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Review Article

Identification and drug susceptibility testing for nontuberculous mycobacteria

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Great progress has recently been made in methodologies for identifying nontuberculous mycobacteria (NTM). Recommendations for drug susceptibility testing (DST) of NTM have been expanded and updated by the Clinical and Laboratory Standards Institute and are crucial in the management of NTM infections. This article summarizes the clinically relevant molecular methods used to discriminate NTM species and updates the information on DST. Furthermore, recent progress on new antimicrobials against NTM infections is reviewed.
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Introduction

The importance of nontuberculous mycobacteria (NTM) has recently been increasingly appreciated. The diagnosis, treatment, and prevention of NTM diseases are challenging because there are more than 150 species that have a similar clinical appearance to *Mycobacterium tuberculosis* complex (MTBC) and because of their intrinsic resistance to antimicrobial agents, their poorly defined therapeutic strategies, their diverse treatment regimens, and the lack of new therapeutic agents. In this article, we explore the relevant molecular methods that can be employed in the identification of NTM and review information on the detection of antimicrobial resistance in clinical practice. This review also details recent advances in chemotherapy for NTM infections that are not currently addressed by the Clinical and Laboratory Standards Institute (CLSI).[1]

Laboratory identification of NTM

Before the 1990s, laboratories relied on traditional non-molecular methods for species identification.[2] However, these traditional methods are more time consuming and less sensitive than molecular testing is.

In the 1990s, the polymerase chain reaction (PCR) and gene sequencing molecular methods were introduced, and gene sequencing has become the reference method for identifying NTM species and subspecies.[3] Gene sequencing has led to more than 150 species being discovered in contrast to the approximately 55 species identified before the 1990s; the quality of available databases are the cornerstones of the success of this methodology.[4] In other words, an identification result is only as good as the quality of the database used.

Matrix-assisted laser desorption/ionization mass spectrometry—time of flight (MALDI-TOF) and whole genome sequencing have recently marked a new era of NTM identification.

Based on their relevance in clinical practice, this article focuses on the following molecular-based methods: (1) commercial nucleic acid probes, (2) PCR restriction-enzyme analysis (PRA), (3) gene sequencing, (4) MALDI-TOF, and (5) the line probe assay.

Commercial nucleic acid probes

Although several commercial nucleic acid probes are available worldwide, many clinical mycobacterial laboratories, especially those in Taiwan, rely on the commercial single-stranded DNA nucleic-acid-probe DR. TBDR/NTM IVD kit (DR. Chip Corporation, Taiwan) for rapid and effective identification of *Mycobacterium* species among positive mycobacterial growth indicator tube (MGIT) cultures and as a crucial epidemiologic tool for diagnosing NTM disease.[5]

The DR. TBDR/NTM IVD kit, involving both DNA amplification and hybridization, was designed toidentify15 common NTM species and MTBC. The 16S—23S *rRNA* gene internal transcribed spacer, RNA polymerase β subunit (*rpoB*) gene, and PCR positive-control genes are amplified using multiplex PCR. Subsequently, species-specific and genotype-specific probes on the chip hybridize to target amplified DNA sequences for identification.[5] The DR. TBDR/NTM IVD kit has an accuracy rate for identification of *Mycobacterium* species of 95.5%.[5]

In the use of commercial nucleic acid probes, the accuracy of species discrimination is generally favorable, the method is not labor intensive, the turnaround time is short, and speedy identification (<24 h) can be achieved, although the method is costly, and less common *Mycobacterium* species that may be encountered as pathogens in clinical practice cannot be identified correctly (e.g. *Mycobacterium palustre* was assigned to MAC, *Mycobacterium saskatchewanense* and *Mycobacterium nebraskense* were identified as *Mycobacterium intracellulare*, and so on).[6—10] Thus, other methodologies for species identification of NTM ought to be made available.

PRA

In this method of NTM species differentiation, the amplified PCR products [*rpoB* gene and *heat-shock protein 65* gene (*hsp65* gene)] are processed through digestion using specific restriction enzymes (MspI, HaeIII, and BstEII), after which analysis is performed using agarose gel electrophoresis.[11—13] The obtained patterns are compared with the species identification algorithms proposed by Kim et al. and the published online database.[12—15]

Using additional restriction enzymes, PRA can differentiate *Mycobacterium abscessus* complex into subspecies levels (*M. abscessus* subsp. *abscessus*, subsp. *massiliense*, and subsp. *bolletii*). A viable culture of NTM that is isolated in a MGIT is not necessary for PRA, although technical problems related to the similar patterns of some closely related NTM species and small size differences between fragments are sometimes encountered, making species discrimination impossible. Furthermore, several newly discovered NTM species have yet to be extensively investigated using PRA.

Gene sequencing

Because of the increasing number of NTM species being discovered, various methods of species identification are being developed. However, such development has led to inconsistency between methodologies, and an accurate method of species differentiation is still required. Gene sequencing is currently considered the gold standard for identifying NTM species, although a qualified database is essential for alignment.[3] Furthermore, one disadvantage of single gene sequencing is that many closely related NTM species are undistinguishable using gene sequencing, meaning that additional species identification techniques are required.[16] Several common target genes for the species identification of NTM are detailed as follows.

(1) *16S rRNA* gene

The sequence at the 5'end of the *16S rRNA* gene, which comprises approximately 500 bps, contains most information on the species-specific sequence in mycobacterial and thus enables taxonomic identification of both slowly growing mycobacteria (SGM) and rapidly growing mycobacteria

(RGM) (partial 500-bp sequencing method).[3] A major disadvantage of the partial 500-bp sequencing method is that it cannot differentiate species with the same hypervariable region as well as between complete *16S rRNA* gene (1500-bp) sequences, such as *Mycobacterium marinum* versus *Mycobacterium ulcerans*, *Mycobacterium kansasii* versus *Mycobacterium gastri*, *Mycobacterium genavense* versus *Mycobacterium simiae*, *M. abscessus* versus *Mycobacterium chelonae* versus *Mycobacterium franklinii*, and *Mycobacterium goodii* versus *Mycobacterium smegmatis*. Note that *M. goodii* and *M. smegmatis* have 4 bps of *16S rRNA* gene difference, making it possible to discriminate between these two species by using the complete 1500-bp sequencing method.[17] However, complete 1500-bp sequencing is impractical for routine species identification.

