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ARTICLE

Antimicrobial susceptibility of rapidly growing mycobacteria using the rapid colorimetric method

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Abstract Drug susceptibility testing (DST) of rapidly growing mycobacteria (RGM) are recommended for guiding the antimicrobial therapy. We have evaluated the use of resazurin in Mueller–Hinton medium (MHR) for MIC determination of RGM and compared the results with those obtained with the reference standard broth microdilution in Mueller–Hinton (MH) and with the resazurin microtiter assay (REMA) in 7H9 broth. The MIC of eight drugs: amikacin (AMI), cefoxitin (FOX), ciprofloxacin (CIP), clarithromycin (CLA), doxycycline (DOX), linezolid (LZD), moxifloxacin (MXF) and trimethoprim-sulfamethoxazole (TMP-SMX) were evaluated against 76 RGM (18 species) using three methods (MH, MHR, and REMA) in a 96-well plate format incubated at 37 °C over 3–5 days. Results obtained in the MH plates were interpreted by the appearance of turbidity at the bottom of the well before adding the resazurin. MHR and 7H9-REMA plates were read by visual observation for a change in color from blue to pink. The majority of results were obtained at day 5 for MH and 1 day after for MHR and 7H9-REMA. However, the preliminary experiment on time to positivity results using the reference strain showed that the resazurin can be added to the MH at day 2 to produce the results at day 3, but future studies with large sets of strains are required to confirm this suggestion. A high level of agreement (kappa 1.000–0.884) was obtained between the MH and the MHR. Comparison of results obtained with 7H9-REMA, on the other hand, revealed several discrepancies and a lower level of agreement (kappa 1.000–0.111). The majority of the strains were resistant to DOX and TMP-SMX, and the most active antimicrobials for RGM were AMI and FOX. In the present study, MHR represented an excellent alternative for MIC determination of RGM. The results could be read reliably, more easily, and more quickly than with the classical MH method.

Introduction

Rapidly growing mycobacteria (RGM) are nontuberculous mycobacteria (NTM) that are widely distributed in the environment and isolated most frequently from soil and water [1]. In the last few years, reports of human infections caused by RGM have increased globally, especially in immunocompromised individuals, although RGM can also affect persons with a competent immune system [2, 3]. They can cause a wide variety of disseminated or localized infections, particularly pulmonary, skin, and soft tissue infections [4–6]. The treatment of choice for NTM infections is antibiotic chemotherapy, which requires multiple antimicrobial agents administered during a long period of time; consequently, the treatment is costly and often associated with drug-related toxicities [7, 8]. RGM are intrinsically resistant to several antibiotics and

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treatment regimens can differ by species. Therefore, RGM infections require individualized treatment based on the results of drug susceptibility testing (DST), which will help to choose the most effective antimicrobial therapy [2, 9].

The Clinical and Laboratory Standards Institute (CLSI) currently recommends the Mueller–Hinton (MH) microdilution broth-based method as the gold standard for determining the minimum inhibitory concentration (MIC) of antimicrobial agents for RGM [10]. On the other hand, the resazurin microtiter assay (REMA), a 7H9 broth-based method, has previously been used for the rapid detection of drug resistance in *Mycobacterium tuberculosis* against first- and second-line drugs [11, 12]. The addition of a redox indicator to the 7H9 broth-based microdilution method shortens the time to results. MIC DST may run in Mueller–Hinton broth with resazurin (MHR), being the preferred culture medium for RGM rather than the 7H9 broth-based method. The objective of the present study was to evaluate, to our knowledge for the first time, the use of resazurin as an indicator of growth in a Mueller–Hinton broth-based method for the DST of RGM. We analyzed the agreement among the gold standard MH method, MHR, and the classical REMA in 7H9 medium.

Materials and methods

Strains

A total of 76 RGM strains (11 type strains, 10 reference strains, and 55 clinical isolates) were used in this study (Tables 1, 2 and 3). These strains were obtained from the CCUG collection (http://www.ccug.se), from the clinical laboratory of St Luc Hospital, Brussels, Belgium, and from the Federal University of São Paulo, Brazil. The strains belonged to the following species: *Mycobacterium abscessus/chelonae* group, *Mycobacterium aurum*, *Mycobacterium cosmeticum*, *Mycobacterium duvalii*, *Mycobacterium fortuitum*, *Mycobacterium goodie*, *Mycobacterium immunogenum*, *Mycobacterium abscessus* subsp. *abscessus*, *Mycobacterium abscessus* subsp. *bolletii*, *Mycobacterium mageritense*, *Mycobacterium neoaurum*, *Mycobacterium peregrinum*, *Mycobacterium phlei*, *Mycobacterium senegalense*, *Mycobacterium smegmatis*, *Mycobacterium vaccae*, and *Mycobacterium wolinskyi*. *Mycobacterium peregrinum* ATCC 700686 was used for quality control, as recommended by the CLSI. Strains were cultured on Löwenstein–Jensen medium. The inoculum was prepared with a small modification according to the CLSI guideline, in distilled water, adjusted to a McFarland tube n° 0.5, and diluted 1:10 in the medium used to perform the broth microdilution method (MH or 7H9).

