacterium intracellul*are, is the most common infection of NTM infection.

3.1.2. Gender and age distribution of cases with NTM infection

Gender and age distribution of cases with NTM infection were further analyzed. Among 242 patients with NTM infection, males (58.26%) were more than females (41.74%). The number of patients was statistically different among the three age groups of <40, 41–60, and >60 years ($p = 0.033$). The mean age was 63.28 ± 12.22 years for male patients and 61.07 ± 14.50 years for female

Table 1
Distribution of 242 strains of NTM.

Strain	Number of strains (n)	Percentage (%)
Slow-growing mycobacterium	211	87.19
Mycobacterium intracellulare	161	66.53
Mycobacterium avium	38	15.70
Mycobacterium kansasii	11	4.55
Mycobacterium smegmatis	1	0.41
Rapid-growing mycobacterium	31	12.81
Mycobacterium fortuitum	4	1.65
Mycobacterium chelonei/abscessus complex	27	11.16
Total	242	100

Table 2
Methods and results of identification of 114 NTM strains for drug susceptibility testing.

Strain	Microarray technology(n)	Melting curve technology(n)	MALDI-TOF-MS(n)	Total(n)
M. avium complex	57	8	11	76
M. abscessus complex	14	5	3	22
M. kansasii	3	0	0	3
M. lentiflavum	0	3	2	5
M. timonense	0	0	3	3
M. fortuitum	1	1	0	2
M. szulgai	0	0	1	1
M. gordonae	0	0	1	1
M. xenopi	0	1	0	1

patients, which was statistically different ($p < 0.05$) (Fig. 1).

3.2. Laboratory test results

Laboratory tests to identify the NTM infection were performed here. Acid-fast staining was conducted on 165 patients, among which 77 patients were positive, with a positive rate of 46.67%. Mycobacterium culture was conducted on 211 patients, and 184 patients (87.20%) had positive results. For the QB-SPOT assay, 79 of 202 patients were positive (>6), with a positivity rate of 39.11%. For the GeneXpert MTB/RIF assay, 24 of 194 patients (12.37%) were positive. Additionally, 41 patients were tested for IgE, where the value < 190 was considered negative, and among them, 34 patients were positive, with a positive rate of 17.07%. Tuberculosis antibodies were tested in 141 patients, with positive results observed for 77 patients (54.61%) (Fig. 2). The culture positivity rate significantly exceeded that of the other five laboratory tests.

3.3. Drug sensitivity test results

3.3.1. Drug sensitivity test results of 114 cases of non-tuberculous mycobacterium infection

A total of 114 experimental strains of mycobacterium was tested for drug sensitivity between 2020 and 2022, and strain identification was performed by gene microarray, melting curves, and MALDI-TOF-MS. The results exhibited that the 114 NTM infections were dominated by *Mycobacterium avium-intracellulare* complex and *Mycobacterium chelonei/abscessus* complex (Table 2).

The 76 strains of *Mycobacterium avium* showed the highest rate of resistance to DOX (100%) and MIN (100%), followed by AZM (93.42%), GAT (92.11%), SMZ (82.89%), TOB (80.26%), LZD (78.95%), AK (56.58%), FOX (40.79%), CLR (43.42%), EMB (38.16%), RFP (25%), RFB (13.16%), and MFX (7.89%). The drug resistance rates of the 22 strains of *Mycobacterium chelonei/abscessus* complex were as follows: DOX (100%), EMB (95.45%), TOB (95.45%), SMZ (95.45%), RFP (90.91%), RFB (90.91%), GAT (86.36%), MIN (86.36%) AZM (86.36%), MFX (68.18%), LZD (63.64%), AK (45.45%), CLR (36.36%), and FOX (31.82%) (Table 3). The 90 strains of slow-growing NTM had the highest rate of resistance to DOX and MIN (97.78%) and the highest rate of sensitivity to MFX (7.78%).

As for the drug resistance of fast-growing NTM, we only analyzed the resistance to fast-growing NTM-sensitive drugs recommended by the Guidelines for the diagnosis and treatment of non-tuberculous mycobacteria diseases (2020 edition). The data revealed the 24 strains of fast-growing NTM were the most resistant to DOX (100%) and more sensitive to FOX (37.5%) and CLR (29.17%). The resistance rate of the remaining antimicrobials was higher than 40%.

