## Letter to the Editor

# Antimicrobial Susceptibility Testing and Molecular Characterization of *Mycobacterium fortuitum* Isolates in China*

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**We performed molecular identification of clinical isolates of *Mycobacterium fortuitum (M. fortuitum)* and conducted drug susceptibility testing to analyze the *in vitro* susceptibility of clinical *M. fortuitum* isolates and potential molecular mechanism conferring resistance to fluoroquinolone and macrolide drugs. The results showed that moxifloxacin had the highest *in vitro* activity against *M. fortuitum*, and most *M. fortuitum* isolates were resistant to clarithromycin and linezolid in China. The loss of genetic mutation in clarithromycin- and amikacin-resistant isolates indicates that some other intrinsic mechanism conferring clarithromycin and amikacin resistance plays an essential role in *M. fortuitum* infection.**

Key words: Nontuberculosis; Genotype; Phenotype; Susceptibility

Despite being considered ubiquitous environmental organisms, rapid growing mycobacteria (RGM) are becoming a significant and increasing health concern worldwide[1]. The opportunistic pathogens cause a wide variety of infections, ranging from pulmonary to skin and soft tissue infections[1]. Among RGM species, *Mycobacterium fortuitum (M. fortuitum)* is one of the most common species causing human diseases, particularly post-surgical infections.

Antimicrobial susceptibility is essential for clinicians to strategize appropriate treatment regimens for diseases caused by pathogenic bacteria. Previous *in vitro* studies revealed that *M. fortuitum* isolates are typically susceptible to several antimicrobial agents, including fluoroquinolones, amikacin, and sulfonamides. In contrast, macrolides, the most effective drugs for treating nontuberculous mycobacteria, should be used with caution for *M. fortuitum* infection because they are associated with its intrinsic resistance conferred by the inducible ermmethylase gene. Thus, understanding the mechanisms of drug resistance is essential for effectively treating infections by this species.

In China, *M. fortuitum* is the second most common cause of RGM disease after *Mycobacterium abscessus*. However, data regarding the drug susceptibility of this species is limited in this region. The aim of this study was to analyze *in vitro* susceptibility of clinical *M. fortuitum* isolates against 21 antimicrobial agents. In addition, we investigated the potential molecular mechanism conferring drug resistance to *M. fortuitum*.

The strains evaluated in this study were isolated from clinical specimens from suspected pulmonary and extra pulmonary tuberculosis patients, collected between 2012 and 2014, at Guangzhou Chest Hospital and Shanghai Pulmonary Hospital, which are the largest tuberculosis (TB)-specialized hospitals in southern and eastern China, respectively. All nontuberculous mycobacterium (NTM) strains identified by conventional biochemical methods were further divided into subspecies by multi-locus sequence analysisof 16S rRNA, *hsp65*, *rpoB*, and 16S-23S rRNA internal transcribed spacer sequences.

The broth dilution method was applied to evaluate the *in vitro* drug susceptibility of *M. fortuitum* strains according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI). Breakpoint values were referenced from CLSI guidelines. *Mycobacterium peregrinum* (ATCC700686) was evaluated in each batch experiment as a control.

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The reference *M. fortuitum* strain (ATCC6481) was also tested in each experiment. In addition, the minimum inhibitory concentration (MIC) values of all strains were evaluated in triplicate.

Crude genomic DNA was prepared by the direct boiling method. Three genes conferring second-line injectable drug, fluoroquinolone, and macrolide resistance, were amplified using primers 16S rRNA F (5'-GCACAAGCGGCGGAGCAT-3') and R (5'-GGTGATC CAGCCGCACCTT-3'), gyrA F (5'-GGAGCCTCTGACCGTA TCGA-3') and R (5'-GCCCGGTCTTGTAGGTGTCC-3'), and 23S rRNA F (5'-CGGTGATCCTATGCTGCCAAGA-3') and R (5'-CCCCAGTTAAACTACCCACCAG-3') with the corresponding primer sets. DNA sequences were compared with published sequences NZ_CP011269.1 for *M. fortuitum* by using DNAstar and BioEdit (version 7.1.3.0) software.

