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ORIGINAL ARTICLE

In vitro subminimum inhibitory concentrations of macrolide antibiotics induce macrolide resistance in *Mycoplasma pneumoniae*

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Abstract

Aim: This study aims to investigate the inducing effect of subminimum inhibitory concentrations of macrolide antibiotics on *Mycoplasma pneumoniae (M. pneumoniae)* resistance to drugs.

Materials and Methods: One *M. pneumoniae* reference strain M129 (ATCC 29342) and 104 clinical isolates were incubated at 37°C for 6-8 days. Genomic DNA of *M. pneumoniae* was extracted using TIANamp Bacteria DNA kit and amplified by polymerase chain reaction (PCR).

Results: Ten sensitive isolates obtained from 104 *M. pneumoniae* clinical isolates were induced by subminimum inhibitory concentrations of macrolide antibiotics. Among them, three were found to possess mutations in L4 and L22 ribosomal proteins. Two cases carried simultaneously the C162A and A430G mutations of L4 and the T279C mutation of L22. In addition, one case had only the A209T mutation of L4.

Conclusions: Repeated in vitro exposure to subminimum inhibitory concentrations of macrolide antibiotics could induce selective mutations in ribosomal genes of *M. pneumoniae* clinical isolates that cause resistance to macrolide antibiotics. Hippokratia 2015, 19 (1): 57-62.

Keywords: Macrolide resistance, *Mycoplasma pneumoniae*, subminimum inhibitory concentration

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Introduction

Mycoplasma pneumoniae (*M. pneumoniae*), the smallest cellular organism that persists as obligate extracellular parasite, is a major causative pathogen of community-acquired respiratory tract infections[1]. Infections caused by *M. pneumoniae* predominantly occur in children and young adults, accounting for 10-30% of cases worldwide[2]. *M. pneumoniae* is insensitive to antibiotic agents acting on cell wall, such as β-lactam and vancomycin, and is resistant to polymyxin, rifampicin, and sulfanilamide groups. Aminoglycosides and chloromycetin indistinctively inhibit *M. pneumoniae*, and are rarely used as clinical chemotherapeutic agents for treating mycoplasma infections. Macrolide, tetracycline and fluoroquinolone antibiotics can significantly inhibit *M. pneumoniae*, but tetracycline and fluoroquinolone antibiotics can also cause serious side effects on children. Therefore, macrolides such as erythromycin and azithromycin, become the first choice of therapeutic agents against *M. pneumoniae* infection. However, macrolide-resistant *M. pneumoniae* has become an emerging problem in several countries, particularly in China[2-9]. Data from previous studies showed that more than 90% of macrolide-resistant *M. pneumoniae* were found in China[6]. It is well-known that macrolide-resistant *M. pneumoniae* causes long duration of fever, requires long-term treatment and leads to stronger side effects. Therefore, macrolide-resistant *M. pneumoniae* attracts more and more concerns.

Several possible mechanisms of resistance to antibiotics of various microorganisms have been investigated, including target site mutation, ribosome methylation, drug efflux or inactivation, and drug-related fire-fighting[10-13]. Among these mechanisms, target site mutation is the most widely accepted and the only described mechanism for macrolide resistance of *M. pneumoniae*. The principal target site of macrolides is domain II and/or domain V of the 23SrRNA, a component of the 50S ribosomal subunit[11]. In addition, ribosomal proteins L4 and L22 encoded by

Table 1. Primers used for polymerase chain reaction assays.

Primer designation	Sequence (5′→3′)	Amplicon size (bp)
Ribosomal protein L4	forward oligo GTGAGTTCTCCCCGACC reverse oligo TCAAGGAGGTGTACAAAAGT	529
Ribosomal protein L22	forward oligo CCCTTGTTTAATTGCTAA reverse oligo AAAGCACCAGCAACATTA	464
23SrRNA 2063 and 2064 region	forward oligo AAGAGTTCATATCGACGGCAG reverse oligo CATCTAAGTGTGAAACTAT	303
23SrRNA 2617 region	forward oligo ACTATAACGGTCCTAAGGTA reverse oligo TTATCATGTAGAGAATAGGT	210

the rplD and rplV genes, respectively, can facilitate the formation of the binding sites[14]. The macrolide resistance is determined by specific point mutations in domain V of 23SrRNA gene, and the mutations at position 2063, 2064 and 2617 are known as common mutations[2,9,15]. High level resistance to 14-membered ring macrolide and low level resistance to 16-membered ring macrolide are induced by a mutation from A to G at position 2063, and mutations that induce high level resistance to 14- and 16-membered ring macrolide and low level resistance to 15-membered ring macrolide are A to G mutation at position 2064[16]. However, the relationship between these mutations and macrolide resistance of *M. pneumoniae* remains obscure, and needs further exploration.

