Original Article

Preliminary Study on Drug Susceptibility Profile and Resistance Mechanisms to Macrolides of Clinical Isolates of Non-tuberculous Mycobacteria from China*

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Abstract

Objective Macrolide susceptibility and drug resistance mechanisms of clinical non-tuberculous mycobacteria (NTM) isolates were preliminarily investigated for more accurate diagnosis and treatment of the infection in China.

Methods Four macrolides, including clarithromycin (CLAR), azithromycin (AZM), roxithromycin (ROX), and erythromycin (ERY), were used to test the drug susceptibility of 310 clinical NTM isolates from six provinces of China with the broth microdilution method. Two resistance mechanisms, 23S rRNA and *erm*, were analyzed with nucleotide sequence analysis.

Results Varied effectiveness of macrolides and species-specific resistance patterns were observed. Most *Mycobacterium abscessus* subsp. *massiliense* were susceptible and all *M. fortuitum* were highly resistant to macrolides. All the drugs, except for erythromycin, exhibited excellent activities against slow-growing mycobacteria, and drug resistance rates were below 22.2%. Only four highly resistant strains harbored 2,058/2,059 substitutions on *rrl* and none of other mutations were related to macrolide resistance. G2191A and T2221C on *rrl* were specific for the *M. abscessus* complex (MABC). Seven sites, G2140A, G2210C, C2217G, T2238C, T2322C, T2404C, and A2406G, were specifically carried by *M. avium* and *M. intracellulare*. Three sites, A2192G, T2358G, and A2636G, were observed only in *M. fortuitum* and one site G2152A was specific for *M. gordonae*. The genes *erm*(39) and *erm*(41) were detected in *M. fortuitum* and *M. abscessus* and inducible resistance was observed in relevant sequevar.

Conclusion The susceptibility profile of macrolides against NTM was demonstrated. The well-known macrolide resistance mechanisms, 23S rRNA and *erm*, failed to account for all resistant NTM isolates, and further studies are warranted to investigate macrolide resistance mechanisms in various NTM species.

Key words: Non-tuberculous mycobacteria; Macrolide; Drug resistance

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INTRODUCTION

Non-tuberculous mycobacteria (NTM) are a large group of mycobacteria that are positive for acid-fast staining except *Mycobacterium tuberculosis* complex (MTBC) and *M. leprae*. More than 160 species of NTM have been identified, and 40 of these were deemed as pathogenic or opportunistic pathogens.

There has been a continuous increase in incidences and disease burden of NTM in many regions[1,2]. The incidence of NTM in the United Kingdom increased from 0.9 per 100,000 population in 1995 to 2.9 per 100,000 population in 2006 and continued to increase to 7.6 per 100,000 in 2012[3,4]. The nationwide surveillance of tuberculosis (TB) in China showed that the average NTM isolation rate increased from 4.3% (29/682) in 1979 to 11.1% (49/441) in 2000 and 22.9% (83/363) in 2010[5,6].

Most NTM strains are intrinsically resistant to the first- and second-line anti-TB agents and difficult to eliminate with treatment regimens commonly used for TB[7,8]. The resistance profile of NTM is highly species-specific and the clinical course and treatment response of NTM disease may be very variable[9]. Hence, antibiotics prescribed for NTM treatment must have demonstrated activities against the specific species in the antibiotic susceptibility testing. As NTM antibiotic susceptibility testing is time-consuming, demanding several days [rapidly growing mycobacteria (RGM)] or weeks [slowly growing mycobacteria (SGM)] to obtain results depending on the bacterial growth time, more data need to be accumulated on the antibiotic susceptibility and resistance mechanism of NTM species to allow rapid detection of antibiotic-resistant genotype and for clinical applications.