4. Discussion

The epidemiology of NTM infection diseases remains currently challenging to study due to the lack of precise information and data in different countries or regions [10]. In clinical practice, the differential diagnosis of NTM diseases is also difficult, and the incidence and prevalence of NTM infection varies significantly among studies [1]. The 2010 epidemiological survey of tuberculosis in China demonstrated an NTM isolation rate of 22.9% [11]. As the onset and detection of NTM are greatly affected by climate and environmental factors, leading to the varying isolation rate of NTM from province to province. For example, the isolation rate in Yunnan Province is approximately 2% [12], while the number rises to 25.8% in 2014 in the Hangzhou [13], and reaches 30% in Guangzhou [14]. In the current study, the isolation rate of NTM is 15.53%, which was markedly lower than the domestic average rate but consistent with the findings by Yu et al. [15]. Among 242 cases with NTM infection, 211 NTM strains were slow-growing mycobacterium, while 31 strains were fast-growing mycobacteria.

The incidence of NTM infection is strongly influenced by age and gender. It is reported the disease prevalence increases with age [16,17], and the risk for NTM infection was significantly higher among persons ≥65 years of age [18]. Of note, our results revealed that NTM infection predominantly occurred in males older than 60 years, with a statistically significant difference, which is consistent with relevant reports [19–21]. We hypothesized that NTM infection might be related to the aging of the population and poor lifestyle habits such as smoking in older men. Further investigations might discover more risk factors in the further.

Among laboratory tests in our study, acid-fast staining had a detection rate of 46.67% for NTM infection. However, this method cannot directly distinguish between *Mycobacterium tuberculosis* and NTM. Isolating culture, one of the most sensitive techniques for NTM detection, demonstrated a higher detection rate of 87.20%. For this method, a positive culture can be further subjected to other

Table 3
The drug resistance rate of NTM (%).

	Number of strains (n)	EMB	CLR	LZD	RFB	RFP	GAT	DOX	FOX	TOB	SMZ	MIN	MFX	AZM	AK
Mycobacterium avium-intracellulare complex	76	38.16	43.42	78.95	13.16	25	92.11	100	40.79	80.26	82.89	100	7.89	93.42	56.58
Mycobacterium chelonei/abscessus complex	22	95.45	36.36	63.64	90.91	90.91	86.36	100	31.82	95.45	95.45	86.36	68.18	86.36	45.45
Mycobacterium kansasii	3	66.67	0	0	0	0	33.33	100	100	100	100	100	33.33	100	66.67
Mycobacterium lentiflavum	5	100	60	80	60	60	60	80	80	80	80	80	0	80	60
Mycobacterium timonense	3	100	100	100	0	33.33	33.33	100	33.33	100	100	100	0	100	100
Mycobacterium fortuitum	2	50	50	100	100	100	100	100	0	100	100	100	0	50	100
Mycobacterium szulgai	1	100	100	100	100	0	100	100	100	100	100	100	0	100	100
Mycobacterium gordonae	1	0	0	0	0	0	100	100	100	100	100	100	0	100	100
Mycobacterium xenopi	1	0	0	0	0	0	0	0	0	0	0	0	0	100	0
The mean drug resistance rate (%)		53.51	42.98	72.81	31.58	40.35	85.96	98.25	42.98	84.21	85.96	95.61	19.3	91.23	56.14

Notes:EMB:ethambutol, CLR:clarithromycin, LZD:linezolid, RFB:rifabutin, RFP:rifampicin, GAT:gatifloxacin, DOX:doxycycline, FOX:cefoxitin, TOB:tobramycin, SMZ:sulfamethoxazole, MIN:minocycline, MFX:moxifloxacin, AZM:azithromycin, AK:amikacin.

