

Journal Pre-proof

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, Florian P. Maurer, Erja Chryssanthou, Louise Fernström, Hanaa Benmansour, Samira Boarbi, Anne Torunn Mengshoel, Peter Michael Keller, Miguel Viveiros, Diana Machado, Margaret M. Fitzgibbon, Simone Mok, Jim Werngren, Daniela Maria Cirillo, Fernando Alcaide, Hanne-Leena Hyyryläinen, Alexandra Aubry, Sönke Andres, Darshaalini Nadarajan, Erik Svensson, John Turnidge, Christian G. Giske, Gunnar Kahlmeter, Emmanuelle Cambau, Jakko van Ingen, Thomas Schön, for the EUCAST AMST and ESCMYC study groups

PII: S1198-743X(23)00060-5

DOI: <https://doi.org/10.1016/j.cmi.2023.02.007>

Reference: CMI 3208

To appear in: *Clinical Microbiology and Infection*

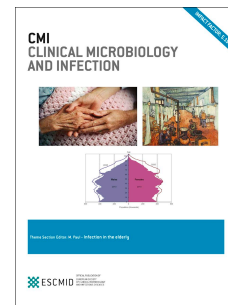
Received Date: 7 December 2022

Revised Date: 6 February 2023

Accepted Date: 7 February 2023

Please cite this article as: Fröberg G, Maurer FP, Chryssanthou E, Fernström L, Benmansour H, Boarbi S, Mengshoel AT, Keller PM, Viveiros M, Machado D, Fitzgibbon MM, Mok S, Werngren J, Cirillo DM, Alcaide F, Hyyryläinen H-L, Aubry A, Andres S, Nadarajan D, Svensson E, Turnidge J, Giske CG, Kahlmeter G, Cambau E, van Ingen J, Schön T, for the EUCAST AMST and ESCMYC study groups, Towards clinical breakpoints for non-tuberculous mycobacteria – Determination of epidemiological cut off values for the *Mycobacterium avium* complex and *Mycobacterium abscessus* using broth microdilution, *Clinical Microbiology and Infection*, <https://doi.org/10.1016/j.cmi.2023.02.007>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that,



during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2023 The Author(s). Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases.

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

4
5 Gabrielle Fröberg^{1,2}, Florian P. Maurer^{3,4}, Erja Chryssanthou^{1,5}, Louise Fernström⁶,
6 Hanaa Benmansour⁷, Samira Boarbi⁸, Anne Torunn Mengshoel⁹, Peter Michael
7 Keller¹⁰, Miguel Viveiros¹¹, Diana Machado¹¹, Margaret M. Fitzgibbon^{12,13}, Simone
8 Mok^{12,13}, Jim Werngren¹⁴, Daniela Maria Cirillo¹⁵, Fernando Alcaide¹⁶, Hanne-Leena
9 Hyyryläinen¹⁷, Alexandra Aubry¹⁸, Sönke Andres³, Darshaalini Nadarajan³, Erik
10 Svensson¹⁹, John Turnidge²⁰, Christian G. Giske^{1,5}, Gunnar Kahlmeter²¹,
11 Emmanuelle Cambau⁷, Jakko van Ingen²² and Thomas Schön²³⁻²⁵ for the EUCAST
12 AMST and ESCMYC study groups.

13
14 ¹ Department of Clinical Microbiology, Karolinska University Hospital, Stockholm,
15 Sweden.

16 ² Division of Infectious Diseases, Department of Medicine Solna, Karolinska Institutet,
17 Stockholm, Sweden.

18 ³ National Reference Center for Mycobacteria, Research Center Borstel, Borstel,
19 Germany.

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

22 ⁵ Division of Clinical Microbiology, Department of Laboratory Medicine, Karolinska
23 Institutet, Stockholm, Sweden.

- 24 ⁶ Department of Internal medicine, Lycksele hospital, Lycksele, Sweden.
- 25 ⁷ AP-HP, GHU Nord, service de Mycobactériologie spécialisée et de référence,
26 laboratoire associé au Centre National de Référence des Mycobactéries et de la
27 Résistance des Mycobactéries aux Antituberculeux, Université Paris Cité, Paris,
28 France.
- 29 ⁸ National Reference Center for Tuberculosis and Mycobacteria, Sciensano,
30 Brussels, Belgium.
- 31 ⁹ Department of Bacteriology, Division of Infection Control, Norwegian Institute of
32 Public Health, Oslo, Norway.
- 33 ¹⁰ Institute for Infectious Diseases, University of Bern, Switzerland.
- 34 ¹¹ Unit of Medical Microbiology, Global Health and Tropical Medicine, Instituto de
35 Higiene e Medicina Tropical, Universidade NOVA de Lisboa, Lisboa, Portugal.
- 36 ¹² Irish Mycobacteria Reference Laboratory, St James's Hospital, Dublin, Ireland.
- 37 ¹³ Department of Clinical Microbiology, School of Medicine, Trinity College, Dublin,
38 Ireland.
- 39 ¹⁴ Department of Microbiology, Unit for Laboratory Surveillance of Bacterial
40 Pathogens, Public Health Agency of Sweden, Solna, Sweden.
- 41 ¹⁵ IRCCS San Raffaele Scientific Institute , Milan, Italy.
- 42 ¹⁶ Department of Clinical Microbiology, Bellvitge University Hospital-IDIBELL,
43 University of Barcelona, L'Hospitalet de Llobregat, Barcelona, Spain.
- 44 ¹⁷ Department of Health Security, Finnish Institute for Health and Welfare, Helsinki,
45 Finland.