Antimicrobial agents and concentrations

Strains were tested against eight drugs: amikacin (AMI), cefoxitin (FOX), ciprofloxacin (CIP), clarithromycin (CLA), doxycycline (DOX), linezolid (LZD), moxifloxacin (MXF), and trimethoprim-sulfamethoxazole (TMP-SMX). The range of antimicrobial concentrations were: AMI (8–256 μg/ml), FOX (16–512 μg/ml), CIP (0.5–16 μg/ml), CLA (1–32 μg/ml), DOX (0.5–16 μg/ml), LZD (4–128 μg/ml), MXF (0.5–16 μg/ml), and TMP-SMX (0.5/9.5–16/304 μg/ml). For quality control with *M. peregrinum* ATCC 700686 all drugs were tested in up to four additional lower dilutions. All antimicrobial agents were obtained from Sigma–Aldrich and were solubilized according to the manufacturer's recommendations. CIP and DOX stock solutions were prepared at a concentration of 1 mg/ml in distilled water, AMI at 2 mg/ml, and FOX at 3 mg/ml. CLA and LZD were prepared at 1 mg/ml in DMSO, and TMP at 1 mg/ml in 0.05 N HCl. SMX stock solutions were prepared at concentrations of 2 mg/ml in 0.05 N NaOH. All stock solutions were filter sterilized, and stored frozen at −20 °C until used, for no more than 3 months. Working solutions were prepared at 4x the final higher test concentration in MH or 7H9 medium according to the method performed.

Antimicrobial susceptibility testing

Susceptibility testing was performed using two 96-well microtiter plates per strain. In one plate the MICs of the drugs were determined using the microdilution broth method according to the guidelines described by the CLSI using cation-adjusted MH broth [10] with some modifications. Serial two-fold dilutions of each drug were prepared directly in the plate and 100 μl of inoculum diluted 1:10 was added to each well. The inoculated plates were covered, sealed in a plastic bag, and incubated at 37 °C in a normal atmosphere for 3–5 days. Although the guideline recommends incubation at 30 °C, plates were incubated at 37 °C for the optimal incubation temperature of the resazurin. All strains grew well at this temperature. The MICs of the MH plates were interpreted by the appearance of turbidity at the bottom of each well and interpreted according to the CLSI guidelines [10]. Plates were examined after 72 h to check if growth appearing as turbidity or a deposit of cells at the bottom of the well in the growth control well was sufficient (at least 2+) to record the MIC. Otherwise, the plates were re-incubated and read daily thereafter (for up to 5 days) until growth was sufficient. The MIC was determined as the lowest concentration of antimicrobial agent at which no growth was observed. After MIC interpretation, 30 μl of 0.01 % resazurin was added to each well of the same plate, sealed again and incubated overnight at 37 °C for color development. A change in color from blue to pink indicated growth of bacteria and the MIC was defined as the

Table 1 Minimum inhibitory concentration (*MIC*; μg/ml) of the type strains for the eight antimicrobial agents tested compared using the three methods

Species	MIC (μg/ml)																							
	AMI			FOX			CIP			CLA			DOX			LZD			MXF			TMP-SMX		
	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9
M. vaccae CCUG 21003^T	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	16	16	16	≤0.5	≤0.5	≤0.5	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	8–152	4–76	4–76
M. neoaurum CCUG 37665 ^{T}	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	≤0.5	≤0.5	≤0.5	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	16–304	4–76	16–304
M. duvalii CCUG 41352 ^{T}	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	16	>32	>32	1	1	4	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	>16–304	8–152	16–304
M. wolinskyi CCUG 47168^T	≤8	≤8	≤8	64	64	32	≤0.5	≤0.5	≤0.5	>32	>32	>32	1	1	8	8	8	8	2	1	1	>16–304	8–152	16–304
M. abscessus subsp. *abscessus* ATCC 19977 ^{T}	≤8	≤8	≤8	32	64	64	1	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	8	32	4	2	2	16–304	8–152	8–152
M. abscessus subsp. *bolletii* CCUG 50184 ^{T}	≤8	≤8	16	32	32	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	8	32	2	2	2	16–304	8–152	8–152
M. chelonae ATCC 35752 ^{T}	≤8	≤8	16	64	64	128	4	4	1	≤1.0	≤1.0	2	>16	>16	>16	8	8	32	2	2	2	16–304	8–152	16–304
M. immunogenum ATCC 700505 ^{T}	≤8	≤8	16	32	32	32	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	8	8	2	2	2	>16–304	16–304	16–304
M. peregrinum DSM43271^T	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	8	16	>16	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	16–304	4–76	8–152
M. fortuitum subsp. *fortuitum* CCUG 27973^T	≤8	≤8	≤8	32	32	64	≤0.5	≤0.5	≤0.5	>32	>32	>32	4	4	16	16	16	8	≤0.5	≤0.5	≤0.5	>16–304	>16–304	>16–304
M. goodii CCUG 58730^T	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	8	>32	>32	1	1	2	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	8–152	4–76	16–304