Macrolides are the preferred choice of the limited antimicrobial agents for the treatment of NTM infections. The *in vitro* susceptibility test and *in vivo* clinical treatment outcome of macrolides are well consistent[1,10]. Resistance to macrolide class of antibiotics is mainly conferred by three different mechanisms as follows: substitution and modification of the 23S rRNA target site by various methyltransferases; drug efflux; and inactivation of the molecules by esterases, hydrolases, transferases, or phosphorylases enzymes[11,12]. However, only two well-known mechanisms have been reported for mycobacteria, namely, mutations in the 23S rRNA-encoding gene, *rrl*, at positions 2,058/2,059 (*Escherichia coli* numbering) for acquired resistance and *erm* genes encoding 23S rRNA methyltransferases for natural resistance[9]. The *erm* genes have been described only in certain NTM species and have five classes as follows: *erm*(37) of *M. tuberculosis* complex, *erm*(38) of *M. smegmatis*, *erm*(39) of *M. fortuitum*, *erm*(40) of *M. mageritense* and *M. wolinskyi*, and *erm*(41) of MABC and *M. fukienense*[13,14]. Aside from the two mechanisms above, Ag85 mutant of *M. smegmatis* displayed increased sensitivity to erythromycin (ERY), indicating that the defects in the enzymes and proteins involved in maintaining the cell wall integrity may increase the susceptibility of the organism to macrolides[15]. Some of genes encoding putative ATP-binding cassette (ABC) transport systems could be involved in macrolide export[16]. However, more evidence is needed to demonstrate the role of Ag85 and ABC transport systems in the development of macrolide resistance in mycobacteria. In this study, 310 NTM clinical isolates belonging to six of the most common species in China were tested to comprehensively compare the susceptibility of NTM to four commonly used macrolides in clinical settings and elucidate the roles of the two known resistance mechanisms of NTM.

MATERIALS AND METHODS

Strains

A total of 310 identified NTM clinical isolates from six provincial TB hospitals of China, including Anhui, Fujian, Jiangxi, Inner Mongolia, Hunan, and Sichuan Provinces, were included in the antibiotic susceptibility testing. All strains had been re-verified up to the species level by sequencing of the genes *rrs*, *rpoB*, *hsp65*, *ITS*, and *sodA*. All tests performed in this study were conducted in the laboratory of Branch of Tuberculosis, National Institute for Communicable Disease Control and Prevention.

Antibiotic Susceptibility Testing

Four macrolides used in this study, including ERY, clarithromycin (CLAR), azithromycin (AZM), and roxithromycin (ROX), were purchased from Sigma-Aldrich Co. (St Louis, MO). Minimum inhibitory concentrations (MICs) were determined with the broth microdilution method based on the Alamar Blue Assay (MABA). Briefly, fresh cultures were completely ground and their densities were adjusted to 0.5 McFarland (1.5×10^8 cells/mL) with

saline. The suspension was 1:200 diluted with culture medium supplemented with or without 10% oleic acid-albumin-dextrose-catalase (OADC) (BD, Franklin Lakes, NJ, USA) for SGM and RGM, respectively. Serial two-fold dilutions of the antibiotic solutions were prepared in Mueller-Hinton broth (Difco, Detroit, MI, USA) and inoculated with bacterial dilutions. The final reaction system comprised 100 μL antibiotic solution and an equal volume of the bacterial suspension in each well. The plates were sealed and incubated at 37 °C. The indicator (20 μL Alamar Blue mixed with 50 μL of 5% Tween-80) was added when the drug-free control which was checked daily showed a color change from blue to pink. MIC testing usually ended at 3 to 6 days for RGM and 7 to 11 days for SGM. The lowest drug concentration that inhibited the strain growth and prevented color change was recorded as the MIC value. All tests for each strain were repeated twice. The dilution concentration range was 0.125-256.000 μg/mL for ERY, ROX, and AZM and 0.063-128.000 μg/mL for CLAR. The interpretive criteria of each drug were 8 μg/mL for RGM and 32 μg/mL for SGM, as suggested by the Clinical and Laboratory Standards Institute (CLSI, M24-A2)[17].