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

49 ¹⁹ International Reference Laboratory of Mycobacteriology, Statens Serum Institut,
50 Copenhagen, Denmark.

51 ²⁰ School of Biological Sciences and Adelaide Medical School, University of Adelaide,
52 Adelaide, South Australia.

53 ²¹ The EUCAST Development Laboratory, Clinical microbiology, Central Hospital,
54 Växjö, Sweden.

55 ²² Department of Medical Microbiology, Radboud University Medical Center,
56 Nijmegen, the Netherlands.

57 ²³ Department of Infectious Diseases, Kalmar County Hospital, Kalmar, Sweden.

58 ²⁴ Department of Biomedical and Clinical Sciences, Linköping University, Linköping,
59 Sweden.

60 ²⁵ Department of Infectious Diseases in Östergötland, Linköping University,
61 Linköping, Sweden.

62

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

65

66

67

- 68 Short title: ECOFFs for NTM
- 69 Type of article: Original article
- 70 Length of abstract: 248/250 words
- 71 Length of main text: 2487/2500 words
- 72 Number of references: 29/30

Journal Pre-proof

73 **Abstract**

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

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

83 *Results.* The clarithromycin ECOFF was 16 mg/L for *M. avium* (n=1271) whereas
84 TECOFFs were 8 mg/L for *M. intracellulare* (n=415) and 1 mg/L for MAB (n=1014)
85 confirmed by analysing MAB subspecies without inducible macrolide resistance
86 (n=235). For amikacin, the ECOFFs were 64 mg/L for MAC and MAB. For
87 moxifloxacin, the WT spanned >8 mg/L for both MAC and MAB. For linezolid, the
88 ECOFF and TECOFF were 64 mg/L for *M. avium* and *M. intracellulare*, respectively.
89 Current CLSI breakpoints for amikacin (16 mg/L), moxifloxacin (1 mg/L) and linezolid
90 (8 mg/L) divided the corresponding WT distributions. For QC *M. avium* and *M.*
91 *peregrinum*, ≥95% of MIC values were well within recommended QC ranges.

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

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

98

99

Journal Pre-proof

100 Introduction

101 Clinically relevant infections with non-tuberculous mycobacteria (NTM) such as the
102 *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* (MAB) are
103 increasing (1). Current treatment regimens are inefficient as illustrated by the
104 treatment duration of at least 12 months for pulmonary disease with cure rates at 40-
105 50% for MAB and 50-70% for MAC with a microbiological recurrence rate of 30% (2-
106 5).

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

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

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

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

141

142

143 **Material and methods**

144 In total 1,686 MAC isolates (1,271 *M. avium*, 415 *M. intracellulare*) and 1,014 MAB
145 isolates from 12 laboratories collected between 2010 and 2022 were included.
146 Identification of species and inducible macrolide resistance (MAB) was performed
147 according to routine procedures by each participating laboratory, which was by line
148 probe assays (GenoType Mycobacterium CM and NTM-DR, Hain Lifescience,
149 Germany) in the majority of cases. The Sensititre™ SLOMYCOI and RAPMYCOI
150 assays were performed according to the instructions for use (17) which are in turn
151 based on CLSI protocol M24-A2 (11). Further details of culture, species
152 determination and BMD are described in the Supplementary file 1. Data are
153 presented as aggregated distributions based on *all* available MIC data from all
154 laboratories. For MAB and macrolides, data are also separated according to
155 subspecies with inducible macrolide resistance (*M. abscessus* subsp. *abscessus* erm
156 28T (n=335) and *M. abscessus* subsp. *bolletii* (n=114)) versus without inducible
157 macrolide resistance (*M. abscessus* subsp. *abscessus* erm 28C (n=52) and *M.*
158 *abscessus* subsp. *massiliense* (n=183)). ECOFFs were set based on the EUCAST
159 SOP 10.2 (16). ECOFFs require at least five valid MIC distributions, which are
160 defined by strict EUCAST criteria including at least 15 isolates per drug, a visible
161 mode, a minimum of 100 isolates in the putative WT distribution and set using
162 ECOFFinder algorithm (18) combined with eye-balling (16). Tentative ECOFFs
163 (TECOFFs) require at least three valid MIC distributions.

164

165 **Results**166 *Wild-type MIC distributions and (T)ECOFFs for MAC*

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

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

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

193

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

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

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

213 above the highest test concentration (>32 mg/L), but with a distinct mode at 16 mg/L,
214 suggesting a putative WT distribution ending at 64 mg/L, while an ECOFF could not
215 be defined. For amikacin, the ECOFF was 64 mg/L (range 2 – 64 mg/L). Imipenem
216 showed a broad WT distribution of $\leq 2 - 64$ mg/L, but with a distinct mode at 16 mg/L
217 and the ECOFF could be set at 64 mg/L. The tigecycline ECOFF was 2 mg/L (range
218 0.03 – 2 mg/L). For the QC *M. peregrinum*, $\geq 99\%$ of MIC values from seven
219 laboratories were well within the QC ranges as recommended by the manufacturer
220 and CLSI for clarithromycin, moxifloxacin, linezolid, amikacin and imipenem (n=336-
221 340, Figure 3 A-F). The majority of QC MICs for moxifloxacin and amikacin were
222 below the testing range (Figure 3), but within the recommended QC ranges which
223 include truncations at the lower end for these drugs.