In the current study, we aim to investigate how *M. pneumoniae* resistance to macrolide antibiotics is related to selective mutations in ribosomal gene of *M. pneumoniae* that are induced by *in vitro* macrolide antibiotics.

Materials and Methods

Samples

One hundred and four unique *M. pneumoniae* clinical isolates were obtained from bronchial aspirations of 104 patients with low level respiratory infections in Shanghai. Informed consent was obtained from all the patients or his/her families. All procedures were approved by the Ethics Committee of the University of South China. One *M. pneumoniae* reference strain M129 (ATCC 29342) and 104 clinical isolates (200 μL for each sample) were inoculated in 2 mL medium in a 10 mL culture tube and incubated at 37°C for 6-8 days. Color change of the medium was observed every day. If the color of the medium changes from red to yellow, mycoplasma growth can be confirmed. By contrast, color change from red to yellow with turbidity indicates bacterial contamination in the medium.

Polymerase chain reaction (PCR) amplification

Genomic DNA of each *M. pneumoniae* clinical isolate was extracted using TIANamp Bacteria DNA kit (Tiangen, China). The absorbance of 50-fold diluted DNA samples was determined at 260 nm and then at 280 nm. DNA concentration was calculated as follows: DNA concentration = absorbance$_{260}$ × 50 μg/L. The ratio of absorbance$_{260}$/absorbance$_{280}$ is an indication of DNA purity. Genomic DNAs of resistant strains before and after induction were successfully prepared as stated above. Primers used to amplify resistance target gene, 23SrRNA (2063, 2064, and 2617), and ribosomal proteins L4 and L22 were designed by ourselves using Primer Premier 5.0 (Table 1). The PCR reaction system was composed of ddH_2O (19 μL), 10×buffer (2.5 μL), dNTP (0.5 μL), forward oligo and reverse oligo (1 μL), DNA template (0.5 μL) and Pfu DNA polymerase (0.5 μL), reaching a total volume of 25 μL. The PCR conditions were: 94°C for 10 min; denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 2 min (30 cycles); termination at 72°C for 10 min. Then, the PCR products were sequenced, and underwent basic local alignment search tool (BLAST) comparison with coding sequences in Gene Bank to analyze gene mutations in 23SrRNA, ribosomal protein L4 and L22 before and after induction.

Microdilution method

The macrolide antibiotic agents were dispensed according to the antibiotic susceptibility test standard set by

Table 2. In vitro minimum inhibitory concentrations (MIC) range, MIC$_{50}$, and MIC$_{90}$ and MIC distribution for the five macrolide antibiotics among the 104 *M. pneumoniae* clinical isolates.

Antibiotic agents	MIC (mg/L)			MIC (mg/L, strain No)																	
	Range	MIC$_{50}$	MIC$_{90}$	*M. pneumoniae* reference strain M129	≤ 0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	> 128
Erythromycin	≤0.004->128	>128	> 128	≤ 0.004	10		1												3	3	8
Azithromycin	≤0.004-64	16	32	≤ 0.004	11								1	1		2	40	31	18		
Josamycin	≤0.004->128	4	8	≤ 0.004	10	1					1	2	4	9	44	26	5		2	1	
Kitasamycin	≤0.004->128	64	> 128	≤ 0.004	1	5	4		1						1	3	2	4	32	50	
Clarithromycin	≤0.004->128	128	> 128	≤ 0.004	11											1	4	5	9	20	54

MIC: minimum inhibitory concentration.

The United States National Committee for Clinical Laboratory Standards in 2008[17]. The minimum inhibitory concentrations (MICs) of the macrolide antibiotic agents for the 104 *M. pneumoniae* clinical isolates were determined by microdilution method.

Briefly, medium containing 10^4-10^5 CFU/mL of *M. pneumoniae* was dispensed into 96-well microplates and incubated at 37°C for 6-8 days. There were 3 controls: medium that was used as the negative control; medium containing the highest concentration of antimicrobial agents was used as the drug control; medium containing only *M. pneumoniae* was used as the drug-free control. The definition of MIC was the lowest concentration of antimicrobial agents at which the metabolism of the *M. pneumoniae* was inhibited as evidenced by the deficiency of color change in the medium when the drug-free control just started to show color change. The MIC values were represented by the average of three test results. The sensitivity of isolates was determined according to the standards prescribed by the United States National Committee for Clinical Laboratory Standards 2008[17]. MIC values of resistant strains were 4 times greater than those of the reference sensitive strains.