Polymerase Chain Reaction (PCR) and Sequencing

Boiled DNA template from the fresh cultures was used. The primer set for the amplification of *rrl* gene was *rrl*-F, 5′-CCT GCA CGA ATG GCG TAA CG-3′ and *rrl*-R, 5′-CAC CAG AGG TTC GTC CGT C-3′[18]. The in-frame primer sets used to detect *erm* genes and the reference sequence used for primer design were as follows: *erm*(37)-F, 5′-CGG TGA GCT CGT GTT TGA CAT C-3′ and *erm*(37)-R, 5′-AGG CCG ACG GTC AGG GTG AAC C-3′ (GenBank accession No. AE000516); *erm*(38)-F, 5′-GAA ATC GTC TCG CGC ACA AAC-3′ and *erm*(38)-R, 5′-TGC TGA CCA ACG TCG TCG AAG-3′ (GenBank accession No. AY154657); *erm*(39)-F, 5′-AGT TCA TCA CGG CCG GCA TGA G-3′ and *erm*(39)-R, 5′-ATC GAA CAA CGC CAC CCA CTG-3′ (GenBank accession No. AY487229); e*rm*(40)-F, 5′-TTG ACG GCC ATC GAG ATC GAC-3′ and *erm*(40)-R, 5′-GAC GGT GTG ATG CCG TTG TG-3′ (GenBank accession No. AY570506); *erm*(41)-F, 5′-GCA CTG CGC GAG AAG CTG GCA-3′ and *erm*(41)-R, 5′-GCG GTG GAT GAT GGA AAG-3′ (GenBank accession No. EU590124). The sequencing primer set of entire *erm*(41) was 5′-GCA CTG CGC GAG AAG CTG GCA-3′ and 5′-GCA CTG CGC GAG AAG CTG GCA-3′. PCR products of *rrl* and *erm*(41) were sequenced (Tsingke BioTech, Beijing, China) and mutations were identified with alignment using MEGA 7.0 software.

RESULTS

Antibiotic Susceptibility Profiles

The antibiotic susceptibility profiles and MIC ranges of 310 NTM clinical isolates are shown in Tables 1 and 2, respectively. The antibiotic resistance of the four macrolides was in the order ERY, AZM, ROX, and CLAR (high to low) and showed obvious species specificity. *M. fortuitum* and *M. gordonae* were the two species with the most distinct difference. *M. fortuitum* isolates were almost 100.0% resistant to all four macrolides, with 32 μg/mL MIC_{50} and ≥ 128 μg/mL MIC_{90}. Hence, the use of macrolides may be excluded for the treatment of infections caused by *M. fortuitum*. On the contrary, *M. gordonae* strains were essentially sensitive to macrolides with ≤ 4 μg/mL MIC_{90}. The clinical isolation rate of *M. massiliense* was much lower than that of *M. abscessus*, and only four isolates were included in this study. Except for the two ERY-resistant isolates, all *M. massiliense* were sensitive to the four macrolides, which showed high antibacterial efficiencies. The resistance observed

Table 1. Resistance (%) of 310 NTM Clinical Isolates from China to Four Macrolides

Species	Number of Strains	ERY (*n*/%)	AZM (*n*/%)	ROX (*n*/%)	CLAR (*n*/%)
RGM					
M. abscessus subsp. *abscessus*	52	14/26.9	12/23.1	12/23.1	7/13.5
M. abscessus subsp. *massiliense*	4	2/50.0	0/0	0/0	0/0
M. fortuitum	9	9/100.0	9/100.0	9/100.0	7/77.8
SGM					
M. avium	63	43/63.5	14/22.2	8/12.7	6/9.5
M. intracellulare	159	58/35.2	17/10.7	10/6.3	5/3.1
M. gordonae	23	2/8.7	2/8.7	1/4.3	0/0

Note. *n*, number of resistant strains.