(2) *rpoB* gene

The *rpoB* gene is an gene with almost 3,600bp, and it encodes the β subunit of RNA polymerase. This gene has been recommended for use as a second-line target for NTM species identification to compensate for the lack of discriminatory power between closely related NTM groups, especially RGM, when sequencing the *16S rRNA* gene.[18,19] One study demonstrated that relative to and unlike INNO-LiPA/*16S rRNA* gene sequencing, *rpoB* gene sequencing can identify a greater number of known and less common NTM species, in addition to having the ability to identify both SGM and RGMspecies.[20]

Unlike the partial 500-bp *16S rRNA* gene sequencing that most clinical laboratories sequence between 450bp and 480bp (at least 300bp) to ensure accuracy of species identification, the problems with *rpoB* gene sequencing include the absence of CLSI-established cutoff values for this gene and the fact that several regions have been proposed in different studies.[21–23] Moreover, the lack of sequence databases of reference strains can result in incorrect alignment and misidentification.

(3) *hsp65* gene

The 65-kDa *hsp65* gene, less conserved among mycobacterial species than the *16S rRNA* gene is, has been used to differentiate closely related NTM species by using the 441-bp Telenti fragment, especially for identifying RGM species, including *Mycobacterium peregrinum*, *Mycobacterium septicum*, *Mycobacterium houstonense*, *Mycobacterium fortuitum*, *Mycobacterium porcinum*, and *Mycobacterium senegalense*.[14] This gene also enables discrimination between *M. chelonae* and *M. abscessus* and between the three subspecies of *M. abscessus* complex.[14] As for SGM, the *hsp65* gene can differentiate *M. marinum* from *M. ulcerans* and *M. kansasii* from *M. gastri*.[24] Moreover, online databases are available for the *hsp65* gene sequence.[25]

(4) *Erythromycin ribosomal resistance methylase (erm)* gene

Several RGM species have been discovered to possess *erm* genes. The *erm*(38) gene is present in *Mycobacterium goodie* and *M. smegmatis*; the *erm*(39) gene in *M. fortuitum*, *M. houstonense*, *M. porcinum*, and *Mycobacterium neworleansense*; the *erm*(40) gene in *Mycobacterium mageritense* and *Mycobacterium wolinskyi*; and the *erm*(41) gene in *M. abscessus* complex.[26–32] The presence of a functional *erm* gene, which is caused by methylation of the 23S rRNA and may predict inducible macrolide resistance, is associated with a higher treatment failure rate. Of the *erm* genes, *erm*(41), discovered in *M. abscessus* complex, is the most clinically feasible. One study reported an approximately 90% response rate in patients with infection by *M. abscessus* complex that harbored a nonfunctional *erm* gene (*M. abscessus* subsp. *massiliense*); a response rate of only approximately 25% was reported in patients with infection by a species with a functional *erm* gene (*M. abscessus* subsp. *abscessus*).[28] Additionally, the *erm*(41) gene was discovered to enable differentiation of the three subspecies of *M. abscessus* complex.[27] Notably, unless the isolate has a 23S rRNA gene mutation, as evidenced by a 3–5-day clarithromycin minimum inhibitory concentration (MIC) $\geq$32 μg/mL, extended incubation is required to assess the inducible macrolide resistance.[1]

MALDI-TOF

MALDI-TOF is a new method of NTM species identification based on the species-specific spectral profiles produced by extracted ribosomal proteins from which specific mass-to-charge ratios are determined.[2,33,34] One advantage of this technique is that it is quicker than traditional identification methods are, although the hands-on time is increased by the need for a pre-inactivated mycobacterial cell extraction procedure, which usually involves bead beating or vortexing in formic acid, ethanol, and acetonitrile.[35] The accuracy of MALDI-TOF depends on the quality of harbored spectra, and achieving high-quality harbored spectra is challenging because of the complexity of the mycobacterial cell wall. The obtained spectra is then compared with a consensus profile for species identification.[36]

Studies have demonstrated that this method can differentiate species of RGM and SGM, although it cannot discriminate between *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*; *M. chelonae* and *M. abscessus*; *Mycobacterium mucogenicum* and *Mycobacterium phocaicum*; the *M. fortuitum* group; *Mycobacterium avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*; or *M. intracellulare* and *Mycobacterium chimaera*.[34,36–40] Furthermore, the detection rate of MTBC was as low as 41.6%.[40] Thus, MTBC should be ruled out, and the nonviable specimen should be provided. Moreover, the procedure should be performed in a biosafety cabinet.

Line probe assay

This method is based on application of nucleic acid amplification and reverse hybridization to detect NTM and its speciation. In short, the target sequence from growth media is amplified by using PCR and biotinylated primers. The products are then reverse hybridized onto a nitrocellulose strip with membrane-bound species-specific

Table 1 Antimicrobials and susceptibility breakpoints for nontuberculous mycobacteria (NTM), as proposed by the Clinical and Laboratory Standards Institute.

