의학박사 학위논문

In-Depth Analysis of Amikacin Susceptibility in *Mycobacterium avium* Complex: Validation of Minimum Inhibitory Concentration in the Presence of Trailing Growth

Mycobacterium avium complex에서 아미카신 감수성 심층 분석: 후행성장이 있는 경우 최소억제농도 검증

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박 보 성

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이 논문을 의학박사 학위 논문으로 제출함

2024년 2월

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Abstract

Background: Amikacin is tested as a first-line drug for *Mycobacterium avium* complex (MAC). MAC often shows trailing growth in amikacin well when broth microdilution minimum inhibitory concentration (MIC) is measured. Although Clinical and Laboratory Standards Institute recommends reading amikacin MIC at the concentration of complete inhibition, trailing growth often causes a subjective variation in MIC readings and a shift in MIC toward false resistance. Therefore, this study was performed to evaluate the effect of trailing growth of MAC on amikacin MIC and suggest the objective criteria to read MIC in trailing growth.

Methods: From November 2021 to April 2022, antimycobacterial susceptibility tests were performed request-based for clinical isolates of MAC using Sensititre SLOMYCOI (Thermo Fisher Scientific, Cleveland, OH, USA). For strains with an amikacin MIC of 32 μg/mL or higher, the AST was re-tested, and the MIC was determined at the point of trailing growth onset. The onset of trailing growth is defined as the point where a notable decrease in bacterial growth is observed compared to the preceding well, and where increasing concentrations of amikacin do not result in any additional inhibition of bacterial growth. Mutations in the *rrs* gene were additionally tested for all isolates included in the study. Amikacin treatment history was searched for patients corresponding to all isolates included in the study. MIC corresponding to

the 50th percentile (MIC_{50}) and 90th percentile (MIC_{90}) of MAC isolates were determined and the epidemiological cut-off (ECOFF) value was also calculated using the ECOFFinder algorithm. The MIC_{50}, MIC_{90} and ECOFF values of the study isolates were validated by comparison with the amikacin MIC distribution in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) deposited data set. This study was approved by the Institutional Review Board of Asan Medical Center (S2022-1595-0001).

Results: A total of 134 isolates including 67 *M. intracellulare*, 65 *M. avium*, and 2 *M. chimaera* were tested for AST and interpretation of amikacin MIC resulted in 44 (32.8%) being categorized as intermediate and 29 (21.6%) as resistant. A total of 72 isolates (53.7%) of *M. intracellulare* and *M. avium*, classified as intermediate and resistant, were re-tested using the SLOMYCOI panel, with MIC reading at the concentration which trailing growth started. Among them, 44 isolates of MAC were changed to more susceptible category when trailing growth was considered no growth; 10 and 4 isolates of *M. intracellulare* changed from resistant to intermediate or susceptible, respectively, and 1 and 4 isolates of *M. avium* changed from resistant to intermediate or susceptible, respectively. Any of MAC isolates including one of resistant category did not carry *rrs* mutation. The MIC_{50}, MIC_{90}, and ECOFF values of the study isolates were in excellent agreement with the distribution of amikacin MICs from EUCAST. Among all patients included in this study, six had a history of amikacin treatment.

Of those, only one had a history of six month treatment with amikacin and MAC isolate from the patient exhibited definite resistant MIC of greater than 64 against amikacin.

Conclusion: At initial readout, amikacin resistance amounted to 21.6% of clinical isolates, however the resistance was not correlated with *rrs* mutations in our study. When CLSI interpretation conditions were strictly followed, amikacin resistance rates were much higher than clinical expectations due to trailing growth. To make the current CLSI breakpoints clinically meaningful, we believe it is best to read the MIC at the concentration trailing growth started.

Keywords: *Mycobacterium avium* complex, Amikacin, Minimum inhibitory concentration, Trailing growth, Sensititre SLOMYCOI panel

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Background

The incidence of nontuberculous mycobacterial pulmonary disease (NTM-PD) is increasing globally [1], and this trend is observed also in South Korea. According to the study using national health insurance data from 2007 to 2016, the prevalence of NTM infection significantly increased from 6.7 per 100,000 population in 2007 to 39.6 per 100,000 population in 2016 [2]. Similarly, the incidence of NTM infection also increased markedly from 6.0 per 100,000 in 2008 to 19.0 per 100,000 in 2016.

Mycobacterium avium complex (MAC) is the most common pathogen causing NTM-PD. Currently, the species and subspecies that constitute the MAC include *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, *Mycobacterium arosiense*, *Mycobacterium bouchedurhonense*, *Mycobacterium chimaera*, *Mycobacterium colombiense*, *Mycobacterium intracellulare*, *Mycobacterium marseillense*, *Mycobacterium paraintracellulare*, *Mycobacterium timonense*, *Mycobacterium vulneris*, *Mycobacterium yongonense* [3]. Among these, *M. avium* subsp. *avium* and *M. intracellulare* are well-known pathogens. Recently, *M. chimaera* has also been recognized as a pathogen [4]. It has been reported in patients undergoing open heart surgery, where *M. chimaera* forms biofilms in the

water tanks of heater-cooler units used during the surgery. This can lead to infection through aerosolized bacteria entering the heart area during open heart surgery.

The first-line drugs to treat MAC-related disease include rifampin (RIF), ethambutol (EMB), macrolide, and/or amikacin [5]. In controlled clinical trials, macrolides have been identified as antimicrobial agents that show a correlation between *in vitro* susceptibility tests for MAC and the clinical response [6]. Additionally, recent reports also indicate a correlation between the minimum inhibitory concentration (MIC) values of amikacin and clinical response [7]. Therefore, macrolides and amikacin are considered first-line drugs for evaluating the susceptibility of MAC isolates.

