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Antimicrobial Susceptibility of *Mycoplasma pneumoniae* Isolates and Molecular Analysis of Macrolide-Resistant Strains from Shanghai, China▽

Yang Liu,[1] Xinyu Ye,[1] Hong Zhang,[2] Xiaogang Xu,[1] Wanhua Li,[2] Demei Zhu,[1] and Minggui Wang[1,3]*

Institute of Antibiotics, Huashan Hospital,[1] and Institute of Biomedical Sciences,[3] Fudan University, Shanghai 200040, China, and Shanghai Children's Hospital, Shanghai Jiaotong University, Shanghai 200040, China[2]

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Fifty-three *Mycoplasma pneumoniae* strains were isolated from pediatric patients in Shanghai, China, from October 2005 to February 2008. Of 53 clinical isolates, 44 (83%) were resistant to erythromycin (MICs of >128 μg/ml for all 44 strains), azithromycin, and clarithromycin. All macrolide-resistant *M. pneumoniae* strains harbored an A-to-G transition mutation at position 2063 in 23S rRNA genes. Forty-five (85%) clinical isolates were classified into the P1 gene restriction fragment length polymorphism type I, and six (11%) were type II.

Mycoplasma pneumoniae is increasingly recognized as common and important pathogen in community-acquired respiratory tract infections (RTIs) and pneumonia, particularly in school-age children and young adults (9, 13). *M. pneumoniae* isolates are usually susceptible to macrolides; thus, macrolides are the most important drugs for treatment of RTI infections, especially in children (13). Fluoroquinolones and tetracyclines, which may be used as alternatives to macrolides, are not recommended for children. Macrolide-resistant *M. pneumoniae* strains have been reported in Japan, China, France, and the United States (6–8, 12, 16, 17).

In this study, 53 strains of *M. pneumoniae* were collected from pediatric patients with RTIs in Shanghai, China. The in vitro activities of macrolides, tetracyclines, and fluoroquinolones against *M. pneumoniae* isolates were determined, and the mechanisms of resistance for macrolide-resistant stains were investigated. In addition, the type of *M. pneumoniae* clinical isolates was determined by PCR-restriction fragment length polymorphism (RFLP) typing of the P1 gene.

***M. pneumoniae* strains.** Fifty-three *M. pneumoniae* isolates were obtained from bronchial aspirations (each specimen collected from one patient) in the Shanghai Children's Hospital from October 2005 to February 2008. There were 6 isolates in 2005, 17 in 2006, 24 in 2007, and 6 in 2008. Culture of *M. pneumoniae* was performed as described previously by Waites (14). All of the strains were identified by colony morphology and PCR assay. PCR amplification of 16S rRNA genes was done to identify *M. pneumoniae* using primers with sequences 5′-GCCACCCTCGGGGGCAGTCAG-3′ and 5′-GAGTCGG GATTCCCCGCGGAGG-3′ as previously described (4).

Antimicrobial susceptibility of *M. pneumoniae*. To determine the MICs, the broth microdilution method with SP4 broth (Remel, Lenexa, KS) was performed as described previously by Waites (14). Every susceptibility testing was repeated three times. *M. pneumoniae* reference strain MPFH (ATCC 15531) was also included. Comparative in vitro activities of 10 antimicrobials are listed in Table 1. All of the *M. pneumoniae* isolates were susceptible to the tetracyclines and fluoroquinolones tested in this study. Moxifloxacin was more active than ciprofloxacin and levofloxacin. The MIC_{50} and MIC_{90} of moxifloxacin were 0.03 μg/ml and 0.06 μg/ml, respectively—much lower than those of ciprofloxacin and levofloxacin.