224

225

226

227

228 Discussion

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

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

253 – an alternative would be to use *M. abscessus subsp. abscessus* ATCC 19977 (*erm*
254 28T) where QC ranges have also been suggested for bedaquiline and omadacycline
255 (20, 22).

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

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

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

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

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

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

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

319 **Transparency declaration**

320 *Conflict of Interest (COI)*

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

322 *Funding*

323 GF; Stockholm Region and Karolinska Institute clinical research grant.

324 FPM; Mukoviszidose Institut gGmbH, Bonn, the German Cystic Fibrosis Association

325 Mukoviszidose e.V. TS; Swedish Heart and Lung Foundation and the Swedish

326 research council.

327 PK; Swiss Innovation Agency Innosuisse.

328 **Authors' contributions**

329 Conceptualization: GK, Jvl and TS; Methodology: TS and GK; Formal Analysis: GF,

330 TS, GK, JT; Resources: All co-authors; Data curation: GF, LF, GK; Writing – original

331 draft: GF and TS; Writing – review and editing: All co-authors; Visualization: GF and

332 GK; Project administration: TS.

333

334

335

336 **References**

- 337 1. Dahl VN, Molhave M, Floe A, van Ingen PJ, Schon PT, Lillebaek PT, et al. Global trends of
338 pulmonary infections with nontuberculous mycobacteria: a systematic review. *Int J Infect Dis.* 2022.
- 339 2. Daley CL, Iaccarino JM, Lange C, Cambau E, Wallace RJ, Andrejak C, et al. Treatment of
340 Nontuberculous Mycobacterial Pulmonary Disease: An Official ATS/ERS/ESCMID/IDSA Clinical
341 Practice Guideline. *Clin Infect Dis.* 2020;71(4):905-13.
- 342 3. Diel R, Nienhaus A, Ringshausen FC, Richter E, Welte T, Rabe KF, et al. Microbiologic
343 Outcome of Interventions Against Mycobacterium avium Complex Pulmonary Disease: A Systematic
344 Review. *Chest.* 2018;153(4):888-921.
- 345 4. Kwak N, Dalcolmo MP, Daley CL, Eather G, Gayoso R, Hasegawa N, et al. Mycobacterium
346 abscessus pulmonary disease: individual patient data meta-analysis. *Eur Respir J.* 2019;54(1).
- 347 5. Kwak N, Park J, Kim E, Lee CH, Han SK, Yim JJ. Treatment Outcomes of Mycobacterium avium
348 Complex Lung Disease: A Systematic Review and Meta-analysis. *Clin Infect Dis.* 2017;65(7):1077-84.
- 349 6. Griffith DE, Brown-Elliott BA, Langsjoen B, Zhang Y, Pan X, Girard W, et al. Clinical and
350 molecular analysis of macrolide resistance in Mycobacterium avium complex lung disease. *Am J*
351 *Respir Crit Care Med.* 2006;174(8):928-34.
- 352 7. Mougari F, Loiseau J, Veziris N, Bernard C, Bercot B, Sougakoff W, et al. Evaluation of the new
353 GenoType NTM-DR kit for the molecular detection of antimicrobial resistance in non-tuberculous
354 mycobacteria. *J Antimicrob Chemother.* 2017;72(6):1669-77.
- 355 8. Bastian S, Veziris N, Roux AL, Brossier F, Gaillard JL, Jarlier V, et al. Assessment of
356 clarithromycin susceptibility in strains belonging to the Mycobacterium abscessus group by erm(41)
357 and rrl sequencing. *Antimicrob Agents Chemother.* 2011;55(2):775-81.
- 358 9. Pasipanodya JG, Ogbonna D, Ferro BE, Magombedze G, Srivastava S, Deshpande D, et al.
359 Systematic Review and Meta-analyses of the Effect of Chemotherapy on Pulmonary Mycobacterium
360 abscessus Outcomes and Disease Recurrence. *Antimicrob Agents Chemother.* 2017;61(11).

