ORIGINAL RESEARCH

Epidemiology of Nontuberculous Mycobacteria in Nanjing and *MAB_0540* Mutations Associated with Clofazimine Resistance in *Mycobacterium abscessus*

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Background: Nontuberculous mycobacteria (NTM) are easily misdiagnosed as multidrug-resistant tuberculosis (MDR-TB), and treatment drugs are very limited. The main objective of our study was to evaluate the minimal inhibitory concentration (MIC) in vitro of bedaquiline (BDQ), clofazimine (CFZ), linezolid (LZD), delamanid (DLM), and pretomanid (PA-824) for treatment of *M. abscessus* and *M. intracellulare*. Furthermore, we determined whether *MAB_1448, MAB_4384, MAB_2299c, MAB_1483, MAB_0540, rplD, rplC,* and *rrl* were related to drug resistance to provide an experimental basis for the use of these five drugs in the treatment of NTM.
Methods: We identified sample characteristics of epidemics in 550 patients with suspected NTM infection in Nanjing from 2019 to 2021 using the PCR-reverse spot hybrid method. Furthermore, we evaluated the MIC of BDQ, CFZ, DLM, LZD, and PA-824 against 155 clinical isolates of NTM using the microbroth dilution method. The resistant isolates were sequenced using Sanger sequencing.
Results: The top three dominant species of NTM distributed in Nanjing were *M. intracellulare, M. avium,* and *M. abscessus*. Notably, the proportion of *M. abscessus* infections increased. The proportion of *M. abscessus* increased from 12% in 2019 to 18% in 2021. Demographic analysis showed that female infection rates were substantialy greater than male for *M. abscessus* (P=0.017, <0.05). Our results demonstrate that NTM are highly sensitive to bedaquiline and clofazimine in vitro. However, delamanid and pretomanid had little effect on *M. abscessus* and *M. intracellulare*. In addition, we found 30–41 nucleotide deletion mutations and some novel point mutations in the *MAB_0540* gene of *M. abscessus* that are resistant to clofazimine.
Conclusion: Bedaquiline, clofazimine, and linezolid were more successful in vitro treatments against *M. abscessus* and *M. intracellulare*. The *MAB_0540* mutation may be associated with resistance of *M. abscessus* to clofazimine.
Keywords: nontuberculous mycobacteria, minimal inhibitory concentration, gene mutations

Introduction

Nontuberculous mycobacteria (NTM), a mycobacterium other than the *Mycobacterium tuberculosis complex* (MTC) and *M. leprae*, are conditional pathogens widely found in the environment.[1] Studies have shown that the probability of NTM transmission is extremely low, and exposure comes primarily from the environment, soil, water, etc.[1] In recent years, lung diseases caused by NTM have attracted widespread attention as disease prevalence and mortality have gradually increased. More than 190 species of NTM have been identified, and after 2015, the dominant species of NTM in China gradually changed from *M. abscessus* to *M. intracellulare*.[1,2] In recent years, NTM have shown growth trends worldwide, especially *M. abscessus*.[3–5] Currently, *M. abscessus* is the only species with evidence of human-to-human transmission. Furthermore, different species of NTM have different drug susceptibilities, especially *M. abscessus*, which is naturally resistant to a variety of first-line antituberculosis drugs.[3] In clinical practice, NTM are easily misdiagnosed as multidrug-resistant tuberculosis, which is a cause for concern.[6] Several studies have shown that immunocompromised elderly people are the primary population of NTM infection.[5,7,8] Patients infected with NTM usually have one or more underlying diseases, like tumors, surgical history, previous tuberculosis, and HIV.[5,9] Due to displaying similar clinical

and radiological characteristics to tuberculosis, NTM lung diseases were misdiagnosed in some cases, causing great inconvenience for clinical treatment and control of the occurrence and increased development of NTM.[6] Macrolides are the drugs of choice for the treatment of NTM infection,[10] but in recent years, the widespread use of macrolides has also predisposed bacteria to drug resistance, so there is a need to find new therapeutic agents.[11]

Bedaquiline (BDQ), clofazimine (CFZ), linezolid (LZD), delamanid (DLM), and pretomanid (PA-824) have been recommended by the WHO for the treatment of multidrug-resistant tuberculosis.[10] In recent years, some studies have attempted to use bedaquiline, clofazimine, and linezolid for NTM.[12–14] However, the in vitro susceptibility of NTM to these five drugs has been poorly reported and limited to local studies. Studies of linezolid resistance in NTM clinical isolates have identified resistance-associated mutations in *23S rRNA* (*rrl*), *rplC*, and *rplD*.[15] The potential resistance genes of *M. abscessus* to bedaquiline are *MAB_1448, MAB_4384*, and *MAB_2299c*.[13,16,17] Whole genome sequencing of *M. abscessus* against clofazimine revealed that the major genes linked to resistance were *MAB 2299c, MAB 1483*, and *MAB 0540*.[18] However, there have been no systematic studies on drug resistance-related gene mutations, and the mechanism of drug resistance remains unclear.

In this study, we investigated suspected NTM infections admitted from January 2019 to December 2021 in Nanjing Public Health Medical Center to explore the relationship between different species and age, sex, and previous medical history, further providing a reliable basis for the clinical treatment of nontuberculosis mycobacterial disease. In addition, we conducted in vitro drug susceptibility studies of NTM to bedaquiline, clofazimine, linezolid, delamanid, and pretomanid and sequenced the possible drug resistance-related genes to provide a basis for the development of new drugs for the treatment of NTM.

Materials and Methods
Methods
Species Identification by PCR-Reverse Spot Hybrid Method
M. tuberculosis and NTM were identified by PCR-reverse spot hybridization.[19] All reagents were obtained from the Mycobacterium species Identification Kit (Shenzhen Yaneng Bio, China).

DNA Isolation
Fresh colonies were picked for inactivation, and then samples were centrifuged at 13,000 rpm for 5 min. The MTB lotion solution was added to the sediment and centrifuged at 10,000 rpm for 2 minutes. Next, 50 μL of lysate was added and it was placed in a boiling water bath for 10 minutes, then centrifuged at 10,000 r/min for 2 minutes. The supernatant was used for PCR amplification.