AMI amikacin, *FOX* cefoxitin, *CIP* ciprofloxacin, *CLA* clarithromycin, *DOX* doxycycline, *LZD* linezolid, *MXF* moxifloxacin, *TMP-SMX* trimethoprim-sulfamethoxazole, *MH* Mueller–Hinton broth, *MHR* Mueller–Hinton broth (CAMH)+resazurin, *7H9* 7H9 medium+resazurin (REMA)

Table 2 The MIC (μg/ml) of reference strains for the eight antimicrobial agents tested compared using the three methods

Species	MIC (μg/ml)																							
	AMI			FOX			CIP			CLA			DOX			LZD			MXF			TMP-SMX		
	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9
M. peregrinum CCUG 28064	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	16	16	>16	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	>16–304	4–76	16–304
M. chelonae ATCC14472	≤8	≤8	≤8	32	32	64	4	4	8	≤1.0	2	16	16	16	>16	≤4	≤4	8	1	1	1	4–76	4–76	16–304
M. fortuitum CCUG 31556	≤8	≤8	≤8	32	32	32	≤0.5	≤0.5	≤0.5	32	32	>32	>16	>16	>16	8	8	32	≤0.5	≤0.5	1	>16–304	>16–304	>16–304
M. abscessus CCUG 41449	16	16	16	64	64	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	32	1	1	1	4–76	8–152	16–304
M. phlei CCUG 28060	≤8	≤8	≤8	32	64	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	≤0.5	≤0.5	1	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	4–76	8–152	>16–304
M. smegmatis CCUG 28063	≤8	≤8	≤8	64	64	64	≤0.5	≤0.5	≤0.5	>32	>32	>32	≤0.5	≤0.5	≤0.5	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	16–304	4–76	8–152
M. senegalense CCUG 59339	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	>32	>32	>32	≤0.5	1	4	8	≤4	8	≤0.5	≤0.5	≤0.5	>16–304	16–304	4–76
M. cosmeticum CCUG 55442	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	16	>16	>16	≤4	≤4	≤4	≤0.5	≤0.5	1	4–76	4–76	16–304
M. mageritense CCUG 51275	256	256	256	≤16	≤16	32	2	2	2	>32	>32	>32	>16	>16	>16	≤4	≤4	8	≤0.5	≤0.5	≤0.5	16–304	8–152	16–304
M. goodii CCGU 52054	≤8	≤8	≤8	32	64	32	≤0.5	≤0.5	≤0.5	>32	>32	>32	≤0.5	≤0.5	≤0.5	8	8	8	≤0.5	≤0.5	≤0.5	≤0.5–9.5	≤0.5–9.5	≤0.5–9.5
M. peregrinum ATCC 700686	≤0.5	≤0.5	1	≤16	≤16	≤16	0.062	≤0.031	0.062	0.062	≤0.062	0.125	0.25	0.25	0.25	2	2	2	0.062	0.062	0.062	0.5–9.5	0.5–9.5	0.5–9.5

Table 3 The MIC (μg/ml) of clinical isolates for the eight antimicrobial agents tested compared using the three methods

