Single-nucleotide polymorphisms related to fluoroquinolone and aminoglycoside resistance in *Mycobacterium avium* isolates

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Objective: The relationships between fluoroquinolone and aminoglycoside resistance and single-nucleotide polymorphisms (SNPs) in *gyrA*, *gyrB*, and *rpsL* genes were investigated in 95 clinical isolates of *Mycobacterium avium* from China.

Methods: Fluoroquinolone and aminoglycoside resistance were determined by the broth microdilution method. *GyrA*, *gyrB*, and *rpsL* were sequenced, SNPs were identified, and the corresponding amino acid mutations were recorded.

Results: The *M. avium* isolates displayed high levels of ofloxacin (93.68%), ciprofloxacin (92.63%), and streptomycin (65.26%) resistance. Moxifloxacin (18.95%) and amikacin (2.11%) were highly active against the strains. Fluoroquinolone resistance involving *gyrA* and *gyrB* gene mutations was identified. For *gyrA*, the most frequent SNPs were T→C (71/95, 74.74%), followed by A→G (64/95, 67.37%) and T→C (62/95, 65.26%). The amino acid mutations occurred mainly at Gly2444Asp (GGT→GAT) (20/95, 21.05%), Ala2445Val (GCC→GTC) (20/95, 21.05%), Val2449Ile (GTC→ATC) (20/95, 21.05%), and Glu2450Gln (GAA→GAG) (20/95, 21.05%). Prominent SNPs in *gyrB* included A→C (69/95, 72.63%), C→T (51/95, 53.68%), and T→G (29/95, 30.53%), and their amino acid substitutions were Ile2160Val (ATT→GTT) (21/95, 22.11%), Ile2160Met (ATT→ATG) (20/95, 21.05%), and Ile2273Leu (ATC→CTC) (11/95, 11.58%). Among the strains with aminoglycoside resistance, SNPs in *rpsL* were identified mostly at position G→A (73/95, 76.84%). G→C (21/95, 22.11%) was commonly seen. The amino acid mutations primarily involved Ala1539983Glu (CAG→CGG) (20/95, 21.05%), His1539984Asn (GCG→GAC) (19/95, 20.00%), His1539986Asp (GAC→GAG) (19/95, 20.00%), and Glu1539988Glu (GAG→GAG) (18/95, 18.95%).

Conclusion: Our study provides valuable information that could be used for the future diagnosis and treatment of *M. avium* disease.

Keywords: *Mycobacterium avium*, drug resistance, single-nucleotide polymorphism, amino acid mutation, minimum inhibitory concentration

Introduction

*Mycobacterium avium* was first isolated from chickens in 1933 as a cavitary disease, which was similar to tuberculosis.1 This species is the most common cause of nontuberculous mycobacterial infections in humans, especially respiratory system diseases.2 The incidence and prevalence of *M. avium* lung diseases have been increasing worldwide.2 *M. avium* lung disease often affects elderly people with chronic lung diseases and may be the manifestation of a complex genetic disorder determined by the interactions of multiple genes as well as environmental factors.2,3 It is difficult to treat and requires carefully individualized analysis of the risks and benefits of treatment.2,3 Despite
extensive research into drug resistance in *Mycobacterium tuberculosis*, few studies have investigated the molecular mechanisms underlying drug susceptibility in *M. avium*. There have been continuous efforts to address problems, such as drug resistance to conventional antituberculosis agents, and research using *M. avium* could be beneficial.

DNA gyrase, the primary target of fluoroquinolone drugs, relaxes DNA supercoiling ahead of the DNA helicase–DNA replication complex. DNA gyrase is encoded by the *gyrA* and *gyrB* genes. In contrast, aminoglycosides inhibit protein synthesis by binding to the ribosome near the A site. The primary mechanism of acquires resistance to aminoglycosides in mycobacteria is based on modification of the 30S subunit of the ribosome as the drug target. This modification is caused by mutations, often in the *rpsL* gene, which encodes the S12 ribosomal protein. Other mutations associated with drug resistance involve single-nucleotide polymorphism (SNP) changes to a single base at a specific position in the genome. The correlation between SNPs and phenotypic diversity has been established for some mycobacterial species, encouraging further correlation analyses to be done to distinguish the SNPs among the strains.

