***In Vitro* Assessment of 17 Antimicrobial Agents Against Clinical *Mycobacterium Avium* Complex Isolates**

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Abstract

Background: Recently, *Mycobacterium avium* complex (MAC) infections have been increasing, especially in immunocompromised and older adults. The rapid increase has triggered a global health concern due to limited therapeutic strategies and adverse effects caused by long-term medication. To provide more evidence for the treatment of MAC, we studied the *in vitro* inhibitory activities of 17 antimicrobial agents against clinical MAC isolates.

Results: A total of 111 clinical MAC isolates were enrolled in the study and they were identified as *M. intracellulare*, *M. avium*, *M. marseillense*, *M. colombiense*, *M. yongonense*, *M. arosiense*, and one isolate was undifferentiated at subspecies level. MAC strains had relatively low (0–21.6%) resistance to clarithromycin, amikacin, bedaquiline, rifabutin, streptomycin, and clofazimine, and the resistant rates to isoniazid, rifampin, linezolid, doxycycline, and ethionamide were very high (72.1–100%). In addition, *M. avium* and *M. intracellulare* had different resistance profiles for clarithromycin, ethambutol, trimethoprim/sulfamethoxazole, amikacin, linezolid, clofazimine, cycloserine, and ethionamide.

Conclusions: Our results supported the current usage of macrolides, rifabutin, and aminoglycosides in the regimens for MAC infection, and also demonstrated the low resistance rate against new drugs, such as clofazimine, tedizolid, and bedaquiline, suggesting the possible implementation of these drugs in MAC treatment.

Background

Members of the *Mycobacterium avium* complex (MAC) are the most common nontuberculous mycobacteria (NTM) species that cause pulmonary, soft tissue, and systemic diseases. MAC tends to cause infection in people with immunodeficiencies or underlying lung diseases. Host factors associated with MAC infection include acquired immunodeficiency syndrome, gene mutations in the interferon gamma (IFN- γ)-interleukin 12 axis, positive anti-IFN- γ autoantibodies, cystic fibrosis, and bronchiectasis[1–3]. Over the last decade, the incidence of MAC infections have increased, along with the emergence of several novel subspecies. *Mycobacterium intracellulare* and *Mycobacterium avium* remain the most important and prevalent pathogens in the MAC, while other subspecies, including *Mycobacterium chimaera*, *Mycobacterium colombiense*, and *Mycobacterium marseillense* in the MAC have been increasingly reported recently.

M. chimaera, one of the subspecies of *M. intracellulare*, is transmitted through contaminated catheters and often causes disseminated and life-threatening infections in people who have undergone open-heart surgery[4, 5]. As for *M. colombiense* and *M. marseillense*, they are genetically different from *M. avium* and *M. intracellulare*[6, 7]. *M. colombiense* was first reported in Colombian patients with human immunodeficiency virus[8], and has since been isolated from both immunocompromised and immunocompetent patients with cutaneous, lymph node, and pulmonary infections[9–11]. *M. marseillense*, which was identified later in 2009, has similar pathogenicity to *M. colombiense*. For Other subspecies, like *Mycobacterium vulneris*, *Mycobacterium timonense*, *Mycobacterium arosiense*, *Mycobacterium yongonense*, and *Mycobacterium bouchodurhonense*, few cases were reported.

MAC infections can be difficult to treat due to multiple factors, including environmental and genetic risk factors and frequent drug-related side effects. A culture conversion rate of 50%-80%, a recurrence rate of 25%-48%, and a reinfection rate of 46%-75% have been observed in patients with MAC lung diseases (MAC-LD)[12–14]. Treatment guidelines for MAC-LD by the American Thoracic Society and the British Thoracic Society recommended a three-drug therapeutic approach that includes macrolides, rifampin, and ethambutol[15]. Additionally, for patients with refractory, severe or macrolide-resistant MAC-LD, parenteral amikacin or streptomycin are recommended treatments. In the MAC treatment regimen, only macrolides and amikacin undergo drug susceptibility testing[16–18], as the other agents lack correlations between *in vitro* testing and *in vivo* clinical response. Recently, a limited number of new antibiotics, including anti-tuberculous agents, such as clofazimine, bedaquiline, and tedizolid, have been introduced to treat MAC.

