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Macrolide-Resistant *Mycoplasma pneumoniae* in Adults in Zhejiang, China

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Mycoplasma pneumoniae* is a major pathogen causing community-acquired pneumoniae (CAP), which is generally treated with macrolides. In recent years, however, although macrolide-resistant *M. pneumoniae* has been reported frequently, particularly in China, very little is known about the prevalence of macrolide-resistant *M. pneumoniae* infection in adults. In this study, we survey the macrolide-resistant *M. pneumoniae* in adults in Zhejiang province and characterize the mechanisms of resistance to macrolide. Six hundred fifty throat swab samples were collected from adult patients with CAP from January 2012 to August 2014. These samples were assayed by nested PCR and then cultivated for *M. pneumoniae*. All isolates were sequenced to determine the mutation in domain V of the 23S rRNA gene. The activities of 10 antibiotics against macrolide-resistant *M. pneumoniae* isolates were also investigated *in vitro*. Moreover, restriction fragment length polymorphism (RFLP) analysis of the amplified P1 gene was used to type 50 resistant strains. One hundred percent (71/71) of *M. pneumoniae* strains isolated from adults with CAP were resistant to erythromycin (MIC = 128 to >256 μg/ml), clarithromycin (MIC = 128 to >256 μg/ml), and azithromycin (MIC = 32 to >64 μg/ml). Furthermore, all macrolide-resistant *M. pneumoniae* strains identified had an A2063G mutation in domain V of the 23S rRNA gene. Forty-six resistant strains (92.0%) were classified into type I strain on the basis of P1 gene PCR-RFLP analysis. According to these findings, it is suggested that macrolide-resistant *M. pneumoniae* infection is very prevalence among adults in Zhejiang province. Thus, there is necessary to perform the epidemiological monitoring of macrolide-resistant *M. pneumoniae* in the future.

Mycoplasma pneumoniae remains an important cause of community-acquired pneumonia (CAP), and this organism accounts for up to 40% of cases (1–3). Although most of these infections are asymptomatic or mild, severe bronchopneumonia and lung abscesses are occurring increasingly (4). Furthermore, *M. pneumoniae* infection may lead to several extrapulmonary conditions, such as myocarditis, pericarditis, meningitis, neuritis, and erythema multiforme, sometimes with a fatal outcome (5, 6). *M. pneumoniae* infection could occur at any age. However, research on *M. pneumoniae* infection in adults has lagged behind that in children. Epidemiological studies demonstrate that *M. pneumoniae* infections account for 20.7% in adults with CAP in China, more than *Streptococcus pneumoniae*, so *M. pneumoniae* is the leading pathogen of CAP (7). Therefore, it is important to study *M. pneumoniae* infection in adults.

Because of the absence of cell walls with *M. pneumoniae*, macrolide antibiotics are recognized generally as the first-choice agents in clinical treatment (3, 8, 9). However, with the widespread use of the drug, increasing numbers of macrolide-resistant *M. pneumoniae* have been reported in the past decade, especially in Asia, Europe, and the United States (6, 10–12). In China, the infection rate of macrolide-resistant *M. pneumoniae* has reached up to 90% (13, 14).

Specific site mutations in domain V of 23S rRNA of *M. pneumoniae* may define the macrolide resistance phenotypes. For instance, the mutations that occurred at both positions 2063 and 2064 led to high-level resistance, whereas positions 2067 and 2617 are associated with low-level resistance to macrolides (3, 15, 16). It was confirmed that the resistance of *M. pneumoniae* to macrolide is mainly caused by mutations in domain V of the 23S rRNA gene, such as A2063G, A2064G, A2063C, A2063T, A2067G, and C2617G, which in turn interfere with the binding of macrolides to rRNA (15, 17). Moreover, a mutation at A2063G is most likely to be present along with these mutations (3, 15, 16).

In this study, 71 *M. pneumoniae*-positive strains were obtained from 650 throat swab samples to evaluate the prevalence of macrolide resistance of *M. pneumoniae* among adults in Zhejiang, China, and characterize the mechanisms of resistance. We identified a significantly high prevalence of macrolide resistance in adults and show that this resistance is associated with the A2063G mutation in domain V of the 23S rRNA gene. Together, these findings highlight the fact that macrolide resistance in *M. pneumoniae* is a serious problem in Zhejiang of China, and local surveillance may play an important role in providing effective therapy against *M. pneumoniae* infection.