PCR Test
PCR was carried out in a 25 μL reaction mixture including 4 μL DNA template. The first step was incubation at 95 °C for 10 min. This was followed by the amplification of the first 30 cycles (95°C for 45s, 68°C for 60s), then perform the amplification of the second 30 cycles (95°C for 30s, 54°C for 30s, 68°C for 60s). Finally, it was extended at 68°C for 10min. The instrument used to perform PCR was the ABI 7500 Quantitative PCR Instrument (Thermo Fisher Scientific, USA).

Hybridization
Next, solution A (2xSSC, 0.1% SDS) is added to the PCR reaction solution and placed in a boiling water bath for 10 min. The solution was removed and put into a hybridization box at 59°C for hybridization for 1.5 hours. The membrane strip was removed and it was continuously washed for 15 min in 40 mL of B solution (0.5 x SSC, 0.1% SDS) pre-warmed to 59°C. Finally, it was washed with supporting reagents and a color developer was added for 10 min to protect it from light. The instrument used in the hybridization process was the Thermostatic Hybridization Instrument (Shenzhen Yaneng Bio, China).

Minimum Inhibitory Concentration (MIC) Testing

NTM isolates (n=155) from 155 patients were cultured and preserved in L-J culture medium (Zhuhai Baso Bio, China) at the Nanjing Public Health Medical Center. Bacterial cultures and related procedures were performed in a negative-pressure laboratory.

NTM in vitro drug susceptibility assays were performed using the CLSI-recommended micro-broth dilution method.[20] Bedaquiline (Meilunbio, Dalian, China), clofazimine (Macklin, Shanghai, China), linezolid (Meilunbio, Dalian, China), delamanid (TargetMol, Boston, USA), and pretomanid (TargetMol, Boston, USA) were dissolved in dimethyl sulfoxide (DMSO). The ranges of drug concentration were as follows: bedaquiline, 0.004 to 2.048 µg/mL; clofazimine, 0.016 to 8 µg/ mL; linezolid, 0.125 to 64 µg/mL; delamanid, 0.063 to 32µg/mL; and pretomanid, 0.125 to 64 µg/mL.

Middlebrook 7H9 liquid medium was supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and catalase solution before clinical use.

Microbroth Dilution Method

A culture plate was prepared on a 96-well plate with negative control set in the first column. Next, 200 µL of medium was added to each well. The second column added 180 µL of 7H9 liquid medium and 20 µL of drug reservoir per well. The third column to the 12th column added 100 µL of medium per well. The positive control is shown in column 12. Starting from column 2, 100 µL of liquid was aspirated and added to the next column, and the drug was diluted multiplicatively.

Bacterial Solution Preparation

Fresh colonies grown for 2–4 weeks were picked with an inoculation loop, and 0.5 McFarland standard concentration was prepared using an ultrasonic dispersion counter.

Inoculation

Except for the negative control wells, the rest of the well plates were incubated by adding 100 µL of bacterial solution, and each bacterium was tested twice in parallel. The rapidly growing Mycobacterium was cultured for 3–5 days, and the slow-growing Mycobacterium was cultured for 7–10 days. In addition, a blank plate was prepared and only 100 µL of culture medium and 100 µL of bacterial solution were added for observation of bacterial growth status.

Observation of Results

Alma Blue solution was prepared with a concentration of 1 mg/mL; 30 µL of Alma Blue solution was added to each well of the 96-well plates, and the results were observed within 24–48h. When the blue color changed to pink or purple, it was judged to be drug resistant, and the drug concentration corresponding to the last blue hole close to the color-changing hole had the lowest inhibitory concentration.

PCR and DNA Sequencing

The primers were designed using NCBI and Primer3plus,[21,22] and synthesized by Universalbiol (Anhui, China). PCR amplification of *MAB_1448, MAB_4384, MAB_0540, MAB_2299c*, and *MAB_1483* for *rplC, rplD*, and *rrl* for *M. abscessus* and *M. intracellulare*, respectively, was conducted under the reaction conditions described in Supplementary Table S1 and Figure S1. The PCR amplification products were purified using 1% agarose gel electrophoresis, the corresponding bands were cut, and the amplification products were sent to the General Biology Company (Anhui, China) for sequencing.

Quality Control

For each MIC experiment, *M. abscessus* was used to determine *Staphylococcus aureus* ATCC29213, and *M. intracellulare* were needed to determine *M. intracellulare* ATCC13950, to ensure that the MIC concentrations of the quality control strains were within the appropriate range.

Statistical Analysis

A bacterial species distribution study was conducted based on the number of detected bacterial species. Demographic data and clinical risk factor analyses were performed based on the number of patients. Statistical analyses were performed using SPSS22.0 software. All tests were two-sided probability tests, and statistical significance was set at $P < 0.05$. Sequencing results were analyzed by sequence analysis using SnapGene 6.0.2 software and compared with the sequences of standard strains on NCBI.[21] Multiple sequence alignment was performed on the website ESPript 3,[23] using *M. tuberculosis* H37Rv protein as the basis for homologous protein sequence alignment.

Results

Distribution of Species in NTM

Between 2019 and 2021, 550 patients at Nanjing Public Health Medical Center were identified as having a NTM infection. Among them, 37 patients were infected with two NTMs simultaneously, three patients with three NTMs, and one patient with four NTMs. Therefore, 597 cases of NTM were identified.

Ten types of NTM were detected between 2019 and 2021 (Figure 1). The results showed that *M. intracellulare* (MIN) accounted for up to 50% of cases (95% CI: 46.4%–54.4%), followed by *M. avium* (MAV) at 17% (95% CI: 14.2%–20.3%), *M. abscessus* (MAB) at 13% (95% CI: 10.2–15.6%), *M. Gordon* (MGO) at 7% (95% CI: 5.1–9.3%), and *M. kansasii* (MKA) at 6% (95% CI: 4.1–7.9%).

In recent three years, *M. intracellulare, M. abscessus*, and *M. avium* have been the dominant species of NTM in Nanjing. There has been a decline in NTM infections; however, it should be noted that the prevalence of different strains has also changed (Figure 2). Infections from *M. abscessus* have shown an increasing trend. The proportion of *M. abscessus* was 12%, 11%, and 18%, respectively, from 2019 to 2021. Furthermore, the proportion of women infected with *M. abscessus* has been increasing annually.

