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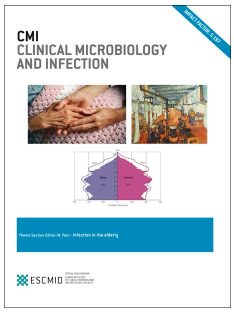

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Towards clinical breakpoints for non-tuberculous mycobacteria – determination of epidemiological cut off values for the *Mycobacterium avium* complex and *Mycobacterium abscessus* using broth microdilution

Gabrielle Fröberg[1,2], Florian P. Maurer [3,4], Erja Chryssanthou[1,5], Louise Fernström[6], Hanaa Benmansour[7], Samira Boarbi[8], Anne Torunn Mengshoel[9], Peter Michael Keller[10], Miguel Viveiros[11], Diana Machado[11], Margaret M. Fitzgibbon[12,13], Simone Mok[12,13], Jim Werngren[14], Daniela Maria Cirillo[15], Fernando Alcaide[16], Hanne-Leena Hyyryläinen[17], Alexandra Aubry[18], Sönke Andres[3], Darshaalini Nadarajan[3], Erik Svensson[19], John Turnidge[20], Christian G. Giske[1,5], Gunnar Kahlmeter[21], Emmanuelle Cambau[7], Jakko van Ingen[22] and Thomas Schön[23-25] for the EUCAST AMST and ESCMYC study groups.

[1] Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden.

[2] Division of Infectious Diseases, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden.

[3] National Reference Center for Mycobacteria, Research Center Borstel, Borstel, Germany.

[4] Institute of Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

[5] Division of Clinical Microbiology, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden.

[6] Department of Internal medicine, Lycksele hospital, Lycksele, Sweden.

[7] AP-HP, GHU Nord, service de Mycobactériologie spécialisée et de référence, laboratoire associé au Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux, Université Paris Cité, Paris, France.

[8] National Reference Center for Tuberculosis and Mycobacteria, Sciensano, Brussels, Belgium.

[9] Department of Bacteriology, Division of Infection Control, Norwegian Institute of Public Health, Oslo, Norway.

[10] Institute for Infectious Diseases, University of Bern, Switzerland.

[11] Unit of Medical Microbiology, Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisboa, Portugal.

[12] Irish Mycobacteria Reference Laboratory, St James's Hospital, Dublin, Ireland.

[13] Department of Clinical Microbiology, School of Medicine, Trinity College, Dublin, Ireland.

[14] Department of Microbiology, Unit for Laboratory Surveillance of Bacterial Pathogens, Public Health Agency of Sweden, Solna, Sweden.

[15] IRCCS San Raffaele Scientific Institute , Milan, Italy.

[16] Department of Clinical Microbiology, Bellvitge University Hospital-IDIBELL, University of Barcelona, L'Hospitalet de Llobregat, Barcelona, Spain.

[17] Department of Health Security, Finnish Institute for Health and Welfare, Helsinki, Finland.

[18] Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux, Centre d'Immunologie et des Maladies Infectieuses, Sorbonne Université, Paris, France.

[19] International Reference Laboratory of Mycobacteriology, Statens Serum Institut, Copenhagen, Denmark.

[20] School of Biological Sciences and Adelaide Medical School, University of Adelaide, Adelaide, South Australia.

[21] The EUCAST Development Laboratory, Clinical microbiology, Central Hospital, Växjö, Sweden.

[22] Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, the Netherlands.

[23] Department of Infectious Diseases, Kalmar County Hospital, Kalmar, Sweden.

[24] Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden.

[25] Department of Infectious Diseases in Östergötland, Linköping University, Linköping, Sweden.

Corresponding author: Thomas Schön, Department of Infectious Diseases, Linköping University, Linköping. Email: thomas.schon@liu.se.

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Abstract

Objective. For non-tuberculous mycobacteria (NTM), minimum inhibitory concentration (MIC) distributions of wild-type isolates have not been systematically evaluated despite their importance for establishing antimicrobial susceptibility testing (AST) breakpoints.