Table 2. Macrolide MIC Range (μg/mL) of NTM

NTM Species	ERY			AZM			ROX			CLAR		
	MIC Range	MIC_{50}	MIC_{90}	MIC Range	MIC_{50}	MIC_{90}	MIC Range	MIC_{50}	MIC_{90}	MIC Range	MIC_{50}	MIC_{90}
RGM												
M. abscessus subsp. *abscessus*	0.030->256.000	0.250	64.000	0.030->256.000	0.500	64.000	0.030->256.000	0.250	64.000	0.030-64.000	0.125	32.000
M. abscessus subsp. *massiliense*	2.000-8.000	4.000	8.000	2.000-4.000	2.000	4.000	2.000-4.000	4.000	4.000	0.125-0.500	0.250	0.500
M. fortuitum	32.000->256.000	64.000	> 256.000	16.000->256.000	32.000	> 256.000	16.000-128.000	32.000	128.000	1.000-32.000	8.000	32.000
SGM												
M. avium	0.250-256.000	32.000	256.000	0.060-64.000	8.000	32.000	0.060-64.000	4.000	32.000	≤ 0.030-32.000	0.500	16.000
M. intracellulare	0.125->256.000	16.000	128.000	0.125->256.000	8.000	32.000	0.125->256.000	2.000	8.000	0.030-128.000	0.250	1.000
M. gordonae	0.030-256.000	0.250	4.000	0.125-64.000	1.000	4.000	0.125-256.000	0.125	4.000	0.030-8.000	0.030	0.500

Note. $MIC_{50/90}$, MICs that inhibit 50% and 90% of the isolates, respectively.

was similar for other three species, *M. abscessus* subsp. *abscessus*, *M. avium,* and *M. intracellulare*. Except for ERY, AZM, ROX, and CLAR showed a resistance rate lower than 24% and MIC_{50} lower than 8 μg/mL.

Genetic Polymorphism of rrl Gene and Relationship with Macrolide Resistance

To explore the role of target-site mutation in the development of macrolide resistance in NTM, the 23S rRNA coding gene, *rrl*, was sequenced and aligned for 143 strains with different MIC levels selected from 310 NTM clinic isolates that were tested for macrolide resistance.

Only one *M. intracellulare* strain harbored A2058T mutation and three *M. abscessus* subsp. *abscessus* harbored A2059G mutations. These four strains were highly resistant to all four macrolides and exhibited an MIC value as follows: ERY > 256 μg/mL, AZM > 256 μg/mL, ROX > 256 μg/mL, and CLAR ≥ 64 μg/mL.

Aside from 2,058/2,059 sites, a total of 193 point mutations different from those in the *E. coli* reference sequence were detected in 143 NTM isolates, wherein 134 sites were common in all NTM isolates tested (Table 3). The other 35 sites existed only in a few of the six NTM species (Table 4). These showed species specificity but were unrelated to macrolide MICs. *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* belonged to the same fast-growing *M. abscessus* species. Their sequences were identical at all sites mentioned above. Although these two subspecies could not be distinguished from each other with *rrl* gene, the two sites G2191A and T2221C allowed differentiation between these species and other four species. The genetic variation in *M. avium* and *M. intracellulare* that belonged to the same *M. avium* complex (MAC) was also very similar. Seven sites, including, G2140A, G2210C, C2217G, T2238C, T2322C, T2404C, and A2406G, were specially carried by these two species, while G2321A was only observed in *M. intracellulare*. Three sites, A2192G, T2358G, and A2636G, were distinct for *M. fortuitum*, while G2152A was specific for *M. gordonae*. The remaining 24 sites were harbored in one or two NTM isolates and these were mutations without any statistical significance. Although we failed to identify any macrolide resistance-related 23S rRNA mutations in this study, these genus- and species-specific sites on *rrl* gene mentioned above may facilitate strain identification up to the species level.