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TABLE 1 Primers used in this study

Primer target and name	Sequence (5′-3′)	Nucleotide position	Product size (bp)
P1 adhesin gene[a]			
Mp-F	ATTCTCATCCTCACCGCCACC	40–331	285
Mp-R	CGTGGTTTGTTGACTGCCACT GCCG	40–331	285
Mpn-F	CAATGCCATCAACCCGCCCTT AACC	178–285	107
Mpn-R	GTTGTCGCGCACTAAGGCCC ACG	178–285	107
23S rRNA gene[b]			
Mp-F1	GTGCTGGAAGGTTAAAGAAG	1845–2777	933
Mp-R1	GATAGTTTCACACTTAGATG	1845–2777	933
Mpn-F1	GAGGTTAGCGCAA GCGAAGC	1865–2206	342
Mpn-R1	ATTAGAACAGCACACAACCA	1865–2206	342
Mpn-F2	AAGAGTTCATATCGACGGCAG	2472–2776	303
Mpn-R2	ATAGTTTCACACTTAGATG	2472–2776	303

[a] Talkington et al. (18).
[b] Lin et al. (20).

MATERIALS AND METHODS

***M. pneumoniae* strains.** A total of 650 throat swab samples were routinely obtained from adult patients aged from 18 to 82 years with CAP from January 2012 to June 2014 at three hospitals in Zhejiang province (The Second Affiliated Hospital of Wenzhou Medical University, Yueqing Third People's Hospital, Zhuji People's Hospital), and all studies were approved by the hospital ethics committee. The diagnosis was mainly confirmed based on clinical signs and symptoms (sore throat, cough, fever, productive sputum, chill, chest pain, dyspnea, or pulmonary rales) and pulmonary radiography.

Rapid detection by nested PCR for *M. pneumoniae* was performed originally using primers based on the P1 gene and methods described previously (18) (Table 1). The *M. pneumoniae* reference strain FH (ATCC 15531) was used as a PCR-positive control.

Positive throat swab specimens identified by nested PCR were cultivated in 2.5 ml of PPLO broth for 10 to 14 days at 37°C with 5% CO_2. The composition of the medium was as described previously (17). When the color of the broth medium changed from red to yellow by the resulting utilization of glucose, 0.2 ml of the suspension was transferred onto the agar medium. The agar medium was incubated at 37°C with 5% CO_2 for 7 to 14 days. Then, a single colony was isolated and subcultivated for three times when typical "fried-egg" colonies on the agar medium were observed under a stereomicroscope. The obtained *M. pneumoniae* strains were identified by nested PCR targeting the P1 gene.

Antimicrobial susceptibility of *M. pneumoniae*. To determine the MICs of 10 antibiotics for *M. pneumoniae* isolates, the microdilution method with pleuropneumonia-like organism (PPLO) broth was performed as described previously (19). These agents are divided into three categories: macrolides (erythromycin, clarithromycin, azithromycin, josamycin, and rokitamycin), tetracyclines (doxycycline and minocycline), and fluoroquinolones (levofloxacin, ciprofloxacin, and gatifloxacin). Every antimicrobial susceptibility test was repeated three times. *M. pneumoniae* reference strain FH (ATCC 15531) was used as a drug-sensitive control.

DNA sequencing. Amplification of domain V of the 23S rRNA gene were performed by nested PCR using primers described by Lin et al. (20) (Table 1). All of the nested PCR products, including the reference strain, were sequenced (Sangon Biotech Co., Ltd., Shanghai, China). The DNA sequences were compared to that of *M. pneumoniae* strain FH (GenBank accession no. CP002077.1) by BLAST. These experiments were performed for three times.

PCR-RFLP typing of the P1 gene. PCR-restriction fragment length polymorphism (RFLP) was performed to type 50 macrolide-resistant strains as described previously (21). Briefly, a fragment of P1 adhesin gene was amplified with the primers ADH1 and ADH2 (21) and then digested with HaeIII restriction endonuclease (NEB, Shanghai, China). The digested samples were analyzed on a 1.2% agarose gel.