Methods. We gathered MIC distributions for drugs used against the *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* (MAB) obtained by commercial broth microdilution (SLOMYCOI and RAPMYCOI) from 12 laboratories. Epidemiological cut-off values (ECOFFs) and tentative ECOFFs (TECOFFs) were determined by EUCAST methodology including quality control (QC) strains.

Results. The clarithromycin ECOFF was 16 mg/L for *M. avium* (n=1271) whereas TECOFFs were 8 mg/L for *M. intracellulare* (n=415) and 1 mg/L for MAB (n=1014) confirmed by analysing MAB subspecies without inducible macrolide resistance (n=235). For amikacin, the ECOFFs were 64 mg/L for MAC and MAB. For moxifloxacin, the WT spanned >8 mg/L for both MAC and MAB. For linezolid, the ECOFF and TECOFF were 64 mg/L for *M. avium* and *M. intracellulare*, respectively. Current CLSI breakpoints for amikacin (16 mg/L), moxifloxacin (1 mg/L) and linezolid (8 mg/L) divided the corresponding WT distributions. For QC *M. avium* and *M. peregrinum*, ≥95% of MIC values were well within recommended QC ranges.

Conclusion. As a first step towards clinical breakpoints for NTM, (T)ECOFFs were defined for several antimicrobials against MAC and MAB. Broad wild-type MIC distributions indicate a need for further method refinement which is now under development within the EUCAST subcommittee for anti-mycobacterial drug

susceptibility testing. In addition, we showed that several CLSI NTM breakpoints are not consistent in relation to the (T)ECOFFs.

Introduction

Clinically relevant infections with non-tuberculous mycobacteria (NTM) such as the *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* (MAB) are increasing (1). Current treatment regimens are inefficient as illustrated by the treatment duration of at least 12 months for pulmonary disease with cure rates at 40-50% for MAB and 50-70% for MAC with a microbiological recurrence rate of 30% (2-5).

For MAC, a macrolide such as clarithromycin or preferably azithromycin is the core drug, combined with a rifamycin and ethambutol, the latter two mainly to prevent the development of macrolide resistance (2, 6). MAB is notoriously difficult to treat (4). Current guidelines recommend using at least 3 active drugs based on antimicrobial susceptibility testing (AST), with an initial phase of intravenous drugs like amikacin, imipenem and tigecycline combined with oral drugs like a macrolide and clofazimine, followed by a continuation phase of 3 active oral or inhaled drugs (2). Within MAB, most isolates are harbouring a functional methyl transferase encoded by the *erm* (41) gene, resulting in inducible macrolide resistance observed after prolonged incubation to 14 days (7). Only *M. abscessus subsp. massiliense* and a minority of *M. abscessus subsp. abscessus* lack inducible macrolide resistance (7, 8). The importance of macrolides is strongly supported by systematic reviews reporting treatment success rates in the range of 27-34% for *M. abscessus* subsp. *abscessus,* and 54-57% for *M. abscessus* subsp. *massiliense* (4, 9).

The role of AST in therapy guidance for MAC and MAB disease has so far only been established for the macrolides and to some extent, amikacin. For decades, it has generally been claimed that AST for NTM is of limited use due to a poor correlation

between MICs and clinical outcome (10, 11). However, this more likely reflects the poor clinical efficacy of some of the available drugs used in NTM treatment in combination with insufficient data on MIC distributions, pharmacokinetic/pharmacodynamics (PK/PD) and clinical outcome data (2, 12-14).

The Clinical and Laboratory Standards Institute (CLSI) recommends using broth microdilution (BMD) in cation adjusted Mueller Hinton broth (CAMHB) for AST of most NTM (10, 11). There is limited data in support of the current CLSI breakpoints in terms of wild-type (WT) MIC distributions, epidemiological cut-off values (ECOFFs), PK/PD and clinical outcome (13, 15). So far, single laboratory studies using commercial BMD plates, such as Sensititre SLOMYCOI and RAPMYCOI (Thermo Fisher Scientific Inc., US) have suggested putative ECOFFs representing the highest MIC value for the phenotypic WT distribution (12, 15). However, to define ECOFFs, valid WT distributions from at least five separate laboratories are required according to European Committee of Antimicrobial Susceptibility Testing (EUCAST) to capture intra- and interlaboratory technical variability (16). Thus, the aim of this study was to define EUCAST ECOFFs for drugs against MAC and MAB in a widely used commercial BMD method as a first step towards EUCAST NTM breakpoints.