Species	MIC (μg/ml)																							
	AMI			FOX			CIP			CLA			DOX			LZD			MXF			TMP-SMX		
	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9
M. fortuitum 4/44	≤8	≤8	≤8	≤16	32	64	≤0.5	≤0.5	≤0.5	4	4	16	>16	>16	>16	16	16	32	≤0.5	≤0.5	≤0.5	16–304	>16–304	>16–304
M. abscessus P-1	≤8	≤8	≤8	32	32	64	>16	>16	8	≤1.0	≤1.0	4	>16	>16	>16	8	8	32	1	≤0.5	≤0.5	4–76	4–76	16–304
M. peregrinum 2/77	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	8	≤4	8	≤0.5	≤0.5	≤0.5	>16–304	4–76	8–152
M. fortuitum 2011/12	≤8	≤8	≤8	≤16	32	32	≤0.5	≤0.5	≤0.5	4	4	16	>16	>16	>16	≤4	≤4	8	≤0.5	≤0.5	≤0.5	>16–304	16–304	16–304
M. chelonae 2012/56	≤8	≤8	32	128	128	128	1	≤0.5	≤0.5	≤1.0	2	32	>16	>16	>16	≤4	≤4	32	2	2	2	16–304	8–152	>16–304
M. chelonae 2012/13	≤8	≤8	≤8	64	64	128	1	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	8	32	4	2	2	16–304	8–152	16–304
M. chelonae 2010/51	≤8	≤8	≤8	32	32	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	16	>16	>16	≤4	≤4	8	2	2	2	16–304	16–304	>16–304
M. chelonae 2013/50	≤8	≤8	≤8	32	32	32	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	8	8	16	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	>16–304	16–304	16–304
M. chelonae 2013/2	≤8	≤8	16	32	32	64	≤0.5	≤0.5	≤0.5	8	16	>32	>16	>16	>16	8	8	8	≤0.5	≤0.5	≤0.5	4–76	4–76	1–19
M. fortuitum 2011/15	≤8	≤8	≤8	≤16	≤16	32	≤0.5	≤0.5	≤0.5	2	2	16	4	4	8	≤4	8	32	≤0.5	≤0.5	≤0.5	16–304	16–304	16–304
M. chelonae 98203	≤8	≤8	≤8	64	64	128	8	4	4	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	8	2	2	2	16–304	8–152	8–152
M. chelonae 96–1705	≤8	≤8	≤8	32	64	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	≤4	1	1	1	16–304	8–152	>16–304
M. chelonae 96–1712	≤8	≤8	≤8	32	32	32	≤0.5	≤0.5	≤0.5	16	16	16	>16	>16	>16	≤4	≤4	8	≤0.5	≤0.5	≤0.5	>16–304	16–304	16–304
M. abscessus/chelonae 6410	≤8	≤8	≤8	32	32	32	1	≤0.5	1	>32	>32	>32	>16	>16	>16	8	8	8	≤0.5	≤0.5	1	>16–304	16–304	8–152
M. abscessus subsp. *bolletii* B67	≤8	≤8	≤8	32	64	64	8	8	4	≤1.0	≤1.0	≤1.0	>16	>16	>16	8	≤4	8	8	4	2	8–152	8–152	16–304
M. abscessus subsp. *bolletii* B61	≤8	≤8	16	32	32	64	1	≤0.5	≤0.5	≤1.0	≤1.0	4	>16	>16	>16	≤4	≤4	16	4	4	4	8–152	4–76	16–304
M. abscessus subsp. *bolletii* INCQS 00594	≤8	≤8	16	32	32	128	1	≤0.5	≤0.5	≤1.0	≤1.0	8	>16	>16	>16	8	8	32	8	4	8	16–304	8–152	>16–304
M. abscessus subsp. *bolletii* B66	≤8	≤8	≤8	32	32	128	4	4	2	8	32	>32	>16	>16	>16	≤4	≤4	32	2	2	2	>16–304	16–304	16–304
M. abscessus subsp. *bolletii* B60	≤8	≤8	16	64	64	128	4	4	2	≤1.0	≤1.0	8	>16	>16	>16	≤4	≤4	32	2	2	2	8–152	4–76	16–304

Table 3 (continued)