Antimicrobial susceptibility testing results for the 51 *M. fortuitum* isolates are shown in Table 1. Moxifloxacin showed the highest *in vitro* activity against *M. fortuitum*, the $MIC_{50}$ and $MIC_{90}$ of which were 0.125 and 1 μg/mL, respectively. On the basis of the CLSI recommendations, the prevalence of moxifloxacin-resistant *M. fortuitum* isolates was 3.9% (2/51). Gatifloxacin also exhibited potent *in vitro* activity, with an $MIC_{50}$ and $MIC_{90}$ of 0.125 and 0.5 μg/mL, respectively, while the other fluoroquinolone, levofloxacin, was four-fold less active than moxifloxacin. The $MIC_{50}$ and $MIC_{90}$ of levofloxacin were 0.5 and 4 μg/mL, respectively. At a breakpoint of 64 μg/mL, amikacin was active against 44 (86.3%) *M. fortuitum* isolates, yielding an $MIC_{50}$ and $MIC_{90}$ of 4 and of 0.5 μg/mL, respectively. In addition, meropenem and cefoxitin showed moderate *in vitro* activity against *M. fortuitum*, and there were 13 (25.5%) and 21 (41.2%) isolates resistant to these two antimicrobial agents, respectively. In contrast, fewer than half of the isolates tested were susceptible to imipenem (56.9%) and clarithromycin (76.5%), and nearly all *M. fortuitum* isolates were resistant to linezolid (84.3%) and tobramycin (100.0%).

**Table 1.** Distribution of *M. fortuitum* Isolates against Antimicrobial Agents

| Antimicrobial Agent[a] | Range | $MIC_{50}$ μg/mL | $MIC_{90}$ μg/mL | Resistance (%) |
|---|---|---|---|---|
| MOX | 0.0625-64 | 0.125 | 1 | 2 (3.9%) |
| AMK | 0.0625-64 | 4 | 64 | 7 (13.7%) |
| MEM | 0.25-256 | 16 | 32 | 13 (25.5%) |
| CFX | 0.25-256 | 64 | 256 | 21 (41.2%) |
| IMP | 0.25-256 | 32 | 256 | 29 (56.9%) |
| CLA | 0.0625-64 | 32 | 64 | 39 (76.5%) |
| LZD | 0.0625-64 | 64 | 64 | 43 (84.3%) |
| TOB | 0.25-256 | 32 | 64 | 51 (100.0%) |
| GAT | 0.0625-64 | 0.125 | 0.5 | - |
| LFX | 0.0625-64 | 0.5 | 4 | - |
| CFM | 0.0625-64 | 4 | 8 | - |
| RFB | 0.0625-64 | 4 | 8 | - |
| CAP | 0.0625-64 | 1 | 8 | - |
| TIG | 0.0625-64 | 1 | 16 | - |
| SM | 0.0625-64 | 64 | 64 | - |
| AZM | 0.0625-64 | 64 | 64 | - |
| RIF | 0.0625-64 | 64 | 64 | - |
| EMB | 0.0625-64 | 2 | 16 | - |
| MIN | 0.25-256 | 16 | 128 | - |
| SFX | 0.25-256 | 128 | 256 | - |
| VCM | 0.25-256 | 256 | 256 | - |

***Note***. [a]clarithromycin (CLA), amikacin (AMK), moxifloxacin (MOX), linezolid (LZD), rifabutin (RFB), tobramycin (TOB), meropenem (MEM), cefoxitin (CFX), capreomycin (CAP), azithromycin (AZM), levofloxacin (LFX), gatifloxacin (GAT), minocycline (MIN), tigecycline (TIG), sulfamethoxazole (SFX), streptomycin (SM), clofazimine (CFM), vancomycin (VCM), ethambutol (EMB), rifampcine (RIF), imipenem (IMP). -These drugs have no breakpoint values.

We further analyzed genetic mutations conferring clarithromycin, amikacin, and moxifloxacin resistance in *M. fortuitum*. As shown in Table 2, of the two moxifloxacin-resistant isolates, both possesseda nonsynonymous mutation in the gyrA gene, including one with Ser→Leu at codon 91 and one with Asp→Gly at codon 95. In contrast, all clarithromycin-resistant and AMK-resistant isolates had a wild-type sequence in the 23S rRNA and 16S rRNA genes, respectively.