Demographic Characteristics of Patients Infected with NTM

Among the 550 patients, 54.2% (298/550) were male and 45.8% (252/550) were female, with a male-to-female ratio of 1.2 to 1 (Table 1). The results showed that the risk factors for NTM infection were related to sex (P=0.003, <0.05), and men were more likely to be infected with NTM. The male age distribution ranged between 30–79 years old, while females were mainly between 50–69 years old. The minimum age of patients infected with NTM was 15 years old, the maximum was 90 years old, the average age was 56.82±16.90, and the median was 60. The population over 50 years of age accounted for 70.7%, and more than 50.5% of the patients were over 60 years old, indicating that NTM infection was
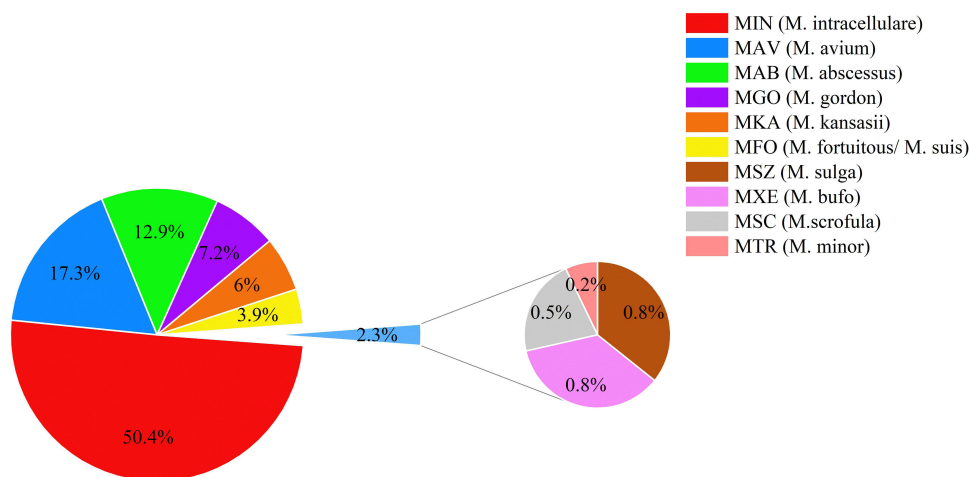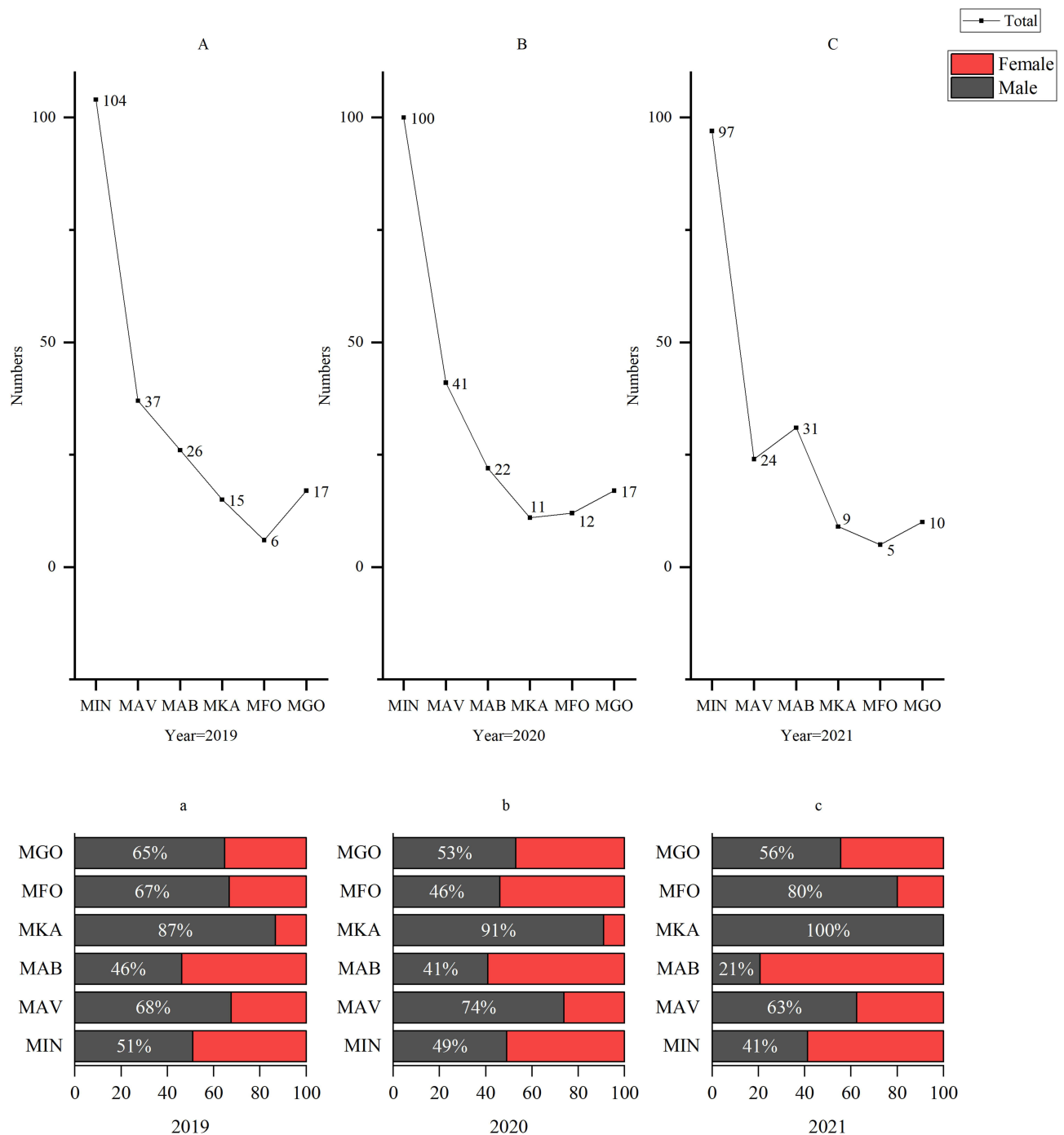

Figure 1 Distribution of NTM species.