Species/complex	CLSI M24, 2nd ed., 2011			CLSI M24, 3rd ed., 2018		
	MIC category (μg/mL)			MIC category (μg/mL)		
	S	I	R	S	I	R
***M. avium* complex**						
Primary						
Clarithromycin	≤8	16	≥32	≤8	16	≥32
Intravenous amikacin[a]	—	—	—	≤16	32	≥64
Inhaled liposomal amikacin[a]	—	—	—	≤64		≥128
Secondary						
Moxifloxacin	≤1	2	≥4	≤1	2	≥4
Linezolid	≤8	16	≥32	≤8	16	≥32
M. kansasii						
Primary						
Clarithromycin[a]	—	—	>16	≤8	16	≥32
Rifampicin[a]	—	—	>1	≤1	—	≥2
Secondary						
Amikacin[a]	—	—	>32	≤16	32	≥64
Ciprofloxacin[a]	—	—	>2	≤1	2	≥4
Ethambutol	—	—	>4	—	—	—
Isoniazid	—	—	—	—	—	—
Linezolid[a]	—	—	—	≤8	16	≥32
Moxifloxacin[a]	—	—	>2	≤1	2	≥4
Rifabutin[a]	—	—	>2	≤2	—	≥4
Streptomycin	—	—	—	—	—	—
Trimethoprim/sulfamethoxazole[a]	—	—	>2/38	≤2/38	—	≥4/76
Doxycycline/minocycline[a]	—	—	—	≤1	2–4	≥8
M. marinum						
Amikacin[a]	—	—	>32	≤16	32	≥64
Ciprofloxacin[a]	—	—	>2	≤1	2	≥4
Clarithromycin[a]	—	—	>16	≤8	16	≥32
Doxycycline/Minocycline[a]	—	—	>4	≤1	2–4	≥8
Ethambutol[a]	—	—	>4	—	—	—
Moxifloxacin[a]	—	—	>2	≤1	2	≥4
Rifabutin[a]	—	—	>2	≤2	—	≥4
Rifampicin[a]	—	—	>1	≤1	—	≥2
Trimethoprim/sulfamethoxazole[a]	—	—	>2/38	≤2/38	—	≥4/76
Linezolid[a]	—	—	—	≤8	16	≥32
Rapidly growing mycobacteria						
Amikacin	≤16	32	≥64	≤16	32	≥64
Cefoxitin	≤16	32–64	≥128	≤16	32–64	≥128
Ciprofloxacin	≤1	2	≥4	≤1	2	≥4
Clarithromycin	≤2	4	≥8	≤2	4	≥8
Doxycycline	≤1	2–4	≥8	≤1	2–4	≥8
Imipenem	≤4	8–16	≥32	≤4	8–16	≥32
Linezolid	≤8	16	≥32	≤8	16	≥32
Meropenem	≤4	8–16	≥32	≤4	8–16	≥32
Moxifloxacin	≤1	2	≥4	≤1	2	≥4
Trimethoprim/sulfamethoxazole	≤2/38	—	≥4/76	≤2/38	—	≥4/76
Tobramycin	≤2	4	≥8	≤2	4	≥8

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible.

[a] Different breakpoints exist in CLSI 2011 versus CLSI 2018.

Table 2 NTM isolates that are clinically significant and non-significant.

Significant	Non-significant
1. NTM isolates recovered from sterile sites, such as the blood, pleural effusion, cerebrospinal fluid, soft tissue, and lymphadenopathy. 2. Isolation of NTM species from an immunocompromised host, especially those on corticosteroids and antitumor necrosis factor therapy. 3. Isolates from multiple samples and in large quantities.	1. Isolation of *M. gordonae*, *M. terrae* complex, *M. chelonae*, the *M. mucogenicum* group, *M. botniense*, *M. chlorophenolicum*, *M. aromaticivorans*, M. hodleri, *M. murale*, *M. pallens*, *M. rufum*, *M. rutilum*, *M. litorale*, M. arabiense, M. sediminis, M. paragordonae, *M. cookii*, M. fredericksbergense, and *M. psychrotolerans* from respiratory samples. 2. The *M. fortuitum* group is rarely a respiratory pathogen except in patients with lipoid pneumonia or achalasia. 3. Isolates recovered in single sample and small numbers of colonies.

fragments. An enzyme-mediated color reaction then occurs. The strips visually analyzed following a colorimetric conjugation step through its comparison with a commercially available chart for NTM species identification.[41]

Three commercial line probe assays are available: INNO-LiPA (Innogenetics, Ghent, Belgium), GenoType Mycobacterium CM/AS (Hain Lifescience, Nehren, Germany), and Speed-Oligo Mycobacteria (Vincel, Granada, Spain), which are based on nucleotide differences in the 16S–23S *rRNA* gene spacer region, 23S *rRNA* gene, and 16S *rRNA* and 16S–23S *rRNA* regions, respectively.[41] No MTBC isolates were wrongly identified as NTM when these three line probe assays were employed.[6]

Drug susceptibility testing for NTM

Principles and timings

The methods of drug susceptibility testing (DST) and proposed MIC breakpoints for NTM that are commonly encountered in clinical practice are constantly changing. The laboratory testing guidelines were revised and expanded in 2011 and more recently in 2018.[1,42] In particular, the broth microdilution method of diluting antimicrobial agents in serial twofold dilutions indexed to base 2 (e.g., 1, 2, 4, 8, and 16 μg/mL) was recommended as the gold standard for DST of NTM isolates.[1,42] Conversely, MIC breakpoints have been updated for some NTM species on the basis of clinical data, the clinical experience of experts in mycobacteriology, the comparative breakpoints used for other organisms, population distribution data, and the importance of gene sequencing in detecting drug resistance (Table 1).[1,42]

The CLSI has emphasized the need to ensure that the clinical significance of NTM isolates is related to the pathogenicity of host factors, clinical presentations, and the NTM isolate.[1,42] Experts generally agree that DST should be performed only for NTM isolates with clinical significance. Additionally, DST should be repeated in patients who have persistent positive culture following 3 months of adequate antimicrobial therapy.[1,43] Table 2 provides comments on assessing the clinical significance of NTM isolates.[1,44]

Broth microdilution

Several DST methods for mycobacteria have been developed, including the absolute concentration method, resistance ratio method, proportion method, disk diffusion/disk elution, broth macrodilution, broth microdilution, the Epsilon test, and the rapid analysis of mycolic acids.[32] This article only introduces broth microdilution because it is currently recommended by the CLSI as the standard method for the DST of NTM.[1,42]

In broth microdilation, the MIC is measured by inoculating small volumes (usually 100 μL) of cation-adjusted Mueller Hinton broth with a standardized inoculum of 5×10^5 CFU in a 96-well plate format. The growth density is detected optically and compared with growth in drug-free control vials to determine the MIC. Because the MIC is determined for twofold dilution, the MIC measured using this method is not an absolute value. Instead, the true MIC is between the two resultant concentrations. For example, if the MIC is reported as 16 μg/mL, then the true MIC is between the reported 16 μg/mL and the next lowest concentration of 8 μg/mL.

DST for SGM

(1) *M. avium* complex

M. avium complex (MAC) is primarily composed of two species, *M. avium* and *M. intracellulare*, although at least nine other species are candidates for inclusion.[2,45,46] Except for *M. avium*,*M. intracellulare*, and *M. chimaera*,

Table 3 Summary of the associations between *in vitro* susceptibility and clinical outcome in patients with NTM infection.