Material and methods

In total 1,686 MAC isolates (1,271 *M. avium*, 415 *M. intracellulare*) and 1,014 MAB isolates from 12 laboratories collected between 2010 and 2022 were included. Identification of species and inducible macrolide resistance (MAB) was performed according to routine procedures by each participating laboratory, which was by line probe assays (GenoType Mycobacterium CM and NTM-DR, Hain Lifescience, Germany) in the majority of cases. The Sensititre™ SLOMYCOI and RAPMYCOI assays were performed according to the instructions for use (17) which are in turn based on CLSI protocol M24-A2 (11). Further details of culture, species determination and BMD are described in the Supplementary file 1. Data are presented as aggregated distributions based on *all* available MIC data from all laboratories. For MAB and macrolides, data are also separated according to subspecies with inducible macrolide resistance (*M. abscessus* subsp. *abscessus erm* 28T (n=335) and *M. abscessus* subsp. *bolletii* (n=114)) *versus* without inducible macrolide resistance (*M. abscessus* subsp. *abscessus erm* 28C (n=52) and *M. abscessus* subsp. *massiliense* (n=183)). ECOFFs were set based on the EUCAST SOP 10.2 (16). ECOFFs require at least five valid MIC distributions, which are defined by strict EUCAST criteria including at least 15 isolates per drug, a visible mode, a minimum of 100 isolates in the putative WT distribution and set using ECOFFinder algorithm (18) combined with eye-balling (16). Tentative ECOFFs (TECOFFs) require at least three valid MIC distributions.

Results

Wild-type MIC distributions and (T)ECOFFs for MAC

Aggregated MIC-distributions for clarithromycin, rifampicin, rifabutin, and ethambutol against MAC are presented in Figure 1. For *M. avium*, clarithromycin ECOFF was 16 mg/L (range 0.06-16 mg/L), one MIC dilution step higher than for *M. intracellulare* (TECOFF 8 mg/L; range 0.06-8 mg/L). The rifampicin WT distribution for both species was broad, without a mode and truncated at the upper end (>8 mg/L). For rifabutin, the WT distribution was instead truncated at the lower end (≤0.25 mg/L) and thus ECOFFs could not be defined. In addition, the QC *M. avium* did not show an on-scale result for 75% (230/307) of recorded MICs for rifabutin. Ethambutol exhibited WT distributions expanding partly above the highest MIC tested (>16 mg/L), but with distinct modes at 8 mg/L for *M. avium* and 4 mg/L for *M. intracellulare*, suggesting a putative WT distribution ending at 32 mg/L, while ECOFFs could not be defined. For the QC *M. avium,* ≥99% of MIC values from four laboratories were well within the QC ranges as recommended by the manufacturer for clarithromycin, rifampicin and rifabutin (n=307-376, Figure 1; A1-D1).

Aggregated MIC distributions of amikacin, moxifloxacin, linezolid and trimethoprim-sulfamethoxazole (TSU) against MAC are presented in Figure 2. Amikacin ECOFF was 64 mg/L (range ≤1 – 64 mg/L) for both *M. avium* and *M. intracellulare*. Moxifloxacin showed WT distributions expanding above the highest MIC tested (>8 mg/L) for both species, but with a distinct mode at 2 – 4 mg/L, suggesting a putative WT distribution ending at 16 mg/L, while ECOFFs could not be defined. Linezolid ECOFF was 64 mg/L (range ≤1 – 64 mg/L) for *M. avium* and with the same TECOFF for *M. intracellulare* (4 valid MIC distributions). For TSU, the TECOFF was 4 mg/L for

M. avium and 8 mg/L for *M. intracellulare* (4 valid distributions for both species). For the QC *M. avium,* ≥95% of the MIC values from four laboratories were well within the QC ranges as recommended by the manufacturer for amikacin, moxifloxacin, linezolid and TSU (n=155-377, Figure 2; E1-H1).