Figure 2 The changes of six major NTM strains from 2019 to 2021. The number of patients infected with each strain of NTM annually from 2019 to 2021 was shown in (**A–C**). The ratio of males to females in each strain of NTM for each year was displayed (**a–c**).
Abbreviations: MIN, *M. intracellulare*; MAV, *M. avium*; MAB, *M. abscessus*; MKA, *M. kansasii*; MFO, *M. fortuitum/M. suis*; MGO, *M. gordonae*.

related to age. In addition, men aged 30–49 years old were more likely to be infected with NTM than were women of the same age ($P < 0.05$).

In addition, there was a significant difference between men and women in *M. avium, M. kansasii*, and *M. abscessus* infections ($P < 0.05$) (Table 2). The infection rates of *M. avium* and *M. kansasii* were higher in males than in females, whereas those of *M. abscessus* showed the opposite trend.

Table 1 Gender and Age of Patients Infected with NTM

Age Group	Male	Female	Total	χ^2	P
<20	3	4	7 (1.27%)	0.143	0.705
20–29	22	16	38 (6.91%)	0.947	0.330
30–39	47	20	67 (12.18%)	10.881	0.001
40–49	32	17	49 (8.91%)	4.592	0.032
50–59	48	63	111 (20.18%)	2.027	0.155
60–69	67	75	142 (25.82%)	0.451	0.502
70–79	52	45	97 (17.64%)	0.505	0.477
≥80	27	12	39 (7.09%)	5.769	0.016
Total	298	252	550 (100%)	21.619	0.003

Past medical history and complications	Number of people	Proportion
Tuberculosis (TB)	256	46.55%
Nontuberculous mycobacteriosis	321	58.36%
Immune deficiency disease	73	13.27%
Bronchiectasis	43	7.82%
Hepatic dysfunction	205	37.27%
Diabetes	20	3.64%
Hypertension	12	2.18%
Tumor + surgical history	22	4.00%
Coronary heart disease (CHD)	7	1.27%
Uremia	2	0.36%
Leucopenia	31	5.64%
Cytomegalovirus infection	8	1.45%

Table 2 Gender Distribution Among Different Species Types

| Species Type	Gender		χ^2	P
	Male	Female		
M. avium	71	32	14.767	0.000
M. kansasii	33	3	25.000	0.000
M. intracellulare	143	158	0.748	0.387
M. abscessus	28	49	5.727	0.017
M. fortuitum/porcine	14	9	1.087	0.297
M. gordonae	25	18	1.140	0.286
M. szulgai	4	1	—	—
M. triviale	1	0	—	—
M. scrofulaceum	2	1	—	—
M. xenia	1	4	—	—

A total of 58.36% (321/550) of the patients were definitively diagnosed with NTM, and 46.55% (256/550) had been diagnosed with or had a history of tuberculosis. Notably, 13.27% of patients had AIDS. Bronchiectasis is a common complication of NTM infections. In addition, 37.27% of the patients had abnormal liver function (Table 1).

MIC Distributions to Bedaquiline and Clofazimine

In this study, the NTM strains saved by Nanjing Public Health Medical Center were collected. The strains cultivated more than once by the same patient were counted as one case, and the redundant, contaminated, and failed strains were deleted, yielding 155 results in total. Among them, there were 30 strains of *M. abscessus*, 117 strains of *M. intracellulare*,

one strain of *M. kansasii*, two strains of *M. avium*, two strains of *M. gordonii*, and three strains of *M. fortuitum / porcine*. Finally, 155 NTM isolates showed MIC results.