In this study, the broth microdilution method recommended by the Clinical and Laboratory Standards Institute was used to test the drug sensitivity including 10 antimicrobial agents of 95 *M. avium* clinical isolates collected from five Chinese provinces between 2005 and 2012. The SNP analysis was performed to determine the mutation characteristics of the strains carrying fluoroquinolone and aminoglycoside resistance. The results of this study should be helpful for improving the diagnosis and treatment of *M. avium* infections in the clinical work.

**Materials and methods**

**Strains**

All 95 *M. avium* clinical isolates were isolated from the sputum samples of patients with suspected tuberculosis between 2005 and 2012, which were collected from Fujian, Hunan, Jiangxi, Sichuan, and Anhui provinces in China. They were part of the routine hospital laboratory procedure. The species were identified via sequence analysis of *hsp65, rpoB*, and the 16S–23S ITS genes.

**Antimicrobial agents**

Ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, sparfloxacin, streptomycin, amikacin, kanamycin, capreomycin, and tobramycin were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All the antibiotics were freshly prepared before they were used.

**Drug susceptibility tests**

*M. avium* isolates were incubated in Difco Middlebrook 7H10 agar from BD (Franklin Lakes, NJ, USA) with 5% oleic acid–albumin–dextrose–catalase. The microdilution method was performed for fluoroquinolone and aminoglycoside susceptibility testing against *M. avium* using cation-adjusted Mueller-Hinton broth with the addition of 5% oleic acid–albumin–dextrose–catalase. The experiments were conducted in 96-well microplates. First, 0.5 McFarland standard bacterial suspensions were prepared. Second, the bacterial solutions and drug dilution mixtures were added to wells with a blank control included. Finally, the 96-well microplates were incubated at 37°C with 5% carbon dioxide.

The minimum inhibitory concentration (MIC), MIC50, and MIC90 values for each antimicrobial agent were determined according to the previous method. Fluoroquinolone and aminoglycoside resistance were identified using the MIC breakpoints.

**PCR amplification and DNA sequencing**

Genomic DNA was isolated from the *M. avium* isolates by heating the bacterial suspensions in Tris–EDTA buffer (pH = 8.0) at 100°C for 30 min. The precipitant was removed by centrifugation at 12,000 rpm for 5 min. A 50-μL polymerase chain reaction (PCR) mixture, which included the following reagents, was then prepared as follows: 2 μL 2× PCR mixture, 1 μM forward primer, 1 μM reverse primer, and 5 μL of genomic DNA, as described previously. The *gyrA, gyrB,* and *rpsL* gene primers used in this study are listed in Table 1. PCR amplification was performed in a thermocycler with an initial denaturation step for 5 min at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 60 seconds at 72°C, with a final extension step of 7 min at 72°C. The amplified products were separated by electrophoresis on 1% agarose gels with ethidium bromide staining and visualized under ultraviolet illumination.

| Gene | Primer | Sequence (5′–3′) | Length of amplified fragment (bp) |
|------|--------|-----------------|---------------------------------|
| gyra | F      | CGGACATGGAGGACCAAGGAA | 991                             |
| gyra | R      | GCGGAACCTCCTCCACCTTAAC |                                |
| gyrb | F      | ACCAAGACCCAAGCTTGGCAACA | 540                             |
| gyrb | R      | CGGAAGCGACGTCGTAACAAA |                                |
| rpsl | F      | ACCATTCGGACCCCTAGA   | 592                             |
| rpsl | R      | CGCCTAACCAGTTAGA     |                                |

**Abbreviations:** F, forward; R, reverse.
by 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, and a 5-min final extension at 72°C. The PCR products were sequenced at Tianyihuiyuan Bio Tech Co., Ltd. (Beijing, China). All the sequencing results were compared with the M. avium 104 complete genome sequences (NC_008595.1) in GenBank using the NCBI BLAST server (www.ncbi.nlm.nih.gov).

Statistical analysis
The data obtained herein were analyzed using the SPSS v.17.0 software. The resistance percentages and the MIC50 and MIC90 values for the fluoroquinolone and aminoglycoside agents were calculated for the M. avium isolates.