TABLE 2 *M. pneumoniae* isolated from three hospitals in Zhejiang, China

Hospital	Total no. of samples	No. of macrolide-resistant samples
Second Affiliated Hospital of Wenzhou Medical University	300	35
Yueqing Third People's Hospital	210	27
Zhuji People's Hospital	140	9
Total	650	71

RESULTS

Clinical isolates of *M. pneumoniae*. A total of 145 (22.3%) *M. pneumoniae*-positive samples were obtained from 650 samples by nested PCR targeting of the P1 adhesion gene. Cultivation for *M. pneumoniae* with PPLO broth and agar was performed further in the 145 PCR-positive samples, and 71 strains were isolated (Table 2).

Antimicrobial susceptibility. Compared to the *in vitro* activities of the *M. pneumoniae* reference strains listed in Table 3, all 71 clinical isolates showed a significantly increase in the degree of MICs against macrolides and resistance to erythromycin and clarithromycin with MICs of >128 μg/ml. The MIC of azithromycin (32 to >64 μg/ml) was lower than that of erythromycin and clarithromycin. The 16-member macrolides rokitamycin and josamycin were more effective than the 14- and 15-member macrolides, and rokitamycin (0.064 to 1 μg/ml) had a more effective MIC than did josamycin (1 to 8 μg/ml).

All of the clinical isolates, as well as *M. pneumoniae* reference strains, were susceptible to the tetracyclines (doxycycline and minocycline) and fluoroquinolones (levofloxacin, ciprofloxacin, and gatifloxacin) in this study. Gatifloxacin, in particular, with an MIC of 0.016 to 0.125 μg/ml was more active than both levofloxacin and ciprofloxacin.

Sequencing analysis of 23S rRNA genes. All 71 macrolide-resistant clinical strains harbored the A2063G mutation in domain V of 23S rRNA genes. Neither a position 2064 nor a position 2617 site mutation in 23S rRNA gene was observed. In addition, a deletion that occurred at 2018A was found in both 71 clinical strains and the *M. pneumoniae* reference strain, which indicates that the 2018A deletion does not correlate with macrolide resistance.

PCR-RFLP typing of the P1 gene. A total of 46 (92.0%) resistant strains were classified as type I on the basis of P1 gene PCR-RFLP analysis, indicating that type I strains were predominant among the tested resistant strains.

DISCUSSION

To our knowledge, this is the first study about the evaluation of macrolide-resistant *M. pneumoniae* infection in adults in Zhejiang, China. During the study period, we found a high rate of

TABLE 3 MICs of 10 antibiotics against *M. pneumoniae* clinical strains and the FH strain

Isolate group[a]	MIC (μg/ml)[b]									
	ERY	CLR	AZM	JOS	RKI	MIN	DOX	LVX	CIP	GAT
Clinical isolates (A2063G)	128 to >256	128 to >256	32 to >64	1 to 8	0.064 to 1	0.031 to 1	0.125 to 1	0.25 to 2	0.5 to 2	0.016 to 0.125
Reference strain FH	0.016	0.008	0.002	0.063	0.01	0.031	0.063	0.5	1	0.125

[a] As characterized by mutation in the 23S rRNA gene.
[b] Abbreviations: ERY, erythromycin; CLR, clarithromycin; AZM, azithromycin; JOS, josamycin; RKI, rokitamycin; DOX, doxycycline; MIN, minocycline; LVX, levofloxacin; CIP, ciprofloxacin; GAT, gatifloxacin. MIC ranges are given for the clinical iolates.

resistance to macrolides for *M. pneumoniae* in adults, and this resistance is associated with the A2063G mutation in domain V of the 23S rRNA gene. Furthermore, the PCR-RFLP results indicated that type I strain was predominant among the resistant strains (92.0%).

M. pneumoniae is one of the most common causes of CAP and leads to about 2 to 30% of CAP in adults (7, 22). In the present study, *M. pneumoniae* infection was identified by nested PCR assay in adult patients. The results showed that 22.3% (175/650) of adults with CAP were infected with *M. pneumoniae*. It is well known that PCR technology is a rapid, easy, accurate method for early diagnosis of *M. pneumoniae* (23–25). Among PCR methods, nested PCR have remarkable advantages over traditional PCR, including superior sensitivity and specificity, because of involvement of the reamplification of a PCR product with a second set of primers (23). Our findings are in agreement with other studies and suggest that nested PCR assess should be considered the preferred method for the diagnosis of *M. pneumoniae* infection.

