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

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*Mycoplasma pneumoniae* is a major pathogen causing community-acquired pneumonias (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 surveyed 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 may lead to 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.
**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, Zhiyui 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, dysnea, or pulmonary rales) and pulmonary radiography.

Rapid detection by nested PCR for *M. pneumoniae* was performed originally using primers based on the P1 adhesin 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 cultured on PPLO broth and agar was performed with PPLO broth and agar was performed as described previously (19). Chromogenic cultures were read once every 24 to 48 hours and red colonies were subcultivated on fresh PPLO agar and PPLO broth (1:100). The broth method with pleuropneumonia-like organism (PPLO) broth was performed as described previously (19).

Antimicrobial susceptibility. 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).

**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.

**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 adhesin 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 23S rRNA gene was more active than both levofloxacin and ciprofloxacin.

PCR-RFLP typing of the P1 gene. PCR-RFLP 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 HaeII restriction endonuclease (NEB, Shanghai, China). The digested samples were analyzed on a 1.2% agarose gel.

**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...
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 assay 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 sharpenly 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.

<table>
<thead>
<tr>
<th>Isolate group†</th>
<th>ERY (µg/ml)</th>
<th>CLR (µg/ml)</th>
<th>AZM (µg/ml)</th>
<th>JOS (µg/ml)</th>
<th>RKI (µg/ml)</th>
<th>MIN (µg/ml)</th>
<th>DOX (µg/ml)</th>
<th>LVX (µg/ml)</th>
<th>CIP (µg/ml)</th>
<th>GAT (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical isolates (A2063G)</td>
<td>128 to &gt;256</td>
<td>128 to &gt;256</td>
<td>32 to &gt;256</td>
<td>1 to 8</td>
<td>0.064 to 1</td>
<td>0.031 to 1</td>
<td>0.125 to 1</td>
<td>0.25 to 2</td>
<td>0.5 to 2</td>
<td>0.016 to 0.125</td>
</tr>
<tr>
<td>Reference strain FH</td>
<td>0.016</td>
<td>0.008</td>
<td>0.002</td>
<td>0.063</td>
<td>0.01</td>
<td>0.031</td>
<td>0.063</td>
<td>0.5</td>
<td>1</td>
<td>0.125</td>
</tr>
</tbody>
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† As characterized by mutation in the 23S rRNA gene.

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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.

REFERENCES


2. Chiromma M, Sallustio A, Esposito S, Perulli M, Chinellato I, Di Bari C,