- 361 10. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official
362 ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial
363 diseases. *Am J Respir Crit Care Med.* 2007;175(4):367-416.
- 364 11. TCaLSI C. Susceptibility Testing of Mycobacteria, *Nocardia* spp., and Other Aerobic
365 Actinomycetes, 3rd Edition. M242020.
- 366 12. Maurer FP, Pohle P, Kernbach M, Sievert D, Hillemann D, Rupp J, et al. Differential drug
367 susceptibility patterns of *Mycobacterium chimaera* and other members of the *Mycobacterium*
368 *avium-intracellulare* complex. *Clin Microbiol Infect.* 2019;25(3):379 e1- e7.
- 369 13. van Ingen J, Egelund EF, Levin A, Totten SE, Boeree MJ, Mouton JW, et al. The
370 pharmacokinetics and pharmacodynamics of pulmonary *Mycobacterium avium* complex disease
371 treatment. *Am J Respir Crit Care Med.* 2012;186(6):559-65.
- 372 14. Kwon BS, Kim MN, Sung H, Koh Y, Kim WS, Song JW, et al. In Vitro MIC Values of Rifampin
373 and Ethambutol and Treatment Outcome in *Mycobacterium avium* Complex Lung Disease.
374 *Antimicrob Agents Chemother.* 2018;62(10).
- 375 15. Schon T, Chryssanthou E. Minimum inhibitory concentration distributions for *Mycobacterium*
376 *avium* complex-towards evidence-based susceptibility breakpoints. *Int J Infect Dis.* 2017;55:122-4.
- 377 16. ECoAST E. SOP 10.2. MIC distributions and the setting of epidemiological cut-off (ECOFF)
378 values. 2021.
- 379 17. ITDSS T. Broth Microdilution (BMD) method. For Rapidly Growing Mycobacteria (RGM),
380 Slowly Growing Nontuberculosis Mycobacteria, *Nocardia* and other Aerobic Actinomycetes. 2013.
- 381 18. Turnidge J, Kahlmeter G, Kronvall G. Statistical characterisation of bacterial wild-type MIC
382 value distributions and the determination of epidemiological cut-off values. *Clin Microbiol Infect.*
383 2006;12(5):418-25.
- 384 19. Nikolayevskyy V, Maurer FP, Holicka Y, Taylor L, Liddy H, Kranzer K. Novel external quality
385 assurance scheme for drug susceptibility testing of non-tuberculous mycobacteria: a multicentre
386 pilot study. *J Antimicrob Chemother.* 2019;74(5):1288-94.

- 387 20. Brown-Elliott BA, Wallace RJ, Jr. In Vitro Susceptibility Testing of Omadacycline against
388 Nontuberculous Mycobacteria. *Antimicrob Agents Chemother.* 2021;65(3).
- 389 21. Schon T, Matuschek E, Mohamed S, Utukuri M, Heysell S, Alffenaar JW, et al. Standards for
390 MIC testing that apply to the majority of bacterial pathogens should also be enforced for
391 *Mycobacterium tuberculosis* complex. *Clin Microbiol Infect.* 2019;25(4):403-5.
- 392 22. Brown-Elliott BA, Wallace RJ, Jr. In Vitro Susceptibility Testing of Bedaquiline against
393 *Mycobacterium abscessus* Complex. *Antimicrob Agents Chemother.* 2019;63(2).
- 394 23. Chew KL, Octavia S, Go J, Ng S, Tang YE, Soh P, et al. In vitro susceptibility of *Mycobacterium*
395 *abscessus* complex and feasibility of standardizing treatment regimens. *J Antimicrob Chemother.*
396 2021;76(4):973-8.
- 397 24. Renvoise A, Bernard C, Veziris N, Galati E, Jarlier V, Robert J. Significant difference in drug
398 susceptibility distribution between *Mycobacterium avium* and *Mycobacterium intracellulare*. *J Clin*
399 *Microbiol.* 2014;52(12):4439-40.
- 400 25. Heifets LB, Lindholm-Levy PJ, Comstock RD. Clarithromycin minimal inhibitory and
401 bactericidal concentrations against *Mycobacterium avium*. *Am Rev Respir Dis.* 1992;145(4 Pt 1):856-
402 8.
- 403 26. Schon T, Werngren J, Machado D, Borroni E, Wijkander M, Lina G, et al. Antimicrobial
404 susceptibility testing of *Mycobacterium tuberculosis* complex isolates - the EUCAST broth
405 microdilution reference method for MIC determination. *Clin Microbiol Infect.* 2020;26(11):1488-92.
- 406 27. Jeong BH, Jeon K, Park HY, Moon SM, Kim SY, Lee SY, et al. Peak Plasma Concentration of
407 Azithromycin and Treatment Responses in *Mycobacterium avium* Complex Lung Disease. *Antimicrob*
408 *Agents Chemother.* 2016;60(10):6076-83.
- 409 28. Tortoli E. Microbiological features and clinical relevance of new species of the genus
410 *Mycobacterium*. *Clin Microbiol Rev.* 2014;27(4):727-52.

411 29. van Ingen J, Turenne CY, Tortoli E, Wallace RJ, Jr., Brown-Elliott BA. A definition of the
412 Mycobacterium avium complex for taxonomical and clinical purposes, a review. *Int J Syst Evol*
413 *Microbiol.* 2018;68(11):3666-77.

414

415

Journal Pre-proof

416 **Legends**

417

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

425

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

433

434 **Figure 3.** MIC distributions for clarithromycin, moxifloxacin, linezolid, amikacin,
435 imipenem and tigecycline for all isolates of *M. abscessus* (MAB) (A-F, black bars)
436 and QC *M. peregrinum* ATCC 700686 (A-F, grey bars). Arrows indicate
437 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.