Species	MIC (μg/ml)																							
	AMI			FOX			CIP			CLA			DOX			LZD			MXF			TMP-SMX		
	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9
M. abscessus subsp. *bolletii* B31	≤8	≤8	≤8	32	32	64	4	4	2	≤1.0	≤1.0	≤1.0	>16	16	>16	≤4	≤4	8	4	4	4	8–152	4–76	16–304
M. abscessus subsp. *abscessus* P2	≤8	≤8	16	32	64	64	4	4	2	≤1.0	≤1.0	2	>16	>16	>16	≤4	≤4	8	2	2	2	4–76	4–76	8–152
M. abscessus subsp. *abscessus* P1	≤8	≤8	16	32	64	128	4	8	4	≤1.0	≤1.0	8	>16	>16	>16	≤4	≤4	32	2	2	2	4–76	8–152	16–304
M. aurum 50518	≤8	≤8	≤8	≤16	≤16	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	1	1	4	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	1–19	≤0.5–9.5	≤0.5–9.5
M. abscessus subsp. *abscessus* EPM 13400	≤8	≤8	≤8	≤16	32	32	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	8	2	2	2	8–152	8–152	16–304
M. abscessus subsp. *abscessus* EPM 13219	≤8	≤8	16	32	64	64	4	4	1	≤1.0	≤1.0	2	>16	>16	>16	≤4	≤4	16	1	2	1	4–76	4–76	8–152
M. abscessus/chelonae D16Q15	16	16	16	128	128	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	32	2	2	2	8–152	4–76	16–304
M. abscessus 8223	≤8	≤8	16	32	32	64	1	1	≤0.5	8	8	>32	>16	>16	>16	8	8	32	2	2	2	16–304	8–152	4–76
M. chelonae 96–443	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	16–304	8–152	4–76
M. chelonae D16R19	≤8	≤8	16	32	64	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	≤4	≤4	32	4	2	2	16–304	8–152	16–304
M. abscessus/chelonae 96–892	≤8	≤8	≤8	32	32	32	≤0.5	≤0.5	≤0.5	16	16	16	>16	>16	>16	8	8	8	2	2	2	>16–304	16–304	8–152
M. abscessus/chelonae D16Q14	≤8	≤8	16	32	32	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	16	16	>16	≤4	≤4	8	≤0.5	1	1	4–76	4–76	8–152
M. abscessus/chelonae D16Q20	≤8	≤8	≤8	32	64	64	2	2	1	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	≤4	1	1	≤0.5	16–304	8–152	8–152
M. chelonae 96–1724	≤8	≤8	≤8	32	32	128	4	4	1	≤1.0	≤1.0	≤1.0	>16	>16	>16	8	8	16	1	1	1	>16–304	16–304	>16–304
M. abscessus/chelonae D16Q19	≤8	≤8	16	32	32	128	1	1	≤0.5	≤1.0	≤1.0	≤1.0	8	8	>16	≤4	≤4	≤4	≤0.5	1	1	16–304	8–152	8–152
M. chelonae 96–1717	≤8	≤8	≤8	32	32	128	1	≤0.5	1	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	8	1	1	1	>16–304	8–152	16–304
M. chelonae D16R24	≤8	≤8	16	64	32	64	1	1	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	8	1	1	1	>16–304	16–304	>16–304
M. chelonae 96–1728	≤8	≤8	≤8	≤16	≤16	≤16	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	≤4	≤0.5	≤0.5	≤0.5	1–19	≤0.5–9.5	≤0.5–9.5

Table 3 (continued)

Species	MIC (μg/ml)																							
	AMI			FOX			CIP			CLA			DOX			LZD			MXF			TMP-SMX		
	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9	MH	MHR	7H9
M. abscessus/chelonae D16Q13	≤8	≤8	≤8	32	64	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	≤4	8	2	2	2	>16–304	4–76	8–152
M. abscessus/chelonae D16R12	≤8	≤8	≤8	32	32	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	≤4	8	2	2	2	16–304	8–152	4–76
M. abscessus/chelonae D16Q16	≤8	≤8	≤8	32	32	32	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	≤4	≤4	8	2	2	2	4–76	4–76	8–152
M. chelonae D16R10	≤8	≤8	16	64	64	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	16	1	1	1	4–76	4–76	8–152
M. chelonae D16R3	≤8	≤8	≤8	32	64	128	1	≤0.5	≤0.5	≤1.0	≤1.0	2	16	>16	>16	8	≤4	32	4	2	2	>16–304	8–152	16–304
M. chelonae D16R7	≤8	≤8	≤8	32	32	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	8	32	2	2	2	>16–304	16–304	8–152
M. abscessus/chelonae D16R18	≤8	≤8	16	32	32	64	1	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	8	≤4	8	2	2	2	4–76	4–76	4–76
M. chelonae D16R9	≤8	≤8	16	32	32	64	1	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	≤4	≤4	8	2	2	2	8–152	8–152	16–304
M. chelonae D16Q24	≤8	≤8	≤8	32	32	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	≤4	1	1	≤0.5	4–76	4–76	4–76
M. chelonae D16R2	≤8	≤8	≤8	32	32	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	8	1	1	1	4–76	4–76	4–76
M. abscessus/chelonae D16R14	16	16	16	128	128	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	≤4	≤4	16	2	2	2	4–76	4–76	8–152
M. abscessus/chelonae D17A2	≤8	≤8	16	32	64	128	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	8	2	2	2	4–76	4–76	8–152
M. chelonae D16R4	≤8	≤8	≤8	32	32	128	2	2	1	≤1.0	≤1.0	≤1.0	2	8	>16	≤4	≤4	8	1	1	2	4–76	4–76	8–152
M. chelonae D16R20	≤8	≤8	16	32	32	32	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	2	>16	>16	>16	8	8	8	2	2	2	>16–304	16–304	16–304
M. abscessus/chelonae D16R27	≤8	≤8	≤8	64	64	64	≤0.5	≤0.5	≤0.5	≤1.0	≤1.0	≤1.0	>16	>16	>16	≤4	≤4	≤4	2	2	2	≤0.5–9.5	1–19	1–19
M. chelonae 961720	≤8	≤8	≤8	32	32	128	1	1	1	≤1.0	≤1.0	≤1.0	16	>16	>16	≤4	≤4	8	1	≤0.5	1	>16–304	8–152	8–152
M. abscessus 52495	≤8	≤8	≤8	64	64	128	>16	>16	>16	≤1.0	≤1.0	4	>16	>16	>16	≤4	≤4	8	16	4	4	16–304	4–76	8–152
M. abscessus 52214	256	256	64	≤16	≤16	32	≤0.5	≤0.5	≤0.5	32	>32	>32	>16	>16	>16	≤4	≤4	8	≤0.5	≤0.5	≤0.5	>16–304	16–304	4–76