On the other hand, for EMB, RIF, and rifabutin (RFB), the correlation between MIC values and clinical response is known to be poor [3]. Therefore, while these are recommended treatment regimens, the Clinical and Laboratory Standards Institute (CLSI) M24-Ed3 does not recommend reporting susceptibility for these drugs due to the inability to establish breakpoints that differentiate susceptible and resistant strains. However, previous studies have indicated that an unfavourable response is associated with MAC-PD patients having MICs ≥8 μg/mL for RIF and EMB, suggesting that reporting MIC values could be useful [8]. Due to insufficient research on streptomycin, it is recommended that only the MIC value be reported if this drug is tested.

As the acquired mutational resistance mechanisms, such as mutations in the 23S rRNA gene, are the same for clarithromycin and azithromycin in MAC isolates, it is cost-effective to test only one macrolide. Due to reasons such as poor solubility at the high concentrations used in testing, clarithromycin is the preferred class drug for macrolide testing, and testing for azithromycin susceptibility is not recommended.

Numerous studies suggest that amikacin resistance in MAC is associated with mutations in the 16S rRNA (*rrs*) gene [9, 10]. Therefore, sequencing the 16S rRNA gene to identify mutations at positions 1408, 1409, and 1411 (*Escherichia coli* numbering) can confirm amikacin resistance in MAC isolates. However, the absence of mutations in these positions does not guarantee susceptibility, as other resistance mechanisms may exist.

For isolates from patients intolerant to macrolide therapy or with macrolide-resistant MAC, antimycobacterial susceptibility testing (AST) for second-line agents such as moxifloxacin and linezolid, following the clinical breakpoints of CLSI M62, should be considered. However, the *in vivo* effectiveness of these agents for MAC disease remains unproven [3]. Due to limited options for treating macrolide-resistant infections, interest in multidrug regimens, including clofazimine, is increasing [11]. Yet, since breakpoints for clofazimine are not established, only MIC values should be reported if AST is performed on this drug.

AST is recommended if a patient exhibits no clinical improvement or deteriorates while remaining culture positive. In cases of disseminated disease, AST should be repeated after three months of treatment, and for chronic pulmonary disease, after six months [12].

CLSI M24-Ed3 recommends using the broth microdilution test for antimycobacterial susceptibility testing of NTM, including MAC [3]. Except for trimethoprim-sulfamethoxazole, the MIC of drugs is determined in a well where visible bacterial growth is completely inhibited [13]. Accurate MIC reading is critical because the assignment of susceptible, intermediate, and resistant depends on which well the MIC is read from, and this decision influences treatment selection.

Sensititre SLOMYCOI panel (Thermo Fisher Scientific, Cleveland, OH, USA) is a commercialised broth microdilution testing kit widely used in clinical laboratories. However, it has been used to evaluate MIC only against *Mycobacterium marinum* [14] and clarithromycin MIC for MAC [15]. Although studies have been conducted to determine the epidemiological cut-off (ECOFF) value for MAC or to identify the distribution of MICs in MAC species using the Sensititre panel, to our knowledge, the evaluation of the amikacin MIC using the Sensititre SLOMYCOI panel in MAC is not well established [16-20]. In the broth microdilution assay for MAC employing a Sensititre SLOMYCOI panel, MIC determination occasionally becomes ambiguous due to non-confluent, faint sporadic trailing growth within the amikacin well.

Difficulty in MIC reading due to trailing growth in broth microdilution is common in the antifungal susceptibility test of *Candida* species to azole drugs, particularly fluconazole, voriconazole, and *Aspergillus* species to itraconazole [21, 22]. Moreover, according to CLSI M07-Ed11 and M24-Ed3, in the context of conducting antimicrobial susceptibility tests using broth microdilution for gram-positive cocci against chloramphenicol, clindamycin, erythromycin, linezolid, tedizolid, and tetracycline, and for *Nocardia* species against linezolid, it is recommended to ignore trailing growth [3, 23]. Difficulties in determining the MIC of amikacin for MAC in broth microdilution assays because of trailing growth have been described in a previous study; however, the study did not use the Sensititre SLOMYCOI panel [9].

There have been reports suggesting variability in reading MAC's MIC depending on the reader's experience [7]. In this study, readers with less than two years of experience tended to record the AST results of the same MAC sample as "±growth," while those with over five years of considerable experience often noted them as negative.

This study aimed to propose a method for amikacin MIC reading in the presence of trailing growth during broth microdilution for MAC using Sensititre SLOMYCOI panels and assess the clinical significance of the findings.

Materials and Methods

Study Population

In this study, a total of 134 strains, isolated more than once from individual patients and identified as MAC by a polymerase chain reaction (PCR)-hybridisation assay (GenoType Mycobacterium CM/AS; Hain Lifescience GmbH, Germany), were included. These isolates were collected between November 2021 and April 2022 at the Asan Medical Center in Seoul, South Korea.

Species Identification by Sequencing Methods

Sequencing of the 16S rRNA, *hsp65*, and *rpoB* genes was carried out on all strains using previous literature primers to determine MAC species (Table 1) [24-26]. Sequences obtained via PCR were analysed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). In cases where the results of the 16s rRNA, *hsp65*, and *rpoB* genes analyses were discordant, an isolate was assigned to a species if the NCBI BLAST results for at least two of these three genes were consistent.

