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Short Communication

Detection and molecular characterisation of amikacin-resistant *Mycobacterium abscessus* isolated from patients with pulmonary disease

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ABSTRACT

Objectives: The aim of this study was to investigate the molecular mechanisms conferring amikacin (AMK) resistance in *Mycobacterium abscessus* clinical isolates.
Methods: A total of 194 *M. abscessus* clinical isolates were collected from patients with pulmonary disease during the period 2012–2017. AMK susceptibility was determined by the broth microdilution method. Whole-genome data were used for identification of mutations in resistance-associated genes. Quantitative reverse transcription PCR (qRT-PCR) was performed to measure the gene transcriptional level.
Results: AMK showed high in vitro killing activity against *M. abscessus*, with an MIC_{50} of 8 mg/L and an MIC_{90} of 16 mg/L. Five isolates (2.6%) were resistant to AMK (MIC > 1024 mg/L), of which four (80.0%) harboured a resistance-associated *rrs* mutation A1408G. qRT-PCR analysis showed that most of the AMK-resistant isolates (4/5; 80.0%) overexpressed the transcriptional regulator gene *whiB7* and the multidrug-efflux transporter gene *tap*. However, overexpression of the aminoglycoside-modifying enzyme gene *eis2* was only observed in one (20.0%) AMK-resistant isolate.
Conclusion: The AMK resistance rate in *M. abscessus* clinical isolates in this study was low (2.6%). The A1408 G mutation in *rrs* and overexpression of WhiB7 and Tap were the predominant mechanisms of AMK resistance in *M. abscessus*.

1. Introduction

Infections caused by nontuberculous mycobacteria (NTM) have increased significantly around the world, raising serious public-health concerns [1]. *Mycobacterium abscessus*, one of the most challenging NTM, has gained increasing medical importance in recent years. Infections caused by *M. abscessus* include chronic pulmonary disease, cutaneous disease, lymphadenitis and disseminated disease, with chronic pulmonary infection encountered the most frequently in clinical practice [2]. Treatment of *M. abscessus* infection is notoriously difficult due to the high degree of intrinsic resistance of the bacterium to many major classes of antibiotics. Therefore, *M. abscessus* has been called an antibiotic nightmare [3].

The aminoglycoside antibiotic amikacin (AMK) exerts its bactericidal activity by targeting the decoding A-site in the bacterial ribosome [4]. AMK possesses good antimycobacterial activity against NTM species, including *M. abscessus*, and is regarded as one of the most active parenteral antibiotics for the treatment of *M. abscessus* infection [5]. Successful use of AMK for the treatment of patients with *M. abscessus* pulmonary disease has been reported [6]. The latest guideline proposed administration of an aminoglycoside, preferably amikacin, and a macrolide, e.g. clarithromycin or azithromycin, in combination with additional compounds, e.g. imipenem, cefoxitin, linezolid, tigecycline and/or

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fluoroquinolones, for *M. abscessus* pulmonary disease [7]. Thus, amikacin is a cornerstone of antimicrobial chemotherapy against *M. abscessus* infection.

However, AMK resistance in *M. abscessus* has been emerging [8]. Resistance to AMK is conferred by several mechanisms, including target mutation, drug modification, and reduced uptake and/or increased efflux [9]. It has been well acknowledged that mutations in the target sites of 16S rRNA (*rrs*) and 30S ribosomal protein S12 (RpsL) are responsible for high-level amikacin resistance in *M. abscessus* [10,11]. In recent years, overexpression of the aminoglycoside-modifying enzyme gene *eis2* and the multidrug efflux transporter gene (*tap*) were also demonstrated to be involved in the AMK resistance in *M. abscessus* [12]. Constitutive activation of these genes is often caused by increased expression of the activator gene *whiB7* [12,13]. However, these discoveries were mainly obtained from studies on the *M. abscessus* type strain ATCC 19977. Large-scale data regarding the prevalence of AMK resistance as well as studies focusing on the investigation of the underlying resistance mechanisms in *M. abscessus* clinical isolates are limited. In this study, the susceptibility to AMK of 194 *M. abscessus* clinical isolates collected from patients with pulmonary disease was determined and molecular characterisation of AMK-resistant isolates was performed. The findings from this work may expand our understanding of AMK resistance among *M. abscessus* clinical isolates.

2. Materials and methods

2.1. Isolation of Mycobacterium abscessus

Collection and identification of *M. abscessus* clinical isolates were performed as described previously [14]. All isolates were stored at −80 °C until use.