Wild-type MIC distributions and (T)ECOFFs for MAB

Aggregated MIC distributions of clarithromycin, moxifloxacin, linezolid, amikacin, imipenem and tigecycline against MAB are presented in Figure 3. For clarithromycin, there was a broad MIC distribution, with a truncation of the WT distribution at the lower end (range ≤0.06 - 1 mg/L) as well as at the higher end of the test range (>16 mg/L). Setting an ECOFF was challenging for clarithromycin even with 1014 MIC observations from 10 separate laboratories (n=21-284 from each laboratory), but a TECOFF could be set at 1 mg/L (4 valid distributions). The distribution was also subdivided according to subspecies with *versus* without inducible macrolide resistance (Figure 4). This analysis confirmed a WT distribution at ≤0.06 - 1 mg/L with TECOFF at 1 mg/L (n=235 isolates from 10 laboratories) for isolates without inducible macrolide resistance. Of note, a substantial number of isolates belonging to MAB subspecies with inducible macrolide resistance (64%, 288/449) showed a MIC below the currently suggested CLSI breakpoint (S ≤ 2 mg/L) when read at day 3-5, in particular for *M. abscessus* subsp. *abscessus erm* 28T. For the other drugs tested, there were no significant differences in MICs among MAB subspecies (Supplementary file 2).

For moxifloxacin, the WT distribution was truncated above the highest concentration tested (>8 mg/L) without a mode. Linezolid also showed a WT distribution expanding

above the highest test concentration (>32 mg/L), but with a distinct mode at 16 mg/L, suggesting a putative WT distribution ending at 64 mg/L, while an ECOFF could not be defined. For amikacin, the ECOFF was 64 mg/L (range 2 – 64 mg/L). Imipenem showed a broad WT distribution of ≤2 - 64 mg/L, but with a distinct mode at 16 mg/L and the ECOFF could be set at 64 mg/L. The tigecycline ECOFF was 2 mg/L (range 0.03 – 2 mg/L). For the QC *M. peregrinum,* ≥99% of MIC values from seven laboratories were well within the QC ranges as recommended by the manufacturer and CLSI for clarithromycin, moxifloxacin, linezolid, amikacin and imipenem (n=336-340, Figure 3 A-F). The majority of QC MICs for moxifloxacin and amikacin were below the testing range (Figure 3), but within the recommended QC ranges which include truncations at the lower end for these drugs.

Discussion

In this European multi-centre study of MIC distributions for MAC and MAB, we could define (T)ECOFFs for several of the antimicrobials included on the most widely adopted commercial BMD panels. Overall, most MIC distributions were broad and spanned at least five dilution steps. Thus, despite several hundred of MICs for MAC and MAB deriving from at least five different laboratories, ECOFFs for NTM were more challenging to define compared to other pathogens. We used the latest EUCAST SOP for definition of valid WT distributions and setting ECOFFs (16). In several cases, truncations of the WT distributions did not permit a definition of (T)ECOFF, even though some antimicrobials such as ethambutol, moxifloxacin (MAC) and linezolid (MAB) displayed distinct modes suggesting putative ends of these distributions. These truncations will unfortunately remain with the implementation of new versions of BMD plates, currently recommended for research use only (SLOMYCO2 and RAPMYCO2). On the other hand, clofazimine is included in both updated commercial plates, where future studies for defining ECOFFs for this drug are warranted (2).

On-scale QC data are essential to assuring the reproducibility of MICs and the validity of AST methods used in clinical routine. There has been low essential and categorical agreement for MAB of 47-76% for clarithromycin and amikacin (19, 20) and the slow uptake of standardized QC testing for mycobacteria was recently discussed (21). Considering MAB and other rapidly growing mycobacteria (RGM), current guidelines recommend QC *M. peregrinum* ATCC 700686. However, recommended QC ranges are broad, usually spanning over four MIC concentrations and without a lower defined range for several drugs including the essential drugs clarithromycin and amikacin (11). As QC isolate for the most clinically important RGM

– an alternative would be to use *M. abscessus subsp. abscessus* ATCC 19977 (*erm* 28T) where QC ranges have also been suggested for bedaquiline and omadacycline (20, 22).