Table 1. Primer sequences used for sequencing in the study

Target	**Primer**	**Sequence**
16S rRNA gene	8FPL	5' AGT TTG ATC CTG GCT CAG 3'
	806R	5' GGA CTA CCA GGG TAT CTA AT 3'
	515FPL	5' TGC CAG CAG CCG CGG TAA 3'
	13B	5' AGG CCC GGG AAC GTA TTC AC 3'
hsp65	HSPFOR	5' ACC AAC GAT GGT GTG TCC AT 3'
	HSPREV	5' CTT GTC GAA CCG CAT ACC CT 3'
rpoB	rpoB_1_F	5' GGC AAG GTC ACC CCG AAG GG 3'
	rpoB_1_R	5'AGC GGC TGC TGG GTG ATC ATC 3'
rrs (16S rRNA)	rrs1-F	5' ATG ACG TCA AGT CAT CAT GCC 3'
	rrs1-R	5' AGG TGA TCC AGC CGC ACC TTC 3'
rrl (23S rRNA)	23SF	5' AAT GGC GTA ACG ACT TCT CAA CTG T 3'
	23SR	5' GCA CTA GAG GTT CGT CCG TCC C 3'

Antimicrobial Resistance Genes

Mutations at positions 1408, 1409, and 1411 (*E. coli* numbering) were investigated via sequencing of the *rrs* gene (16S rRNA gene) associated with amikacin resistance [9, 27]. Additionally, in alignment with findings from previous studies, we investigated mutations in other regions of the *rrs* gene, beyond positions 1408, 1409, and 1411, to rule out amikacin resistance arising from mutations in these alternative areas of the gene.

Similarly, mutations at positions 2058 and 2059 (*E. coli* numbering) of the *rrl* gene (23S rRNA gene) associated with macrolide resistance were investigated [10, 27-29].

Antimycobacterial Susceptibility Test and Trailing Growth

Antimycobacterial susceptibility test (AST) was performed for all strains via the broth microdilution method using Sensititre SLOMYCOI (Thermo Fisher Scientific, Cleveland, OH, USA) and cation-adjusted Mueller-Hinton broth with 5% oleic albumin dextrose catalase.

The obtained MIC results were read when the bacterial growth was completely inhibited. For strains with an amikacin MIC of 32 μg/mL or higher, the AST was re-conducted, and the MIC was determined at the point of trailing growth onset, which is defined as the juncture at which a notable decrease in bacterial growth, as illustrated in Figure 1, becomes evident. Beyond this

point, no further inhibition of bacterial growth is observed in wells with increasing amikacin concentrations.

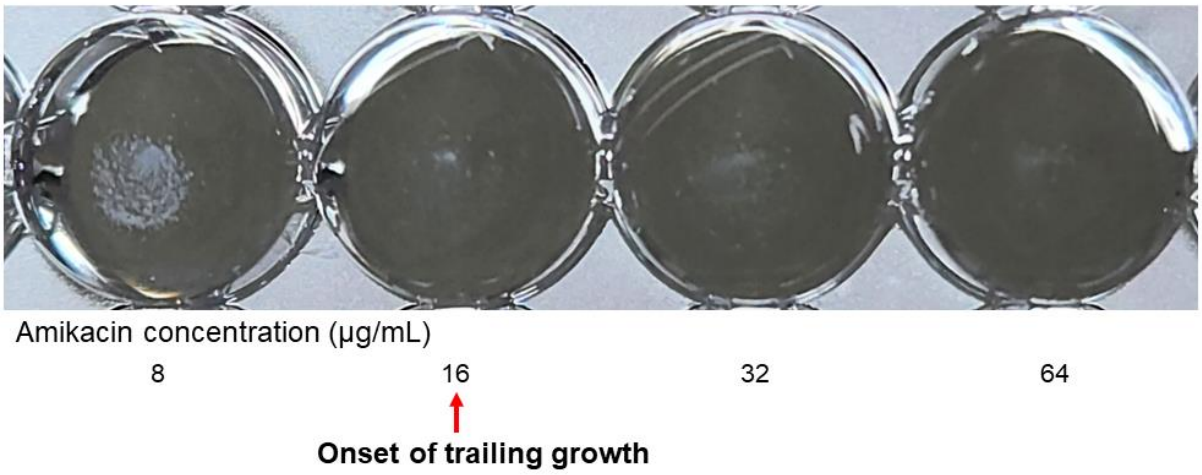

Figure 1. Image of the Sensititre SLOMYCOI plate's amikacin wells illustrating the point of trailing growth onset. In this image, a marked reduction in bacterial growth is observed as the amikacin concentration increases from 8 µg/mL to 16 µg/mL. Beyond this concentration, up to 64 µg/mL, no further inhibition of bacterial growth is apparent. When the MIC is read at the onset of trailing growth, the MIC for this case is determined to be 16 µg/mL.

Review of Amikacin Treatment History

For the 134 isolates corresponding to the research participants, the history of amikacin usage in the related patients was investigated by reviewing electronic medical records. Drug susceptibility was re-tested for strains resistant to clarithromycin, and previous macrolide treatment history was investigated.

Calculation of MIC metrics

The MIC data obtained from the re-tested strains were combined with the MIC data of specimens not re-tested below MIC 16 μg/mL for joint analysis. From these combined data, we calculated the modal MIC, MIC_{50}, and MIC_{90}, as well as the 99.9% ECOFF value according to the ECOFFinder algorithm [30]. The modal MIC represents the MIC value at the median when the strains are arranged in order of their MICs. MIC_{50} is the MIC at which 50% of isolates are inhibited, while MIC_{90} is the MIC inhibiting 90% of isolates. The European Committee of Antimicrobial Susceptibility Testing (EUCAST) recommends the use of John Turnidge's ECOFFinder algorithm for ECOFF calculation. The ECOFFinder algorithm statistically determines the ECOFF by utilizing the principle that the distribution of the base-2 logarithm of MICs for any species follows a Gaussian distribution. The program for the ECOFFinder

algorithm can be downloaded from the EUCAST website (https://www.eucast.org/mic_and_zone_distributions_and_ecoffs) and the CLSI website (https://clsi.org/meetings/susceptibility-testing-subcommittees/ecoffinder/).

Similarly, using the MIC data from the isolates read at the point of complete bacterial growth inhibition (following the CLSI guidelines), we calculated the modal MIC, MIC_{50}, MIC_{90}, and the 99.9% ECOFF value.