Although *in vitro* drug susceptibility testing of MAC is routine, novel drugs are rarely tested. In addition, the prevalent MAC subspecies differ by regions, which could cause different resistance profiles of MAC from different regions. Therefore, we conducted subspecies identification and drug susceptibility testing on the MAC strains collected from patients admitted to our hospital in Shanghai, China. In addition to the frequently used drugs, we also tested clofazimine, bedaquiline, tedizolid, and cycloserine, with the aim of exploring the effectiveness of antimicrobials against MAC.

Methods

Study design, isolate collection and species identification

Between January 2017 and December 2020, a total of 111 MAC clinical isolates were collected from Huashan Hospital affiliated to Fudan University, Shanghai, China. The MAC isolates were cultured in the Middlebrook 7H9 media supplemented with 10% oleic acid/dextrose/catalase (OADC). The MAC subspecies were identified by partial sequences of the *hsp65* and *rpoB* genes and a phylogenetic tree was analyzed based on these genes. The *hsp65* gene was amplified with primers TB11 (5'-AGTTTGATCCTGGCTCAG-3') and TB12 (5'-GGTTACCTTGTTACGACTT-3')[47] and the *rpoB* gene was amplified with primers MycoF (5'-CGATGCGGTAAAGGTGACATTG-3') and MycoR (5'-CCTTGACAGTGGACACCTTGA-3')[48]. The phylogenetic tree was built using the MEGA software version 10.0 by the Neighbor joining method with a bootstrap value 1,000. The sequences of *hsp65* and *rpoB* of MAC type strains, *M. avium subsp. avium* ATCC25291, *M. intracellulare* ATCC13950, *M. intracellulare* FLAC0133, *M. intracellulare* FLAC0181, *M. intracellulare* MOTT-02, *M. marseillense* DSM45437, *M. yongonense* 05-1390, *M. colombiense* CECT3035, *M. vulneris* DSM45247, *M. mantonii* DSM45255, *M. arosiense* DSM45069, *M. paraintracellulare* MOTT-64, and *M. intracellulare subsp. chimaera* DSM44623 were used as references.

Drug susceptibility testing

The Sensititre Myco susceptibility plate for slow-growing mycobacteria (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to test the susceptibility of the following antimicrobial agents: clarithromycin, rifabutin, ethambutol, isoniazid, moxifloxacin, rifampin, trimethoprim/sulfamethoxazole, amikacin, linezolid, ciprofloxacin, streptomycin, doxycycline and ethionamide, according to the manufacturer protocol. Bedaquiline was purchased from AmBeed Inc.

(Arlington Heights, IL, USA). Clofazimine, tedizolid and cycloserine were purchased from Aladdin (Shanghai, China). The drug susceptibility testing of bedaquiline, clofazimine, tedizolid, and cycloserine was performed using broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) protocol M24-A3. The 111 clinical MAC isolates were cultured on Middlebrook 7H11 agar for 7–14 days. *M. intracellulare* ATCC13950, *Staphylococcus aureus* ATCC29215, and *Mycobacterium smegmatis* ATCC19420 were used as controls. Then isolates were transferred to the Middlebrook 7H9 media supplemented with 10% OADC and cultured for one week at 37°C. The bacterial suspension was adjusted to a 1 McFarland standard with sterile demineralized water and was transferred to the Middlebrook 7H9 media with 10% OADC at a ratio of 1:100. For tests using the Sensititre Myco susceptibility plate, 100 μ L of the inoculum solution was added to each well of the 96-well microtitre plate containing lyophilized antibiotics. For the other four antimicrobial agents, 100 μ L of both inoculum solution and serial dilutions of the agents were added to the 96-well plates. Plates were covered with adhesive seals and incubated at 37°C in ambient air for 14 days. Results were read manually by visual growth readings according to the CLSI M24 guidelines and illustrations of various growth patterns. The minimum inhibitory concentration (MIC) values were the lowest concentrations that completely inhibited growth except for trimethoprim/sulfamethoxazole, for which the MIC value was read as the lowest concentration that inhibited 80% of the growth compared to the positive control. MIC breakpoints of the antibiotics for MAC are shown in Table 1.