lowest concentration of the drug that prevented this change in color. In the second plate, the MICs of the drugs were determined by the REMA in Middlebrook 7H9 medium supplemented with 10 % OADC (oleic acid albumin dextrose catalase), 0.5 % glycerol, and 0.1 % casitone and performed as described by Palomino et al. [11]. In both plates a growth control (without any drug added) and a sterile control (only medium) were also included for each strain tested. Sterile water was added to all perimeter wells to avoid evaporation during the incubation. Strains giving discordant results among the methods were reevaluated.

Time to positivity of MHR

To investigate the time to positivity (TTP) of the MHR method and offer time-saving results compared with the CLSI recommendation, we used three MHR plates to test *M. peregrinum* ATCC 700686 against the eight antimicrobial drugs. At day 2, resazurin was added to the plate. Plates were visually read after 3, 4, and 5 days of growth to assess for any variability of results according to the length of incubation.

Data analysis

Using the break points defined by CLSI, strains were classified as resistant, intermediate or susceptible. Kappa (κ) statistic was used to determine the overall agreements in the classification of strains based on their susceptibilities determined by MHR and 7H9-REMA compared with the gold standard, MH. For this purpose, data were collapsed into 2×2 tables: intermediate susceptibility was classified as resistant [S, (I+R)]. Kappa was calculated using MedCalc Statistical Software (version 14.10.2; MedCalc Software, Ostend, Belgium). Comparative analysis of MIC was carried out using categorical and essential agreement between MH/MHR and MH/7H9. Essential agreement (EA) between the two methods (MH/MHR and MH/7H9) was calculated as the percentage of the isolates giving the same results or varying by ±2 log dilutions. Categorical agreement (CA) was defined as the percentage of agreement regarding each breakpoint category (susceptible, intermediate, or resistant) obtained by the two methods. A very major error (VME) constitutes a resistant isolate by MHR or 7H9-REMA-designated susceptible by the reference method MH. A major error (ME) constitutes a susceptible isolate by MHR or 7H9-REMA designated resistant by MH. Minor errors (mE) were designated to intermediate results according to MHR or 7H9-REMA and found either sensitive or resistant by MH.

Results

A total of 76 RGM strains were evaluated in this study. The results of the antimicrobial susceptibility testing are presented in Table 1 (type strains), Table 2 (reference strains), and Table 3 (clinical isolates).

For the type strains (Table 1), there was excellent agreement between the MH method and the MHR assay, with 100 % essential and categorical agreement respectively for all drugs, except for 2 mE found for MOX. When comparing results between MH and 7H9-REMA assays, the EA was 100 % for all drugs except 91 % for DOX (10/11). The CA was 100 % for AMI, CLA and TMP-SMX respectively. However, we found classification errors for the other drugs: 1 mE was found for FOX, 1 VME for CIP, 3 mE and 1 VME for DOX, 1 mE and 3 VME for LZD, and 2 mE for MX.

For the reference strains (Table 2), there was similarly excellent agreement between the MH method and the MHR assay, with 100 % EA and CA for all drugs. When comparing results using the MH and 7H9-REMA methods, EA was 100 % for all drugs, except for CLA and DOX for which it was 90 % (9 out of 10). The CA was 100 % for AMI, CIP, MX and TMP-SMX respectively. Two mE were found for FOX, 1 VME for CLA and DOX, and 2 VME for LZD.

For the clinical isolates (Table 3), EA was excellent (100 %) between the MH and MHR methods for all drugs. The CA was also excellent (100 %) for AMI, CIP, CLA, LZD, and TMP-SMX. One mE was found for DOX (1.8 %), 3 mE for FOX (5.4 %), and 4 mE for MX (7.2 %). When comparing the MH and 7H9-REMA methods, EA was 100 % for AMI, FOX, CIP, MX, and TMP-SMX. For CLA, five discordances were found, giving an EA of 91 %. For DOX the EA was 98 % and for LZD 87 %. The CA was very good only for AMI (100 %). For FOX, 24 mE were found, giving a CA of 56 %, for CIP 6 mE and 2 ME were found, for DOX 3 mE, for LZD, 6 mE and 13 VME, for MX 5 mE, and finally for TMP-SMX 1 ME.