	Antimicrobials with *in vitro* resistance	Treatment outcome
M. avium complex infection	Clarithrimycin and amikacin	Unfavorable
M. kansasii infection	Rifampicin	
M. abscessus lung disease	Clarithromycin	
Extrapulmonary *M. abscessus* infection	Cefoxitin, amikacin, and cotrimoxazole	

the species of MAC have not been identified as respiratory pathogens.[46]

Treatment regimens for MAC primarily include macrolides, rifamycin (e.g., rifampicin or rifabutin), and ethambutol with or without amikacin depending on disease severity and the existence of clarithromycin resistance.[43,47] However, no clinical correlations with *in vitro* MICs have been discovered except for clarithromycin and amikacin (azithromycin is not a candidate because high concentrations cannot be achieved when testing) (Table 3).[1,48–51] Other antibiotics, such as moxifloxacin and linezolid, are increasingly used in the treatment of MAC and may also be tested until studies find no correlation of *in vitro* MICs with treatment outcomes.[1,42] However, moxifloxacin and linezolid exhibit limited activity and should not be recommended as substitutes for first-line antimicrobials for the initial treatment of MAC. Table 1 presents the CLSI-recommended MIC breakpoints for these relevant agents in the treatment of MAC.

Generally, untreated MAC strains are susceptible to macrolides, and the *in vitro* MICs of clarithromycin can also apply to azithromycin.[52,53] In MAC, macrolide resistance is defined as clarithromycin MIC $\geq$32 μg/mL, whereas intermediate macrolide resistance as defined as clarithromycin MIC = 16 μg/mL, which suggests impending macrolide resistance or a mixed population of MAC isolates.[1] Therefore, for patients with clarithromycin MIC = 16 μg/mL, repeating the culture and DST and carefully monitoring the possibility of emerging macrolide resistance as well as the patient's treatment response should be considered. Moreover, the detection of clarithromycin resistance mutations in the 23S *rRNA* gene at adenine position 2058 or 2059 can be used as confirmation when encountering clarithromycin MIC $\geq$32 μg/mL in patients with MAC infection.[53–56]

The breakpoints of MAC to amikacin were revised in the CLSI 2018 recommendations.[1] Separate MIC values for intravenous and inhaled liposomal amikacin have been proposed (Table 1). Studies have reported that the typical acquired resistance to amikacin namely 16S *rRNA* gene A1408G mutation did not present in strains treated with inhaled liposomal amikacin with MICs $\leq$64 μg/mL but did present in isolates with MICs >64 μg/mL. The treatment response was also unfavorable in patients with isolates with amikacin MICs >64 μg/mL. Thus, the resistance breakpoint of inhaled liposomal amikacin should be > 64 μg/mL for MAC.[57,58] In other words, the MIC cutoff value of MAC for resistance to inhaled liposomal amikacin should be $\geq$ 128 μg/mL (Table 1). However, patients who do not respond to intravenous amikacin as well as having the amikacin mutation in the 16S *rRNA* gene had amikacin MICs = 64 μg/mL.[58] That is, the MIC cutoff value of MAC for resistance to intravenous amikacin is $\geq$ 64 μg/mL (Table 1).

(2) *M. kansasii*

The standard antimicrobial treatment regimen involves rifampicin, ethambutol, and isoniazid, although the current CLSI recommendations only recommend DST of *M. kansasii* as a test for susceptibility to rifampicin and clarithromycin because treatment failure is generally related to rifampicin resistance(Tables 1 and 3).[1,42,43,47,59] Because isolates susceptible to rifampicin are cross-susceptible to rifabutin, no additional MIC testing is necessary.[1] For initial *M. kansasii* isolates susceptible to rifampicin, DST is not necessary for other agents. However, if the isolate is discovered to be resistant to rifampicin, the following second-line antimicrobials should be tested: amikacin, ciprofloxacin, moxifloxacin, linezolid, rifabutin, doxycycline/minocycline, and trimethoprim sulfamethoxazole (Table 1).[1,42]

(3) *M. marinum*

M. marinum has an intermediate growth rate but is classified as at type of SGM. This is because the same DST as that for SGM is performed except that a lower temperature is used because it is optimal for the growth of *M. marinum*. Incubation lasting 7 days at 30 °C is recommended for the DST of *M. marinum*. By contrast, MAC isolates should be incubated for 7–14 days at 35°C-37 °C.[41,60]

Antimicrobials used to treat *M. marinum* infections include doxycycline, minocycline, trimethoprim/sulfamethoxazole, rifampicin plus ethambutol, and clarithromycin.[43] The recommended treatment regimen should comprise at least two active agents.[43] In a previous version of its recommendations, the CLSI did not recommend routine DST for *M. marinum* because of its susceptibility to all drugs[42] However, increasing evidence has indicated that some isolates are resistant to doxycycline/minocycline and ciprofloxacin. Thus, the CLSI recommends that the same methodology (broth microdilution) and antimicrobials as used for rifampicin-resistant *M. kansasii* be employed for *M. marinum*, especially when the patient with *M. marinum* disease does not respond favorably to initial treatment (Table 1).[1]

If the treatment response is poor and the patient is not culture-negative after 3 months of appropriate therapy, DST should be repeated, and the treatment regimen should be adjusted based on the DST reports.[1,43]

(4) Miscellaneous SGM

The CLSI recommendations are best applied to three SGM species—MAC, *M. kansasii*, and *M. marinum*—but DST should be considered if SGM isolates have clinical significance (Table 2).[1] The details of DST for SGM isolates other than MAC and *M. kansasii* refer to those for *M. marinum* in the CLSI recommendations (Table 1).