Ethical Considerations

This study was approved by the Institutional Review Board of Asan Medical Center (S2022-1595-0001). Consent from participants was waived.

Results

Identification of MAC Species

All 65 isolates identified as *M. avium* in the PCR-hybridisation assay were identified as *M. avium* through multiple gene sequencing. Of the 69 isolates identified as *M. intracellulare* using the PCR-hybridisation assay, 67 were identified as *M. intracellulare* and 2 as *M. chimaera* via the sequencing method.

Results of Antimycobacterial Susceptibility Tests for Antimicrobials with Established Breakpoints

According to the results of the AST, in which the MIC was measured at the point of complete bacterial growth inhibition, 97.0% of *M. avium* strains were susceptible to clarithromycin, whereas 1.5% were resistant (Table 2). Furthermore, 10.8% of *M. avium* strains were resistant to amikacin, and more than 60 % were resistant to linezolid and moxifloxacin. All *M. intracellulare* strains were susceptible to clarithromycin with no resistant isolates, and 32.9%, 86.6%, and 92.5% were resistant to amikacin, linezolid, and moxifloxacin, respectively.

Table 2. Minimum inhibitory concentration (MIC) values for *Mycobacterium avium* complex clinical isolates, read at complete bacterial growth inhibition

	Amikacin				Clarithromycin				Linezolid				Moxifloxacin			
MIC (μg/mL)	No. (%) of isolates			Susp	No. (%) of isolates			Susp	No. (%) of isolates			Susp	No. (%) of isolates			Susp
	Mavi	Mint	Mchi		Mavi	Mint	Mchi		Mavi	Mint	Mchi		Mavi	Mint	Mchi	
0.5	–	–	–		2 (3.1)	1 (1.5)	–	S	–	–	–		3 (4.6)	–	–	S
1	–	–	–		9 (13.9)	2 (3.0)	–	S	–	–	–		6 (9.2)	–	–	S
2	–	–	–		23 (35.4)	35 (52.2)	1 (50.0)	S	–	–	–		14 (21.5)	5 (7.5)	1 (50.0)	I
4	–	–	–		23 (35.4)	26 (38.8)	1 (50.0)	S	1(1.5)	–	1 (50.0)	S	17 (26.2)	19 (28.4)	–	R
8	3 (4.6)	2 (3.0)	–	S	6 (9.2)	3 (4.5)	–	S	7 (10.8)	1 (1.5)	–	S	21 (32.3)	36 (53.7)	1 (50.0)	R
16 or >8 (Moxi)	33 (50.8)	22 (32.8)	1 (50.0)	S	1 (1.5)	–	–	I	18 (27.7)	8 (11.9)	–	I	4 (6.2)	7 (10.4)	–	R
32	22 (33.8)	21 (31.3)	1 (50.0)	I	–	–	–		21 (32.3)	39 (58.2)	1 (50.0)	R	–	–	–	–
64	5 (7.7)	16 (23.9)	–	R	–	–	–		18 (27.7)	17 (25.4)	–	R	–	–	–	–
>64	2 (3.1)	6 (9.0)	–	R	1 (1.5)	–	–	R	–	2 (3.0)	–	R	–	–	–	–
Total	65 (100.0)	67 (100.0)	2 (100.0)		65 (100.0)	67 (100.0)	2 (100.0)		65 (100.0)	67 (100.0)	2 (100.0)		65 (100.0)	67 (100.0)	2 (100.0)	

Data are shown as n (%). Susp, Susceptibility; Mavi, *M. avium*; Mint, *M. intracellulare*; Mchi, *M. chimaera*; Moxi, Moxifloxacin; S, Susceptible; I, Intermediate; R, Resistant

Antimicrobial Resistance Gene Mutations

An *M. avium* strain resistant to clarithromycin remained resistant upon re-testing, and trailing growth was not observed in the clarithromycin well. A well-known A2058T mutation in the *rrl* gene was detected in the *M. avium* strain that was resistant to clarithromycin; however, no mutations in the *rrl* gene were observed in other isolates.[1] The patient from whom the clarithromycin-resistant strain had a MIC >64 μg/mL was isolated with a 5-year history of azithromycin treatment.

The *rrs* gene, which is associated with amikacin resistance in the *M. avium* complex, underwent sequencing, and all strains, including those showing resistance to amikacin, were identified as having the wild-type version of the gene.[2]

[1] Among the total of 134 strains included in the study, only one strain (*M. avium*) exhibited the widely recognized A2058T mutation in the *rrl* gene (23S rRNA gene).

[2] In all study isolates, the *rrs* gene (16S rRNA gene) was found to be wild type, with no mutations observed.

Results of Antimycobacterial Susceptibility Tests for Rifampin, Rifabutin, and Ethambutol

The percentages of *M. avium*, *M. intracellulare*, and *M. chimaera* strains with a MIC of ≥8 μg/mL were 43.9 %, 62.7 %, and 100 % for RIF, and 96.9%, 94.0%, and 100% for EMB, respectively (Table 3).