The MIC distributions of *M. abscessus* and *M. intracellulare* to BDQ and CFZ are shown in Figure 3A–D. There were 30 isolates of *M. abscessus* which showed drug-susceptibility testing for bedaquiline, and most of the MICs were concentrated at 0.256 μg/mL to 1.024 μg/mL. The MIC_{50} was 0.128μg/mL, and the MIC_{90} was 2.048μg/mL. There were three strains of *M. abscessus* having MIC >2.048 μg/mL. No bedaquiline-resistant strains of *M. intracellulare* were detected. For *M. intracellulare*, the MIC_{50} was 0.004μg/mL and the MIC_{90} was 0.032μg/mL of bedaquiline. The MIC_{50} and MIC_{90} of bedaquiline and clofazimine against *M. abscessus* were higher than those against *M. intracellulare*. A total of 11 strains of *M. abscessus* were determined to be clofazimine-resistant. The MIC of clofazimine against *M. abscessus* was mainly distributed between 0.25μg/mL to 8μg/mL, with a MIC_{50} at 2μg/mL and MIC_{90} at 8μg/mL. The MIC of clofazimine against *M. intracellulare* was mainly distributed from 0.015μg/mL to 0.5μg/mL, with a MIC_{50} and MIC_{90} of 0.125μg/mL and 0.5μg/mL, respectively.
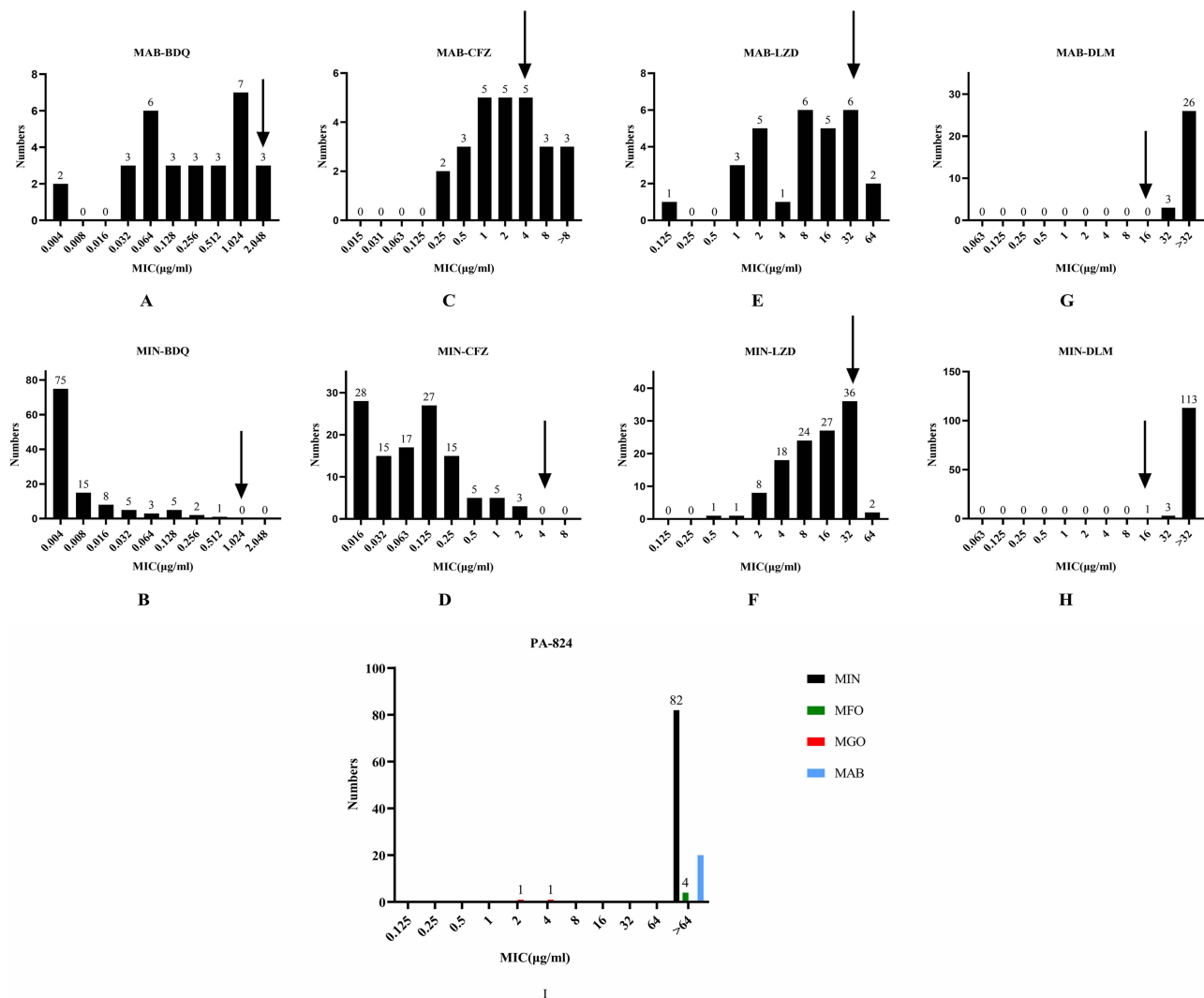
Figure 3 MICs distributions of *M. abscessus* and *M. intracellulare* to bedaquiline, clofazimine, linezolid, delamanid and pretomanid. (**A**) The MIC distributions of *M. abscessus* against bedaquiline. (**B**) The MIC distributions of *M. intracellulare* against bedaquiline. (**C**) The MIC distributions of *M. abscessus* against clofazimine. (**D**) The MIC distributions of *M. intracellulare* against clofazimine. (**E**) The MIC distributions of *M. abscessus* against linezolid. (**F**) The MIC distributions of *M. intracellulare* against linezolid. (**G**) The MIC distributions of *M. abscessus* against delamanid. (**H**) The MIC distributions of *M. intracellulare* against delamanid. (**I**) The MIC distributions of NTM against pretomanid. The position indicated by the arrow indicates the critical concentration value of resistance.
Abbreviations: MAB, *M. abscessus*; MIN, *M. intracellulare*; MFO, *M. fortuitum/porcine*; MGO, *M. gordonii*; BDQ, bedaquiline; CFZ, clofazimine; LZD, linezolid; DLM, delamanid; PA-824, pretomanid.

MIC Distributions to Linezolid, Delamanid, and Pretomanid

The MIC ranges of linezolid were the same for *M. abscessus* and *M. intracellulare*, and 8 strains of *M. abscessus* and 38 strains of *M. intracellulare* showed linezolid resistance (Figure 3E and F). The MIC$_{50}$ of *M. abscessus* was 8 μg/mL, and MIC$_{90}$ was 32 μg/mL. Meanwhile, the MIC$_{50}$ of *M. intracellulare* was 16 μg/mL, and MIC$_{90}$ was 32 μg/mL. Linezolid required higher concentrations against *M. intracellulare* than against *M. abscessus*.

As shown in Figure 3G and H, the *M. intracellulare* and *M. abscessus* included in the study were resistant to delamanid and had MICs ≥ 32 μg/mL. A total of 108 NTM isolates were tested for pretomanid MIC, as shown in Figure 3I. The *M. intracellulare* and *M. abscessus* were resistant to pretomanid and had MICs ≥ 64 μg/mL. Notably, two strains of *M. gordonii* were sensitive to pretomanid.

M. abscessus Resistant Mutations to BDQ, CFZ, LZD

There were 3 strains of *M. abscessus* strains were resistant to BDQ. *MAB_1448, MAB_4384, and MAB_2299c* were also sequenced. The results showed that one strain had a base C insertion at position 13 in *MAB_1448*, which caused a frameshift mutation. In addition, the other two strains failed repeated amplification in *MAB_4384*. Synonymous mutations were observed only in *MAB_4384* and *MAB_2299c* (Table 3).

There were 11 strains of *M. abscessus* resistant to CFZ. *MAB_2299c* is associated with cross-resistance between bedaquiline and clofazimine;[17] no amino acid mutations have been found in *MAB_2299c*. We observed no cross-resistance between bedaquiline and clofazimine in the *MAB_2299c* mutant. In *MAB_1483*, nucleotide mutations alone did not cause amino acid mutations. It was worth noting that a large amount of nucleotide mutation deletion was found in *MAB_0540*. Six of the 11 clofazimine-resistant isolates displayed base deletions between positions 30 and 41 in *MAB_0540*. No mutations were detected in either strain.