Table 1
Breakpoints of 17 antibiotics.

Antimicrobial agent	MIC breakpoints (μ g/mL)		
	Susceptibility	Intermediate	Resistance
CLA ^a	≤ 8	16	≥ 32
RFB ^b	≤ 2	-	≥ 4
EMB ^c	-	-	> 5
INH ^c	-	-	> 0.2
MXF ^a	≤ 1	2	≥ 4
RIF ^b	≤ 1	-	≥ 2
SXT ^b	$\leq 2/38$	-	$\geq 4/76$
AMI ^a	≤ 16	32	≥ 64
LZD ^a	≤ 8	16	≥ 32
CIP ^b	≤ 1	2	≥ 4
STR ^d	≤ 16	32	≥ 64
DOX ^b	≤ 1	2-4	≥ 8
ETH ^c	-	-	> 5
TZD ^e	-	-	> 8
CFZ ^d	≤ 1	2	≥ 4
BDQ ^f	-	-	> 0.25
CS ^d	≤ 16	32	≥ 64

Results

A total of 111 MAC isolates were collected and were identified as *M. intracellulare* (n = 81), *M. avium* (n = 13), *M. marseillense* (n = 7), *M. colombiense* (n = 7), *M. yongonense* (n = 1), and *M. arosience* (n = 1) (Fig. 1). One isolate, named 18-T1838, was similar to both *M. vulneris* and *M. colombiense* in the phylogenetic tree and could not be identified to the subspecies level.

We tested the antimicrobial activities of 17 antimicrobial agents against 111 MAC isolates (Table 2). The detailed MIC values of different subspecies were listed in Table S1. The MAC isolates showed a low resistance rate to commonly used drugs, such as clarithromycin (4.5%), amikacin (2.7%), rifabutin (21.6%), and streptomycin (17.1%). However, they were highly resistant to most anti-tuberculosis drugs, such as isoniazid (100%), rifampin (82.9%), linezolid (72.1%), doxycycline (98.2%), and ethionamide (91.9%). Specifically, all the MAC isolates were resistant to isoniazid. Besides, ciprofloxacin also showed a poor inhibitory effect on MAC isolates which had a resistance rate of 87.4%. Furthermore, the MAC isolates showed an intermediate resistance rate (54.1%, 62.2%, and 60.4%) for ethambutol, trimethoprim/sulfamethoxazole, and moxifloxacin, respectively. Interestingly, the MAC isolates showed a low resistance rate for all four newly used drugs: bedaquiline (0%), clofazimine (19.8%), tedizolid (26.1%), and cycloserine (30.6%).

Table 2
Drug resistant rates of different MAC subspecies.