Detection of erm Gene

While detecting 23S rRNA mutations, the presence of five types of *erm* genes, *erm*(37) to *erm*(41), related to mycobacteria were screened in these 143 NTM isolates. PCR amplicons of expected size (-750 bp) with *erm*(39) primer sets were observed in all *M. fortuitum*. Amplicons of the expected size (-650 bp) with *erm*(41) primer sets were obtained in all 46 *M. abscessus* subsp. *abscessus* isolates, while those of smaller size (-300 bp) were detected in four *M. abscessus* subsp. *massiliense* isolates. Sequencing result showed that *erm*(41) of *M. massiliense* had two deletions (nucleotides 64 and 65; 276 bp after nucleotide 158) as compared with *M. abscessus* and, thus, lacked most of the functional domains of ribosomal RNA adenine dimethylases. No *erm* gene was detected in either of the three SGM species, *M. avium*, *M. intracellulare*, or *M. gordonae*.

As T/C polymorphism at 28th nucleotide on *erm*(41) correlated with inducible macrolide resistance[19,20], we further compared the 3-day and 14-day antibiotic susceptibility of strains that carried *erm*(41) genes. Three *M. abscessus* subsp. *abscessus* strains harboring A2059G substitution were excluded, as their 3-day MICs had already reached the highest concentration. As shown in Table 5, 34 of 43 *M. abscessus* subsp. *abscessus* isolates harbored thymine at 28th nucleotide position (T28 sequevar) corresponding to Trp10 in the amino acid sequence. The other nine isolates were C28 sequevar (cytosine, Arg10). MICs of all T28 sequevar isolates reached the highest detection macrolide concentration at 14 days, irrespective of these strains being sensitive or resistant to macrolide at 3 days. In contrast, MICs of the nine C28 sequevar isolates were essentially the same at 3 and 14 days. Thus, *erm* gene was responsible for inducible macrolide resistance of *M. abscessus* subsp. *abscessus*; the resistance observed at 3 days was out of the interpretable range for 23S rRNA and *erm* gene. Although all four *M. massiliense* isolates were T28 sequevars, these were sensitive to macrolides and had no change in MICs upon prolongation of the antibiotic susceptibility test to 14 days owing to the deletion and loss of function of *erm*(41).

Table 3. Common Point Mutations of NTM that Are Different from *E. coli* Reference Sequence

Serial Number	Position	Mutant	Number of Strains	Serial Number	Position	Mutant	Number of Strains
1	2019	A→C	107	23	2102	G→T	107
2	2023	C→A	107	24	2103	C→A	90
3	2024	G→C	107			C→T	17
4	2025	C→G	107	25	2105	T→G	107
5	2026	T→A	107	26	2106	T→G	107
6	2029	G→A	107	27	2107	G→T	107
7	2037	A→T	107	28	2108	A→T	107
8	2038	G→C	107	29	2128	G→A	107
9	2039	T→G	107	30	2138	G→A	103
10	2040	G→T	107			G→C	1
11	2044	C→G	107			G→T	3
12	2052	A→G	107	31	2139	T→C	80
13	2057	G→A	107			T→G	23
14	2070	A→G	107			T→-	4
15	2076	T→C	107	32	2153	C→T	103
16	2088	A→G	107			C→G	4
17	2089	C→T	107	33	2160	C→T	107
18	2091	C→T	107	34	2163	A→T	107
19	2094	A→G	106	35	2165	C→G	107
		A→T	1	36	2178	C→T	107
20	2095	A→T	90	37	2181	T→G	107
		A→C	17	38	2182	-→A	107
21	2096	C→G	107	39	2184	A→C	107
22	2097	A→T	101	40	2185	A→G	107
		A→C	6	41	2187	G→A	107