2.2. Amikacin susceptibility testing

AMK susceptibility was determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines and the results were interpreted according to CLSI breakpoints as follows: susceptible, minimum inhibitory concentration (MIC) ≤16 mg/L; intermediate, MIC = 32 mg/L; and resistant, MIC ≥ 64 mg/L [15]. *Mycobacterium peregrinum* ATCC 700686 (American Type Culture Collection, Manassas, VA, USA) and *Staphylococcus aureus* ATCC 29213 (American Type Culture Collection) served as control reference strains.

2.3. Whole-genome sequencing

The whole genomes of all 194 strains were sequenced by us previously and are available at DDBJ/ENA/GenBank under the BioProject nos. **PRJNA448987**, **PRJNA398137** and **PRJNA488058** [14].

2.4. Sequence comparison of rrs and rpsL among Mycobacterium abscessus isolates

The sequences of wild-type (WT) *rrs* and *rpsL* genes were extracted from a corresponding publication [16]. Sequences were then aligned with each of the 194 *M. abscessus* genomes to identify the presence of mutations in each gene.

2.5. RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Methods of RNA extraction and qRT-PCR have been described previously [14]. Primer pairs for amplification of each gene were as follows: *whiB7* (Mab_3508c), GTTGAAGTGGAGGCCCGAAG/CACAATGGTCCCCTGCTCAA; *eis2* (Mab_4532c), GTTGTCGGGACAGGTACGAG/ACCTTGCCGGATTCCTTCTG; and *tap* (Mab_1409c), GGACGTCGGAGAAGATCGAC/CATCGGCAACGGTGTCTCTA. The *sigA* (Mab_3009) gene served as the endogenous reference gene (primer, AGCGTGAGCTGCTACAGGAC/TGGATTTCCAGCACCTTCTC). Clinical *M. abscessus* strain A126, with an AMK MIC of 2 mg/L, was used as the reference strain for gene expression analysis. The fold change in target gene expression in a target sample relative to strain A126 was calculated by the $2^{-\Delta\Delta CT}$ method.

3. Results

3.1. Amikacin minimum inhibitory concentration distribution among 194 Mycobacterium abscessus clinical isolates

The MICs of AMK against 194 *M. abscessus* isolates ranged from 2 mg/L to >1024 mg/L, with an MIC_{50} (MIC required to inhibit 50% of the isolates) of 8 mg/L and an MIC_{90} (MIC required to inhibit 90% of the isolates) of 16 mg/L (Fig. 1), suggesting that AMK has high in vitro killing activity against *M. abscessus*. One isolate was intermediate-resistant to AMK (MIC = 32 mg/L). Five isolates (2.6%) were highly resistant to AMK (MIC > 1024 mg/L), therefore the AMK resistance rate in *M. abscessus* clinical strains isolated in this study was low.

3.2. Identification of mutations in the amikacin target sites

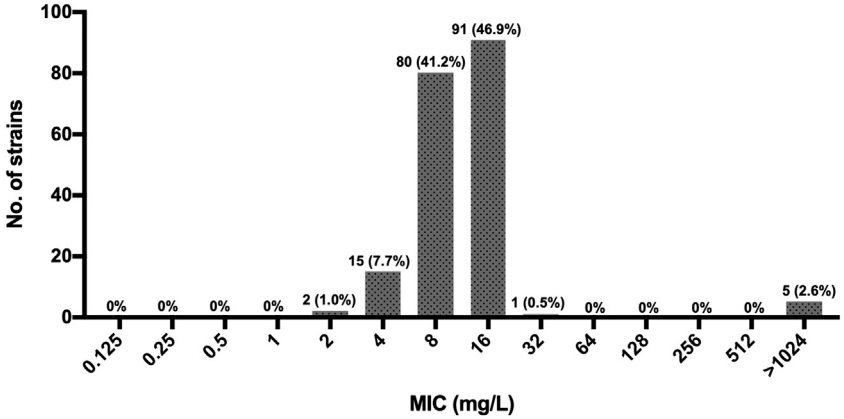

Fig. 1. Distribution of amikacin minimum inhibitory concentrations (MICs) against 194 *Mycobacterium abscessus* clinical isolates. The number and proportion of isolates are labelled on the top of each bar. *Mycobacterium peregrinum* ATCC 700686 and *Staphylococcus aureus* ATCC 29213 served as the control reference strains.