Mutations in Genes of *M. intracellulare* to LZD

There were 8 strains of *M. abscessus* resistant to LZD. Table 3 presents the results. There were 38 *M. intracellulare* strains of linezolid with MIC >16 μg/mL classified as phenotypically resistant according to CLSI.[20] Five strains that were not amplified were excluded from the analysis. The *rplC, rplD*, and *rrl* genes of 33 phenotypically resistant strains were sequenced. The results are presented in Figure 4 and Table 4. The known *rplC* and *rplD* protein crystal structures of Mycobacterium tuberculosis H37Rv were used as the basis for multiple sequence alignment of *M. abscessus* and *M. intracellulare*, respectively, and the alignment results are shown in Supplementary Figures S2 and S3. The mutation site of *MAB_rplD* is in a highly variable region, which is poorly correlated with drug resistance.

Table 3 NTM Mutation *M. abscessus* of BDQ, CFZ, and LZD

| Drug	Gene	Strains Numbers	Nucleotid Mutation	Amino Acid Change
BDQ	*MAB_1448*	1	13insertC	Frameshift mutation
		2	WT	WT
	MAB_4384	1	A180G, C264T, A549G	WT
		2	Failure of amplification	–
	MAB_2299c	2	A264C, T612G	WT
		1	A264C, T612G, A15C, A57G, A99G, G165C, G270C, C300G, C303T, G309C, A339G, C345T, T366C, A513G, C573G, G630A, A225G	WT

(*Continued*)

Table 3 (Continued).

Drug	Gene	Strains Numbers	Nucleotid Mutation	Amino Acid Change
CFZ	MAB_2299c	2	WT	WT
		1	A264C, T612G, A15C, A57G, A99G, G165C, G270C, C300G, C303T, G309C, A339G, C345T, T366C, A513G, C573G, G630A, A225G	WT
		1	A264C	WT
		4	A264C, T612G	WT
	MAB_1483	2	T183C, G228C, A243G, C252T, T267C, A282G, C378T, A435G, C483T, T489C, C501T, C576T, C636T, T687C, G735A	WT
		7	WT	WT
		1	G285A	WT
		1	T183C, G228C, A282G, C288T, C342T	WT
	MAB_0540	2	C29G, T105C, T219C, C387A, G391A, C687T, G771C, G909A, A135G, C369T	Missense mutations
		2	WT	WT
		1	C29G, T105C, T219C, C387A, G391A, C687T, G771C, G909A	Missense mutations
		2	T105C, T219C, G391A, C687T, G771C, A135G, 30–41 deletion, A42G, C69A, G78A, C96A, C133A, A151G, T159G, A261G, G316T, A385G, G391A, C412T, G429A, T438C, C453T, C462T, A510C, T521C, G576C, C609G, A804G, C717T, G741A, C757T, A846G, G882A, C918A, C933T, C972T, T978C	Missense mutations
		1	T105C, T219C, C387A, G391A, C389T, G771C, A135G, 30–41 deletion, C687T, G909A	Missense mutations
		3	T12G, T105C, T219C, G391A, C687T, G571T, A135G, 30–41 deletion, C795T, G909A	Missense mutations
LZD	rplC	9	WT	WT
		1	T240G	WT
	rplD	9	WT	WT
		1	T91C, C303T, G348C, T366C, G431C, T484C, C522T, G529C, T576C	Missense mutations
	rrl	9	WT	WT
		1	C634T, A1717G, T3001C	WT

Abbreviations: WT, wild type; BDQ, bedaquiline; CFZ, clofazimine; LZD, linezolid.

Discussion

Recently, NTM have attracted increasing attention. However, NTM do not meet the mandatory reporting measures for infectious diseases, and there has been no systematic investigation or research internationally or domestically. A previous study showed that the separation rate of NTM in Jiangsu Province in 2008 was 3.37%, slightly lower than the average level in Shanghai.[24] However, in the study by Hu et al, the isolation rate of NTM in Nanjing was 15% from 2017 to 2018, indicating that the infection rate of NTM in Nanjing was on the rise.[25]
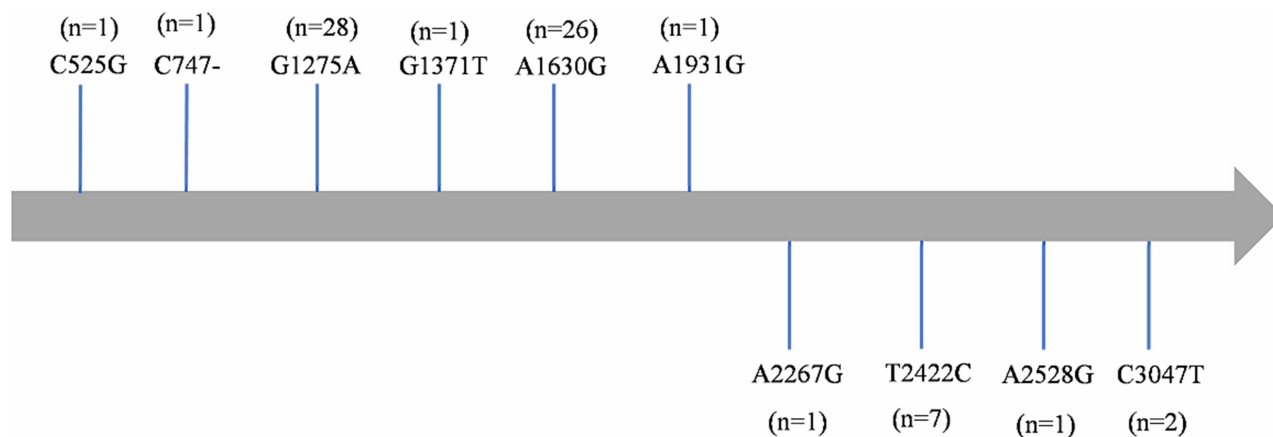
Figure 4 Gene mutations of linezolid in the *23S rRNA* (*rrl*) among *M. intracellulare* clinical isolates.
Abbreviations: n, the number of mutation isolates. -, Base deletion.