Antimycobacterial susceptibility testing of RGM

Of the many RGM groups and complexes responsible for human infections, *M. abscessus* complex (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*) is the most common and critical.[61] The antimicrobials and their MIC breakpoints recommended by the CLSI for RGM are listed in Table 1. Among these antibiotics, tigercycline and clofazimine may be tested, although breakpoints have not been established for RGM because of insufficient data. Thus, if requested, an MIC should be reported.[1] Furthermore, the identification of RGM at no higher than the species level, or the subspecies

level in the case of *M. abscessus* complex, is strongly recommended because the DST results for some antimicrobials may only apply to specific species.[1]

Incubation times for adequate RGM growth in broth vary from 2 to 5 days. However, isolates of *M. abscessus* complex, especially those from patients treated with multiple antibiotics over time, often grow slowly in broth, and incubation beyond 5 days should thus be considered. The other exception to ≤5 days of incubation is when detecting inducible macrolide resistance in RGM.[26,29] Extending the incubation of clarithromycin to 14 days is necessary to detect inducible macrolide resistance—unless MIC ≥16 μg/mL at an earlier time point is discovered—and the final result should be reported. However, the test should be repeated if clarithromycin MIC = 4 or 8 μg/mL; additionally, the isolate should be sent to an experienced laboratory for confirmation.[1,32]

Detection of clarithromycin resistance is essential in patients with *M. abscessus* lung disease, as indicated by a case series study that revealed that resistance to clarithromycin was independently associated with treatment failure (Table 3).[62] One mechanism of macrolide resistance, called acquired macrolide resistance, is associated with mutation of the 23S *rRNA* gene; this mutation mostly occurs because of a single base-pair mutation at adenine 2058 or 2059. This type of resistance is usually detected in the initial MIC reading (3–5 days).[1] By contrast, in approximately 80% of *M. abscessus* subsp. *abscessus* cases, the *erm*(41) gene is acquired and used to predict inducible macrolide resistance, although approximately 20% of the isolates have a nonfunctional *erm*(41) gene (also designated as a hybrid type or known as having sequevar II) and are still susceptible to clarithromycin after 14 days incubation.[32] Table 4 presents a summary of clarithromycin susceptibility based on *erm* gene types.

Table 4 Presence of inducible macrolide resistance based on *erm* gene types in rapidly growing mycobacteria.

	Functional	Nonfunctional
Clarithromycin MIC reports beyond 5-day incubation	Resistant	Susceptible
Species or subspecies/ gene types	*M. goodie*/*erm(38)* *M. smegmatis*/*erm*(38) *M. fortuitum*/*erm*(39) *M. houstonense*/*erm*(39) *M. porcinum*/*erm*(39) *M. neworleansense*/*erm*(39) *M. mageritense*/*erm*(40) *M. wolinskyi*/*erm*(40) *M. abscessus*subsp. *abscessus*/*erm*(41) *M. abscessus*subsp. *bolletii*/*erm*(41)	*M. abscessus* subsp. *massiliense* *M. chelonae* *M. immunogenum* *M. mucogenicum* group *M. peregrinum* *M. senegalense*

Abbreviations: see Table 1.

The detection of amikacin resistance is also important in the selection of a treatment regimen for RGM, because *in vitro* resistance to cefoxitin, amikacin, and cotrimoxazole is related to treatment failure in patients with extrapulmonary *M. abscessus* disease.[63] Amikacin resistance is related to a single substitution (A to G) at position 1408 in the 16S *rRNA* gene for the *M. abscessus* complex and *M. chelonae* complex.[57]

New antimicrobials for treatment of NTM

Because treatment outcomes have been unfavorable in cases where the currently recommended regimens have been used, additional antimicrobial agents—including clofazimine, bedaquiline, delamanid, and tedizolid—have been explored for their *in vitro* activity against NTM, although these agents have not yet been addressed by the CLSI.[1] One study demonstrated that *M. intracellulare* and *M. avium* were susceptible to clofazimine in 97.3% and 12.5% of cases, respectively, when the MICs were tentatively grouped into the three categories of susceptible, intermediate, and resistant, defined by values ≤ 1, 2, and ≥4 μg/mL, respectively.[40,64] In another study, approximately 90% of MAC isolates had bedaquiline MICs ≤0.008 μg/mL.[65] Furthermore, MAC strains, including *M. intracellulare* and *M. avium*, exhibited low MICs against delamanid, ranging from 0.013 to 0.4 μg/mL.[66] Compared with linezolid, tedizolid exhibited equivalent or lower MIC_{50} and/or MIC_{90} values against *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, *M. fortuitum*, *M. chelonae*, *M. marinum*, *M. kansasii*, MAC, and other less common species.[67] Taken together, these antibiotics are potential treatment options for NTM.

Role of *in vitro* synergy between current antimicrobials

Synergistic activity mostly occurs because the increased cell wall permeability induced by one drug causes an increased uptake for the second drug.[68] Evidence of *in vitro* synergy between the various antibiotics used to treat NTM diseases has been obtained, although the CLSI only recommends the DST of single agents.[1] Several drugs—including clarithromycin, clofazimine, and ethambutol—have been widely investigated for their synergistic effects.

Clarithromycin was discovered to have synergistic activity with rifabutin, ethambutol, and clofazimine against MAC.[69]As for RGM, tigercycline and clarithromycin had synergistic activity against 92.9%, 68.8%, 100%, 35.7%, and 46.2% of *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, *M. abscessus* subsp. *bolletii*, *M. fortuitum*, and *M. chelonae* isolates, respectively.[70]

When combined with amikacin, clofazimine had a synergistic effect against 100%, 100%, and 48% of *M. abscessus*, *M. fortuitum*, and *M. chelonae* isolates, respectively.[71] Moreover, clofazimine and isepamicin had *in vitro* synergistic activity against 100% of *M. intracellulare* isolates but only against 50% of *M. avium* isolates.[40]

In vitro synergy against MAC has been discovered between ethambutol and several antimycobacterial agents, including rifampicin, streptomycin, quinolones, and macrolides.[69,72–77] The best known combination is that of ethambutol with rifampicin.

Although synergy appears to be clinically valuable against individual NTM strains, the clinical implications of *in vitro* synergy remain unclear.

Conclusions and future perspectives

The identification of NTM species is not straightforward. New molecular-based methods enable superior discrimination between NTM, have shorter turn-around times, and will gradually replace traditional methods of NTM identification. In particular, the gene sequencing approach may become crucial. Moreover, the selection of appropriate antimicrobial treatment regimens for NTM diseases is imperative. The true role of DST for NTM remains to be uncovered, preferably by clinical trials.

