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Antimicrobial susceptibility and MIC distribution of 41 drugs against clinical isolates from China and reference strains of nontuberculous mycobacteria

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ABSTRACT

To treat nontuberculous mycobacteria (NTM) infections more optimally, further research pertaining to mycobacterial susceptibility to antimicrobial agents is required. A total of 82 species of NTM reference strains and 23 species of NTM clinical isolates were included. Minimum inhibitory concentrations (MICs) for 41 drugs were determined using the microdilution method in cation-adjusted Mueller–Hinton broth. The results showed that most of the NTM were susceptible to aminoglycosides, quinolones, three macrolides (clarithromycin, azithromycin and roxithromycin), cefmetazole, linezolid and capreomycin. Rapidly growing mycobacterium strains were additionally susceptible to cefoxitin, clofazimine, rifapentine, doxycycline, minocycline, tigecycline, meropenem and sulfamethoxazole, whereas slowly growing mycobacterium strains were additionally susceptible to rifabutin. This study on the susceptibility of NTM includes the largest sample size of Chinese clinical isolates and reference strains. NTM species-specific drug susceptibility patterns suggested that it is urgent to identify the species of NTM, to normalise the treatment of NTM infectious disease and to clarify the resistance mechanisms of NTM.

1. Introduction

Nontuberculous mycobacteria (NTM) are a group of environmental mycobacteria that are found predominantly in water, food, soil and dust [1,2]. More than 160 species of NTM have been identified [3]. They are considered as opportunistic pathogens [4] and at least 42 species are associated with NTM infections [5], including pulmonary and extrapulmonary infections. These infection types include skin and soft-tissue infections and lymphatic, disseminated and nosocomial infections that occur post-surgery [6–8]. The incidence and prevalence of NTM diseases have increased globally. Possible reasons for the increase include increased exposure to the infectious agent along with increased risk factors. Improved diagnostic methods are also likely to have facilitated improved detection rates [9].

Tests associated with antituberculosis drug resistance of *Mycobacterium tuberculosis* have been conducted extensively in China [10,11]. However, far fewer studies have been conducted pertaining to the drug susceptibility of NTM. In addition, the sample sizes associated with these studies are relatively small (<100) [12–15]. NTM drug susceptibility tests are predominantly based on the Lowenstein–Jensen (LJ) plate method, which is recommended by the World Health Organization (WHO). The Clinical and Laboratory Standards Institute (CLSI) recommends using broth-based microdilution for drug susceptibility test pertaining to NTM [16]. In this study, the broth-based microdilution method was used to test the drug susceptibility of 397 NTM clinical isolates and 82 NTM reference strains in relation to 41 different drugs. The 397 clinical isolates belong to 23 species and were collected from eight Chinese provinces between 2005 and 2012. The aim of this study was to provide information that will facilitate greater efficacy in the treatment of NTM diseases.

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2. Materials and methods

2.1. Nontuberculous mycobacteria strains

Between 2005 and 2012, more than 3500 mycobacterial isolates were collected from hospitals involved in tuberculosis (TB) treatment in eight Chinese provinces, including Anhui, Fujian, Hunan, Gansu, Jiangxi, Sichuan, Xinjiang and Inner Mongolia. A total of 397 isolates were identified as NTM. Species-level identification was conducted using two steps. First, selective culture medium containing either *p*-nitrobenzoic acid (500 μg/mL) or thiophen-2-carboxylic acid hydrazide (5 μg/mL) was used to test for the presence of *M. tuberculosis*. Second, multilocus PCR was performed to determine the NTM species [17]. In addition, sequencing of the genes *rpoB*, *hsp65*, *ITS*, *gnd*, *glpK*, *secA* and *sodA* was performed to further identify the NTM to species level.

The reference strains that were used as part of this analysis included 41 rapidly growing mycobacterium (RGM) and 41 slowly growing mycobacterium (SGM) obtained either from the American Type Culture Collection (ATCC) or the German Collection of Microorganisms (DSM).

2.2. Antibiotics and culture media

The 41 antibiotics used in this study are listed in Table S1. The antibiotics were purchased from Sigma-Aldrich Co. (St Louis, MO), apart from gatifloxacin and rifabutin that were from Toronto Research Chemicals Inc. (Toronto, Canada) and that was from thioacetazone J&K Chemical Ltd. (Shanghai, China). Middlebrook 7H10 broth, cation-adjusted Mueller–Hinton broth (CA-MHB) and albumin–dextrose–catalase (ADC) supplement were purchased from Difco (Detroit, MI). AlamarBlue® was purchased from AbD Serotec (Oxford, UK).

