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# Antibiotic Sensitivity of 40 *Mycoplasma pneumoniae* Isolates and Molecular Analysis of Macrolide-Resistant Isolates from Beijing, China

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**MICs of eight antibiotics were detected with 40 Chinese *Mycoplasma pneumoniae* isolates. Thirty-eight isolates (95%) were macrolide resistant. Each macrolide-resistant isolate harbored an A2063G or A2064G point mutation in the 23S rRNA gene. All 40 isolates (100%) were type I strains, but they might have originated from different clones.**

*Mycoplasma pneumoniae* is a common pathogen causing community-acquired pneumonia (CAP) (4, 9, 12). Macrolides are the drugs of primary choice for the treatment of *M. pneumoniae* infections. Macrolide resistance rates of *M. pneumoniae* have increased rapidly in recent years, especially in Asia (3, 10, 11). In 2009, studies from China found that 83% (44/53) and 92% (46/50) of *M. pneumoniae* strains isolated from pediatric patients in Shanghai (6) and Beijing (14) were resistant to macrolides, respectively. In 2010, the macrolide resistance rate of *M. pneumoniae* isolated from ambulatory adult patients was 69% (46/67) in Beijing (1).

In this study, 40 *M. pneumoniae* isolates were collected from CAP patients in Beijing, China. The antibiotic resistance patterns of this pathogen were surveyed with eight agents, and the mechanisms of resistance for macrolide-resistant isolates were investigated with 23S rRNA gene analysis.

***M. pneumoniae* strains.** Forty *M. pneumoniae* isolates were obtained from 182 CAP patients in the Beijing, Dongcheng, and Xicheng Centers for Disease Control and Prevention from January 2011 to June 2011. The clonalities of all *M. pneumoniae* isolates were determined by the filtration-cloning technique and identified by colony morphology and real-time PCR assays (2).

**Antimicrobial susceptibility testing of isolates.** The MICs of eight antibiotics were determined by broth microdilution methods with SP4 broth (Remel). *M. pneumoniae* reference strain M129 (ATCC 29342) was tested as an antibiotic-sensitive control. Thirty-eight (95%) isolates were macrolide resistant. The $MIC_{50}$ values of isolates for erythromycin, clarithromycin, azithromycin, and josamycin were greater than 256 $\mu$g/ml, 256 $\mu$g/ml, 32 $\mu$g/ml, and 4 $\mu$g/ml, respectively. The $MIC_{90}$ values of the isolates with the four macrolides above were greater than 256 $\mu$g/ml, 256 $\mu$g/ml, 32 $\mu$g/ml, and 8 $\mu$g/ml, respectively. All *M. pneumoniae* isolates were susceptible to tetracycline and fluoroquinolones (Table 1). Gatifloxacin was more active than ciprofloxacin and levofloxacin. The $MIC_{90}$ of gatifloxacin (0.064 $\mu$g/ml) was much lower than those of ciprofloxacin (1 $\mu$g/ml) and levofloxacin (1 $\mu$g/ml).

**Sequencing analysis of the 23S rRNA gene.** Genomic DNA of each isolate was extracted with the QIAamp DNA minikit (Qiagen). Domains II and V of the 23S rRNA gene were amplified by methods described previously (8). All the amplicons were sequenced by the Beijing Genomics Institute (BGI). Thirty-seven isolates harbored an A2063G mutation in domain V of the 23S rRNA gene, while one isolate harbored an A2064G mutation in domain V of the 23S rRNA gene. No mutations were found in domain II (Table 1).

**Typing of the p1 gene.** All 40 *M. pneumoniae* isolates from CAP patients were genotyped by the PCR method described previously (5). PCR analysis of the p1 genes showed that all (100%) isolates were characteristic of type I. The variable-number tandem repeat (VNTR) sequence in the p1 gene (15) was amplified using the primer pair VNTR-F (5′-GATACCGCTACCGTACCTCG-3′) and VNTR-R (5′-TGAGAATAGCAGCAAACAAGGA-3′). The reaction conditions were 30 cycles of 98°C for 15 s, 56°C for 15 s, and 72°C for 30 s. PCRs were performed using the PrimeSTAR Kit (TaKaRa). Sequence analysis showed that the stable "AGT" VNTR in the p1 gene of each *M. pneumoniae* isolate appeared 4 to 10 times (Table 2).