Our data support previous single laboratory studies of MIC determinations which showed WT distributions in the same range as in the present study (12, 15, 23, 24). However, the broad MIC distributions indicate a need for refinement of both species identification and methodology used for MIC determination for NTM. This is the case in particular for the key drug clarithromycin, where MAB subspecies identification is crucial regarding inducible resistance and MIC testing is dependent on the pH (25). Future development of the EUCAST AMST reference method for NTM should take this into account, but also include proper MIC ranges, standardized preparation of the inoculum and a more thorough growth control like in the EUCAST AMST reference method for *M. tuberculosis* (26). An additional point for discussion is whether clarithromycin is the most suitable macrolide representative, given that current treatment guidelines specifically advocate the use of azithromycin (2) and therapeutic drug monitoring including MIC determination for azithromycin may help to predict and improve treatment outcome although the stability of azithromycin during AST may need consideration (27).

Of note, the clarithromycin TECOFF for *M. intracellulare* (8 mg/L) was lower than the ECOFF for *M. avium* (16 mg/L), which has been observed previously in single laboratory studies (12, 23) with MIC data in the same range as in our study. Another concern is that the MIC distributions were in general broader for *M. intracellulare* than *M. avium*. This could be due to the identification methods used in this study, where current commercial line probe assays such as Hain Genotype CM and NTM-DR can separate *M. avium* from *M. intracellulare* and further *M. intracellulare* from *M.*

chimaera but are not able to separate all subspecies within MAC. Thus, more rare species, such as *M. marseillense, M. colombiense* and *M. arosiense* are lumped together as *M. intracellulare* and differences in between these species may be undefined (28, 29), even though it has been shown that MIC distributions of closely related MAC species are comparable (12). Even so, the relevance of these differences in MIC distributions between *M. avium* and *M. intracellulare* remains to be investigated but indicates the importance of thorough species confirmation when correlating the clinical outcome to MIC data.

We found that the CLSI breakpoints for amikacin (16 mg/L), moxifloxacin (1 mg/L) and linezolid (8 mg/L) divided the corresponding WT distributions. For both MAC and MAB, the WT distributions expanded well above these breakpoints, splitting the WT distributions and causing substantial reproducibility concerns due to the inherent technical variability of MIC testing of up to ±one MIC dilution step. Consequently, the SIR-classification of “susceptible, at standard dosing (S)”, “susceptible at increased exposure (I)” and “resistant (R)” based on these breakpoints is dependent on method variability rather than a prediction of the efficacy of the drug. This is further substantiated by a very low categorical agreement (54%) between laboratories in the SIR classification of linezolid for MAB in quality assessment studies for NTM (19). For moxifloxacin and linezolid, clinical efficacy data for both MAC and MAB in support of the current CLSI breakpoints (1 and 8 mg/L, respectively) are very scarce (2, 11). Additionally, the CLSI breakpoints for moxifloxacin and linezolid were both two MIC dilution steps higher than the non-species related PK/PD breakpoints as defined by EUCAST (0.25 and 2 mg/L, respectively). This is of particular concern for linezolid because of the potential severe side effects from long term use such as anemia and polyneuropathy. We strongly suggest that current breakpoints for moxifloxacin and

linezolid against MAC and MAB should be removed until a reproducible AST is in place supported by both PK/PD and clinical outcome data.

Our study has several limitations as previously indicated. First, WT distributions for many drugs were broad indicating a need for improvement of the method and species identification. Additionally, more MIC results could have facilitated the definition of ECOFFs for some of the drugs. Second, the truncated testing range for several drugs is not suitable for use along with the ECOFFinder algorithm (18). Third, it should be noted that even if ECOFFs are a first step towards clinical breakpoints, there is still a need for PK/PD targets and clinical outcome data. Fourth, potential MIC trailing for drugs such as TSU and linezolid and technical challenges such as antimicrobial instability as for imipenem needs further study.