In this study, we analyzed the characteristics of NTM infections at Nanjing Public Health Medical Center from 2019 to 2021. *M. intracellulare* was the main species causing NTM infections in patients in Nanjing from 2019 to 2021, followed by *M. avium* and *M. abscessus*. Zhou et al conducted a systematic review study and meta-analysis on the prevalence of NTM in Mainland China from 2000 to 2019, and the study showed that the dominant species in China changed from *M. abscessus* to *M. intracellulare* after 2015.[2] Hu et al studied *M. intracellulare, M. abscessus*, and *M. avium* in patients diagnosed with NTM lung disease in Nanjing from 2017 to 2018, and found that NTM infection was related to age but not gender.[25] Our study found that without distinguishing between NTM colonized patients and confirmed patients, there was a significant gender difference in the population with detected NTM, suggesting that the chance of NTM infection was gender-related. Especially in 30–49-year-olds, males are more likely to be infected with NTM than females.

Table 4 NTM Mutation *M. Intracellulare* of LZD

Drug	NTM	Gene	Strains Numbers	Nucleotid Mutation	Amino Acid Change
LZD	*M. intracellulare*	*rplC*	19	WT	WT
			7	G360A	WT
			4	T393C	WT
			1	G372C, T393C	WT
			2	G126C, G150T, C162T, G207A, C222T, G231T, G237C, T241C, C261T, G274A, G279C, C309G, C342G, C345G, G360A, G372C, T393C, C397A, T402C, G444C, C477G, G492C, A534G, T609C, G618C	Missense mutations
		rplD	1	WT	WT
			1	C213T, C276T, G443A, A457G, G468C, T534G, C606T, A607T, G608C, T668C	Missense mutations
			2	A45G, A63C, C129G, C138G, C156T, C195G, T201C, C237G, C252G, C276T, C282T, C294T, C327G, T349C, T357C, C381T, G384A, G397A, A405G, A406C, C408G, C411G, G412T, C414G, C417T, C423G, G429C, G437A, A442C, G468C, G470A, A495G, G507A, G510C, T534G, A547G, G555T, C561T, C585G, G600C, C618G, C639G, T668C	Missense mutations
			17	T668C	WT
			8	G555A, T668C	WT
			1	G437A, T668C	WT
			4	C366G, T668C	WT

Abbreviations: WT, wild type; LZD, linezolid.

There was a significant difference between in the rate of *M. avium* and *M. kansasii* infection between males and females (P=0.000, < 0.05). Males had a higher rate of infection with *M. avium* and *M. kansasii*. Among those infected with *M. avium* and *M. kansasii*, 46.6% and 22.2% of patients had a history of HIV infection, respectively. Studies have shown that patients with immunodeficiency diseases are more susceptible to *M. avium* and *M. kansasii* diseases.[7,26] This study found that there was no significant difference in the rate of *M. intracellulare* infection between males and females, and *M. abscessus* infection was more likely to occur in female patients. Other studies have shown that NTM is more likely to occur in older female patients.[25,27] In this study, *M. abscessus* infection was more likely to occur in older female patients, which may be related to the NTM species.[25,27]

In recent years, NTM infection has increased both within China and worldwide, especially *M. abscessus* infection, which has aroused widespread concern.[28–30] Studies have shown that *M. abscessus* can be transmitted from person to person.[3,5] From 2019 to 2021, the number of NTM infected patients in Nanjing decreased, which may be related to the COVID-19 epidemic. Both have aerosol transmission routes, and the risk of NTM infection may be reduced if specific protective measures are taken. However, in this study, it is worth noting that the number of *M. abscessus* infections has increased, indicating that *M. abscessus* infection is still not negligible.

In this study, we measured the in vitro activities of bedaquiline, clofazimine, linezolid, delamanid, and pretomanid against *M. abscessus* and *M. intracellulare*. We also sequenced correlations between drug resistance-related genes. Bedaquiline showed a great effect on *M. intracellulare* in vitro, with MICs between 0.004 and 0.512 μg/mL. Bedaquiline is a diarylquinoline compound that mainly acts on the ATP synthase of bacteria, which in turn affects the synthesis of ATP in bacteria.[16] In recent years, in vitro studies of bedaquiline against NTM have been reported.[31–33] In addition, in these studies, bedaquiline was more active against NTM, with MICs of <1 μg/mL for *M. intracellulare* and *M. avium*, and MIC <2 μg/mL for *M. abscessus*.[31–34] Bedaquiline had a two-fold higher MIC against *M. abscessus* than against *M. intracellulare*,[31] which was also confirmed in our study. Clofazimine, a drug used to treat *Mycobacterium leprae*, is currently used to treat NTM. In several in vitro drug susceptibility studies,[35,36] clofazimine has shown strong activity against NTM. In the present study, we measured the activity of clofazimine against *M. abscessus* and *M. intracellulare*. In a study in Korea, the MIC_{50} and MIC_{90} for *M. abscessus* were 4 mg/L and 8 mg/L, respectively.[35] In our study, MIC_{50} at 2μg/mL and MIC_{90} at 8μg/mL to *M. abscessus*, whereas clofazimine has a MIC_{50} and MIC_{90} of 0.125μg/mL and 0.5μg/mL for *M. intracellulare*. We found that the MIC_{50} and MIC_{90} values of clofazimine *for M. abscessus* were greater than those for *M. intracellulare*. Furthermore, in the study in Korea, the MIC of clofazimine is closely related to the clinical prognosis of NTM pulmonary (NTM-PD), and an MIC ≤ 0.25 μg/mL of clofazimine was used for NTM-PD patients.[35] In a prospective study on the use of clofazimine against NTM, the addition of clofazimine to the treatment regimen for drug-resistant NTM infection resulted in successful treatment in most patients.[33] Linezolid is an oxazolidi-none compound used primarily for the treatment of diseases caused by gram-positive bacteria and has been used in recent years for the treatment of multidrug-resistant tuberculosis. In this study, the MIC_{50} of linezolid against *M. abscessus* was 8 μg/mL and MIC_{90} was 32 μg/mL. In some studies, linezolid showed general activity against NTM.[37–40] In research by Yu et al and Wen et al,[37,38] the susceptibility of slow-and rapidly growing mycobacteria to linezolid was investigated in Beijing, where linezolid had a low MIC value against *M. kansasii*. An MIC_{90} value of 32 μg/mL was determined for *M. intracellulare* as well as Mycobacterium avium complex, along with an MIC_{90} of 16 μg/mL for *M. abscessus*. In our study, the results showed an MIC_{90} of 32 μg/mL for *M. abscessus* and *M. intracellulare* for linezolid. Delamanid showed some activity against slow-growing mycobacteria, but little activity against fast-growing mycobacteria, especially *M. abscessus*. Our study was in agreement with the results of Yu et al.[32] Delamanid had strong activity only against *M. kansasii*.[41] Delamanid was active against some slow-growing mycobacteria and almost inactive against fast-growing mycobacteria. A few studies have suggested that delamanid has some activity against the *M. avium* complex in vitro.[42] However, in this study, the MIC of delamanid was >32 μg/mL against *M. avium, M. intracellulare*, and *M. abscessus*. In a study by Yu et al, *M. gordonii* showed sensitivity to delamanid.[32] The two strains of *M. gordonii* used in our study also showed delamanid sensitivity. PA-824 is a drug of the same type as delamanid and is used for the treatment of multidrug-resistant tuberculosis. In a study conducted in Beijing, all NTM included in the study were resistant to PA-824.[43] The findings in our study for *M. intracellulare* and *M. abscessus* were consistent with this study. Notably, two strains of *M. gordonii* were found to have low MIC values for PA-824 and delamanid.