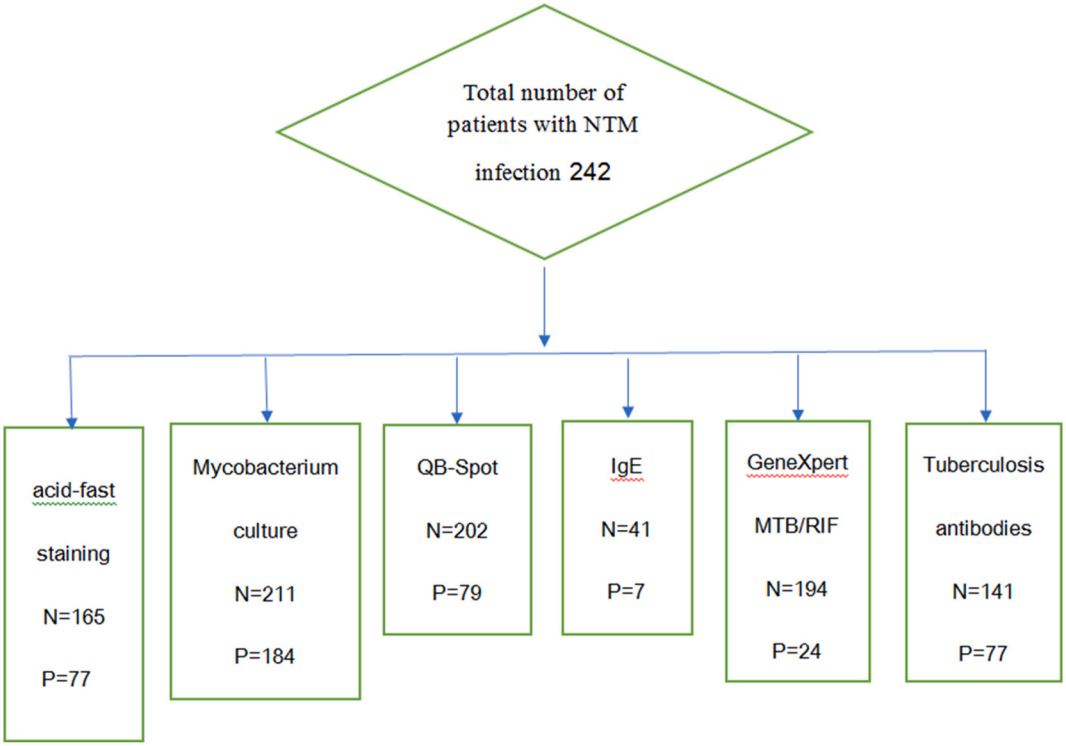

Fig. 1. The diagram showing the gender and age distribution of cases with NTM infection.

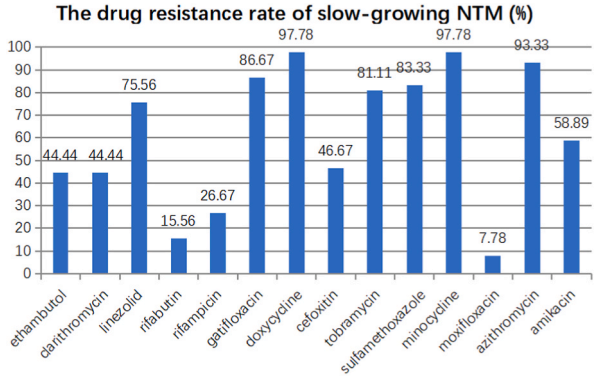

Fig. 2. Bar chart of the laboratory test results.

relevant tests, such as the drug sensitivity test or molecular drug sensitivity test, using the positive strains for further identification. Accordingly, it can be recommended in the clinic use for patients with suspected NTM infection, with the lesion specimens obtained for isolating culture of mycobacterium. For the QB-SPOT assay, 39.11% of patients were positive, which was inconsistent with the principle of this assay. The analysis of our clinical data elucidated that among 202 cases receiving the QB-SPOT assay, the positive rate was 34.48% (60/174) after excluding 28 patients with mixed *Mycobacterium tuberculosis* infection, indicating that when the result of the QB-SPOT assay is positive in the clinic, tuberculosis infection cannot be considered alone, and the false positive rate is relatively high in patients with NTM infection. The positive rate of the GeneXpert MTB/RIF assay was 12.37%, again contrary to the principle of this assay. Through the analysis of clinical data, it was found that all 24 positive patients suffered from mixed *Mycobacterium tuberculosis* infection. As reported, the specificity of the GeneXpert MTB/RIF assay was 100% for the detection of NTM infection [22]. In combination of clinical data with positive results of acid-fast staining, the negative results of the GeneXpert MTB/RIF assay illustrate that NTM infection may be considered [23]. Serum IgE level is mainly upregulated in allergic diseases. In our study, the positive rate of IgE elevation was 17.07% in patients with NTM infection. Hence, this laboratory index cannot be reliably used for the laboratory monitoring of patients with NTM infection. In the tuberculosis antibody test, 77 patients exhibited positive results, and the positive rate of tuberculosis antibodies was 48.8% (61/125) in patients with NTM infection after excluding 16 cases of mixed *Mycobacterium*

tuberculosis infection, suggesting that the false positive rate of the tuberculosis antibody test is relatively high in patients with NTM infection.