Continued

Serial Number	Position	Mutant	Number of Strains	Serial Number	Position	Mutant	Number of Strains
42	2190	T→G	107	87	2387	C→A	107
43	2193	T→C	107	88	2388	A→C	103
44	2195	T→A	81			A→T	4
		T→C	23	89	2400	T→C	107
		T→-	3	90	2401	G→A	107
45	2204	T→C	107	91	2402	G→C	107
46	2207	C→A	107	92	2403	T→C	107
47	2213	A→T	107	93	2409	A→G	107
48	2220	G→T	107	94	2418	C→G	107
49	2222	T→C	107	95	2419	C→T	107
50	2223	G→A	107	96	2420	A→G	107
51	2233	T→C	107	97	2443	T→C	107
52	2248	G→A	107	98	2463	A→C	107
53	2260	C→T	107	99	2464	C→T	107
54	2261	T→G	107	100	2465	C→T	107
55	2271	G→A	107	101	2466	G→C	107
56	2272	A→T	107	102	2476	T→C	107
57	2283	A→C	107	103	2488	C→G	107
58	2286	A→C	107	104	2489	G→A	107
59	2288	G→A	107	105	2490	G→T	107
60	2295	G→C	107	106	2491	T→G	107
61	2296	G→C	107	107	2515	A→G	107
62	2299	A→C	107	108	2518	A→G	107
63	2301	T→A	107	109	2532	A→G	107
64	2307	T→A	107	110	2535	T→C	107
65	2311	A→C	107	111	2549	A→T	107
66	2312	C→A	107	112	2550	T→G	107
67	2319	A→T	107	113	2562	A→C	107
68	2323	T→G	107	114	2563	T→A	107
69	2331	T→G	103	115	2570	T→C	107
		T→A	4	116	2573	T→C	107
70	2332	G→T	107	117	2613	C→T	107
71	2336	T→C	107	118	2619	T→C	107
72	2340	C→G	107	119	2624	T→C	107
73	2341	C→G	107	120	2626	G→C	107
74	2356	C→A	107	121	2630	C→T	107
75	2357	G→C	107	122	2631	T→C	107
76	2359	G→T	107	123	2632	G→A	107
77	2362	G→A	107	124	2635	G→A	107
78	2363	G→A	101	125	2637	A→C	107
		G→T	6	126	2638	C→T	107
79	2364	C→G	107	127	2644	G→A	107
80	2365	G→T	107	128	2645	G→A	107
81	2367	G→A	100	129	2646	G→A	107
		G→-	7	130	2647	G→C	101
82	2374	T→G	107			G→T	6
83	2375	G→A	107	131	2651	C→T	107
84	2382	C→T	107	132	2652	T→C	107
85	2383	A→C	107	133	2672	A→G	107
86	2386	T→G	107	134	2673	G→A	107