Results
Antimicrobial resistance in the M. avium clinical isolates
The M. avium isolates displayed high resistance to ofloxacin (89/95, 93.68%) and ciprofloxacin (88/95, 92.63%). Moxifloxacin (18/95, 18.95%) showed the best activity in the strains tested. In terms of their MIC50 and MIC90 values, ofloxacin had the highest values and moxifloxacin had the lowest ones compared with the other fluoroquinolones. With the aminoglycosides, amikacin (2/95, 2.11%) demonstrated excellent activity against the isolates, whereas streptomycin (62/95, 65.26%) exhibited the worst activity. The MIC50 and MIC90 values of tobramycin were 1 and 32 μg/mL, respectively (Table 2).

### Table 2 MIC and resistance prevalence of Mycobacterium avium clinical isolates to fluoroquinolone and aminoglycoside agents

| Drugs | M. avium |
|-------|----------|
|       | MIC50 (μg/mL) | MIC90 (μg/mL) | Prevalence of resistant strains (%) |
| OF    | 1 to >32      | 16            | 32       | 93.68 (89/95) |
| CIP   | 0.5 to >32    | 8             | 32       | 92.63 (88/95) |
| LEV   | 0.5 to >32    | 8             | 32       | 83.16 (79/95) |
| MXF   | 0.06 to 16    | 2             | 4        | 21.05 (20/95) |
| SPA   | 0.13 to <32   | 2             | 16       | 25.26 (24/95) |
| SM    | 1 to >256     | 8             | 16       | 65.26 (62/95) |
| AM    | 1 to >32      | 8             | 32       | 2.11 (2/95) |
| KN    | 0.25 to >32   | 2             | >32      | 17.89 (17/95) |
| CPM   | 0.13 to 16    | 2             | 16       | 0 (0/95) |
| TOB   | <0.25 to 256  | 1             | 16       | 21.05 (20/95) |

**Abbreviations:** OF, ofloxacin; CIP, ciprofloxacin; LEV, levofloxacin; MXF, moxifloxacin; SPA, sparfloxacin; SM, streptomycin; AM, amikacin; KN, kanamycin; CPM, capreomycin; TOB, tobramycin.

Mutations in gyrA and gyrB genes and fluoroquinolone resistance
Fluoroquinolone resistance in the M. avium isolates was identified by screening for mutations in the gyrA and gyrB genes of this bacterium. Connections between fluoroquinolone resistance and gyrA and gyrB gene mutations were investigated. Among the 18 strains showing completely resistance to all the five fluoroquinolones, the most frequent SNPs were T→C (71/95, 74.74%), followed by A→G (64/95, 67.37%) and T→C (62/95, 65.26%) in the gyrA region of DNA gyrase (Table 3). The amino acid mutations occurred mainly at the sites of Gly2444Asp (GGT→GAT) (20/95, 21.05%), Ala2445Ser (GCC→TCC) (20/95, 21.05%), Ala2447Val (GCC→GTC) (20/95, 21.05%), Val2449Ile (GTC→ATC) (20/95, 21.05%), and Glu2450Gln (GAA→CAA) (20/95, 21.05%) (Table 4). Prominent SNPs in gyrB included A→C (69/95, 72.63%), C→T (51/95, 53.68%), and T→G (29/95, 30.53%) (Table 5), and their corresponding amino acid
substitutions were Ile2160Val (ATT→GTG) (21/95, 22.11%), Ile2160Met (ATT→ATG) (20/95, 21.05%), and Ile2273Leu (ATC→CTC) (11/95, 11.58%) (Table 6).

Mutations in the rpsL gene and aminoglycoside resistance
Aminoglycoside resistance in M. avium isolates was identified from the mutation in the rpsL gene. Associations between aminoglycoside resistance in the isolates and rpsL gene mutation were focused on. Among the strains with aminoglycoside resistance, SNPs were detected mostly at position G→A (73/95, 76.84%) in rpsL. G→C (21/95, 22.11%) was commonly seen (Table 7). The amino acid mutations primarily involved Ala1539985Thr (GCC→ACC) (19/95, 20.00%), His1539992Asp (CAC→GAC) (19/95, 20.00%), and Gln1539983Glu (CAG→GAG) (18/95, 18.95%) (Table 8).

Discussion
In this study, the presence of SNPs associated with fluoroquinolone and aminoglycoside resistance in M. avium isolates was described. Among fluoroquinolones, ofloxacin was the worst activity and moxifloxacin was the best one against the M. avium isolates. Within aminoglycoside agents, streptomycin was the highest and capreomycin was the lowest resistance to the M. avium strains. Herein the SNPs, there were few reports about the M. avium species, but most in the M. tuberculosis.