To conclude, we established MIC distributions and ECOFFs for several first-line drugs used against MAC and MAB. A robust reference method for NTM is now under development within the EUCAST subcommittee for anti-mycobacterial drug susceptibility testing (AMST) to facilitate the definition of ECOFFs and ensure reproducibility for drugs used against NTM.

Transparency declaration

Conflict of Interest (COI)

None of the authors declared any COI affecting the results in this study.

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Authors' contributions

Conceptualization: GK, JvI and TS; Methodology: TS and GK; Formal Analysis: GF, TS, GK, JT; Resources: All co-authors; Data curation: GF, LF, GK; Writing – original draft: GF and TS; Writing – review and editing: All co-authors; Visualization: GF and GK; Project administration:TS.

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Legends

Figure 1. MIC distributions for clarithromycin, rifampicin, rifabutin and ethambutol for *M. avium* (A1-D1, black bars) and *M. intracellulare* (A2-D2; black bars) including all available data. *M. avium* ATCC 700898 was included as a QC (A1-D1; grey bars). Arrows indicate ECOFFs/TECOFFs (black/grey) set on valid distributions and according to EUCAST criteria. Dotted vertical lines indicate current CLSI breakpoints, which are presented in Table 1 together with EUCAST PK/PD breakpoints and recommended QC ranges.

Figure 2. MIC distributions for amikacin, moxifloxacin, linezolid and trimethoprim-sulfamethoxazole (TSU) for *M. avium* (E1-H1, black bars) and *M. intracellulare* (E2-H2, black bars). *M. avium* ATCC 700898 was included as a QC (E1-H1, grey bars). Arrows indicate ECOFFs/TECOFFs (black/grey) set on valid distributions and according to EUCAST criteria. Dotted lines indicate current CLSI breakpoints, which are together with EUCAST PK/PD breakpoints and recommended QC ranges presented in Table 1.

Figure 3. MIC distributions for clarithromycin, moxifloxacin, linezolid, amikacin, imipenem and tigecycline for all isolates of *M. abscessus* (MAB) (A-F, black bars) and QC *M. peregrinum* ATCC 700686 (A-F, grey bars). Arrows indicate ECOFFs/TECOFFs (black/grey) set on valid distributions and according to EUCAST

438 criteria. Dotted vertical lines indicate current CLSI breakpoints, which are presented
439 together with EUCAST PK/PD breakpoints and recommended QC ranges in Table 1.

440

441 **Figure 4**. MIC distribution for clarithromycin of MAB read at day 3-5, divided into
442 subspecies with inducible macrolide resistance (*M abscessus* subsp. *abscessus erm*
443 28T and *M. abscessus* subsp. *bolletii*) (black bars) and without (*M. abscessus* subsp.
444 *abscessus erm* 28C and *M abscessus* subsp. *massiliense)* (grey bars). The arrow
445 indicates the TECOFF of MAB without inducible macrolide resistance. Dotted vertical
446 line indicates current CLSI breakpoints.

447

448 **Table 1.** Current CLSI breakpoints, EUCAST PK/PD breakpoints, ECOFFs,
449 TECOFFs (within brackets), test concentrations for the SLOMYCO/RAPMYCO 1+2
450 plates and recommended QC MIC ranges. *by manufacturer, **by manufacturer and
451 CLSI, NA; not applicable.