Declaration of Competing Interest

The authors have no conflicts of interest relevant to this article.

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References

1. Clinical and Laboratory Standards Institute. *Susceptibility testing of mycobacteria, Nocardia spp., and other aerobic actinomycetes, 3rd ed, CLSI standard document M24:*. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2018.
2. Tortoli E. Microbiological features and clinical relevance of new species of the genus Mycobacterium. *Clin Microbiol Rev* 2014;**27**:727–52.
3. Tortoli E. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev* 2003;**16**:319–54.
4. Turenne CY, Tschetter L, Wolfe J, Kabani A. Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous Mycobacterium species. *J Clin Microbiol* 2001;**39**:3637–48.
5. Lee MR, Cheng A, Huang YT, Liu CY, Chung KP, Wang HC, et al. Performance assessment of the DR. TBDR/NTM IVD kit for direct detection of Mycobacterium tuberculosis isolates, including rifampin-resistant isolates, and nontuberculous Mycobacteria. *J Clin Microbiol* 2012;**50**:3398–401.
6. Tortoli E, Pecorari M, Fabio G, Messinò M, Fabio A. Commercial DNA probes for mycobacteria incorrectly identify a number of less frequently encountered species. *J Clin Microbiol* 2010;**48**: 307–10.
7. Cook VJ, Turenne CY, Wolfe J, Pauls R, Kabani A. Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. *J Clin Microbiol* 2003;**41**:1010–5.
8. Reisner BS, Gatson AM, Woods GL. Use of Gen-Probe AccuProbes to identify Mycobacterium avium complex, Mycobacterium tuberculosis complex, Mycobacterium kansasii, and Mycobacterium gordonae directly from BACTEC TB broth cultures. *J Clin Microbiol* 1994;**32**:2995–8.
9. Lebrun L, Espinasse F, Poveda JD, Vincent-Levy-Frebault V. Evaluation of nonradioactive DNA probes for identification of mycobacteria. *J Clin Microbiol* 1992;**30**:2476–8.
10. Goto M, Oka S, Okuzumi K, Kimura S, Shimada K. Evaluation of acridinium-ester-labeled DNA probes for identification of Mycobacterium tuberculosis and Mycobacterium avium-Mycobacterium intracellulare complex in culture. *J Clin Microbiol* 1991;**29**:2473–6.
11. Simner PJ, Stenger S, Richter E, Brown-Elliott BA, Wallace Jr RJ, Wengenack NL. Mycobacterium: clinical and laboratory characteristics of slowly growing mycobacteria. In: *Manual of clinical microbiology, vol. 1. 11th ed*. . Washington, DC: ASM Press; 2015.
12. Kim BJ, Hong SK, Lee KH, Yun YJ, Kim EC, Park YG, et al. Differential identification of Mycobacterium tuberculosis complex and nontuberculous mycobacteria by duplex PCR assay using the RNA polymerase gene (rpoB). *J Clin Microbiol* 2004;**42**:1308–12.
13. Huang CC, Chen JH, Hu ST, Chiou CS, Huang WC, Hsu JY, et al. Combined rpoB duplex PCR and hsp65 PCR restriction fragment length polymorphism with capillary electrophoresis as an effective algorithm for identification of mycobacterial species from clinical isolates. *BMC Microbiol* 2012;**12**:137.
14. Telenti A, Marchesi F, Balz M, Bally F, Böttger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993;**31**:175–8.
15. Shen GH, Hung CH, Hu ST, Wu BD, Lin CF, Chen CH, et al. Combining polymerase chain reaction restriction enzyme analysis with phenotypic characters for mycobacteria identification in Taiwan. *Int J Tubercul Lung Dis* 2009;**13**: 472–9.
16. Macheras E, Roux A-L, Bastian S, Leão SC, Palaci M, Sivadon-Tardy V, et al. Multilocus sequence analysis and rpoB sequencing of Mycobacterium abscessus (sensu lato) strains. *J Clin Microbiol* 2011;**49**:491–9.
17. Brown BA, Springer B, Steingrube VA, Wilson RW, Pfyffer GE, Garcia MJ, et al. Mycobacterium wolinskyi sp. nov. and Mycobacterium goodii sp. nov., two new rapidly growing species related to Mycobacterium smegmatis and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy. *Int J Syst Bacteriol* 1999;**49**(Pt 4):1493–511.
18. Tortoli E. Phylogeny of the genus Mycobacterium: many doubts, few certainties. *Infect Genet Evol* 2012;**12**:827–31.
19. Adékambi T, Colson P, Drancourt M. rpoB-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. *J Clin Microbiol* 2003;**41**:5699–708.
20. de Zwaan R, van Ingen J, van Soolingen D. Utility of rpoB gene sequencing for identification of nontuberculous mycobacteria in The Netherlands. *J Clin Microbiol* 2014;**52**:2544–51.
21. Kim BJ, Lee SH, Lyu MA, Kim SJ, Bai GH, Chae GT, et al. Identification of mycobacterial species by comparative