Table 3. *In vitro* minimum inhibitory concentrations (MICs) of rifampin, rifabutin, and ethambutol for 134 *Mycobacterium avium* complex clinical isolates

Species	Antimicrobial agents	Minimum inhibitory concentrations (MICs)								No. (%) of isolates with MIC≥8 (μg/mL)
		0.25 (RIF) or ≤0.25 (RFB)	0.5	1	2	4	8	>8 (RIF) or 16 (EMB)	>16	
Mavi	Rifampin	1	1	10	16	15	9	13	–	22 (33.8)
	Rifabutin	39	17	8	–	1	–	–	–	0 (0.0)
	Ethambutol	–	–	–	–	2	16	41	6	63 (96.9)
Mint	Rifampin	–	–	1	9	15	28	14	–	42 (62.7)
	Rifabutin	7	31	23	4	1	–	–	–	0 (0.0)
	Ethambutol	–	–	–	1	3	49	11	3	63 (94.0)
Mchi	Rifampin	–	–	–	–	–	2	–	–	2 (100.0)
	Rifabutin	–	–	1	1	–	–	–	–	0 (0.0)
	Ethambutol	–	–	–	–	–	2	–	–	2 (100.0)

Data are shown as number of isolates. RIF, rifampin; RFB, rifabutin; EMB, ethambutol; Mavi, *M. avium*; Mint, *M. intracellulare*; Mchi, *M. chimaera*

Re-evaluation of Amikacin MIC after Re-test of Antimycobacterial Susceptibility Test

For the strains that exhibited intermediate or resistant MIC values of 32 μg/mL or higher for amikacin, AST was carried out again by determining the MIC at the onset of trailing growth; the results are described in Table 4. In the case of *M. avium*, when the MIC was assessed following the complete inhibition of bacterial growth (as per CLSI M24-Ed3 guidelines), 22 strains initially exhibited an MIC of 32 μg/mL. However, upon re-evaluation, where the MIC was read at the onset of trailing growth, these strains exhibited a diverse range of MIC values spanning from 4 μg/mL to 64 μg/mL. Among these, 36.4% maintained the original MIC reading of 32 μg/mL, whereas 54.5% presented a lower MIC than initially determined. Similarly, among the five strains previously characterized with an MIC of 64 μg/mL, 40.0% maintained the same MIC value upon retesting, whereas the remaining three strains (60.0%) exhibited a lower MIC. The two strains which had initially shown an MIC >64 μg/mL, revealed MICs of 8 μg/mL and 16 μg/mL, respectively when re-tested.

Table 4. Comparison of amikacin MIC readings for *M. avium* and *M. intracellulare* obtained by two different methods

Amikacin MIC at complete growth inhibition (μg/mL)	*M. avium* amikacin MIC at the start of trailing growth (μg/mL)							
	4	8	16	32	64	>64	Undetermined*	Total
32	1 (4.5)	2 (9.1)	9 (40.9)	8 (36.4)	1 (4.5)	0 (0.0)	1 (4.5)	22 (100.0)
64	0 (0.0)	1 (20.0)	1 (20.0)	1 (20.0)	2 (40.0)	0 (0.0)	0 (0.0)	5 (100.0)
>64	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)
	M. intracellulare amikacin MIC at the start of trailing growth (μg/mL)							
32	0 (0.0)	1 (4.8)	8 (38.1)	8 (38.1)	3 (14.3)	0 (0.0)	1 (4.8)	21 (100.0)
64	0 (0.0)	0 (0.0)	3 (18.8)	10 (62.5)	3 (18.8)	0 (0.0)	0 (0.0)	16 (100.0)
>64	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	4 (66.7)	1 (16.7)	0 (0.0)	6 (100.0)

Data are shown as n (%). *Contamination or no growth of mycobacteria in the control well even after re-testing.

Regarding *M. intracellulare*, out of the 21 strains initially presenting an MIC of 32 μg/mL, only 38.1% retained this value upon re-evaluation, whereas 42.9% demonstrated a reduced MIC value. Among the 16 strains that had previously shown resistance with an MIC of 64 μg/mL, only 18.8% retained the original MIC upon re-evaluation. As for the six strains initially recording an MIC >64 μg/mL, only one strain (16.7%) remained resistant upon re-examination, with four strains presenting an MIC of 64 μg/mL and the remaining strain showing an MIC of 16 μg/mL.

History of Prolonged Amikacin Treatment

A history of prolonged amikacin treatment lasting 6 months was identified in only one patient (Table 5). The isolate from this patient had an amikacin MIC >64 μg/mL, regardless of the reading method used. Furthermore, an *M. intracellulare* isolate from a patient with a 1-week exposure to amikacin showed a two-fold decrease in MIC to 16 μg/mL upon re-testing.

Table 5. Clinical information regarding patients with a history of prior amikacin exposure

Case	Species of MAC	Prior amikacin exposure	MIC with susceptibility at complete growth inhibition (μg/mL)	MIC with susceptibility ignoring trailing growth (re-test) (μg/mL)	Other comorbidities	Treatment regimen	Post-treatment AFB conversion
1	*M. intracellulare*	6 months	>64, R	>64, R	-	Az+R+E+INH	Not Converted
2	*M. intracellulare*	1 week	64, R	16, S	DM, Bladder cancer, 3VD, Squamous cell carcinoma	Az+R+E	Converted
3	*M. intracellulare*	3 months	32, I	16, S	-	Az+R+E+Am	Not Converted
4	*M. intracellulare*	3 months	8, S	Not done	Schwannoma, Lung	Az+R+E	Converted
5	*M. avium*	4 months	32, I	16, S	-	Az+E+Am+INH	Not Converted
6	*M. avium*	3 months	32, I	32, I	-	Az+E+Am	Not Converted

AFB, acid-fast bacilli; DM, diabetes mellitus; 3VD, three-vessel disease; Az, azithromycin; R, rifampin; E, ethambutol; INH, isoniazid; Am, amikacin; MIC, minimum inhibitory concentration; MAC, *Mycobacterium avium* complex

Difference in MIC Distribution Depending on Interpretation of Trailing Growth

The difference in MIC distribution when reading the MIC either by ignoring trailing growth or at the point of complete growth inhibition is illustrated in Figure 2. Comparison of the two distinct MIC reading methods revealed a general trend: when trailing growth was ignored, rather than taking readings at the point of complete growth inhibition, lower MIC values were generally observed for both *M. avium* and *M. intracellulare* species. Consequently, the number of strains classified as intermediate or resistant decreased, whereas the count of susceptible strains increased.