This study describes the drug susceptibility profiles of *M. fortuitum* isolates in China. Of the antimicrobial agents tested, moxifloxacin, showed the highest activity against *M. fortuitum* (96.1%), which was higher than those reported in Taiwan (25%)[2] and Iran (29%)[3], but similar to that reported in the UK (100%)[4]. There were several potential reasons for this diversity in results in different regions. A previous study by Swenson et al.[5] revealed that different subspecies of the *M. fortuitum* group showed significant differences in their resistance to fluoroquinolones. Thus, one possible explanation for this difference may be related to the regional diversity of *M. fortuitum* subsepcies in previous studies. In contrast, the various drug susceptibility testing methods applied may have contributed to this difference. In a report from Iran[3], despite applying the broth microdilution method, the application of medium supplemented with nutritional supplements may have increased the MIC values of *M. fortuitum* isolates. Consistently with our observation, Cremades et al.[6] found that moxifloxacin was the most effective antibiotic against *M. fortuitum*, both alone and in combination with other antimicrobial agents. Taken together, our data indicate the potential of moxifloxacin for the treatment of patients infected with *M. fortuitum* in China.

Several studies have shown that amikacin has excellent activity against *M. fortuitum* and other RGM[7-8]. In agreement with previous studies, our results revealed that amikacin showed favorable activity against *M. fortuitum*, which inhibited the growth of 86.3% of *M. fortuitum* isolates. In contrast, clarithromycin, a cornerstone drug used to treat NTM, showed poor *in vitro* activity against *M. fortuitum* in the current study, which was significantly different from previous observations that approximately 20% of *M. fortuitum* isolates were resistant to clarithromycin[2]. Similar to *M. fortuitum*, the proportion of macrolide-resistant mycoplasma was significantly higher than those reported in other countries, which may be attributed to the abuse of macrolides in the treatment of respiratory tract infection[9]. Considering that NTM is a widely distributed opportunistic pathogen, over-exposure to macrolides may lead to the high emergence of drug-resistant bacteria. Similarly, a strikingly higher rate of clarithromycin-resistant *M. kansasii* isolates was described in a recent study from China. Given the remarkable potency of clarithromycin in the clinical treatment of NTM infections, the high prevalence of clarithromycin-resistance in NTM indicates that improved use of currently available antibiotics is necessary.

Drug resistance in bacteria is thought to be primarily mediated by chromosomal mutations. In the current study, we identified two nonsynonymous mutations in the quinolone resistance-determining region of the *gyrA* gene, which yielded a sensitivity of 100% for detecting MOX resistance of *M. fortuitum* isolates. Given the small sample number for moxifloxacin-resistant isolates, further evaluation is needed to validate the diagnostic value of gyrA sequencing for predicting moxifloxacin susceptibility. Macrolide resistance is exclusively attributed to mutations in the *23S rRNA* gene in several NTM species. In contrast, no mutation was found in the clarithromycin-resistant *M. fortuitum* isolates in our study. Similar to clarithromycin-resistant isolates, *M. fortuitum* isolates carried genetic mutations in the *16S rRNA* gene conferring amikacin resistance. A previous report revealed that *M. fortuitum* harbors

**Table 2.** Sequencing Results of *M. fortuitum* Isolates Resistant to Clarithromycin, Amikacin, and Moxifloxacin

| Antimicrobial Agents | Locus | Nucleotide Substitution | Amino Acid Substitution | No. of Isolates (%) |
|---|---|---|---|---|
| Clarithromycin | *23S rRNA* | NA | - | 39 (100.0) |
| Amikacin | *16S rRNA* | NA | - | 7 (100.0) |
| Moxifloxacin | *gyrA* | C272T | Ser91Leu | 1 (50.0) |
| | | A284G | Asp95Gly | 1 (50.0) |

two copies of the rRNA operon, which may make conventional drug resistance mechanisms more complicated[10]. Our data indicate that some other intrinsic mechanism conferring clarithromycin and amikacin resistance plays an essential role in *M. fortuitum* infection.

There were several limitations to this study. First, all data in this study were obtained *in vitro*. Further experiments will be carried out in animal model to assess the *in vivo* effectiveness of promising drugs for clinical practice. Second, the strains were only obtained from two TB-specialized hospitals. Although the patients may have been from different regions of China, the results may have been biased. Finally, the interactions of different drug combinations were not tested in this study.

In conclusion, our data demonstrate that moxifloxacin and amikacin exhibit favorable *in vitro* activity against *M. fortuitum* isolates, while most *M. fortuitum* isolates were resistant to clarithromycin and linezolid in China. The loss of genetic mutation in clarithromycin- and amikacin-resistant isolates indicates that some other intrinsic mechanism conferring clarithromycin and amikacin resistance plays an essential role in *M. fortuitum* infection.

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