Table 4. Species-specific Point Mutations in *rrl* Gene from 143 NTM Strains

Serial Number	Positions	*E. coli*	Mutant	*M. abscessus* (*n* = 46)	*M. massiliense* (*n* = 4)	*M. fortuitum* (*n* = 9)	*M. avium* (*n* = 49)	*M. intracellulare* (*n* = 31)	*M. gordonae* (*n* = 4)
1	2081	T	T→C	-	-	9	49	31	-
2	2083	G	G→A	-	-	9	49	31	-
3	2099	T	T→C	-	-	9	49	31	4
4	2101	A	A→G	46	4	-	49	31	4
5	2131	T	T→G	45	4	9	-	31	4
			T→A	1	-	-	-	-	-
6	2136	G	G→A	-	-	-	-	31	1
7	2137	T	T→C	46	4	9	49	-	4
8	2140	G	G→C	-	-	9	-	-	4
			G→A	-	-	-	49	31	-
9	2141	G	G→C	46	4	9	-	-	-
10	2150	C	C→G	46	4	9	-	-	-
			C→T	-	-	-	49	31	4
11	2151	T	T→G	-	-	9	-	-	4
12	2152	G	G→A	-	-	-	-	-	4
			G→T	46	4	9	-	-	-
13	2154	A	A→G	46	4	9	49	-	4
14	2162	G	G→A	46	4	-	-	-	-
15	2164	C	C→T	-	-	9	49	31	4
16	2191	G	G→A	46	4	-	-	-	-
17	2192	A	A→G	-	-	9	-	-	-
18	2196	T	T→C	-	-	-	49	31	4
19	2202	G	G→C	46	4	9	-	-	-
20	2206	A	A→G	46	4	9	-	-	-
21	2210	G	G→C	-	-	-	49	31	-
22	2212	-	-→A	46	4	9	-	-	4
23	2215	T	T→C	-	-	-	-	8	-
24	2217	C	C→G	-	-	-	49	31	-
25	2221	T	T→C	46	4	-	-	-	-
26	2224	C	C→G	46	4	9	-	-	-
27	2238	T	T→C	-	-	-	49	31	-
28	2267	T	T→C	46	4	9	-	-	-
29	2321	G	G→A	-	-	-	-	31	-
			G→T	46	4	9	-	-	4
30	2322	T	T→C	-	-	-	49	31	-
31	2328	C	C→T	-	-	9	49	31	4
32	2358	T	T→C	41	4	-	-	-	1
			T→G	-	-	9	-	-	-
33	2404	T	T→C	-	-	-	49	31	-
34	2406	T	T→C	-	-	-	49	31	-
35	2636	A	A→G	-	-	9	-	-	-

DISCUSSION

Pulmonary infections with NTM are becoming an increasing concern in many countries. Macrolides are one of the most important drugs used for the treatment of NTM, especially MAC infections. In the present study, the result of an *in vitro* antibiotic susceptibility testing confirmed the efficacy of macrolides against NTM. The resistant rates and MIC_{50} of all strains except *M. fortuitum* to AZM, ROX, and CLAR were lower than 24% and 8 μg/mL, respectively. In addition, these drugs were two- to four-fold more active than ERY *in vivo*[21]. CLAR was the most potent agent against NTM strains, with a resistance rate of 8.1% (25/310), and served as the only agent with high clinical efficacy in the susceptibility test[22]. These results are in line with those reported by Foo et al.[23], wherein CLAR inhibited *M. abscessus* and MAC at a resistance rate of 20% and 10%, respectively. AZM was administrated once daily and is beneficial for patient compliance, especially for the long-term treatment over 6 months commonly required in mycobacterial infections[24]. High efficiency of AZM against NTM was also observed. However, comparative studies with CLAR and AZM for NTM treatment are limited. In our study, AZM concentration required to inhibit NTM was higher than that of CLAR. However, some conflicting results were observed. Choi et al.[25]. found that AZM is a weaker inducer of *erm*(41) gene expression than CLAR and should therefore be preferred for *M. abscessus* infections. In contrast, the findings reported by Maurer[26] contradict this suggestion, as high median MICs of ≥ 256 μg/mL on day 12 were observed for the two drugs. Thus, further studies are warranted to determine the clinical efficacy of CLAR and AZM against NTM. The activity of ROX was reported to be comparable to that of CLAR both *in vitro* and *in vivo* in animal models[27]. Consistent with former reports, we observed a similar activity for ROX in our results. ROX was successfully used for the treatment of cutaneous *M. chelonae* infections[28].

The results of the antibiotic susceptibility testing demonstrated the great difference in sensitivity between different species, particularly between *M. gordonae* and *M. fortuitum* as well as *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*. In our study, most of *M. abscessus* and *M. massiliense* were susceptible to macrolides on day 3. However, resistance was evident only in *M. abscessus* after 14 days of incubation. Truncated *erm*(41) was observed in *M. massiliense* and *erm*(41) polymorphism in *M. abscessus* was related to inducible macrolide resistance. These results support the findings that macrolide-containing regimens were more effective for the treatment of *M. massiliense* infections than *M. abscessus*[29]. In our study, 77.8% *M. fortuitum* were found to be resistant to CLAR and all carried *erm*(39). Esteban et al.[30] showed that 84.3% (75/89) *M. fortuitum* clinical isolates harbored *erm* and only 52.8% of these were resistant to CLAR. This variation may be associated with the differences in the methodology employed and regions. Thus, genetic polymorphism of *erm*(39) gene may exist in *M. fortuitum*, necessitating further