The antibiotic agents we used for inducing macrolide resistance of *M. pneumoniae* were erythromycin, azithromycin, and kitasamycin. The agent concentrations were diluted to 1/2 of the minimum inhibitory concentration. *M. pneumoniae* was inoculated to 2 mL medium containing 1/2 MIC of erythromycin, azithromycin and kitasamycin, respectively, and then incubated at 37°C before being inoculated to medium containing 1/2 MIC of macrolide antibiotic agents when the medium color changed from red to yellow and clear. *M. pneumoniae* repeatedly underwent subculture for 10 generations, and then continued to extend in medium containing the original drug concentration. MIC values of *M. pneumoniae* were determined after macrolide induction. When MIC values were increased 4-fold than before, resistance induction was successful. The reference strains inoculated in the absence of drug medium after subculture for 10 generations were used as control. In the induction process, 0.1 mL bacteria liquid was regularly inoculated to solid medium. Colony morphology of *M. pneumoniae* was observed under the microscope to ensure no contamination from bacteria.

Results

Repeated in vitro exposure to subminimum inhibitory concentrations of macrolide antibiotics causes resistance to macrolide antibiotics in M. pneumoniae

To test the resistance of 104 *M. pneumoniae* strains to macrolide antibiotics, the MICs for five macrolide antibiotic agents on 104 *M. pneumoniae* isolates were measured. After screening 104 *M. pneumoniae* isolates, 11 sensitive isolates were found not resistant to macrolide, and then induced by subminimum inhibitory concentrations of erythromycin, azithromycin, or kitasamycin for 10 generations (Table 2). After erythromycin induction, one isolate (P42) was dead when passaged to the sixth generation, but the other 10 isolates were induced for 10 generations, in which erythromycin MIC values of eight (80%) isolates after induction were four times greater than before (Table 3). In addition, azithromycin-induced resistance showed that P5, P42 and P74 sensitive isolates could not survive when passaged to the third generation. Among the other eight isolates that were induced in the same way as erythromycin, the azithromycin MIC values in three out of eight (37.5%) isolates were increased fourfold or more compared with that before induction (Table 3). However, after induction by kitasamycin, P4 was dead when passaged to the fourth generation. Among the other ten isolates treated by kitasamycin the same as erythromycin, the kitasamycin MIC values of six out of 10 (60%) isolates were fourtimes greater than those before induction (Table 3). These data suggested that repeated in vitro exposure to subminimum inhibitory concentrations of macrolide antibiotics could cause resistance to macrolide antibiotics in *M. pneumoniae*.

Table 3. Drug susceptibility after induction with erythromycin, azithromycin and kitasamycin.

Strains	MIC (mg/L) of erythromycin			MIC (mg/L) of azithromycin			MIC (mg/L) of kitasamycin		
	Before induction	After induction	Folds	Before induction	After induction	Folds	Before induction	After induction	Folds
P2	0.004	0.016	4	0.004	0.004	0	0.004	0.032	8
P4	0.002	0.016	8	0.001	0.001	0	0.016	0.032	2
P5	0.002	0.032	16				0.008	0.032	4
P7	0.004	0.016	4	0.002	0.008	4			
P14	0.004	0.008	2	0.002	0.008	4	0.016	0.064	4
P21	0.004	0.008	2	0.002	0.008	4	0.008	0.032	4
P28	0.002	0.032	16	0.001	0.002	2	0.008	0.032	4
P42							0.008	0.016	2
P74	0.004	0.016	4				0.016	0.032	2
P97	0.004	0.016	4	0.002	0.002	0	0.016	0.032	2
P99	0.016	0.064	4	0.001	0.002	2	0.064	0.256	4

MIC: minimum inhibitory concentration.

Selective mutations in ribosomal genes of M. pneumoniae clinical isolates contribute to resistance to macrolide antibiotics

To analyze the resistant genes of the induced macrolide-resistant *M. pneumoniae* isolates, PCR amplification and GenBank sequence comparison were per-

Table 4. Mutations of 10 induced macrolide-resistant *M. pneumoniae* clinical isolates.