From our assay, in gyrA, we found that the nonsynonymous mutations consisted of GCC→TCC (Ala→Ser) and

### Table 5 SNPs in the gyrB gene

| ReferBase | SampleBase | ReferPos | SamplePos | Strain numbers |
|-----------|------------|----------|-----------|---------------|
| C to T    | 6597→913   | 51       |           |               |
| A to G    | 6474→69    | 11       |           |               |
| A to G    | 6478→73    | 21       |           |               |
| A to G    | 6501→96    | 21       |           |               |
| A to G    | 6666→261   | 21       |           |               |
| T to G    | 6495→90    | 21       |           |               |
| T to G    | 6750→347   | 29       |           |               |
| A to C    | 6465→60    | 21       |           |               |
| A to C    | 6558→154   | 69       |           |               |
| T to G    | 6495→90    | 21       |           |               |

**Notes:** ReferBase indicates the base in Mycobacterium avium 104. SampleBase denotes the base in the tested isolates. ReferPos indicates the position in M. avium 104. SamplePos denotes the position in the isolates tested.

**Abbreviation:** SNPs, single nucleotide polymorphisms.

### Table 6 Amino acid mutations in the gyrB gene from the Mycobacterium avium clinical isolates

| Base mutation | Mutation in ReferPos | Amino acid mutation | Mutation numbers |
|---------------|----------------------|---------------------|------------------|
| CAG→CGG      | 6416                 | Gln2139Arg          | 10               |
| CTC→TTG      | 6436                 | Leu2146Phe          | 2                |
| ATT→GTG      | 6478                 | Ile2160Val          | 21               |
| ATT→ATG      | 6480                 | Ile2160Met          | 20               |
| ATC→CTC      | 6817                 | Ile2273Leu          | 11               |
| ACC→GCC      | 6820                 | Thr2274Ala          | 4                |
| CTG→CGG      | 6902                 | Leu2301Arg          | 4                |

**Note:** ReferPos indicates the position in M. avium 104.

**Abbreviations:** Gin, glutamine; Leu, leucine; Phe, phenylalanine; Ile, isoleucine; Val, valine; Met, methionine; Thr, threonine; Ala, alanine; Arg, arginine.

### Table 7 SNPs in the rpsL gene

| ReferBase | SampleBase | ReferPos | SamplePos | Strain numbers |
|-----------|------------|----------|-----------|---------------|
| C to G    | 4619974→136| 21       |           |               |
| G to A    | 4620032→205| 73       |           |               |
| G to A    | 4620070→232| 21       |           |               |
| A to G    | 4620184→346| 21       |           |               |
| C to G    | 4620203→365| 21       |           |               |
| T to C    | 4620207→369| 21       |           |               |
| C to G    | 4620214→376| 19       |           |               |
| A to G    | 4620217→379| 21       |           |               |
| G to C    | 4620257→418 | 21       |           |               |
| G to C    | 4620272→433 | 21       |           |               |
| G to A    | 4620281→443 | 21       |           |               |
| T to C    | 4620284→446 | 10       |           |               |
| T to C    | 4620287→449 | 21       |           |               |
| G to A    | 4620289→451 | 10       |           |               |
| G to T    | 4620295→456 | 21       |           |               |
| C to T    | 4620306→467 | 21       |           |               |
| G to C    | 4620310→471 | 21       |           |               |
| T to C    | 4620326→487 | 21       |           |               |
| G to C    | 4620329→490 | 21       |           |               |
| G to A    | 4620330→491 | 20       |           |               |
| A to T    | 4620386→546 | 10       |           |               |
| C to G    | 4620389→549 | 21       |           |               |

**Notes:** ReferBase indicates the base in Mycobacterium avium 104. SampleBase denotes the base in the tested isolates. ReferPos indicates the position in M. avium 104. SamplePos denotes the position in the isolates tested.