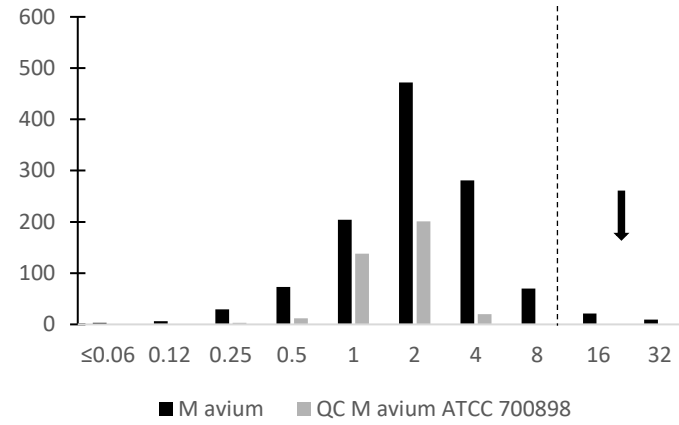
452

453

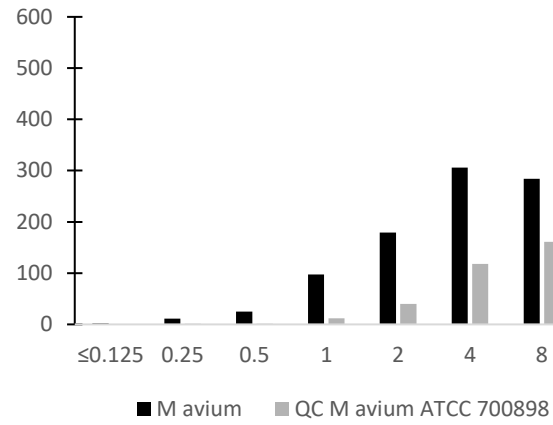
MAC	CLSI	EUCAST	<i>M.avium/</i>	<i>M.avium/</i>	SLOMYCO1	SLOMYCO2	<i>M.avium</i>
			<i>M.intracellulare</i>	<i>M.intracellulare</i>			ATCC700898
	NTM						Recommended
Drug	breakpoints	PK/PD	(T)ECOFF	WT distribution	Test range	Test range	QC range*
Clarithromycin	8≤16≥32	NA	16/(8)	0.06-16/8	0.06 - 64	0.06 - 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	CLSI	EUCAST	MAB	MAB	RAPMYCO1	RAPMYCO2	<i>M.peregrinum</i>
							ATCC700686
	NTM						Recommended
Drug	breakpoints	PK/PD	(T)ECOFF	WT distribution	Test range	Test range	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	CLSI	EUCAST	<i>M.avium/</i>	<i>M.avium/</i>	SLOMYCO1	SLOMYCO2	<i>M.avium</i>
			<i>M.intracellulare</i>	<i>M.intracellulare</i>			ATCC700898
	NTM						Recommended
Drug	breakpoints	PK/PD	(T)ECOFF	WT distribution	Test range	Test range	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	CLSI	EUCAST	MAB	MAB	RAPMYCO1	RAPMYCO2	<i>M.peregrinum</i>
							ATCC700686
	NTM						Recommended
Drug	breakpoints	PK/PD	(T)ECOFF	WT distribution	Test range	Test range	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