The MIC value for the reference strain *M. peregrinum* ATCC 700686 observed in the three methods (MH, MHR, 7H9-REMA) correspond to the MIC values provided by the CSLI guideline. The majority of the strains tested were resistant to DOX and TMP-SMX, while the most active antimicrobial agents were AMI, FOX, LZD, and MXF. Results are expressed as susceptible, intermediate or resistant, according to the MIC breakpoint recommended by the CLSI guidelines (Table 4). The MICs of all antimicrobial agents obtained using the MHR method correlated well with those obtained with the standard MH microdilution method. For AMI, MH classified 74 isolates as susceptible and 2 as resistant. The MHR showed 100 % agreement with the MH, while the 7H9-REMA interpreted 1 isolate as susceptible, while MH considered it intermediate. For FOX, 18 isolates were found to be susceptible by MH, 3 resistant, and 55 considered intermediate.

Table 4 Break points of the eight antimicrobial agents and the results of the antimicrobial susceptibility testing of 76 RGM strains by microdilution assay in MH, MH with resazurin, and by 7H9-REMA

Drugs	MIC break points (μg/ml)			Medium										
	S	I	R	MH			MHR			K	7H9-REMA			κ
				S	I	R	S	I	R		S	I	R	
AMI	≤16	32	≥64	74	0	2	74	0	2	1.000	73	1	2	0.793
FOX	≤16	32–64	≥128	18	55	3	15	58	3	0.884	12	38	26	0.753
CIP	≤1	2	≥4	60	3	13	60	3	13	1.000	65	5	6	0.776
CLA	≤2	4	≥8	57	2	17	57	2	17	1.000	48	3	25	0.727
DOX	≤1	2–4	≥8	10	3	63	10	2	64	1.000	5	4	67	0.635
LZD	≤8	16	≥32	74	2	0	74	2	0	1.000	52	5	19	0.111
MXF	≤1	2	≥4	42	25	9	42	29		1.000	42	30	4	1.000
TMP-SMX	≤2–38	–	≥4–76	4	0	72	4	0 5	72	1.000	5	0	71	0.882

κ kappa

When FOX was evaluated using MHR, there were 3 susceptible isolates classified as intermediate. When these results were compared with those for 7H9-REMA, more discordant results were obtained, with 12 isolates classified as susceptible, 38 as intermediate, and 26 as resistant. For CIP, 100 % isolates gave the same results by MH and MRH with 60 isolates categorized as susceptible, 3 as intermediate, and 13 as resistant. Discrepant results were obtained using 7H9-REMA, with 65 isolates found susceptible, 5 as intermediate, and 6 as resistant. For CLA, 100 % of isolates also gave the same results regarding each break point category by MH and MRH, with 57 isolates determined to be susceptible, 2 intermediate, and 17 resistant. There were susceptibility changes in category when CLA was evaluated using 7H9-REMA. Ten of the 57 susceptible isolates were categorized as resistant and 1 as intermediate. For DOX, 98.6 % of isolates showed the same results between MH and MHR. Only one isolate classified as intermediate by MH was categorized as resistant by MHR. Discrepant results were found with 7H9-REMA, which classified only 5 isolates as susceptible, 4 as intermediate, and 67 as resistant (Table 4). For LZD, 100 % of isolates revealed the same results between MH and MRH with 74 isolates found susceptible, 2 intermediate, and no isolates were found to be resistant to LZD. However, using 7H9-REMA, 19 isolates were categorized as resistant, 52 isolates as susceptible, and 5 as intermediate. For MOX, the three methods classified 42 isolates as susceptible. Twenty-five isolates were found to be intermediate by MH, 29 by MHR and 30 by 7H9-REMA. Nine, 5, and 4 isolates were classified as resistant by MH, MHR, and 7H9-REMA respectively. Finally for TMP-SMX, both methods, MH and MHR, showed 100 % the same results, with 4 isolates susceptible and 72 isolates classified as resistant. No intermediate isolates were found. One isolate found to be resistant by MH and MHR was found to be susceptible using 7H9-REMA. The majority of the strains were read at day 5 for the MH method and 1 day later for the MHR and 7H9-REMA methods.

Overall, our level of agreement between the MH and MHR methods was very good to excellent, as demonstrated by the kappa values (Table 4), which varied from 1.000 to 0.884. Agreement between the MH and 7H9-REMA methods was lower, with poor kappa values between 1.000 and 0.111 poor.