Antimicrobial agent	No. of resistant isolates (%)							
	All isolates n=111	M. intracellulare n = 81	M. avium n = 13	M. marseillense n=7	M. colombiense n=7	M. yongonense n=1	M. arosience n=1	18-T1838 n=1
CLA	5(4.5%)	3(3.7%)	2(15.4%)	0(0%)	0(0%)	0	0	0
RFB	24(21.6%)	17(21.0%)	2(15.4%)	1(14.3%)	3(42.9%)	0	1	0
EMB	60(54.1%)	33(40.7%)	12(92.3%)	6(85.7%)	7(100%)	1	1	0
INH	111(100%)	81(100%)	13(100%)	7(100%)	7(100%)	1	1	1
MXF	67(60.4%)	43(53.1%)	8(61.5%)	6(85.7%)	7(100%)	1	1	1
RIF	92(82.9%)	64(79.0%)	11(84.6%)	7(100%)	7(100%)	1	1	1
SXT	69(62.2%)	45(55.6%)	10(76.9%)	6(85.7%)	5(71.4%)	1	1	1
AMI	3(2.7%)	1(1.2%)	2(15.4%)	0(0%)	0(0%)	0	0	0
LZD	80(72.1%)	53(65.4%)	11(84.6%)	7(100%)	6(85.7%)	1	1	1
CIP	97(87.4%)	70(86.4%)	10(76.9%)	7(100%)	7(100%)	1	1	1
STR	19(17.1%)	17(21.0%)	2(15.4%)	0(0%)	0(0%)	0	0	0
DOX	109(98.2%)	79(97.5%)	13(100%)	7(100%)	7(100%)	1	1	1
ETH	102(91.9%)	75(92.6%)	10(76.9%)	7(100%)	7(100%)	1	1	1
TZD	29(26.1%)	24(29.6%)	4(30.8%)	0(0%)	1(14.3%)	0	0	0
CFZ	22(19.8%)	14(17.3%)	4(30.8%)	2(28.6%)	2(28.6%)	0	0	0
BDQ	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0	0	0
CS	34(30.6%)	21(25.9%)	9(69.2%)	1(14.3%)	2(28.6%)	1	0	0

Most agents showed similar antimicrobial activities against the two main MAC subspecies, *M. intracellulare* and *M. avium*. However, *M. avium* had a higher resistance rate than that of *M. intracellulare* for clarithromycin (3.7% vs 15.4%), ethambutol (40.7% vs 92.3%), trimethoprim/sulfamethoxazole (55.6% vs 76.9%), amikacin (1.2% vs 15.4%), linezolid (65.4% vs 84.6%), clofazimine (17.3% vs 30.8%), and cycloserine (25.9% vs 69.2%). *M. intracellulare* had a higher resistance to ethionamide than *M. avium*. All or almost all the *M. marseillense* and *M. colombiense* isolates were resistant to ethambutol, isoniazid, moxifloxacin, rifampin, trimethoprim/sulfamethoxazole, linezolid, ciprofloxacin, doxycycline, and ethionamide, while none of them were resistant to clarithromycin, amikacin, streptomycin, or bedaquiline (Table 2). The other agents showed good inhibitory activities against the two subspecies which had a resistance rate ranging from 0–42.9%. *M. yongonense*, *M. arosience*, and 18-T1838 isolates had similar resistance profiles against the 17 antimicrobial agents, except for that *M. yongonense* was resistant to cycloserine (MIC >64 µg/mL) and *M. arosience* was resistant to rifabutin.

Discussion

Our antibiotic susceptibility testing results supported the current recommendation of using macrolides, rifamycins, and aminoglycosides to treat MAC infections. In our study, clarithromycin showed good inhibitory activity against all MAC isolates, consistent with previous studies[19, 20]. The resistance rate of MAC isolates against rifampin was 82.9% (92/111), which was in agreement (78.9%; 216/274) with a previous study[21]. Unlike rifampin, rifabutin showed a better antimicrobial activity and was recommended as an alternative to rifampicin, especially for disseminated MAC infections, for patients infected with MAC[22]. However, in a recent study, neither rifampin or rifabutin inhibited MAC growth *in vitro*[23]. Therefore, further clinical trials are still needed to determine the best choice among different rifamycins for treating MAC diseases. The intermediate resistance against ethambutol was comparable with that of a previous study (58.1%;159/274)[21]. These results do not support the usage of ethambutol for MAC. Among the aminoglycosides, amikacin may be better for treating MAC infections than streptomycin, with an overall low resistant rate of 2.7% (24/111), which is as low as shown in previous studies[24, 25]. Streptomycin is a potentially good choice for treatment of MAC isolates. In a study in a Taiwanese district, the resistance rate of MAC isolates against streptomycin was even lower (4.8%; 4/83)[26]. This difference may be regional (different geographies) or may be due to the inconsistent proportions of MAC subspecies collected in the studies.

