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Short Communication

In vitro activity of clarithromycin in combination with other antimicrobial agents against *Mycobacterium abscessus* and *Mycobacterium massiliense*

Zhijian Zhang [a,b,1], Jie Lu [c,1], Min Liu [d,1], Yufeng Wang [b], Yanlin Zhao [b,*], Yu Pang [b,**]

[a] *Respiratory Diseases Department of Nanlou, Chinese People's Liberation Army General Hospital, Beijing, China*
[b] *National Center for Tuberculosis Control and Prevention, Chinese Center for Disease Control and Prevention, No. 155 Chang Bai Road, Changping District, Beijing 102206, China*
[c] *Beijing Key Laboratory for Pediatric Diseases of Otolaryngology, Head and Neck Surgery, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, Beijing, China*
[d] *Liaoning Provincial Center for Disease Control and Prevention, Shenyang, China*

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ABSTRACT

Macrolides, especially clarithromycin (CLA), remain the cornerstone of therapy for *Mycobacterium abscessus* complex infections. The purpose of this study was to gather results from in vitro drug susceptibility testing of *M. abscessus* and *Mycobacterium massiliense* for the combination of CLA with various other agents, including linezolid (LZD), moxifloxacin (MOX), amikacin (AMK) and tigecycline (TGC). A total of 40 *M. abscessus* complex isolates were studied, comprising 20 *M. abscessus* and 20 *M. massiliense* strains. In vitro drug susceptibility testing revealed that the percentage of TGC-resistant isolates among *M. massiliense* was significantly lower than that among *M. abscessus* ($P = 0.047$). In addition, 17 (85.0%) of 20 *M. massiliense* isolates showed a synergistic effect for the CLA + MOX combination, which was significantly higher than for *M. abscessus* (1/20; 5.0%) ($P < 0.001$). Similarly, synergy for the CLA + TGC combination was found in 5 (25.0%) *M. abscessus* isolates and 13 (65.0%) *M. massiliense* isolates, with a significant difference between the two subspecies ($P = 0.038$). For CLA + LZD and CLA + AMK combinations, statistical analysis demonstrated that there was no significant difference in the proportion of synergistic effect between the two subspecies ($P > 0.05$). In conclusion, these data demonstrate that *M. abscessus* and *M. massiliense* exhibit significant differences in TGC susceptibility. In addition, the activity of CLA in combination with MOX or TGC showed better synergistic activity against *M. massiliense* than against *M. abscessus*.

1. Introduction

Rapidly growing mycobacteria are increasingly recognised as important pathogens causing a wide range of human infections [1–3]. *Mycobacterium abscessus* is the most common mycobacteria involved in pulmonary infections [3,4]. *Mycobacterium abscessus* is notorious for being one of the most resistant organisms to chemotherapeutic agents, which is responsible for unsatisfactory treatment outcomes [2,5]. Recently, this species has been subclassified into *M. abscessus*, *Mycobacterium massiliense* and *Mycobacterium bolletii* [1]. Further clinical observations reveal that these subspecies differ from each other in their drug susceptibility profiles and disease progression [1,6,7]. Therefore, subspecies differentiation within the *M. abscessus* complex will provide important indications for clinical practice.

Macrolides, especially clarithromycin (CLA), have remained the cornerstone of therapy for *M. abscessus* complex infections [3,4]. By attaching to domain V of the peptidyl transferase ring of ribosomal RNA, CLA inhibits protein synthesis in bacteria at an early stage of translation [4]. It is well documented that treatment response rates to antibiotic therapy against *M. abscessus* infection are associated with CLA resistance [4]. To improve the treatment efficacy for *M. abscessus* complex, combination of CLA with two additional agents is now recommended [8]. Notably, resistance to CLA is more frequently observed in *M. abscessus* than *M. massiliense* [9]. Hence, it is interesting to investigate whether the synergy between CLA and other antimicrobial agents shows different profiles between *M. abscessus* and *M. massiliense*, which will provide new insights for initiating an effective regimen containing CLA. The purpose of this study was to gather results from in vitro drug susceptibility testing

* Corresponding author. National Center for Tuberculosis Control and Prevention, Chinese Center for Disease Control and Prevention, No. 155 Chang Bai Road, Changping District, Beijing 102206, China. Fax: +86 10 5890 077.
E-mail address: zhaoyanlin@chinatb.org (Y. Zhao).
** Corresponding author. National Center for Tuberculosis Control and Prevention, Chinese Center for Disease Control and Prevention, No. 155 Chang Bai Road, Changping District, Beijing 102206, China. Fax: +86 10 5890 0779.
E-mail address: pangyu@chinatb.org (Y. Pang).
[1] These three authors contributed equally to this study.

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of *M. abscessus* and *M. massiliense* against the combination of CLA with various other agents, including linezolid (LZD), moxifloxacin (MOX), amikacin (AMK) and tigecycline (TGC).

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains included in this study were isolated from patients diagnosed with non-tuberculous mycobacteria lung disease between January 2011 and December 2012 from four specialised tuberculosis hospitals in China (Guangzhou Chest Hospital, Lianyungang Forth Hospital, Kaifeng Pulmonary Hospital and Affiliated Yongchuan Hospital of Chongqing Medical University) as previously described [10]. All of the strains identified as *M. abscessus* complex were further divided into subspecies by multilocus sequence typing (MLST), including 16S rRNA, 16S–23S rRNA internal transcribed spacer (ITS) sequence, *hsp65* and *rpoB*. A total of 20 *M. abscessus* and 20 *M. massiliense* isolates were randomly selected for the determination of in vitro synergistic effects between CLA and other antimicrobial agents. Prior to carrying out phenotypic drug susceptibility testing, the strains were recovered on Löwenstein–Jensen (L–J) (Baso Diagnostics Inc., Zhuhai, China) slants for 3 days at 37 °C.