sequence analysis of the RNA polymerase gene (rpoB). *J Clin Microbiol* 1999;**37**:1714—20.
22. Lee H, Bang H-E, Bai G-H, Cho S-N. Novel polymorphic region of the rpoB gene containing Mycobacterium species-specific sequences and its use in identification of mycobacteria. *J Clin Microbiol* 2003;**41**:2213—8.
23. Clinical and Laboratory Standards Institute. *Interpretive criteria for identification of bacteria and fungi by DNA target sequencing: approved guideline, 2 nd ed, CLSI document MM18*. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2018.
24. Turenne CY, Semret M, Cousins DV, Collins DM, Behr MA. Sequencing of hsp65 distinguishes among subsets of the Mycobacterium avium complex. *J Clin Microbiol* 2006;**44**: 433—40.
25. Dai J, Chen Y, Lauzardo M. Web-accessible database of hsp65 sequences from Mycobacterium reference strains. *J Clin Microbiol* 2011;**49**:2296—303.
26. Nash KA, Brown-Elliott BA, Wallace Jr RJ. A novel gene, erm(41), confers inducible macrolide resistance to clinical isolates of Mycobacterium abscessus but is absent from Mycobacterium chelonae. *Antimicrob Agents Chemother* 2009;**53**: 1367—76.
27. Kim HY, Kim BJ, Kook Y, Yun YJ, Shin JH, Kim BJ, et al. Mycobacterium massiliense is differentiated from Mycobacterium abscessus and Mycobacterium bolletii by erythromycin ribosome methyltransferase gene (erm) and clarithromycin susceptibility patterns. *Microbiol Immunol* 2010;**54**:347—53.
28. Koh WJ, Jeon K, Lee NY, Kim BJ, Kook YH, Lee SH, et al. Clinical significance of differentiation of Mycobacterium massiliense from Mycobacterium abscessus. *Am J Respir Crit Care Med* 2011;**183**:405—10.
29. Nash KA, Andini N, Zhang Y, Brown-Elliott BA, Wallace Jr RJ. Intrinsic macrolide resistance in rapidly growing mycobacteria. *Antimicrob Agents Chemother* 2006;**50**:3476—8.
30. Nash KA. Intrinsic macrolide resistance in Mycobacterium smegmatis is conferred by a novel erm gene, erm(38). *Antimicrob Agents Chemother* 2003;**47**:3053—60.
31. Nash DR, Wallace Jr RJ, Steingrube VA, Udou T, Steele LC, Forrester GD. Characterization of beta-lactamases in Mycobacterium fortuitum including a role in beta-lactam resistance and evidence of partial inducibility. *Am Rev Respir Dis* 1986; **134**:1276—82.
32. Brown-Elliott BA, Vasireddy S, Vasireddy R, Iakhiaeva E, Howard ST, Nash K, et al. Utility of sequencing the erm(41) gene in isolates of Mycobacterium abscessus subsp. abscessus with low and intermediate clarithromycin MICs. *J Clin Microbiol* 2015;**53**:1211—5.
33. Buckwalter SP, Olson SL, Connelly BJ, Lucas BC, Rodning AA, Walchak RC, et al. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of Mycobacterium species, Nocardia species, and other aerobic actinomycetes. *J Clin Microbiol* 2016;**54**:376—84.
34. Saleeb PG, Drake SK, Murray PR, Zelazny AM. Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 2011;**49**:1790—4.
35. Rodríguez-Sánchez B, Ruiz-Serrano MJ, Ruiz A, Timke M, Kostrzewa M, Bouza E. Evaluation of MALDI biotyper mycobacteria library v3.0 for identification of nontuberculous mycobacteria. *J Clin Microbiol* 2016;**54**:1144—7.
36. Lotz A, Ferroni A, Beretti J-L, Dauphin B, Carbonnelle E, Guet-Revillet H, et al. Rapid identification of mycobacterial whole cells in solid and liquid culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 2010;**48**:4481—6.
37. Hettick JM, Kashon ML, Slaven JE, Ma Y, Simpson JP, Siegel PD, et al. Discrimination of intact mycobacteria at the strain level: a combined MALDI-TOF MS and biostatistical analysis. *Proteomics* 2006;**6**:6416—25.
38. Lefmann M, Honisch C, Böcker S, Storm N, von Wintzingerode F, Schlötelburg C, et al. Novel mass spectrometry-based tool for genotypic identification of mycobacteria. *J Clin Microbiol* 2004;**42**:339—46.
39. Tan N, Sampath R, Abu Saleh OM, Tweet MS, Jevremovic D, Alniemi S, et al. Disseminated Mycobacterium chimaera infection after cardiothoracic surgery. *Open Forum Infect Dis* 2016;**3**:1—3.
40. Huang CC, Wu MF, Chen HC, Huang WC. In vitro activity of aminoglycosides, clofazimine, d-cycloserine and dapsone against 83 Mycobacterium avium complex clinical isolates. *J Microbiol Immunol Infect* 2018;**51**:636—43.
41. Griffith DE. *Nontuberculous mycobacterial disease. A comprehensive approach to diagnosis and management*. Springer; 2018.
42. Clinical and Laboratory Standards Institute. *Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes: approved standard, 2nd ed, CLSI document M24-A2*. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2011.
43. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* 2007;**175**:367—416.
44. Brown-Elliott BA, Wallace Jr RJ. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin Microbiol Rev* 2002;**15**:716—46.
45. Turenne CY, Wallace Jr R, Behr MA. Mycobacterium avium in the postgenomic era. *Clin Microbiol Rev* 2007;**20**:205—29.
46. van Ingen J, Turenne CY, Tortoli E, Wallace Jr RJ, Brown-Elliott BA. A definition of the Mycobacterium avium complex for taxonomical and clinical purposes, a review. *Int J Syst Evol Microbiol* 2018;**68**:3666—77.
47. Haworth CS, Banks J, Capstick T, Fisher AJ, Gorsuch T, Laurenson IF, et al. British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). *Thorax* 2017;**72**:ii1.
48. Brown-Elliott BA, Iakhiaeva E, Griffith DE, Woods GL, Stout JE, Wolfe CR, et al. In vitro activity of amikacin against isolates of Mycobacterium avium complex with proposed MIC breakpoints and finding of a 16S rRNA gene mutation in treated isolates. *J Clin Microbiol* 2013;**51**:3389—94.
49. Babady NE, Hall L, Abbenyi AT, Eisberner JJ, Brown-Elliott BA, Pratt CJ, et al. Evaluation of Mycobacterium avium complex clarithromycin susceptibility testing using SLOMYCO Sensititre panels and JustOne strips. *J Clin Microbiol* 2010;**48**:1749—52.
50. Brown BA, Wallace Jr RJ, Onyi GO. Activities of clarithromycin against eight slowly growing species of nontuberculous mycobacteria, determined by using a broth microdilution MIC system. *Antimicrob Agents Chemother* 1992;**36**:1987—90.
51. Eisenberg E, Barza M. Azithromycin and clarithromycin. *Curr Clin Top Infect Dis* 1994;**14**:52—79.
52. Meier A, Heifets L, Wallace Jr RJ, Zhang Y, Brown BA, Sander P, et al. Molecular mechanisms of clarithromycin resistance in Mycobacterium avium: observation of multiple 23S rDNA mutations in a clonal population. *J Infect Dis* 1996;**174**:354—60.
53. Meier A, Kirschner P, Springer B, Steingrube VA, Brown BA, Wallace Jr RJ, et al. Identification of mutations in 23S rRNA gene of clarithromycin-resistant Mycobacterium intracellulare. *Antimicrob Agents Chemother* 1994;**38**:381—4.
54. Brown-Elliott BA, Nash KA, Wallace Jr RJ. Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. *Clin Microbiol Rev* 2012;**25**:545—82.
55. Nash KA, Inderlied CB. Genetic basis of macrolide resistance in Mycobacterium avium isolated from patients with