Strains	Ribosomal proteins				23SrRNA			
	L4			L22	II domain		V domain	
	162	430	209	279				
M129	C	A	A	T	-	-	-	-
P2	-	-	-	-	-	-	-	-
P4	-	-	-	-	-	-	-	-
P5	-	-	-	-	-	-	-	-
P14	-	-	-	-	-	-	-	-
P21	C→A	A→G		T→C	-	-	-	-
P28	C→A	A→G		T→C	-	-	-	-
P42	-	-	-	-	-	-	-	-
P74	-	-	-	-	-	-	-	-
P97	-	-	-	-	-	-	-	-
P99	-	-	A→T	-	-	-	-	-

- : indicates no mutation compared with M129 (*M. pneumoniae* reference strain).

formed. Using PCR, we amplified 210 bp and 303 bp fragments of 23SrRNA domain II and V genes, respectively (Figure 1A and B), and 529 bp and 424 bp fragments of ribosomal proteins L4 and L22, respectively (Figure 2A and B). Then, the comparison of the sequenced PCR products with coding sequences in GenBank showed that three out of 10 induced macrolide-resistant *M. pneumoniae* isolates were found to possess mutations in L4 and L22 ribosomal proteins, which had not been detected in these isolates prior to the induction. Two cases carried simultaneously the C162A and A430G mutations of L4 and the T279C mutation of L22. In addition, 1 case had only the A209T mutation of L4 (Table 4). These results indicated that selective mutations in ribosomal genes of *M. pneumoniae* clinical isolates contributed to resistance to macrolide antibiotics.

Discussion

Macrolide antibiotics are usually considered as the first-choice agents against *M. pneumoniae* infections both in children and young adults. However, macrolide-resistant *M. pneumoniae* emerge as a tough clinical problem all over the world. It has been reported that the highest resistance rate in the world was found in China (90%), whereas the resistance rates in Japan and the United States were >30% and 8.2%, respectively, and the resistance rates in Europe ranged from 10% to 26%[2,4,6,8,18,19]. In our study, we found that, only 11 out of 104 unique *M. pneumoniae* clinical isolates were susceptible to the five macrolide antibiotics, but the other 93 isolates (89.4%) were resistant to the five macrolide antibiotics, with macrolide resistance rates being consistent with previous reports[6,20-22]. Among the five macrolide antibiotics, erythromycin had the lowest susceptibility for *M. pneumoniae*, with its MIC_{50} and MIC_{90} values being greater than 128 mg/L. By contrast, josamycin showed the best antimycoplasmal activity, with its MIC_{50} and MIC_{90} being 4 mg/L and 8 mg/L, respectively.

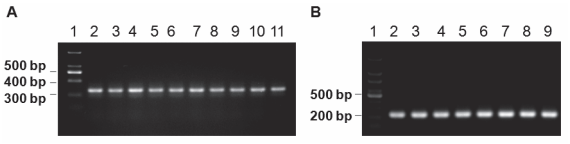

Figure 1. A) Polymerase chain reaction (PCR) amplification of 23SrRNA gene including 2063 and 2064 from M. pneumoniae clinical isolates. 1: DNA marker; 2: M. pneumoniae reference strain M129; 3-11: PCR products (303 bp) of 23SrRNA gene (including 2063 and 2064). B) PCR amplification of 23SrRNA gene including 2617 from M. pneumoniae clinical isolates. 1: DNA marker; 2: M. pneumoniae reference strain M129; 3-9: PCR products (210 bp) of 23SrRNA gene (including 2617).

Subminimum inhibitory concentrations of erythromycin (14-membered macrolide), azithromycin (15-membered macrolide) and kitasamycin (16-membered macrolide) were used for the induction of macrolide resistance in 11 *M. pneumoniae* clinical isolates that were sensitive to macrolide antibiotics. Several induced isolates were resistant to macrolide antibiotics, whereas the MIC values of *M. pneumoniae* reference strains cultured in drug-free medium were unchanged after macrolide induction for 10 generations. By comparing the MIC values of isolates before and after macrolide induction, we found that the MIC values were increased fourfold, and even 16 times. Therefore, we concluded that repeated exposure to subminimum inhibitory concentrations of macrolide antibiotics *in vitro* could induce low levels of drug resistance. In addition, one strain of *M. pneumoniae*-sensitive isolate died during the process of induction by erythromycin and azithromycin, and three strains died in kitasamycin induction process. However, detailed causes are not clear yet.