Among 53 strains, 44 (83%) were resistant to erythromycin with a MIC_{90} of >128 μg/ml to either erythromycin or clarithromycin. These strains also showed high MICs to azithromycin and josamycin, with MIC_{90}s of 64 μg/ml and 4 μg/ml, respectively (Table 1). All 44 macrolide-resistant strains had erythromycin MICs of >128 μg/ml. The 16-member macrolide josamycin possessed lower MICs than the 14- and 15-member macrolides with MICs of 1 to 8 μg/ml. Of 53 *M. pneumoniae* strains, only 9 were susceptible to erythromycin. Most of these nine strains had MICs of ≤0.007 to 0.015 μg/ml for four macrolides tested, but two strains had MICs of 0.5 μg/ml for josamycin (Table 2). The resistance rates of *M. pneumoniae* isolates from 2005 to 2008 were 16.7% (1/6), 76.5% (13/17), 100% (24/24), and 100% (6/6), respectively.

TABLE 1. In vitro activities of 10 antimicrobial agents against 53 *M. pneumoniae* strains

Antimicrobial	MIC (μg/ml) for[a]:			
	Clinical isolates			Reference strain MPFH
	Range	50%	90%	
Erythromycin	≤0.007–>128	>128	>128	≤0.007
Clarithromycin	≤0.007–>128	128	>128	≤0.007
Azithromycin	≤0.007–>128	16	64	≤0.007
Josamycin	≤0.007–8	2	4	≤0.007
Tetracycline	0.015–0.25	0.06	0.125	0.06
Doxycycline	≤0.007–0.125	0.06	0.125	0.03
Minocycline	≤0.007–0.125	0.06	0.125	0.06
Ciprofloxacin	0.015–1	0.5	1	0.25
Levofloxacin	0.015–1	0.25	0.5	0.25
Moxifloxacin	≤0.007–0.06	0.03	0.06	0.06

[a] 50% and 90%, MIC_{50} and MIC_{90}, respectively.

* Corresponding author. Mailing address: Institute of Antibiotics, Huashan Hospital, Fudan University, 12 M. Wulumuqi Rd., Shanghai 200040, People's Republic of China. Phone: (86-21) 52888195. Fax: (86-21) 62482859. E-mail: mgwang@fudan.edu.cn.

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TABLE 2. Distribution of MICs of 10 antimicrobial agents for 53 *M. pneumoniae* strains

Antimicrobial	No. of strains with MIC (μg/ml) of:															
	≤0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Erythromycin	4	4		1												44
Clarithromycin	6	3												17	7	20
Azithromycin	4	3	2									7	10	27		
Josamycin	7						2	3	16	24	1					
Tetracycline		12	7	15	18	1										
Doxycycline	1	15	7	16	14											
Minocycline	2	8	6	11	26											
Ciprofloxacin		2				9	36	6								
Levofloxacin		2		1		24	24	2								
Moxifloxacin	1	11	30	11												

Sequencing analysis of 23S rRNA genes. Primer pairs MP23SF (5′-CAATAAGTTACTAAGGGCTTATGGTGGATGC-3′) and MP23SR (5′-TCCAATAAGTCCTCGAGCAATTAGTATTACTCAG-3′) were used to amplify and sequence the full length of the 23S rRNA fragment. The PCR conditions were 10 min at 94°C first, followed by 30 s at 94°C for denaturation, 30 s at 61°C for annealing, and 4 min at 72°C for elongation for 40 cycles followed by 10 min at 72°C at the end of 40th cycle. The L4 and L22 ribosomal protein genes were also amplified and sequenced by methods described previously (11). Among 53 *M. pneumoniae* strains, all 44 macrolide-resistant strains harbored an A-to-G transition mutation at position 2063 in domain V of the 23S rRNA genes. In addition, an A1290G transition mutation was found in all 53 tested strains regardless of sensitivity or resistance to erythromycin, except for the reference strain, MPFH, which indicates the A1290G change is not related to macrolide resistance. No mutation was found in the L4 and L22 ribosomal protein genes.