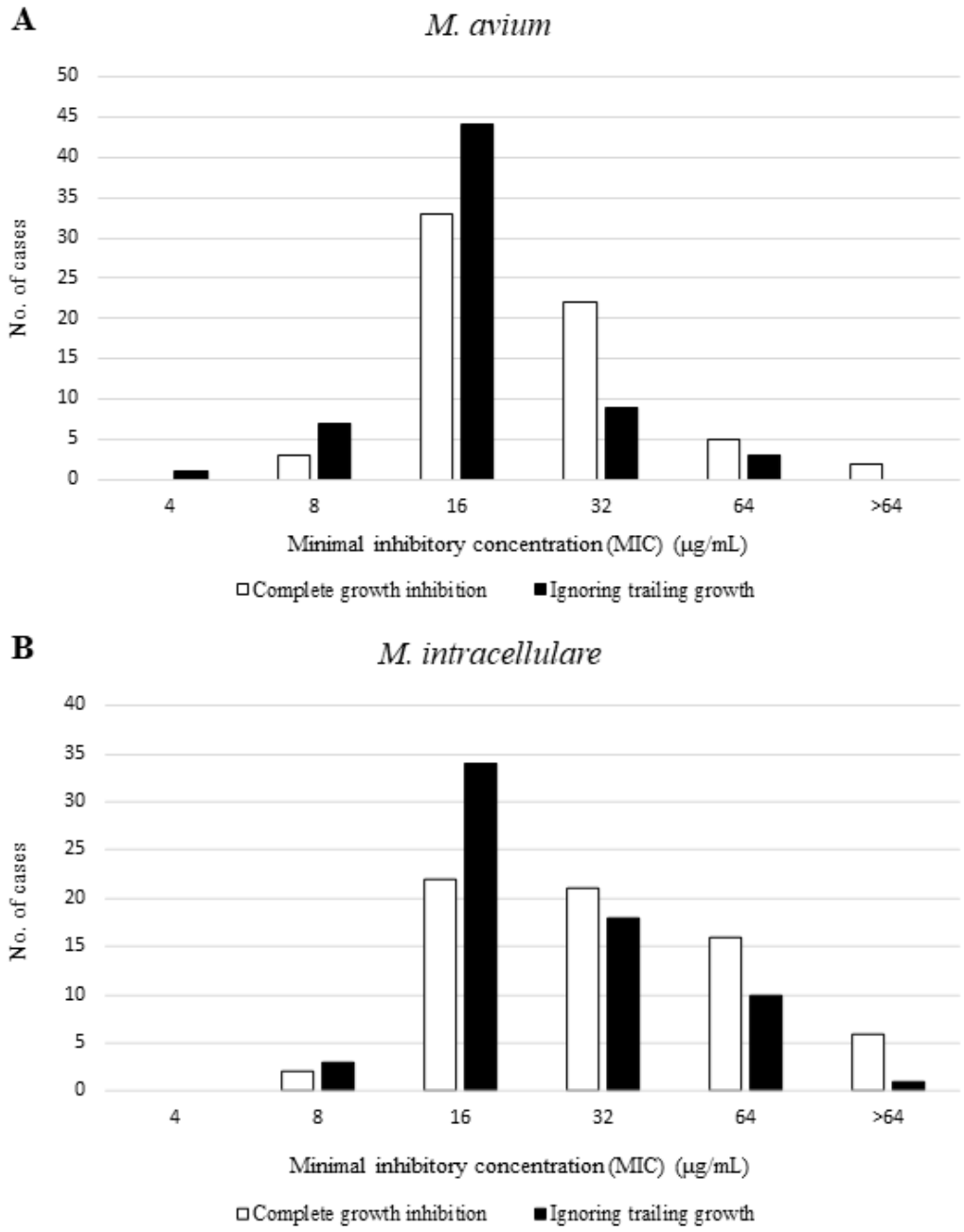

Figure 2. Comparative distribution of MIC values for (A) *M. avium* and (B) *M. intracellulare* read using two different methods. The MIC values ignoring trailing growth were re-tested only for cases with an initial MIC of 32 µg/mL or above, and the results are presented in addition to the cases with MIC values of 16 µg/mL or below.

For *M. avium*, ignoring trailing growth, had no impact on the modal MIC and MIC_{50}, with both remaining at 16 μg/mL. However, the MIC_{90} decreased from 64 to 32 μg/mL, and the 99.9 % ECOFF decreased from 128 to 64 μg/mL (Table 6). In the case of *M. intracellulare*, the exclusion of trailing growth did not affect the modal MIC, which remained at 16 μg/mL, but the MIC_{50} decreased from 32 to 16 μg/mL. The MIC_{90} remained at 64 μg/mL, whereas the 99.9 % ECOFF decreased from 256 to 128 μg/mL.

Table 6. Comparison of MIC reading methods and associated MIC metrics for *M. avium* and *M. intracellulare*

Species	MIC reading methods	Modal MIC	MIC_{50}	MIC_{90}	99.9% ECOFF
M. avium	Complete growth inhibition	16	16	64	128
	Ignoring trailing growth	16	16	32	64
M. intracellulare	Complete growth inhibition	16	32	64	256
	Ignoring trailing growth	16	16	64	128

All MIC metrics values are expressed in μg/mL. ECOFF, epidemiological cut-off value.

Discussion

The MIC values read at the onset of trailing growth during re-testing showed a low concordance rate compared with the original MIC values read at the point when bacterial growth was completely inhibited. For *M. avium*, the concordance rates were as follows: MIC = 32 μg/mL, 36.4%; 64 μg/mL, 40.0%; and >64 μg/mL, 0.0%. In the case of *M. intracellulare*, the concordance rates were MIC = 32 μg/mL, 38.1%; 64 μg/mL, 18.8%; and >64 μg/mL, 16.7%.