**Abbreviation:** SNPs, single nucleotide polymorphisms.

### Table 8 Amino acid substitutions in the rpsL gene from the Mycobacterium avium clinical isolates

| Base mutation | Mutation in ReferPos | Amino acid mutation | Mutation numbers |
|---------------|----------------------|---------------------|------------------|
| ACC→GCC      | 4619935              | Thr1539979Ala       | 11               |
| CAG→GAG      | 4619947              | Gin1539983Glu       | 18               |
| GCC→ACC      | 4619953              | Ala1539985Thr       | 19               |
| CAC→GAC      | 4619974              | His1539992Asp       | 19               |
| GCC→ACC      | 4620001              | Ala1540001Thr       | 13               |
| GCG→AGC      | 4620004              | Ala1540002Thr       | 17               |
| GGA→CGA      | 4620019              | Gly1540007Arg       | 17               |

**Note:** ReferPos indicates the position in M. avium 104

**Abbreviations:** Thr, threonine; Ala, alanine; Gin, glutamine; Glu, glutamic acid; His, histidine; Asp, aspartic acid; Gly, glycine; Arg, arginine.
GCC→GTC ( Ala→Val), which were in agreement with the previous studies for M. tuberculosis. However, our findings were demonstrated that GTC→ATC (Val→Ile), GAA→CAA (Glu→Gln), and GGT→GAT (Gly→Asp) may be in the hotspot mutation region and crucially responsible for the fluoroquinolone resistance. Some studies indicated that the gyrA mutations of M. tuberculosis were in Ala90Thr, Asp94His, Asp94Val, Thr95Ser, Thr80Ala, and Ser91Pro, which were not found in our studies. According to the gyrB mutations, some studies were elucidated that there were the mutations in the Asn499Thr, Asp461Asn, and Gly512Arg. In our research, SNPs in the gyrB commonly included ATT→GTT (Ile→Val) and ATT→ATG (Ile→Met), which mutated in the 2160th position (it may be a hotspot mutation position) of the reference M. avium 104. Otherwise isoleucine might be the critical mutation amino acid.

Aminoglycoside resistance in mycobacteria usually occurs in the rpsL gene. In our current study, the SNPs were mostly described as G to A substitution in rpsL, followed by C to G substitution. The most common amino acid mutation mainly involved Ala→Thr and His→Asp. In previous studies, AAG→AGG (Lys43Arg) in rpsL was the most common mutation among the resistant M. tuberculosis isolates that showed an MDR phenotype, whereas an AAG→AGG (Lys88Arg) substitution was found to usually play a minor role and occur much less frequently than Lys43Arg.

On the one hand, we found some isolates contained SNPs, but they were synonymous mutations. Some strains were resistant to fluoroquinolones and aminoglycosides in the absence of amino acid mutations, whose drug-resistant mechanism should be explored in the future. Furthermore, strains with higher drug resistance phenotypes had nonsynonymous mutations in gyrA and gyrB and rpsL genes. On the other hand, the isolates with synonymous mutations were resistant to drugs. There were 48 fluoroquinolone-resistant isolates with gyrA synonymous mutations and 52 resistant strains with gyrB synonymous mutations. In terms of aminoglycoside resistance, there were 12 drug-resistant strains with rpsL synonymous mutation.

It is known that drug resistance may be caused by mutations in the target regions of antitymocellular drugs. The fluoroquinolone and aminoglycoside resistance regions need to be accurately identified in order to study the SNPs and amino acid mutations occurring in them. It also should be deeply researched whether DNA extracted from specimens may lack the resistant genotype and lead to a false low detection rate. Nevertheless, sequencing the regions of genes can be a convenient way to quickly identify antituberculosis drug resistance, thus enabling the selection of effective drugs for treatment of the disease.

In addition to SNPs, there could be other drug resistance mechanisms, which should be discussed in the future. For one thing, the permeability in the cell wall to drugs and the existence of efflux pumps probably account for fluoroquinolone and aminoglycoside resistance in other resistant isolates that lack gyrA and gyrB and rpsL mutations. For another thing, fluoroquinolones can inhibit bacterial topoisomerase IV, which may inhibit DNA replication and transcription, and cause drug resistance. Furthermore, M. avium is known to have subspecies, such as M. avium subsp. avium, M. genavense, and M. avium subsp. paratuberculosis. These subspecies may differ in their drug resistance and SNP profiles, making them worthy of further research.

**Conclusion**

The fluoroquinolone and aminoglycoside resistance patterns and the SNP profiles of the clinical isolates of M. avium were considered. These data should be helpful for optimizing the treatment and preventing further transmission of drug-resistant M. avium strains. These findings have emphasized the need for the implementation of drug susceptibility along with accurate and rapid molecular tests for the detection of resistant mutations once M. avium is diagnosed.

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**Disclosure**

The authors report no conflicts of interest in this work.

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