As second-line drugs for MAC disease, the clinical efficacy of moxifloxacin and linezolid remains uncertain[27]. In our study, both had limited activity against MAC isolates, which is comparable with previous studies in Korea[28], Sweden[25], and China[29]. However, unlike the poor activity *in vitro*, a recent study has shown that fluoroquinolone-containing regimens could achieve similar clinical improvement with the standard regimen and could be an alternative for patients who cannot tolerate the standard regimen[30]. As for the other tested anti-tuberculosis drugs, such as isoniazid, ciprofloxacin, doxycycline, and ethionamide, the MAC isolates showed high resistance, which supported the consensus that these drugs should not be used in the treatment of MAC diseases as shown in a previous study[20]. The comparison of drug resistance rate of recommended agents for MAC isolates from different studies were shown in Table 3.

Table 3
The comparison of drug resistance rate of recommended agents for MAC isolates from different studies.

Nation/district	Year	Isolate	CLA	RIF	EMB	MXF	RFB	AMI	LZD	STR
This study	2021	111	5(4.5%)	92(82.9%)	60(54.1%)	67(60.4%)	24(21.6%)	3(2.7%)	80(72.1%)	19(17.1%)
Germany	2020	98	1(1.2%)	-	-	38(44.7%)	-	0(0%)	57(67.1%)	-
Germany	2019	683	17(2.5%)	-	-	430(63.1%)	-	-	511(75.0%)	-
Korea	2018	1883	95(5.0%)	1080(57.4%)	1691(89.8%)	1054(56.0%)	-	166(8.8%)	805(42.8%)	-
Sweden	2017	229	6(2.6%)	210(91.7%)	-	112(48.9%)	-	11(4.8%)	118(51.5%)	-
Taiwan	2018	83	0(0%)	-	-	72(86.7%)	-	2(2.4%)	61(73.5%)	4(4.8%)
UK	2016	-	248(19.9%)	686(55.7%)	391(31.9%)	-	58(5.9%)	100(8.2%)	-	498(53.0%)

In our study, the new oxazolidinone, tedizolid, had a significantly lower resistance rate than linezolid, supporting the previous results which indicated that tedizolid has enhanced *in vitro* activities against several NTM species[31]. In addition, it has less side effects in long-term therapy, compared with linezolid and has a concentration-dependent activity against *M. avium*. Its efficacy can be enhanced by ethambutol, which suggests its potential role in the treatment of MAC diseases[32].

Clofazimine, which also had a low resistance rate in our study, has been recently proven to be an effective agent for the treatment of MAC both in patients and mouse models[33, 34]. A recent study conducted in Korea found that a lower MIC value of clofazimine (≤ 0.25 mg/L) was associated with negative conversion of sputum culture in patients with NTM lung diseases[35]. Another study in Korea demonstrated that clofazimine, together with inhaled amikacin, could provide favorable outcomes in patients with refractory MAC-LD[36]. Nevertheless, the adverse effects of clofazimine are a major concern that affects its application in patients.