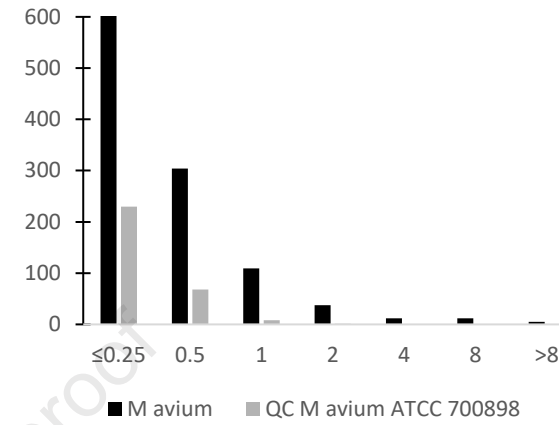
A1. Clarithromycin



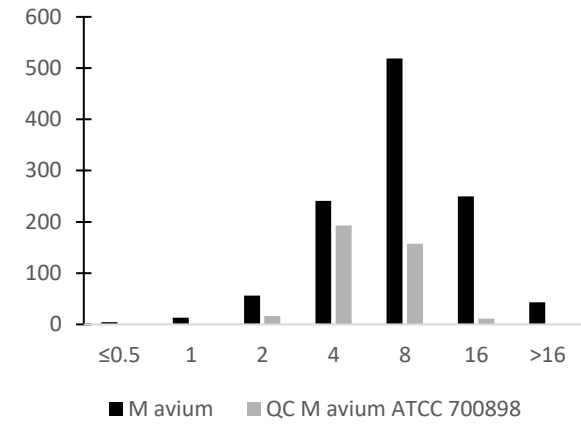
B1. Rifampicin



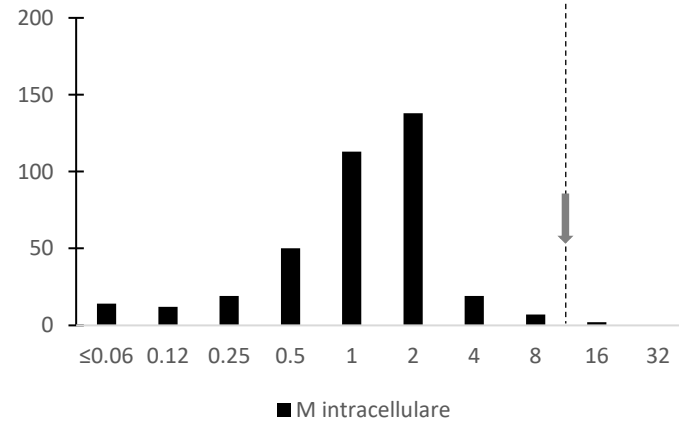
C1. Rifabutin



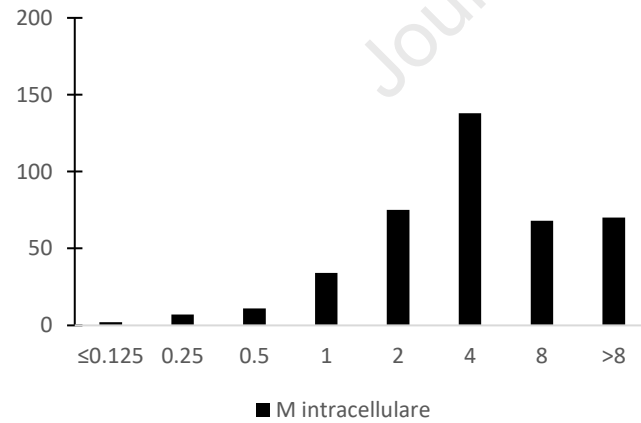
D1. Ethambutol



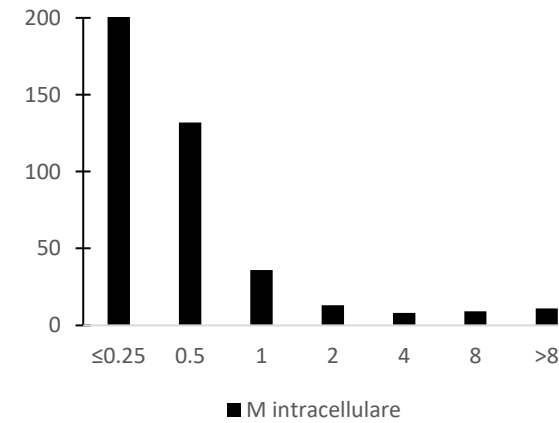
A2. Clarithromycin



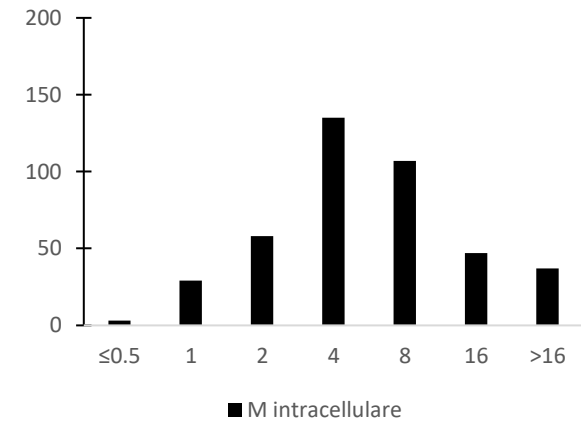
B2. Rifampicin



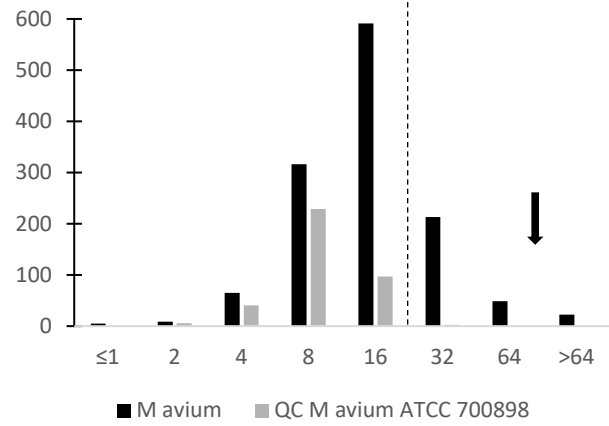