Mutations in the 16S rRNA gene (*rrs*) and 30S ribosomal protein S12 (RpsL) are known mechanisms leading to AMK resistance. The DNA sequence of the entire *rrs* gene and the amino acid sequence of the entire RpsL were extracted from the whole-genome sequence data and were compared with WT sequences. A total of six kinds of mutations in *rrs* were observed, with five mutations (G249C, T711C, C946T, A975G and C976T) occurring in 34/188 (18.1%) AMK-susceptible strains (Table 1). One point mutation of A1374 G was present in AMK-resistant strains (A233, G142, G179 and G192) with high proportion (4/5; 80%). Furthermore, secondary structure analysis of *rrs* revealed that the mutation site of A1374G corresponded to the A1408G site according to the *Escherichia coli* numbering system, which has been demonstrated to caused high-level AMK resistance in multiple species [17]. No non-synonymous mutation in *rpsL* was found in any of the AMK-resistant strains.

3.3. Analysis of other resistance mechanisms

The transcriptional regulator WhiB7 is required for multidrug resistance both in *M. abscessus* and *Mycobacterium smegmatis*. Among the *whiB7*-dependent transcripts, the aminoglycoside acetyltransferase gene *eis2* and the multidrug efflux transporter gene *tap* have been reported to confer amikacin resistance in *M. abscessus* [12,13]. Thus, qRT-PCR was used to assess the transcriptional levels of these genes in six AMK-non-susceptible isolates [one intermediate-resistant (A254) and five fully resistant (A8, A233, G179, G142 and G192)]. As shown in Fig. 2, except for strain A233, AMK-resistant strains had a >10-fold increase in transcriptional levels of whiB7 compared with AMK-susceptible strain A126. All of the *whiB7*-overexpressing strains showed high-level expression of *tap*. Overexpression of *eis2* was only observed in one strain (A8), in which the sequence of *rrs* is WT.

4. Discussion

AMK is a front-line drug used in combination with other antibiotics against *M. abscessus* infection. Resistance to AMK in *M. abscessus* has emerged, but large-scale data on the prevalence of AMK resistance among *M. abscessus* clinical isolates are limited. Several mechanisms of resistance have been elucidated in the *M. abscessus* laboratory type strain ATCC 19977. However, the molecular mechanisms conferring AMK resistance in *M. abscessus* clinical isolates remain underexplored. In this study, 194 *M. abscessus* strains were isolated from recent pulmonary infection cases in mainland China. Using these isolates, the in vitro susceptibility to AMK of *M. abscessus* was determined and resistance mechanisms were investigated.

The AMK resistance rate of *M. abscessus* in this study was as low as 2.6% (5/194), which is consistent with a previous study [18], suggesting that AMK is still an ideal drug for the treatment of infections caused by *M. abscessus*. One interesting discovery of this study was that the MIC distribution of AMK was bimodal, indicating that strains in this study included *M. abscessus* WT isolates that are innately susceptible to AMK (trailing into the 2–16 mg/L region) and a non-WT population possessing acquired resistance to AMK (>1024 mg/L region). Of the five non-WT isolates, four (80.0%) harboured the A1408 G mutation in *rrs*. Further phylogenetic analysis showed that these isolates belong to a different clone (data not shown), indicating that the appearance of the A1408G mutant was not due to clonal spread. Several kinds of *rrs* mutations were also observed in AMK-susceptible strains, implying that spontaneous mutations occur even though *rrs* is highly conserved. Hence, regular detection of mutations in *rrs* in clinical practice, especially for the A1408G site, is required for determination of AMK resistance and to optimise the use of antibiotics for therapy.

It has been reported that *M. abscessus* WhiB7, a transcriptional activator, is required for intrinsic resistance of *M. abscessus* to multiple antibiotics. Deletion of *whiB7* resulted in multidrug susceptibility, such as to amikacin, clarithromycin, erythromycin, and tetracycline. Pryjma et al. demonstrated that transcription of *whiB7* in *M. abscessus* was induced by subinhibitory concentrations of clarithromycin [12]. In the current study, AMK-resistant strains (A8, G179, G142 and G192) isolated from patients who received continuous clarithromycin treatment for >3 months showed overexpression of *whiB7*. However, the expression level of *whiB7* was not elevated in AMK-resistant strain A233, which was isolated from a patient before initial clarithromycin therapy. These results support the effect of clarithromycin pre-exposure on amikacin resistance. Clinical use of clarithromycin may induce increased resistance to amikacin. As clarithromycin and amikacin are both cornerstones for *M. abscessus* therapy and are routinely co-administered, the emergence of cross-resistance to amikacin by clarithromycin has complicated the treatment of *M. abscessus* infections. Further investigations are desperately required to increase the killing efficacy of AMK and clarithromycin against *M. abscessus*, such as developing inhibitors of WhiB7.