Regarding time to positivity results, after 3 days of incubation the *M. peregrinum* ATCC 700686 control strain was found to be susceptible to all the antibiotics evaluated using the MRH method. After 4 or 5 days of incubation *M. peregrinum* ATCC 700686 remained susceptible to all antibiotics. There was no difference in the MICs after 3, 4 or 5 days of incubation and were comparable with those recommended by the CSLI guideline.

Discussion

In the present study, we evaluated the use of resazurin as an indicator of growth in the gold standard MH microdilution method to determine the MICs of eight antimicrobial agents in a set of 76 RGM. We investigated the agreement among the gold standard MH method, the MHR method, and the classical REMA in 7H9 broth method. The CLSI recommends the MH broth microdilution method for the DST of RGM. This technique seems to be easy and reliable; however, Broda et al. [13] have reported difficulties in interpreting the results using this method. The growth of RGM does not appear as a defined button at the bottom of the microtiter well, but as a hazy suspension and therefore, difficult for the reader to make an accurate determination of the MIC by visual turbidity reading, making the test subjective and variable [13]. To overcome this

problem, we proposed to add the redox indicator resazurin to the broth medium. The results could be also read visually and the indicator improves the end-point readability by a color change from blue to pink. Further, the addition of resazurin to the broth microdilution method could shorten the time for results, with an incubation time of 3 days compared with 5 days.

The procedure used to perform the MHR method was the same as that of the original MH microdilution method described by the CLSI, in terms of medium preparation, inoculum, and drug concentrations. The only extra step was the addition of resazurin to the MH broth at a final concentration of 0.01 %. When resazurin is added to the medium, bacterial growth results in a pink color, making it easy to interpret the results. An excellent level of agreement in results between MH and MHR was obtained for all drugs and strains. On the other hand, the classical REMA in 7H9 medium showed poor results compared with the gold standard, MH. RGM results in MH medium were more reproducible and end-point readings were easier than in 7H9 medium. Previously, several reports have shown the usefulness of Alamar blue microdilution method in 7H9 broth for the DST of *Mycobacterium avium* isolates [14–16]. On the other hand, Jadaun et al. [17] showed that resazurin can also be used in 7H9 broth for the DST of *M. avium* isolates. Resazurin is a non-proprietary reagent and an inexpensive alternative to Alamar blue for the DST of mycobacterial isolates. Cavusoglu et al. [18] evaluated the commercial Sensititre RAPMYCO plate (Trek Diagnostic Systems) to test the activities of several antimicrobial agents against RGM; however, this method also uses the growth turbidity at the bottom of the well as end point reading. The present study evaluates for the first time an alternative approach of using resazurin in MH medium for MIC determination of RGM. There was an excellent essential and categorical agreement between the reference method MH and the MHR assay with no major or very major errors. However, EA and CA was lower between MH and 7H9 methods with classification errors for many drugs. The majority of our strains were found resistant to DOX and TMP-SMX. This is in agreement with findings described in previous studies [9, 13, 19]. In this study, the most active drugs were AMI and CIP [20, 21]. Traditionally, AMI has been the best antimicrobial agent against RGM. Furthermore, AMI and CIP are considered the drugs of choice for the treatment of RGM infections [16, 22, 23]. Some studies, however, have reported high resistance to CIP [9, 13]. The treatment of infections caused by RGM is difficult and limited by the small number of effective drugs available. In this context, each species and strain must be individually evaluated. In our study, after sequence analysis of protein encoding genes, some isolates of *M. abscessus/chelonae* group did not allow us to identify these isolates as one of the established species. Also, there is a possible inducible resistance to macrolides among several NTM species; the final reading for clarithromycin should be at least 14 days unless resistance is recognized for *M. abscessus*. The results for CLA in the present study were obtained after no more than 5 days and should be considered as not definitive as it was not the objective of this study.

The preliminary results of the time to positivity evaluation with the fully susceptible strain *M. peregrinum* ATCC 700686 showed that there was no difference in the MIC results obtained after 3, 4, and 5 days of growth when using resazurin added to the MH medium after 2 days and read the day after. This means that clear and easy to interpret results appear achievable after 3 days of growth with the MHR method, while it is often necessary to wait until 5 days of growth before interpreting the results using the CLSI standard method. However, future studies with large sets of strains are required to confirm this suggestion.

In summary, in the present study, the MHR method could be an excellent alternative for the DST of RGM. The test could offer the MIC of antimicrobial agents in 3 days. The method has the advantage of being versatile and able to test many drug concentrations of choice.

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Conflict of interest The authors declare that they have no conflicts of interest.

Compliance with ethical standards We confirm that no human participants or animals were included in this study. Consequently, no informed consent was needed.

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