disseminated disease. *Antimicrob Agents Chemother* 1995;**39**:2625–30.
56. Nash KA, Inderlied CB. Rapid detection of mutations associated with macrolide resistance in Mycobacterium avium complex. *Antimicrob Agents Chemother* 1996;**40**:1748–50.
57. Prammananan T, Sander P, Brown BA, Frischkorn K, Onyi GO, Zhang Y, et al. A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in Mycobacterium abscessus and Mycobacterium chelonae. *J Infect Dis* 1998;**177**:1573–81.
58. Griffith DE, Eagle G, Thomson R, Aksamit TR, Hasegawa N, Morimoto K, et al. Amikacin liposome inhalation suspension for treatment-refractory lung disease caused by Mycobacterium avium complex (CONVERT). A prospective, open-label, randomized study. *Am J Respir Crit Care Med* 2018;**198**:1559–69.
59. Ahn CH, Wallace Jr RJ, Steele LC, Murphy DT. Sulfonamide-containing regimens for disease caused by rifampin-resistant Mycobacterium kansasii. *Am Rev Respir Dis* 1987;**135**:10–6.
60. Jernigan JA, Farr BM. Incubation period and sources of exposure for cutaneous Mycobacterium marinum infection: case report and review of the literature. *Clin Infect Dis* 2000;**31**:439–43.
61. Brown-Elliott BAPJ. *Rapidly growing mycobacteria*. Tuberculosis and nontuberculous mycobacterial infections; 2017. p. 703–23.
62. Jeon K, Kwon OJ, Lee NY, Kim BJ, Kook YH, Lee SH, et al. Antibiotic treatment of Mycobacterium abscessus lung disease: a retrospective analysis of 65 patients. *Am J Respir Crit Care Med* 2009;**180**:896–902.
63. Wallace Jr RJ, Swenson JM, Silcox VA, Bullen MG. Treatment of nonpulmonary infections due to Mycobacterium fortuitum and Mycobacterium chelonei on the basis of in vitro susceptibilities. *J Infect Dis* 1985;**152**:500–14.
64. Abate G, Miorner H, Ahmed O, Hoffner SE. Drug resistance in Mycobacterium tuberculosis strains isolated from re-treatment cases of pulmonary tuberculosis in Ethiopia: susceptibility to first-line and alternative drugs. *Int J Tubercul Lung Dis* 1998;**2**:580–4.
65. Brown-Elliott BA, Philley JV, Griffith DE, Thakkar F, Wallace Jr RJ. In vitro susceptibility testing of bedaquiline against Mycobacterium avium complex. *Antimicrob Agents Chemother* 2017;**61**. e01798-16.
66. Krieger D, Schonfeld N, Vesenbeckh S, Bettermann G, Bauer TT, Russmann H, et al. Is delamanid a potential agent in the treatment of diseases caused by Mycobacterium avium-intracellulare? *Eur Respir J* 2016;**48**:1803–4.
67. Brown-Elliott BA, Wallace Jr RJ. In vitro susceptibility testing of tedizolid against nontuberculous mycobacteria. *J Clin Microbiol* 2017;**55**:1747–54.
68. van Ingen J, Boeree MJ, van Soolingen D, Mouton JW. Resistance mechanisms and drug susceptibility testing of nontuberculous mycobacteria. *Drug Resist Updates* 2012;**15**:149–61.
69. Piersimoni C, Tortoli E, Mascellino MT, Passerini Tosi C, Sbaraglia G, Mandler F, et al. Activity of seven antimicrobial agents, alone and in combination, against AIDS-associated isolates of Mycobacterium avium complex. *J Antimicrob Chemother* 1995;**36**:497–502.
70. Huang CW, Chen JH, Hu ST, Huang WC, Lee YC, Huang CC, et al. Synergistic activities of tigecycline with clarithromycin or amikacin against rapidly growing mycobacteria in Taiwan. *Int J Antimicrob Agents* 2013;**41**:218–23.
71. Shen GH, Wu BD, Hu ST, Lin CF, Wu KM, Chen JH. High efficacy of clofazimine and its synergistic effect with amikacin against rapidly growing mycobacteria. *Int J Antimicrob Agents* 2010;**35**:400–4.
72. Banks J, Jenkins PA. Combined versus single antituberculosis drugs on the in vitro sensitivity patterns of non-tuberculous mycobacteria. *Thorax* 1987;**42**:838–42.
73. Heifets LB. Synergistic effect of rifampin, streptomycin, ethionamide, and ethambutol on Mycobacterium intracellulare. *Am Rev Respir Dis* 1982;**125**:43–8.
74. Hoffner SE, Svenson SB, Kallenius G. Synergistic effects of antimycobacterial drug combinations on Mycobacterium avium complex determined radiometrically in liquid medium. *Eur J Clin Microbiol* 1987;**6**:530–5.
75. Hoffner SE, Kratz M, Olsson-Liljequist B, Svenson SB, Kallenius G. In-vitro synergistic activity between ethambutol and fluorinated quinolones against Mycobacterium avium complex. *J Antimicrob Chemother* 1989;**24**:317–24.
76. Hoffner SE, Svenson SB, Beezer AE. Microcalorimetric studies of the initial interaction between antimycobacterial drugs and Mycobacterium avium. *J Antimicrob Chemother* 1990;**25**:353–9.
77. Yajko DM, Sanders CA, Madej JJ, Cawthon VL, Hadley WK. In vitro activities of rifabutin, azithromycin, ciprofloxacin, clarithromycin, clofazimine, ethambutol, and amikacin in combinations of two, three, and four drugs against Mycobacterium avium. *Antimicrob Agents Chemother* 1996;**40**:743–9.