In general, macrolides are used as the first-choice therapeutic agent for treating *M. pneumoniae* infections in children, as well as in adults. The macrolide resistance rate has been shown to be very high in *M. pneumoniae* strains isolated in China in recent years (1, 6, 14). This study showed a macrolide resistance rate of 95% in Beijing. Our previous study found that adult and adolescent patients infected with macrolide-resistant *M. pneumoniae* required significantly longer durations of antibiotic therapy and needed longer times to recuperate from fever (1). Although *M. pneumoniae* isolates were susceptible to fluoroquinolones, this class of drugs is not ordinarily recommended for children, except in particular cases.

Macrolide resistance in *M. pneumoniae* is strongly associated with mutations in the 23S rRNA gene (7, 13). In fact, A2063G and A2064G mutations have been shown to be responsible for high-level macrolide resistance in *M. pneumoniae* (8). The A2063G mutation in domain V of the 23S rRNA gene is recognized to be the most prevalent in macrolide-resistant *M. pneumoniae* isolates (8). More than 95% of the reported macrolide-resistant *M. pneu-*

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**TABLE 1** Genotype characteristics and MIC ranges of eight antimicrobial agents against 40 *M. pneumoniae* clinical isolates

| Sequence type of p1 gene | Mutation in 23S rRNA gene | No. of isolates | MIC[a] (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | ERY | CLR | AZM | JOS | TET | CIP | LVX | GAT |
| Type I | A2063G | 37 | 256–>256 | 64–>256 | 8–64 | 2–8 | 0.032–0.125 | 0.5–2 | 0.25–2 | 0.032–0.125 |
| Type I | A2064G | 1 | >256 | >256 | 32 | 4 | 0.125 | 1 | 1 | 0.125 |
| Type I | None | 2 | 0.008–0.016 | <0.008 | <0.008 | 0.064 | 0.125 | 0.5–1 | 1 | 0.125 |

[a] MICs were determined by microdilution methods using SP4 broth. The MIC of each agent was defined as the lowest concentration of each antibiotic preventing the color change. ERY, erythromycin; CLR, clarithromycin; AZM, azithromycin; JOS, josamycin; TET, tetracycline; CIP, ciprofloxacin; LVX, levofloxacin; GAT, gatifloxacin.

*moniae* isolates in China had this mutation, including those in this study. Only one isolate with the A2064G mutation was detected in this study; this mutation locus is rarely reported.

Since 2007, the predominant *M. pneumoniae* strains isolated from China have been type I (1, 6, 14). Most macrolide-resistant isolates have also been found to belong to this type. In this study, all of the macrolide-resistant isolates belong to type I strains. In a previous study, a shift from type II to type I was found in Japan from 2000 to 2004, and macrolide-resistant isolates were also found in both types (8). It has been suggested that the macrolide resistance for *M. pneumoniae* is not associated with a specific p1 gene type but the current predominant isolate. Although all macrolide-resistant isolates belong to type I in this study, different numbers of stable "AGT" VNTRs (15) in the p1 gene of each isolate appeared (Table 2). Based on the VNTR data, these type I macrolide-resistant *M. pneumoniae* isolates spread in Beijing belonged to different resistant clones. Since the numbers of stable "AGT" VNTRs in the p1 gene are different among the macrolide-resistant isolates, the question mentioned in previous studies (1, 6) that asks whether or not all *M. pneumoniae* isolates identified with the same p1 type are derived from the same clone has now been answered. This also may be a better locus for the multilocus VNTR analysis (MLVA) typing method.

In summary, 95% (38/40) of *M. pneumoniae* isolates from CAP patients were macrolide resistant. Each macrolide-resistant isolate harbored a point mutation in the 23S rRNA gene. All *M. pneumoniae* isolates in this study were type I, which is the predominant type in Beijing. Based on the different numbers of stable "AGT" VNTRs in the p1 gene, these macrolide-resistant *M. pneumoniae* isolates spread in Beijing were shown to belong to different resistant clones.

**TABLE 2** VNTRs in all 40 *M. pneumoniae* isolates

| No. of "AGT" VNTRs | No. detected |
|---|---|
| 4 | 2 |
| 5 | 6 |
| 7 | 16 |
| 8 | 11 |
| 9 | 2 |
| 10 | 3 |

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