Bedaquiline is a diarylquinoline antibiotic, acting through an antimicrobial mechanism by inhibiting F1Fo-ATP synthase, an enzyme that is essential in *Mycobacterium tuberculosis*[36]. Although several clinical studies have found increased sputum conversion rates with bedaquiline in patients with multidrug-resistant tuberculosis, its efficacy in the treatment of MAC-LD is currently controversial. In some studies, bedaquiline is considered to be a good candidate for refractory or relapsing diseases caused by MAC[38, 39], while in other studies, bedaquiline treatment in patients with MAC-LD were not favorable due to the emergence of resistance and the decreased systemic exposure caused by rifamycin through the induction of cytochrome P450[40, 41]. In our study, most MAC isolates showed low MIC values (0.015–0.12 μ g/mL) for bedaquiline, which is in agreement with previous studies[42–44]. Clinical trials are warranted to correlate the *in vitro* susceptibility of MAC to bedaquiline with the clinical outcome.

Cycloserine is mainly used to treat drug-resistant *M. tuberculosis*, and there are few reports on its effect on NTM. MAC isolates were completely sensitive to cycloserine in several studies[45], with an MIC breakpoint of 80 μ g/mL. However, in our study, the resistant rates (≥ 64 μ g/mL) are 28.9% and 42.9% for *M. intracellulare* and *M. avium*, respectively. Considering the side effects of long-term use of cycloserine and the intermediate resistance rate *in vitro*, it is necessary to be cautious and more data are needed to test its effect upon clinical application as a candidate drug.

In this study, drug susceptibilities of *M. avium* and *M. intracellulare* (which has been less reported) to several agents were different. *M. avium* had a higher resistance rate than *M. intracellulare* for clarithromycin, ethambutol, trimethoprim/sulfamethoxazole, amikacin, linezolid, clofazimine, and cycloserine. However, since the number of resistant *M. avium* isolates was small in our study, the calculations of the resistance rates of clarithromycin and amikacin using only two isolates might be inaccurate. In another study in Germany[20], higher resistance rates of *M. avium* to trimethoprim/sulfamethoxazole and linezolid were also reported. Contrary to our results, in a recent study in Shanghai[46], *M. intracellulare* had higher resistance rates than *M. avium* for most tested antimicrobials. The difference may be related to different identification methods between these studies. As for the other subspecies, few studies have reported their resistance profiles and more research is needed in the future.

Conclusions

In conclusion, clarithromycin, rifabutin, amikacin, and streptomycin showed good *in vitro* antimicrobial activities against the MAC isolates, with resistance rates of less than 25%. However, isoniazid, rifampin, linezolid, doxycycline, and ethionamide had poor inhibitory activities, which is consistent with previous studies, and thus, not suitable to treat MAC diseases. In addition, new drugs, such as clofazimine, tedizolid, bedaquiline, and cycloserine also showed good antimicrobial activities *in vitro* and could be introduced to treat MAC in the future. Besides, different resistance profiles for several drugs were seen for *M. avium* and *M. intracellulare*, but further studies are still needed to confirm these differences.

Abbreviations

CLSI: Clinical and Laboratory Standards Institute; IFN- γ : interferon gamma; MAC: *Mycobacterium avium* complex; MAC-LD: *Mycobacterium avium* complex lung diseases; NTM: nontuberculous mycobacteria; OADC: oleic acid/dextrose/catalase; CLA, clarithromycin; RFB, rifabutin; EMB, ethambutol; INH, isoniazid; MXF, moxifloxacin; RIF, rifampin; SXT, trimethoprim/sulfamethoxazole; AMI, amikacin; LZD, linezolid; CIP, ciprofloxacin; STR, streptomycin; DOX, doxycycline; ETH, ethionamide; TZD, tedizolid; CFZ, clofazimine; BDQ, bedaquiline; CS, cycloserine.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

JC, LS, and WZ designed the study. SL, WH, SW, YZ, and XC performed the experimental work. SW and HL collected the data. SL, WH, and JC analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Figures

Figure 1

Phylogenetic analyses of concatenated *hsp65* and *rpoB* gene sequences of clinical MAC isolates, using the neighbor-joining method by MEGA10 software. The scale bar indicated the number of substitutions per nucleotide position. The type strains were indicated in each cluster, if included.

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