Table 5. Number of Resistant Strains and MIC Range at 3 and 14 Days for Two Types of *erm*(41) Sequevar Strains of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* Absenting *rrl* Mutation (A2059G)

M. abscessus **Subspecies**	**Sequevar**	**Antibiotic**	**Number of Resistant Strains (*n*)**		**MIC Range (μg/mL)**	
			Day 3	Day 14	Day 3	Day 14
M. abscessus subsp. *abscessus*	T28	ERY	25	34	0.3-128.0	> 256.0
	(*n* = 34)	AZM	10	34	0.3-128.0	> 256.0
		ROX	7	34	0.3-64.0	64.0->256.0
		CLAR	3	34	0.3-16.0	8.0->128.0
	C28	ERY	4	4	0.3-32.0	2.0-32.0
	(*n* = 9)	AZM	1	1	0.3-16.0	1.0-16.0
		ROX	1	1	0.3-16.0	1.0-16.0
		CLAR	0	0	0.3-4.0	1.0-4.0
M. abscessus subsp. *massiliense*	T28	ERY	2	2	< 1.0-16.0	1.0-16.0
	(*n* = 4)	AZM	0	0	< 1.0	1.0
		ROX	0	0	< 1.0	1.0
		CLAR	0	0	< 1.0	1.0

studies. SGM species were more susceptible to macrolide than RGM, probably owing to the absence of *erm* genes in these species. We found that *M. avium* seemed less susceptible than *M. intracellulare* to macrolide, consistent with the results of previous reports[31,32]. As macrolide susceptibility of MAC has been correlated with clinical treatment outcomes, one may expect more therapeutic failures in treating *M. avium* infections than *M. intracellulare* infections. These species-specific sensitivity suggests the importance of the identification of NTM isolates to subspecies level to design specific treatment regimens. The species-specific sites of *rrl* gene, such as G2321A of *M. intracellulare*, A2192G, T2358G, and A2636G of *M. fortuitum*, and G2152A of *M. gordonae*, identified in this study may be employed as a rapid identification method for NTM.

The most striking observation of this study is the low detection rate of the two well-known macrolide resistance mechanisms. Several reports have suggested that point mutations at 2,058/2,059 position in *rrl* gene were the most common mechanisms to confer macrolide resistance in MAC strains and were observed in > 90% of the reported resistant mutants[22]. However, a significant fraction of resistant strains who failed to harbor any previously identified mutations were observed in former reports. In this study, the proportion of strains with 2,058/2,059 substitutions in 23S rRNA was particularly low and none of other substitutions related to macrolide resistance was identified. This observation was similar to *M. abscessus* strains isolated from Korea, wherein most strains with acquired resistance had no 2,058/2,059 substitutions in *rrl*[33]. In this study, *erm*(39) and *erm*(41) were detected in all *M. fortuitum* and MABC and inducible resistance was observed in relevant sequevars. However, the resistance observed at 3 days could not be explained by 23S rRNA and *erm* mechanisms. Therefore, the well-known macrolide resistance mechanisms failed to cover NTM isolates and meet the requirement for developing fast diagnostic methods for resistant strains to guide rational and individualized medication. The unknown mechanisms responsible for macrolide resistance in NTM remain to be elucidated.

AUTHORS' CONTRIBUTIONS

LI Fu designed and performed the experiments, analyzed data, and prepared the manuscript; LI Gui Lian, PANG Hui, LIU Hai Can, XIAO Tong Yang, LI Shuang Jun, and LUO Qiao participated in the experiments; WANG Rui Bai and WAN Kang Lin designed and reviewed the draft of the manuscript. All authors read and approved the final manuscript.

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