The main genes known to be associated with resistance in *Mycobacterium tuberculosis* to bedaquiline are *atpE, Rv0678, Rv1979C, and pepQ*.[12,44] In *M. abscessus, MAB_1448, MAB_4384*, and *MAB_2299c* are the genes reported to be potentially associated with bedaquiline resistance.[13,17] For most strains, the minimal bactericidal concentration (MBC) of bedaquiline was higher than the MIC, indicating strong bacterial inhibition.[34] In the study by Aguilar-Ayala et al, they sequenced the atpE gene of all strains, and results indicated that mutations in the atpE gene were associated with bedaquiline resistance.[34] In a study on the distribution of the MIC of bedaquiline for *M. abscessus* and determination of the mechanism of reduced drug susceptibility, no atpE mutation was found in *M. abscessus*.[45] MAB_4384 of *M. abscessus* was homologous to MmpS5/MmpL5 and Rv0678 of Mycobacterium tuberculosis.[45] This suggests that the mechanism of resistance to bedaquiline in *M. abscessus* is different from that of *Mycobacterium tuberculosis*. Mutations in MAB_4384 resulted in reduced susceptibility to bedaquiline.[45] In this study, we found no mutations in *MAB_4384*. Only one *M. abscessus* isolate was found to have a code-shifting mutation with a base insertion at site 13 of *MAB_1448*.

Among the *M. abscessus* mutations of clofazimine resistance, the main mutated genes were *MAB_0540, MAB_1483*, and *MAB_2299c*,[18] of which *MAB_2299c* was confirmed to be associated with cross-resistance to bedaquiline and clofazimine.[17] In vitro activity studies of clofazimine against nontuberculous Mycobacterium avium isolated from Beijing revealed unidirectional cross-resistance between bedaquiline- and clofazimine-resistant strains.[46] In our study, clofazimine showed strong antibacterial activity against *M. abscessus* and *M. intracellulare*. In novel mutation studies of clofazimine resistance in *M. abscessus, MAB_2299c, MAB_1483*, and *MAB_0540* are the major mutated genes.[18] In a genome-wide study, a large deletion between position 0 and 45 was found in *MAB_0540*,[18] and a deletion between position 30 and 41 was also found in our sequencing study. In addition, mutations at other sites of *MAB_0540* were found in our study, which may have caused amino acid changes. Unfortunately, how this gene affects the mechanism of drug action remains unclear and further studies are needed.

The primary genes associated with linezolid resistance were *rplC, rplD*, and *rrl*. Ye et al[15] performed a molecular analysis of *M. abscessus* associated with linezolid resistance and compared it with the standard *M. abscessus* ATCC19977 strain and found nine mutations in the *23SrRNA* gene associated with linezolid resistance. Other mutations in both resistant and sensitive strains were found which were not associated with linezolid resistance.[15] In our study, none of the strains of *M. abscessus* resistant to linezolid were found to have mutations associated with linezolid resistance. In a Korean study, molecular analysis of the linezolid-resistant Mycobacterium avium complex identified two novel mutations associated with the *23SrRNA* gene, G2599A and A2137T.[47] In our study, neither of these mutations was found in linezolid-resistant strains of *M. intracellulare*.

Conclusion

In conclusion, we clarified the distribution of NTM in Nanjing from 2019 to 2021 and determined the in vitro drug susceptibilities of NTM to bedaquiline, clofazimine, linezolid, delamanid, and pretomanid. Furthermore, we sequenced the major resistance genes in the drug-resistant strains. From 2019 to 2021, *M. intracellulare* was the most prevalent strain in Nanjing, followed by *M. avium* and *M. abscessus*. *M. intracellulare* and *M. abscessus* were more susceptible to bedaquiline, clofazimine, and linezolid in vitro. In addition, we found 30–41 nucleotide deletion mutations and other novel point mutations in the *MAB_0540* gene of *M. abscessus* resistant to clofazimine.

Abbreviations

NTM, nontuberculous mycobacteria; MDR-TB, multi-drug resistant tuberculosis; MIC, minimal inhibitory concentration; BDQ, bedaquiline; CFZ, clofazimine; LZD, linezolid; DLM, delamanid; PA-824, pretomanid; MTC, *Mycobacterium tuberculosis complex*; CI, confidence interval; HIV, Human immunodeficiency virus; NTM-PD, nontuberculous mycobacteria pulmonary.

Data Sharing Statement

Data relating to this study are contained and presented in this document. Other materials are available from the corresponding author on reasonable request.

Ethics Approval and Informed Consent

The study protocol was approved by Nanjing Center for Disease Control and Prevention Ethics Committee (approval number: PJ2020-A001-04; approval date: 17 August, 2020). Patient information collected in the case system did not

contain name, address or other personal information, so the patient's written informed consent was exempt. The study was conducted in accordance with the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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