2.3. Minimum inhibitory concentration (MIC) determination

MIC testing was performed using the CA-MHB-based microdilution method according to the approved CLSI guidelines [16]. Inocula were prepared from actively growing bacteria collected from LJ slants or Middlebrook 7H10 agar plates. Inocula were adjusted with saline to a cell density of 1.5×10^8 cells/mL (0.5 McFarland standard) and were then diluted (1:200) using CA-MHB for RGM or CA-MHB + 5% ADC supplement (CA-MHB-S) for SGM. Antibiotics were serially diluted two-fold in 100 μL of CA-MHB or CA-MHB-S. The final reaction volume was 200 μL (100 μL of the antibiotic solution and 100 μL of the bacterial suspension). Three negative controls were used as part of this study: medium without antibiotics was used to determine the addition time for alamarBlue; Medium without inoculum was used to determine the interference to alamarBlue by the medium; and a series of concentration gradients for each drug were used to determine the interference to alamarBlue by the drug–medium mixture colour. The plates were sealed in individual Ziploc bags and were incubated at 37 °C.

After 24 h for RGM or 6 days for SGM, the first drug-free growth control wells were examined using an indicator (20 μL of alamarBlue and 50 μL of sterile 5% Tween-80). The plates were then re-incubated for 24 h. If the control well changed to a pink colour, all of the wells containing drugs were supplemented with the indicator. After a further 24 h of incubation, the colour of all of the wells was recorded. Each MIC for the tested drug was recorded on a daily basis between the third and sixth days for RGM and between the seventh and eleventh days for SGM. The MIC was defined as the lowest drug concentration that prevented a change in colour. The final MIC result for each drug in association with the tested strains was the mean value from two or three tests. The MIC breakpoints of the drugs, which indicate sensitivity, moderate susceptibility and resistance, were interpreted according to previous references [16,18–23]. As shown in Table S1, the breakpoints for the majority of the drugs analysed are not available for most of the NTM species. Therefore, in this study the breakpoints of representative drugs of each class of antibiotics associated with the following reference species were used: *Mycobacterium avium*; *M. avium* complex (MAC); *Mycobacterium kansasii*; *Mycobacterium marinum*; RGM; *M. tuberculosis*; or *Nocardia* and other aerobic actinomycetes [16,19].

3. Results

Of the >3500 mycobacterial clinical isolates collected, 397 were identified as NTM. The 397 isolates belonged to 23 species, including 312 SGM and 85 RGM (Table 1).

The MIC results for NTM species with nine or more clinical isolates are detailed in Table 2. The MIC_{50} and MIC_{90} values (MICs at which 50% and 90% of the strain populations were susceptible, respectively) of the 41 drugs that were tested are recorded in Table S2. The susceptibility patterns for species where less than five isolates were analysed are recorded in Table 3, and the MICs of these strains are given in Table S3.

MIC testing was subsequently performed for the 82 reference strains (including 41 RGM and 41 SGM strains) against 32 drugs (Tables 4 and 5) in order to compare the susceptibility between the clinical isolates and the reference strains. Five species were included only among the NTM clinical isolates but not the reference strains, including *Mycobacterium marseillense*, *M. kansasii*, *Mycobacterium colombiense*, *Mycobacterium monacense* and *Mycobacterium phocaicum.*

3.1. Macrolide susceptibility patterns

Susceptibility to the macrolides (clarithromycin, azithromycin and roxithromycin) was common in the majority of reference strains and clinical isolates analysed. Only 8 of the 82 reference strains that were analysed (*Mycobacterium houstonense*, *Mycobacterium boenickei*, *Mycobacterium chitae*, *Mycobacterium flavescens*, *Mycobacterium goodii*, *Mycobacterium porcinum*, *Mycobacterium setense* and *Mycobacterium wolinskyi*) were resistant to the macrolides. However, five

Table 1
Classification of the nontuberculous mycobacteria (NTM) clinical isolates.

NTM species	No. of isolates
Slowly growing mycobacteria	
M. avium	97
M. intracellulare	172
M. gordonae	24
M. lentiflavum	3
M. marseillense	3
M. kansasii	2
M. szulgai	2
M. triplex	2
M. colombiense	2
M. shimoidei	2
M. setense	1
M. kumamotonense	1
M. mantenii	1
Rapidly growing mycobacteria	
M. abscessus	53
M. massiliense	9
M. chelonae	3
M. fortuitum	9
M. parascrofulaceum	3
M. neoaurum	2
M. septicum	2
M. saskatchewanense	2
M. monacense	1
M. phocaicum	1
Total	397