PCR-RFLP typing of the P1 gene. P1 gene PCR-RFLP typing was performed as previously described (2). Briefly, a fragment of the P1 gene was amplified using primers ADH1 and ADH2 (2) and then digested with HpaII restriction endonuclease (TaKaRa Biotechnology, Dalian, China). The resulting fragments were analyzed on a 2% agarose gel. Forty-five (85%) clinical isolates were classified into type I, 6 (11%) were classified into type II, and the other 2 (4%) could not be classified by this method. Thus, type I was predominant among the isolates tested. Forty-one (91.1%) of 45 type I strains were resistant to erythromycin; however, only 1 (16.7%) out of 6 type II strains was resistant to erythromycin. A shift in *M. pneumoniae* since 2006 from type II to type I was observed. All but two unclassified *M. pneumoniae* strains isolated in 2007, all strains isolated in 2008, and almost all strains isolated in 2006 were found belong to type I.

Both fluoroquinolones and tetracyclines have good activities against *M. pneumoniae* strains isolated from children in Shanghai; however, the resistance rate to erythromycin was very high. Macrolide-resistant *M. pneumoniae* isolates were first found in 1968 and have been spreading since 2000 (6, 7, 10). About 17% (13/76) and 6% (12/195) of *M. pneumoniae* clinical strains isolated from 2000 to 2004 in Japan were resistant to erythromycin (6, 7). A recent report from Japan showed that erythromycin-resistant strains increased from 0% (0/47) in 2002 to 30.6% (37/121) in 2006 (8). In addition, a recent report showed that the erythromycin resistance rate was 92% (46/50) in *M. pneumoniae* strains isolated in Beijing, China (17). In this study, 44 (83%) out of 53 *M. pneumoniae* strains isolated from children showed resistance to erythromycin, and the resistance rates increased from 17% in 2005 to 76% in 2006 and 100% in 2007 and 2008. Thus, macrolide-resistant *M. pneumoniae* isolates seem to spread rapidly in certain parts of Asia.

Macrolide resistance in *M. pneumoniae* is associated with mutations at 23S rRNA or ribosomal proteins L4 and L22 (5, 15). The A2063G mutation is found to be most prevalent in macrolide-resistant *M. pneumoniae* isolates, followed by the A2064G mutation. Other mutations like A2617G and mutations in ribosomal proteins L4 and L22 were very rare (1, 15). Among 13 erythromycin-resistant *M. pneumoniae* strains isolated in Japan from 2000 to 2003, 10 (77%) strains had an A2063G transition and 1 (8%) each had an A2063C, A2064G, or A2617G transition (6). Morozumi et al. (7, 8) identified 67 macrolide-resistant *M. pneumoniae* isolates from Japan between 2002 and 2006 in two different reports. An A2063G transition was found in 59 (88%) strains and an A2064G transition in 7 (10%) strains, and no mutation was found in 1 strain. In this study, the A-to-G transition mutation at position 2063 in domain V of 23S rRNA genes was found in all 44 erythromycin-resistant *M. pneumoniae* isolates.

A strong association between the P1 gene PCR-RFLP type and erythromycin resistance has been demonstrated in this study. When the erythromycin resistance rates increased markedly since 2006, a shift from type II to type I of the P1 gene type was found in this study: all strains isolated in 2007 and 2008 that were resistant to erythromycin were found belong to type I, except for two unclassified strains. Although *M. pneumoniae* isolates identified with the same P1 subtype may not necessarily be the same clone, it is possible the same resistant clone belonged to type I spread in Shanghai. Further studies of clonal spread are under investigation by pulsed field gel electrophoresis (7) and multiple-locus variable number of tandem repeat analysis (3).

In summary, clinical strains of *M. pneumoniae* isolated from children in Shanghai, China, are found to have high resistance rate to macrolides. Mutation at A2063G in domain V of 23S rRNA is associated with macrolide resistance. The P1 gene RFLP type I is predominant among the clinical isolates of *M. pneumoniae* tested.

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