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MAC Drug	CLSI NTM breakpoints	EUCAST PK/PD	*M.avium/ M.intracellulare* (T)ECOFF	*M.avium/ M.intracellulare* WT distribution	SLOMYCO1 Test range	SLOMYCO2 Test range	*M.avium* ATCC700898 Recommended QC range*
Clarithromycin	8≤16≥32	NA	16/(8)	0.06-16/8	0.06 - 64	0.06 - 64	0.25 - 4
Rifampicin		NA	NA/NA	0.25 - >8	0.125 - 8	0.004 - 4	≥1
Rifabutin		NA	NA/NA	≤0.25	0.25 - 8	0.12 - 4	≤0.25 - 1
Ethambutol		NA	NA/NA	≤0.5 - >16	0.5 - 16	NA	NA
Amikacin	16≤32≥64	S ≤ 1	64/64	≤1 - 64	1 - 64	1 - 256	2 - 16
Moxifloxacin	1≤2≥4	S ≤ 0.25	NA/NA	0.25 - >8	0.125 - 8	0.015 - 4	0.25 - 4
Linezolid	8≤16≥32	S ≤ 2	64/(64)	≤1 - 64	1 - 64	1 - 32	8 - 32
TSU		NA	(4)/(8)	≤0.125/0.5 - 4/8	0.125 - 8	0.25 - 4	0.25 - 2
MAB **Drug**	**CLSI** **NTM breakpoints**	**EUCAST** **PK/PD**	**MAB** **(T)ECOFF**	**MAB** **WT distribution**	**RAPMYCO1** **Test range**	**RAPMYCO2** **Test range**	***M.peregrinum* ATCC700686** **Recommended QC range****
Clarithromycin	2≤4≥8	NA	(1)	≤0.06 - 1	0.06 - 16	0.06 - 16	≤0.06 - 0.5
Moxifloxacin	1≤2≥4	S ≤ 0.25	NA	≤0.25 - >8	0.25 - 8	0.015 - 4	≤0.06 - 0.25
Linezolid	8≤16≥32	S ≤ 2	NA	≤1 - >32	1 - 32	1 - 32	1 - 8
Amikacin	16≤32≥64	S ≤ 1	64	2 - 64	1 - 64	1 - 256	≤1 - 4
Imipenem	4≤8-16≥32	S ≤ 2	64	≤2 - 64	2 - 64	0.008 - 32	2 - 16
Tigecycline		S ≤ 0.5	2	0.03 - 2	0.015 - 4	0.03 - 2	NA

MAC Drug	CLSI NTM breakpoints	EUCAST PK/PD	*M.avium/ M.intracellulare* (T)ECOFF	*M.avium/ M.intracellulare* WT distribution	SLOMYCO1 Test range	SLOMYCO2 Test range	*M.avium* ATCC700898 Recommended QC range*
Clarithromycin	8≤16≥32	NA	16/(8)	0.06-16/8	0.06 - 64	0.06 - 64	0.25 - 4
Rifampicin		NA	NA/NA	0.25 - >8	0.125 - 8	0.004 - 4	≥1
Rifabutin		NA	NA/NA	≤0.25	0.25 - 8	0.12 - 4	≤0.25 - 1
Ethambutol		NA	NA/NA	≤0.5 - >16	0.5 - 16	NA	NA
Amikacin	16≤32≥64	S ≤ 1	64/64	≤1 - 64	1 - 64	1 - 256	2 - 16
Moxifloxacin	1≤2≥4	S ≤ 0.25	NA/NA	0.25 - >8	0.125 - 8	0.015 - 4	0.25 - 4
Linezolid	8≤16≥32	S ≤ 2	64/(64)	≤1 - 64	1 - 64	1 - 32	8 - 32
TSU		NA	(4)/(8)	≤0.125/0.5 - 4/8	0.125 - 8	0.25 - 4	0.25 - 2
MAB **Drug**	**CLSI** **NTM breakpoints**	**EUCAST** **PK/PD**	**MAB** **(T)ECOFF**	**MAB** **WT distribution**	**RAPMYCO1** **Test range**	**RAPMYCO2** **Test range**	***M.peregrinum* ATCC700686** **Recommended QC range****
Clarithromycin	2≤4≥8	NA	(1)	≤0.06 - 1	0.06 - 16	0.06 - 16	≤0.06 - 0.5
Moxifloxacin	1≤2≥4	S ≤ 0.25	NA	≤0.25 - >8	0.25 - 8	0.015 - 4	≤0.06 - 0.25
Linezolid	8≤16≥32	S ≤ 2	NA	≤1 - >32	1 - 32	1 - 32	1 - 8
Amikacin	16≤32≥64	S ≤ 1	64	2 - 64	1 - 64	1 - 256	≤1 - 4
Imipenem	4≤8-16≥32	S ≤ 2	64	≤2 - 64	2 - 64	0.008 - 32	2 - 16
Tigecycline		S ≤ 0.5	2	0.03 - 2	0.015 - 4	0.03 - 2	NA