2.2. Determination of minimum inhibitory concentrations (MICs) and the fractional inhibitory concentration index (FICI)

Five antimicrobial agents, including CLA, LZD, MOX, AMK and TGC, were used in this study. All antimicrobial agents were purchased from Sigma-Aldrich (St Louis. MO). MICs were determined by the broth microdilution method based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [11]. First, the MIC of each single antibiotic was tested in the panel with final drug concentrations of 0.031–128 μg/mL. Clones were harvested from the surface of L–J slants. After vigorous mixing on a vortexer for 1 min, the bacterial suspension was adjusted to match a turbidity equivalent to a 1.0 McFarland standard tube. The 1.0 McFarland suspension was further diluted 1:400 in cation-adjusted Mueller–Hinton broth (Becton Dickinson & Co., Cockeysville, MD) and was then inoculated into 96-well microtitre plates (Corning Inc., Corning, NY) containing serial doubling dilutions of the antimicrobial agents. Following incubation at 37 °C for 72 h, all plates were read manually with a Sensititre™ Vizion™ system (Thermo Fisher Scientific, Basingstoke, UK). For CLA, plates were submitted to an extended incubation period with reading after 14 days of incubation to identify inducible resistance. The MIC was defined as the lowest drug concentration capable of inhibiting visible growth of mycobacteria. The MIC_{50} and MIC_{90} were defined as the concentrations required to inhibit the growth of 50% and 90% of the strains, respectively. The reference strain *M. abscessus* ATCC 19977 was used as a control in all MIC determinations. All experiments were performed in triplicates.

After determining the MICs of single agents, each of the five drugs was tested in combination of CLA at the MIC and at four two-fold dilutions lower than the MIC as previously described. The combined FICI was calculated as [MIC of drug A tested in combination/MIC of drug A alone] + [MIC of drug B tested in combination/MIC of drug B alone]. Synergy was defined when the FICI was ≤0.5, indifference was defined as an FICI of >0.5–2 and antagonism as an FICI of >2 [12].

2.3. Statistical analysis

The χ^2 test was used to compare the percentage of drug resistance and synergy among different drug combinations, except for tables with an expected cell count <5. In the latter cases, Fisher's exact test was applied to analyse the difference. All statistical analyses were performed with SPSS v.14.0 (SPSS Inc., Chicago, IL) and differences were considered to be statistically significant at a *P*-value of <0.05.

3. Results

3.1. Minimum inhibitory concentrations

A total of 40 *M. abscessus* complex isolates were studied, consisting of 20 (50.0%) *M. abscessus* and 20 (50.0%) *M. massiliense* strains. The MIC range and the MIC_{50} and MIC_{90} values of each antimicrobial agent for *M. abscessus* and *M. massiliense* isolates are shown in Table 1.

For *M. abscessus* isolates, CLA was the most potent agent on Day 3, with MIC_{50} and MIC_{90} values of 0.13 μg/mL and 1 μg/mL, respectively. However, the MICs increased to ≥16 μg/mL by Day 14 in 15 isolates (75.0%), indicating inducible CLA resistance. In addition, LZD and AMK showed moderate activity against *M. abscessus* isolates, and 5.0% of isolates (1/20) were resistant to LZD and AMK, respectively. Comparatively, MOX and TGC exhibited less activity against *M. abscessus*, with percentages of resistant strains both for MOX and TGC of 25.0% (5/20).

Similar to *M. abscessus*, MOX resistance was also the most frequently observed resistance in *M. massiliense* isolates (20.0%; 4/20). In contrast, no *M. massiliense* isolate was resistant to LZD, AMK or TGC. Statistical analysis revealed that the percentage of TGC-resistant isolates among *M. massiliense* was significantly lower than that among *M. abscessus* ($P = 0.047$), whereas no significant difference was found for resistance to LZD, AMK or MOX ($P > 0.05$). After

Table 1
Drug susceptibility of *Mycobacterium abscessus* and *Mycobacterium massiliense* isolates included in the study.

Species	MIC (μg/mL)	CLA		MOX	LZD	AMK	TGC
		Day 3	Day 14				
M. abscessus ($n = 20$)	MIC range	0.06–2	0.06–64	0.13–4	0.5–32	0.5–64	0.12–64
	MIC_{50}	0.13	32	2	2	2	4
	MIC_{90}	1	64	4	16	32	16
	No. (%) resistant [a]	0 (0.0)	15 (75.0)	5 (25.0)	1 (5.0)	1 (5.0)	5 (25.0)
M. massiliense ($n = 20$)	MIC range	0.06–16	0.06–16	0.13–4	0.5–8	0.13–8	0.13–4
	MIC_{50}	0.25	0.25	2	2	2	0.5
	MIC_{90}	8	8	4	4	2	2
	No. (%) resistant [a]	2 (10.0)	2 (10.0)	4 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MIC required to inhibit the growth of 50% and 90% of the strains, respectively; CLA, clarithromycin; MOX, moxifloxacin; LZD, linezolid; AMK, amikacin; TGC, tigecycline.

[a] Breakpoint values are referenced from Clinical and Laboratory Standards Institute (CLSI) recommendations (8 μg/mL for CLA, 4 μg/mL for MOX, 32 μg/mL for LZD and 64 μg/mL for AMK) and from a previous report (8 μg/mL for TGC).