Macrolides usually are used as the first-line choice therapeutic agent for the treatment of *M. pneumoniae* infections in both children and adults (3). In our study, the resistance rate to macrolides was extremely high in Zhejiang, China, because all *M. pneumoniae* strains isolated from adult patients showed resistance to macrolides. In 2000, the first macrolide-resistant *M. pneumoniae* strain was isolated in Japan (19). Since then, the frequency of macrolide-resistant *M. pneumoniae* cases has increased rapidly throughout the world, including Europe, eastern Asia, and the Americas (6). Between 2002 and 2008, a progressive increase in macrolide resistance from 5 to 39% among *M. pneumoniae* isolates was observed in Japan and even reached 87% in a recent year (3, 26, 27). Several Chinese studies reported a higher proportion of macrolide-resistant *M. pneumoniae* strains, ranging 63 to 92% (13, 28–30), obtained between 2003 and 2012 from patients with respiratory tract infections. Although it has been reported that the prevalence of macrolide-resistant *M. pneumoniae* is relatively lower in Europe and the United States, ranging from 3.6% in Germany (31) to 25.6% in Italy (2), the rate of resistance has also increased in these areas. For instance, Peuchant et al. (10) reported that the resistance rate increased from 0% before 2005 to 9.8% in 2007 in France. In the United States, the resistance rate also increased from 5% in 2008 to 8.2% in 2012 (12). Obviously, macrolide-resistant *M. pneumoniae* is spreading sharply throughout the world, especially in eastern Asia. In our study, the prevalence of macrolide-resistant *M. pneumoniae* is particularly severe in adults in Zhejiang, China, and poses a great challenge to the selection of appropriate antibiotics for the treatment of *M. pneumoniae* infection. This is most likely attributed to the widespread empirical use of macrolides for respiratory tract infections.

Macrolide resistance in *M. pneumoniae* is highly relevant to mutations in domain V of the 23S rRNA gene. In particular, the point mutation in the peptidyl transferase region of 23S rRNA was considered the main mechanism of macrolide resistance because the mutation blocks the capacity of macrolides to bind the 23S rRNA components of the ribosome (15, 17). The A2063G mutation is recognized as the most prevalent mutation, followed by A2064G. Other mutation types—such as A2063C, A2063T, C2617A and A2067G—are rare (3, 15). In our study, a total of 71 macrolide-resistant *M. pneumoniae* strains harbored an A-to-G transition mutation at position 2063 of the 23S rRNA gene. We found no isolates with the locus 2064 or 2617 mutation. Based on our results, the A2063G transitions are responsible for high-level resistance to 14- and 15-member ring macrolides, such as erythromycin (128 to >256 μg/ml), clarithromycin (128 to >256 μg/ml), and azithromycin (32 to >64 μg/ml) in *M. pneumoniae*. However, 16-member ring macrolides, such as josamycin and rokitamycin, retained activity, with MICs of ≤1 μg/ml, against clinical strains with the A2063G mutation. The data from the susceptibility test also revealed that all of the *M. pneumoniae* isolates were sensitive to tetracyclines (doxycycline and minocycline) and fluoroquinolones (levofloxacin, ciprofloxacin, and gatifloxacin). The new fluoroquinolone gatifloxacin, with MIC of 0.016 to 0.125 μg/ml, was more active than levofloxacin and ciprofloxacin. Taken together, these findings suggest that these antibiotics might be used as alternative medicines for the treatment of *M. pneumoniae* infection in cases of high macrolide resistance in Zhejiang, China.

In conclusion, macrolide resistance of *M. pneumoniae* in Zhejiang, China, was at a high level among adult patients, and a A2063G transition in domain V of 23S rRNA was found in all macrolide-resistant *M. pneumoniae* isolates. All adult patients infected with macrolide-resistant *M. pneumoniae* can treated with fluoroquinolones or minocycline instead of macrolides. This finding also highlights the fact that local surveillance would be significant in determining the prevalence of macrolide resistance among *M. pneumoniae* strains and may provide important information regarding effective therapy for *M. pneumoniae* infections.

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We declare that the experiments performed and described here comply with the current laws of the People's Republic of China.

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