C2. Rifabutin



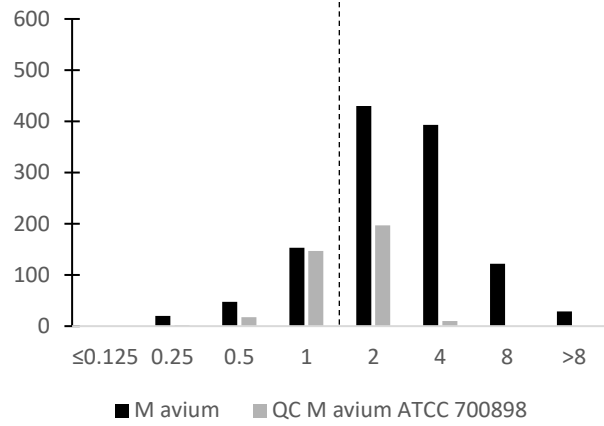
D2. Ethambutol



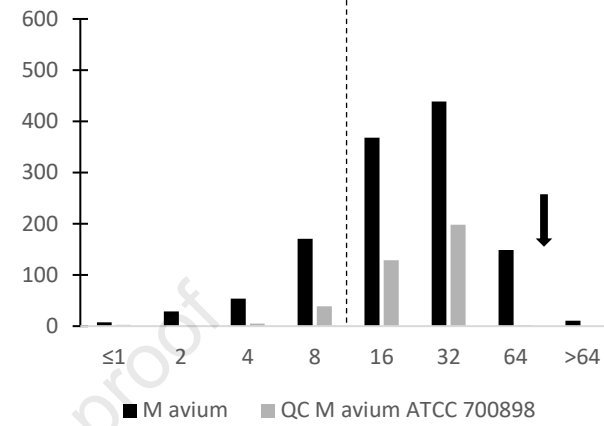
E1. Amikacin



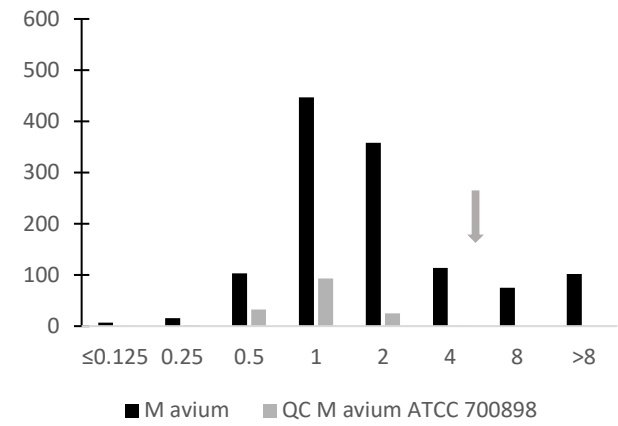
F1. Moxifloxacin



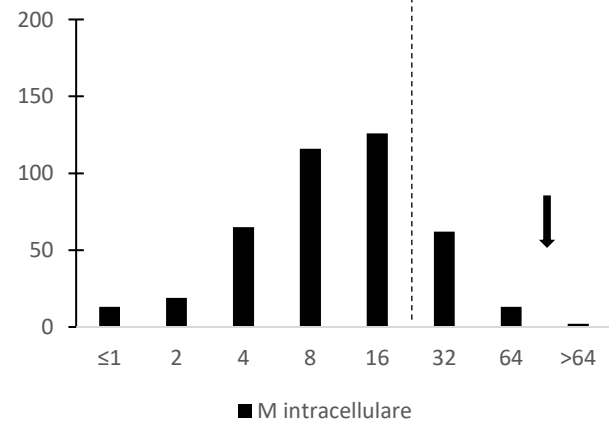
G1. Linezolid



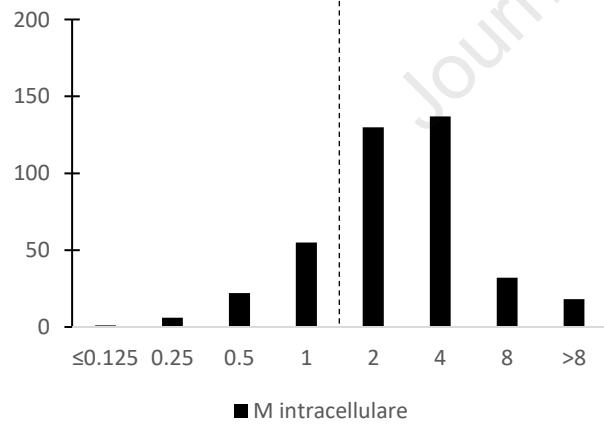
H1. TSU



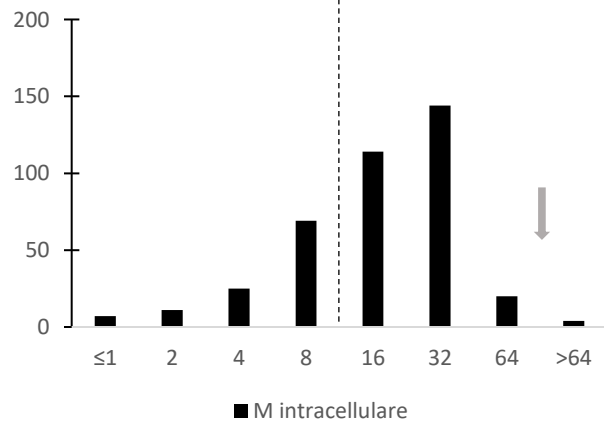
E2. Amikacin



