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ORIGINAL RESEARCH

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%), 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%). 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 Ala1539985Thr (GCC→ACC) (19/95, 20.00%), His1539992Asp (CAC→GAC) (19/95, 20.00%), and Gln-1539983Glu (CAG→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 nontuberculosis mycobacterial infections in humans, especially respiratory system diseases.[1] 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

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extensive research into drug resistance in *Mycobacterium tuberculosis*, few studies have investigated the molecular mechanisms underlying drug susceptibility in *M. avium*.[4–6] 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.[7,8] 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.[9,10] This modification is caused by mutations, often in the *rpsL* gene, which encodes the S12 ribosomal protein.[11] 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.[11,12]

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.[12] 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.[13,14]

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.[12,15] 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.[12,15] 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.[12,15] 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.[12] Fluoroquinolone and aminoglycoside resistance were identified using the MIC breakpoints.[12,16]

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.[17] 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.[17] 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

Table 1 The primers and sequence lengths for *gyrA*, *gyrB*, and *rpsL* genes

Gene	Primer	Sequence (5′–3′)	Length of amplified fragment (bp)
gyrA	gyrA-F	CGAGATGGACGCCAAGGAA	991
	gyrA-R	GCGAATCCTCTTCCACCTCAAC	
gyrB	gyrB-F	ACCAAGACCAAACTGGGCAACA	540
	gyrB-R	CGGAACAGCAGCGTCAACAA	
rpsL	rpsL-F	ACCAGTTGCGACCCGTAGA	592
	rpsL-R	CGCCTAACCGTAAGGAAGTGAA	

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.[17] 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 16 μg/mL and kanamycin were 2 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*			
	MIC range (μg/mL)	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 fluoroquinolone agents, 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

Table 3 SNPs in the *gyrA* gene

ReferBase⇔SampleBase	ReferPos⇔SamplePos	Strain numbers
G⇔A	7330⇔226	20
G⇔C	7730⇔596	57
G⇔T	7335⇔231	20
G⇔A	7347⇔243	20
C⇔T	7364⇔260	20
A⇔G	7520⇔386	62
A⇔G	7541⇔407	64
T⇔C	7625⇔491	52
T⇔C	7649⇔515	62
T⇔C	7652⇔518	71
T⇔C	7655⇔521	51

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 4 Amino acid mutations in the *gyrA* gene from the *Mycobacterium avium* clinical isolates

Base mutation	Mutation in ReferPos	Amino acid mutation	Mutation numbers
GGT⇔GAT	7330	Gly2444Asp	20
GCC⇔TCC	7335	Ala2445Ser	20
GCC⇔GTC	7339	Ala2447Val	20
GTC⇔ATC	7347	Val2449Ile	20
GAA⇔CAA	7350	Glu2450Gln	20
AAC⇔ACC	7768	Asn2590Thr	5

Note: ReferPos indicates the position in *M. avium* 104.
Abbreviations: Gly, glycine; Asp, aspartic acid; Ala, alanine; Ser, serine; Val, valine' Ile, isoleucine; Glu, glutamic acid; Gln, glutamine; Asn, asparagine; Thr, threonine.

Table 5 SNPs in the *gyrB* gene

ReferBase⇔SampleBase	ReferPos⇔SamplePos	Strain numbers
C⇔T	6597⇔193	51
A⇔G	6474⇔69	11
A⇔G	6478⇔73	21
A⇔G	6501⇔96	21
A⇔G	6666⇔261	21
T⇔G	6495⇔90	21
T⇔G	6750⇔347	29
A⇔C	6465⇔60	21
A⇔C	6558⇔154	69
T⇔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⇔TTC	6436	Leu2146Phe	2
ATT⇔GTT	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: Gln, glutamine; Leu, leucine; Phe, phenylalanine; Ile, isoleucine; Val, valine; Met, methionine; Thr, threonine; Ala, alanine; Arg, arginine.

substitutions were Ile2160Val (ATT→GTT) (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 Gln-1539983Glu (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 7 SNPs in the *rpsL* gene

ReferBase⇔SampleBase	ReferPos⇔SamplePos	Strain numbers
C⇔G	4619974⇔136	21
G⇔A	4620052⇔205	73
G⇔A	4620070⇔232	21
A⇔G	4620184⇔346	21
C⇔G	4620203⇔365	21
T⇔C	4620207⇔369	21
C⇔G	4620214⇔376	19
A⇔G	4620217⇔379	21
G⇔C	4620257⇔418	21
G⇔C	4620272⇔433	21
G⇔A	4620281⇔443	21
T⇔C	4620284⇔446	10
T⇔C	4620287⇔449	21
G⇔A	4620289⇔451	10
G⇔T	4620295⇔456	21
C⇔T	4620306⇔467	21
G⇔C	4620310⇔471	21
T⇔C	4620326⇔487	21
G⇔C	4620329⇔490	21
G⇔A	4620330⇔491	20
A⇔T	4620386⇔546	10
C⇔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	Gln1539983Glu	18
GCC⇔ACC	4619953	Ala1539985Thr	19
CAC⇔GAC	4619974	His1539992Asp	19
GCC⇔ACC	4620001	Ala1540001Thr	13
GCG⇔ACG	4620004	Ala1540002Thr	17
GGA⇔CGA	4620019	Gly1540007Arg	17

Note: ReferPos indicates the position in *M. avium* 104
Abbreviations: Thr, threonine; Ala, alanine; Gln, 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*.[11,18–22] However, our findings were demonstrated that GTC→ATC (Val→Ile), GAA→CAA (Glu→Gln), and GGT→GAT (Gly→Asp) may be in the hotpot 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.[23–28] According to the *gyrB* mutations, some studies were elucidated that there were the mutations in the Asn499Thr, Asp461Asn, and Gly512Arg.[29–31] 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 hotpot 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.[32–34] 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.[32,35–37]

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 antimycobacterial 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.[18,38]

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.[11] Furthermore, *M. avium* is known to have subspecies, such as *M. avium* subsp. *avium*, *M. genavense*, and *M. avium* subsp. *paratuberculosis*.[29–31,39] 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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