Since NTM infectious patients present nonnegligible proportion of all tuberculosis patients, and NTM infection often exhibit significant drug resistance. Therefore, the accurate diagnosis of NTM infection is essential for finding more effective treatment for patients. Our study first compared the detection rates of NTM with different testing methods and found that mycobacterium culture is the most specific laboratory diagnostic method, which we believe could provide clinical diagnosis with effective guidance. More importantly, through drug sensitivity tests on NTM strains isolated from patients, we discovered that slow-growing NTM is most sensitive to MFX, while fast-growing NTM is most sensitive to CLR. These results provide drug selective guidance for NTM patients and also provide important clinical clues for the following resistance mechanism researches in the future.

The mechanism of NTM resistance is complex, which may be attributed to factors such as barrier action of cell wall, efflux pump system, drug inactivation enzyme, and mutation or absence of drug action targets, which make it highly resistant to most anti-tuberculosis drugs. The results of the drug sensitivity test elaborated that the clinical isolates of NTM generally had a high resistance rate, with the resistance rate varying from strain to strain [24,25]. Among the drugs recommended by the guidelines for the diagnosis and treatment of non-tuberculous mycobacteria infection (2020 edition), the resistance rate of fast-growing NTM to cefoxitin was relatively low (29.17%). Slow-growing NTM had a lower drug resistance rate than fast-growing NTM and could be treated with a combination of anti-tuberculosis drugs, such as RFB and MFX.

However, there are still some limitations of our study. Firstly, the number of patients is relatively small, as we only collected samples of patients from 2020 to 2022, and from Henan Provincial Chest Hospital. In the future, we are going to expand the sample size and engage in multi-center collaboration to investigate the potential impact of regional factors on our findings. Besides, the mechanisms of how NTM engaged drug resistance is less explored in our study, which is also less investigated compared to the researches around tuberculous mycobacterium. Thus, we aim to perform the genomics and transcriptomics analysis of drug-resistant NTM, to identify the drivers of drug resistance in NTM.

5. Conclusion

Our analysis of clinical data highlighted the notable proportion of mixed *Mycobacterium tuberculosis* infection in patients with NTM infection that should not be ignored. Specifically, the structural changes in lungs occur during the treatment of tuberculosis patients, with co-infection with NTM based on bronchial lesions, or patients are co-infected with these two bacteria at the onset of the disease, both of which require high clinical attention during treatment. In conclusion, the epidemiological survey and drug sensitivity test of NTM infection should be promptly conducted and the accurate judgment is needed in clinical practice.

Ethics approval and consent to participate

This study was designed in accordance with the Declaration of Helsinki and approved by the ethics committee of Henan Provincial Chest Hospital, Zhengzhou University, Henan Province Clinical Medical Research Center for Infectious Diseases (Tuberculosis). Informed consent was waived for this retrospective study design.

Consent for publication

Not applicable.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Jiao Tan: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Yachun Wang:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Zheng Li:** Writing – review & editing, Methodology, Data curation. **Shuang Xia:** Writing – review & editing, Validation, Formal analysis. **Zhen Guo:** Writing – review & editing, Methodology, Conceptualization. **Wenbo Li:** Writing – review & editing, Validation, Data curation. **Yingying Yuan:** Writing – review & editing, Data curation, Conceptualization. **Jingcai Gao:** Writing – review & editing, Methodology, Formal analysis. **Wei Wang:** Writing – review & editing, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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List of abbreviations

NTM	*non-tuberculous mycobacterium*
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
EMB	ethambutol
CLR	clarithromycin
LZD	linezolid
RFB	rifabutin
RFP	rifampicin
GAT	gatifloxacin
DOX	doxycycline
FOX	cefoxitin
TOB	tobramycin
SMZ	sulfamethoxazole
MIN	minocycline
MFX	moxifloxacin
AZM	azithromycin
AK	amikacin
IgE	Immunoglobulin E

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28665.

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