Several genes have been demonstrated to be upregulated by WhiB7 and contribute to AMK resistance. These genes include *eis2* and *tap* [12,13]. In the current study, a dramatically elevated expression level of *tap* was observed in all of the *whiB7*-overexpressing isolates, which further confirmed the role of the *whiB7–tap* signal pathway in AMK resistance. In contrast, no increased expression level of *eis2* was observed in AMK-resistant isolates, except for A8, suggesting that regulation of *eis2* involves a more complex signal transduction pathway. Maurer et al. demonstrated that drug-mediated ribosomal inhibition of protein synthesis is required to upregulate expression of the aminoglycoside-modifying enzyme Aac(2') in *M. smegmatis* [19]. They found that upregulation of *aac(2')* expression is only observed in the *rrs* WT strain but not in the *rrs* A1408G mutant strain, in which the antibiotic's inhibitory activity against the bacterial ribosome was abolished. Similarly, Nash et al. demonstrated that the mRNA transcript level of another aminoglycoside-modifying enzyme, *erm* (41), which confers macrolide resistance by methylation of 23S rRNA *rrl*, increased only in the *rrl* WT strain [20]. In the current study, overexpression of *eis2* was only observed in the *rrs* WT strain A8. No increased expression of *eis2* was found in any of the *rrs* A1408 G mutant strains (A233, G179, G142 and G192), suggesting that the expression level of *eis2* might also be associated with the *rrs* genotype in *M. abscessus*.

In addition, one AMK-intermediate-resistant strain (A254) without any overexpression of *whiB7*, *tap*, *eis2* or *rrs* mutation was observed, suggesting the presence of an unknown resistance

Table 1
Mutation information of the *rrs* gene among 194 *Mycobacterium abscessus* clinical isolates according to amikacin (AMK) susceptibility.[a]

Mutation in *rrs*[b]	*n* (%)	
	AMK-susceptible (*n* = 188)	AMK-resistant (*n* = 5)
G249C	1 (0.5)	0 (0)
T711C	1 (0.5)	0 (0)
C946T	1 (0.5)	0 (0)
A975G	7 (3.7)	0 (0)
C976T	24 (12.8)	0 (0)
A1374G[c]	0 (0)	4 (80.0)

[a] One isolate was intermediate-resistant to AMK with no *rrs* mutation.
[b] Nucleotide bases were numbered from 1 to 1504 of the *rrs* gene in *M. abscessus* (Mab_r5051).
[c] A1374G corresponds to A1408G in the *Escherichia coli* numbering system.

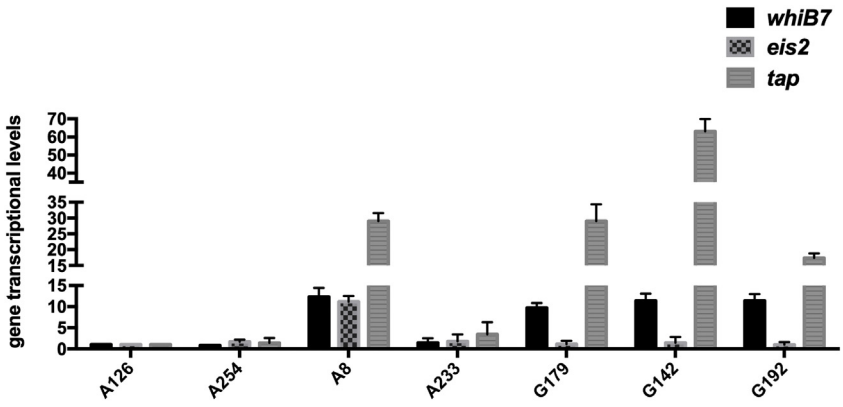

Fig. 2. Quantitative reverse transcription PCR (qRT-PCR) assessment of transcriptional levels of the genes *whiB7*, *tap* and *eis2*. Error bars represent the standard error of each data point. Clinical *Mycobacterium abscessus* strain A126, with an amikacin minimum inhibitory concentration of 2 mg/L, was used as the reference strain for gene expression analysis.

mechanism. We are currently in the process of investigating the resistance mechanism in this strain.

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Competing interests

None declared.

Ethical approval

Not required.

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