In several studies, the MIC_{50} and MIC_{90} for amikacin against *M. avium* and *M. intracellulare* have been reported to be 16 μg/mL and 32 μg/mL, respectively [7, 31]. In this study, when we excluded trailing growth for *M. intracellulare*, a decrease in MIC_{50} from 32 μg/mL to 16 μg/mL was observed. Similarly, for *M. avium*, the MIC_{90} showed a reduction from 64 μg/mL to 32 μg/mL (Table 6). Therefore, these findings suggest that by ignoring trailing growth, the MIC values closely align with those previously reported in the literature.

Recently, efforts have been made to determine the ECOFF values for MAC against amikacin [20, 32]. Beyond single studies, EUCAST has been actively gathering MIC distribution data

from various researchers and subsequently publishing an aggregated MIC distribution along with the 99.9% ECOFF value on their official platform (http://mic.eucast.org/Eucast2/). Based on the cumulative data gleaned from previous studies and the MIC distribution data provided by EUCAST, the ECOFF values for *M. avium* and *M. intracellulare* stand at 64 μg/mL. Moreover, our findings indicated that when trailing growth was disregarded, the ECOFF for *M. avium* decreased from 128 to 64 μg/mL, aligning seamlessly with the values reported in the EUCAST MIC distribution. Concurrently, the ECOFF for *M. intracellulare* saw a reduction from 256 to 128 μg/mL, which is close to the value reported by EUCAST. However, the classification of susceptibility by the CLSI M24-Ed3 breakpoint considers MICs of 16 μg/mL or below as susceptible, 32 μg/mL as intermediate, and 64 μg/mL or above as resistant, resulting in a discrepancy between the EUCAST ECOFF and the CLSI breakpoint. This finding suggests that the CLSI guidelines for the amikacin breakpoint for MAC should be revised if the MIC is to be read at the point of complete growth inhibition, as has traditionally been done. If CLSI is to maintain its current breakpoint, the MIC should be read, ignoring trailing growth.

The detection of the A2058T mutation in the *rrl* gene in clarithromycin-resistant isolates is consistent with previous research findings [10]. Previous studies have indicated that mutations in the *rrs* gene were rarely observed in strains with an amikacin MIC of 64 μg/mL. However,

they were predominantly detected in strains with a MIC >64 μg/mL. Therefore, the absence of *rrs* gene mutations in the eight isolates with a MIC >64 μg/mL, as determined at the point of complete inhibition of bacterial growth in this study, contradicts previous findings [7, 9, 10]. In contrast, the MIC results obtained at the onset of trailing growth provided a more plausible explanation for the absence of *rrs* gene mutations. One patient, whose isolate exhibited an amikacin MIC exceeding 64 μg/mL at the onset of trailing growth, had a history of amikacin treatment for 6 months, which is consistent with previous reports indicating that patients with MAC isolates demonstrating an amikacin MIC greater than 64 μg/mL had received treatment for over 6 months [7]. However, no *rrs* gene mutation was observed in this *M. intracellulare* isolate. Although the exact MIC of this strain was not determined, previous studies have provided strong evidence of *rrs* gene mutations in cases where amikacin MIC >256 μg/mL [9, 10]. However, the number of strains with MIC = 128 μg/mL or 256 μg/mL in previous studies was relatively small. Additionally, other resistance mechanisms to amikacin, such as aminoglycoside acetyltransferase, enhanced intracellular survival protein (eis), and drug efflux pumps, may be present in these strains alongside *rrs* gene mutations [9, 10]. Furthermore, in another study focused on *Mycobacterium abscessus* and *Mycobacterium massiliense*, *rrs* mutations were only detected in strains with amikacin MIC >2,048 μg/mL, suggesting that *rrs* mutations may be involved in resistance only in highly resistant strains [27]. Although the

clinical significance of trailing growth in the amikacin well of the Sensititre panel has not been reported, our study demonstrated that ignoring trailing growth and reading the MIC at the point of trailing growth onset is more consistent with the patient's treatment history and genotyping results. Further evaluations with more cases may be necessary to ascertain the cause of trailing growth and its clinical implications.

Among the 69 isolates identified as *M. intracellulare* via PCR-hybridisation, two isolates were identified as *M. chimaera* after sequencing of the 16S rRNA, *hsp65*, and *rpoB* genes; the remaining 67 isolates were identified as *M. intracellulare*. PCR-hybridisation and sequencing showed identical results for *M. avium* strains. As the PCR-hybridisation assay using GenoType Mycobacterium CM/AS cannot distinguish between *M. chimaera* and *M. intracellulare* [33], sequencing species-specific genes is useful for accurate differentiation.

Limitations

If a larger sample size had been used in this study, it is possible that more strains exhibiting resistance with MICs >64 μg/mL when the MIC was read while ignoring trailing growth would have been identified. This would have potentially allowed for the confirmation of alternative genotypic resistance mechanisms beyond the *rrs* gene. The failure to accomplish this due to the limited sample size constitutes a limitation of this study, necessitating further investigation in subsequent research.

Conclusions

For accurate species identification of MAC, sequencing of the 16S rRNA, *hsp65*, and *rpoB* genes was conducted, enabling differentiation between *M. intracellulare* and *M. chimaera*, which were indistinguishable by the previous PCR-hybridization method. The sequencing of 16S rRNA, *hsp65*, and *rpoB* genes effectively identified *M. chimaera*.

As 132 of 134 MAC isolates were susceptible to clarithromycin, macrolides could be used as first-line drugs without AST in patients with MAC disease. Only 7.5% of susceptible MAC strains were found for linezolid and 6.7% for moxifloxacin. Therefore, when considering these antimicrobials for MAC infection treatment, it is essential to perform antimycobacterial susceptibility testing and tailor the therapeutic approach according to these findings.