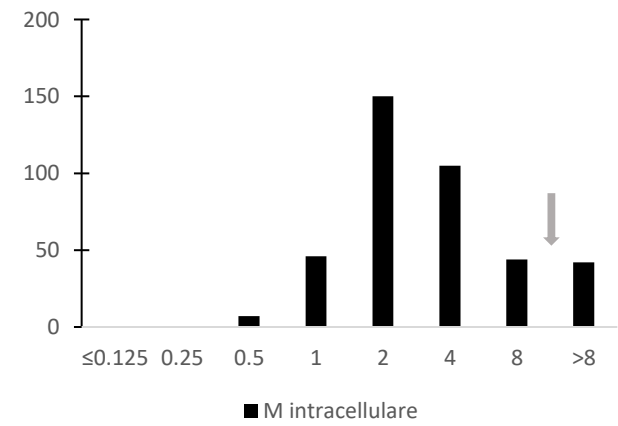
F2. Moxifloxacin



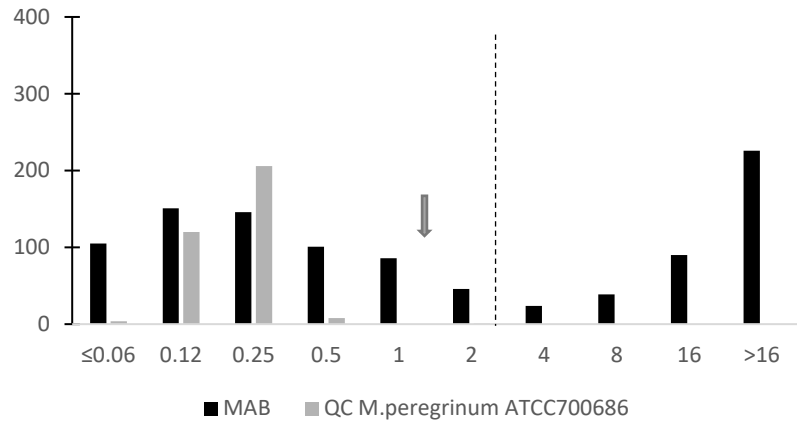
G2. Linezolid



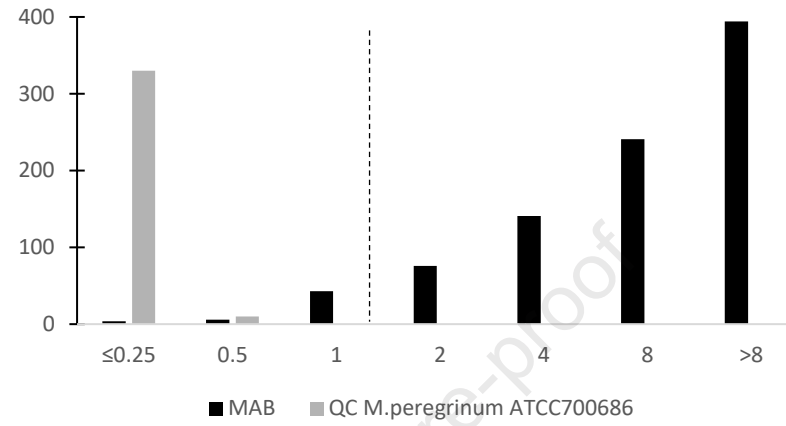
H2. TSU



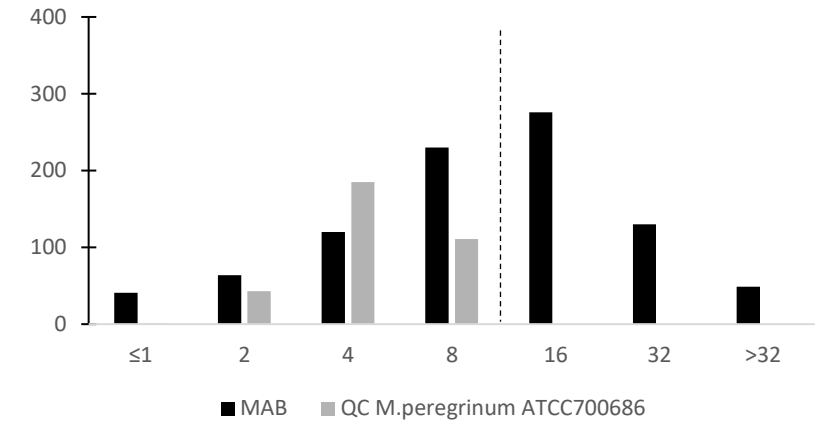
A. Clarithromycin



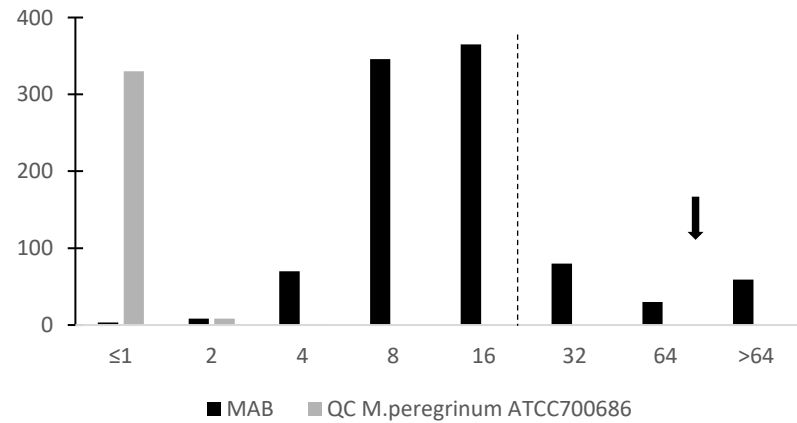
B. Moxifloxacin



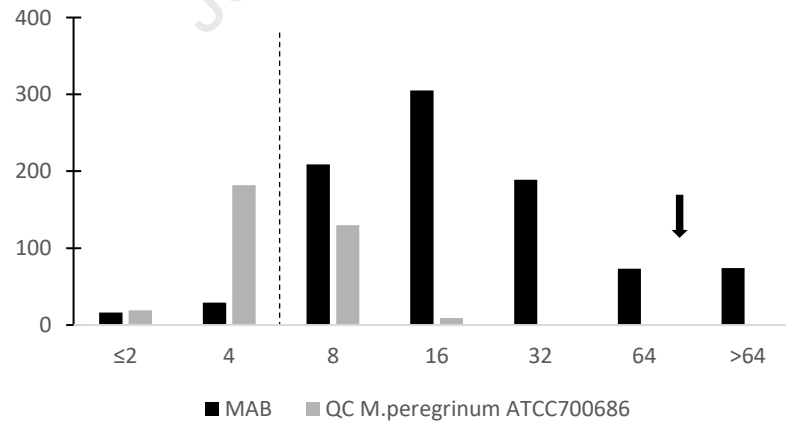
C. Linezolid



D. Amikacin



E. Imipenem



F. Tigecycline