Macrolide antibiotics inhibit protein synthesis by binding to domains II and V of 23SrRNA[13,23]. Specifically, it has been clearly shown that ribosomal mutations in domains II and V of 23SrRNA and mutations in ribosomal proteins L4 and L22 are associated with the resistance to macrolide antibiotics[24,25]. The L22 protein is important for the assembly of the 50S ribosomal subunit as well as the folding of 23SrRNA[26]. Mutations in L22 protein are usually located in a β-hairpin extension at the C-terminus of the protein[21,27], whereas, L4 mutations perturb the three-dimensional structure of 23SrRNA at multiple sites and hence, hypothetically preventing macrolide binding by af-

fecting the opening of the nascent peptide exit tunnel[28]. Although the modification of ribosomal protein L4 or L22 genes has been reported in *Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus pyogenes*, *Rickettsia* genus, *Haemophilus influenzae, Ureaplasma parvum, Mycoplasma hominis* and *M. pneumoniae*[29-38], few reports described the mutations in ribosomal protein L4 or L22 genes in *M. pneumoniae* clinical isolates that are related to macrolide resistance. In 2004, Pereyre *et al* reported that in vitro amino acid changes in ribosomal proteins L4 and L22 could be induced arising as H70R or H70L replacement, and one, two, or three G insertion in site 60 in L4 as well as P112R and A114T replacement or $_{111}$IPRA$_{114}$ deletion in L22. However, no mutations were found in domain II of 23SrRNA in this study and it remains unknown whether the mutations induced in vitro arise in clinical *M. pneumoniae* isolates[38]. Moreover, Matsuoka *et al*. reported that of 13 erythromycin (ERY) resistant *M. pneumoniae* strains, 12 were highly ERY resistant and one was weakly resistant, 10 strains had an A2063G transition, one strain showed A2063C transversion, one strain showed an A2064G transition, and the weakly ERY-resistant strain showed C2617G transversion of domain V. Domain II and ribosomal proteins L4 and L22 were not associated with the ERY resistance of these clinical *M. pneumoniae* strains[39]. In addition, Liu *et al*. reported that 70 ERY resistant *Mycoplasma pneumoniae* strains all showed 2063 or 2064 site mutation in domain V of the 23S rRNA but no mutations in domain II. Site mutations of L4 or L22 can be observed in either resistant or sensitive strains, although it is not known whether this mutation is associated with drug resistance[21].

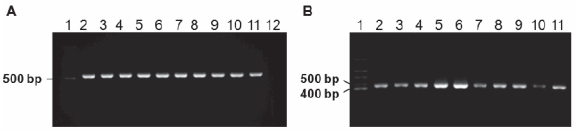

Figure 2. A) Polymerase chain reaction (PCR) amplification of ribosomal protein L4 gene from M. pneumoniae clinical isolates. 1: DNA marker; 2: M. pneumoniae reference strain M129; 3-11: PCR products (464 bp) of ribosomal protein L4 gene; 12: Negative control. B) PCR amplification of ribosomal protein L22 gene from M. pneumoniae clinical isolates. 1: DNA marker; 2: M. pneumoniae reference strain M129; 3-11: PCR products (529 bp) of ribosomal protein L22 gene.

In the current study, we detected that after induction with subminimum inhibitory concentrations of macrolide antibiotics, two (P28 and P99) out of the eight strains of induced erythromycin-resistant *M. pneumoniae* showed mutations in ribosomal proteins L4 (C162A, A430G and A209T) and L22 (T279C), one (P21) out of three strains of induced azithromycin-resistant *M. pneumoniae* showed mutations in ribosomal protein L4 (C162A and A430G), three (P21, P28, and P99) out of the six cases of induced kitasamycin-resistant *M. pneumoniae* showed mutations in ribosomal proteins L4 (C162A, A430G and A209T) and L22 (T279C). However, no mutation was found in domains II and V of 23SrRNA (including 2063, 2064, and 2617) in macrolide-resistant *M. pneumoniae*.

Conclusion

Repeated exposure to subminimum inhibitory concentrations of macrolide antibiotics (erythromycin, azithromycin, and kitasamycin) in vitro could induce low levels of macrolide resistance and selective mutations in ribosome that are related to resistance to macrolides in *M. pneumoniae* clinical isolates, which might help understand the resistance mechanism of *M. pneumoniae*. In addition, it should be noted that no mutation was found in domains II and V of 23SrRNA (including 2063, 2064, and 2617), despite a four- or more fold increase of MIC. To comprehensively investigate the complicated resistance mechanisms of *M. pneumoniae*, more investigation needs to be performed to detect additional mechanisms, including mutations elsewhere in the 23SrRNA or ribosomal protein, or a drug efflux system that may lead to the resistant phenotype of this mutation. Characterization of the mechanisms of resistance to macrolides is important for the clinical management of antibiotic therapy and can provide alternative choices for treating *M. pneumoniae* infection.

Conflict of interest

Authors declare no financial or non-financial competing interests.

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