For Rifampin and Ethambutol, no breakpoints exist to distinguish between susceptibility, intermediate, and resistance. However, there have been reports of unfavorable outcomes with MIC values of 8 or above. In this study, the percentage of isolates with an MIC of 8 or above

was 33.8% for *M. avium* with Rifampin and 96.9% for Ethambutol, while for *M. intracellulare*, it was 62.7% for Rifampin and 94.0% for Ethambutol. Therefore, when considering treatment with these drugs, it is crucial to conduct susceptibility testing to determine the MIC.

When trailing growth is interpreted as false growth and MICs are read accordingly, the resultant MICs for MAC against amikacin are generally lower than when trailing growth considered as true growth. Furthermore, these adjusted MICs, which disregard trailing growth, provide a more accurate explanation of the *rrs* genotype results, amikacin treatment history, and EUCAST's MIC distribution than the MICs according to the CLSI guidelines. Therefore, CLSI guidelines should be updated to ignore trailing growth when reading the amikacin MIC for MAC.

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국문 요약

배경: 아미카신은 *Mycobacterium avium* complex(MAC)에 대한 항균제 감수성 검사를 시행할 때 평가해야하는 1차 약제이다. MAC은 액체배지 미량희석법(broth microdilution)으로 최소억제농도(minimum inhibitory concentration, MIC)를 측정할 때 아미카신 well에서 후행성장(trailing growth)을 종종 보인다. Clinical and Laboratory Standards Institute (CLSI) 가이드라인에서는 균의 성장이 완전히 억제되는 농도에서 아미카신의 MIC를 읽을 것을 권장하지만, 후행성장은 종종 MIC 판독에서 주관적인 차이를 발생시키게 되고, 위내성 쪽으로 MIC를 높이는 원인이 된다. 따라서, 이 연구는 MAC의 후행성장이 아미카신 MIC에 미치는 영향을 평가하고, 후행성장에서 MIC를 객관적으로 판독하는 기준을 제시하기 위해 수행되었다.

대상 및 방법: 2021년 11월부터 2022년 4월까지 MAC의 임상 분리 균주에 대한 항균제감수성시험(antimycobacterial susceptibility test, AST)는 Sensititre SLOMYCOI

(Thermo Fisher Scientific, Cleveland, OH, USA)를 사용하여 요청 기반으로 수행되었다. 아미카신 MIC가 32 μg/mL 이상인 균주에 대해서는 AST를 재검사하였고, MIC는 끌림성장의 시작 지점에서 결정되었다. 끌림성장의 시작은 이전 well과 비교하여 박테리아 성장이 눈에 띄게 감소하고 아미카신 농도를 증가시켜도 추가적인 균 성장 억제가 관찰되지 않는 지점으로 정의하였다. 모든 연구대상 균주에 대한 *rrs* 유전자 변이가 추가적으로 검사되었으며, 모든 연구 대상 환자들의 아미카신 치료력 또한 조사되었다. 연구에 포함된 MAC 균주의 50번째 백분위수(MIC_{50})와 90번째 백분위수(MIC_{90})의 최소억제농도가 결정되었고, 역학적 판정 기준치(epidemiological cut-off value, ECOFF)도 ECOFFinder 알고리즘을 사용하여 계산되었다. 연구에 포함된 균주의 MIC_{50}, MIC_{90} 및 ECOFF 값은 유럽 항균제 감수성 검사 위원회 (European Committee on Antimicrobial Susceptibility Testing, EUCAST)에 제출된 데이터세트의 아미카신 MIC 분포와 비교하여 검증되었다. 이 연구는 아산병원 기관 윤리위원회의 승인을 받았다(S2022-1595-0001).

결과: 총 134주의 임상 분리 균주(*M. intracellulare* 67주, *M. avium* 65주 및 *M.*

chimaera 2주)에 대한 AST가 시행되었으며, 아미카신 MIC 판독이 중간인 범주는 44주(32.8%), 내성인 범주는 29주(21.6%)로 분류되었다. 감수성이 중간 및 내성으로 분류된 총 72주(53.7%)의 *M. intracellulare* 및 *M. avium* 균주들을 SLOMYCOI를 사용하여 재검사하였고, 후행성장의 시작지점을 MIC로 판독하였을 때, MAC 44주는 더 감수성인 범주로 변경되었다. 아미카신 내성이었던 *M. intracellulare* 균주 중 10주는 중간, 4주는 감수성으로 변경되었으며, 내성이었던 *M. avium* 균주 중 1주는 중간, 4주는 감수성으로 변경되었다. 처음 내성 범주였던 MAC 중 *rrs* 유전자 변이를 가진 균주는 발견되지 않았다. 균성장에서 후행성장을 제외하고 MIC를 판독하는 경우 MIC_{50}, MIC_{90} 및 ECOFF값은 EUCAST 감수성 누적 데이터 상의 아미카신 MIC 분포와 잘 일치하였다. 6명의 환자만 아미카신 치료력이 있었고, 단 한 명만 아미카신으로 6개월 동안 장기 치료를 받았으며, 치료기간 중 분리된 MAC 균주는 아미카신 MIC가 64 μg/mL 이상으로 나타나 확실한 내성으로 판독하였다.

결론: CLSI 가이드라인의 판독 조건에 따르면 아미카신에 대한 내성 비율이

21.6%에 달하였지만 *rrs* 유전자 변이가 동반된 경우가 없고, 아미카신 사용력은 1명에서만 관찰되어 위내성이 의심되었다. 초기 판독에서 아미카신 감수성이 아닌 균주들에서 후행성장이 관찰되었으며, 후행성장을 균자람에서 제외할 때 아미카신 내성률이 임상적으로 납득할만한 수준으로 낮아졌다. 결과적으로 후행성장이 있는 균주들의 MIC는 후행성장이 시작되는 지점을 읽는 것이 CLSI 감수성 판정기준을 적용했을 때 위내성을 방지할 수 있을 것으로 판단되었다.