REVIEW ARTICLE

# Macrolide-resistant *Mycoplasma pneumoniae*: characteristics of isolates and clinical aspects of community-acquired pneumonia

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**Abstract** *Mycoplasma pneumoniae* is one of the main pathogens causing community-acquired respiratory tract infections in children and adults. Macrolide (ML) antibiotics are recognized generally as first-choice agents for *M. pneumoniae* infections, and these antibiotics were thought to have excellent effectiveness against *M. pneumoniae* for many years. In 2000, however, *M. pneumoniae* showing resistance to macrolides was isolated from clinical samples obtained from Japanese pediatric patients with community-acquired pneumonia (CAP). Since then, prevalence of ML-resistant *M. pneumoniae* isolates in pediatric patients has increased rapidly. In 2007, ML-resistant *M. pneumoniae* isolates were obtained from Japanese adults with CAP; numbers of such isolates also have gradually increased in Japan. Recently, similar antimicrobial resistance in *M. pneumoniae* has begun to emerge worldwide. In this review, we focus on changes of ML-resistant *M. pneumoniae* from year to year and consider resistance mechanisms as well as clinical features of patients with resistant *M. pneumoniae* infection.

## Introduction

Sixteen *Mycoplasma* species have been isolated from human respiratory and urogenital specimens [1]. One of these species, *M. pneumoniae*, is a main pathogen in respiratory tract infections (RTI) acquired in the community. In school-aged children and young adults with community-acquired pneumonia (CAP), *M. pneumoniae* accounts for as many as 10–30% of cases [2–8]. In distinction, *M. pneumoniae* pneumonia in younger children and in the elderly is infrequent but not rare [9–15].

*M. pneumoniae* pneumonia is diagnosed based on characteristic chest radiographic abnormalities, patient symptoms, and clinical laboratory data. Diagnosis ultimately is confirmed using serologic tests performed upon paired sera obtained during both acute and convalescent phases. Conventional culture using pleuropneumonia-like organism (PPLO) broth, which requires more than 2 weeks, has not been carried out routinely. As a consequence, antibiotic choice usually is empirical.

Development of molecular methods such as polymerase chain reaction (PCR) assays to be used in combination with conventional diagnostic tests using serology and culture have contributed to improved diagnosis and characterization of *M. pneumoniae* infection in pediatric and adult patients [16–28]. Macrolides (ML) such as clarithromycin (CAM) and azithromycin (AZM) generally are recognized as first-choice agents against *M. pneumoniae* [29–33]. However, in parallel with increased use of oral ML with a

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14-membered ring (14-ML) and AZM for RTI, *M. pneumoniae* showing resistance to ML has been isolated increasingly in clinical samples from Japanese pediatric patients with CAP [31, 34, 35]. Prevalence of ML-resistant (ML$^r$) *M. pneumoniae* in pediatric patients has increased rapidly [35, 36] and ML$^r$ *M. pneumoniae* is beginning to be isolated worldwide [37–41].

In this review, we summarize the emergence and increase of ML$^r$ *M. pneumoniae* isolates, resistance mechanisms, and clinical features of ML$^r$ *M. pneumoniae* infection.

## Diagnosis of infection

*M. pneumoniae* infection tends to show cyclic epidemics every 3–5 years; these outbreaks are particularly likely to occur in summer or early fall [42–46]. Although the incidence of *M. pneumoniae* pneumonia is highest in children 5–9 years old, such pneumonia also occurs frequently in younger children and in the elderly. Prevalence of *M. pneumoniae* infection also may vary according to population and diagnostic methods used.

Isolation of *M. pneumoniae* by culture from clinical samples such as throat or nasopharyngeal swabs was considered standard for diagnosis several years ago. However, *M. pneumoniae* is difficult to grow in liquid culture; cultures using PPLO broth require at least 2 weeks for completion. Compared with serologic tests or molecular techniques, including PCR, sensitivity of cultures may be <60% or 70%, even in laboratories with expertise [6, 18, 20]. Therefore, culture methods are only used rarely for routine diagnosing *M. pneumoniae* infection.

Serologic methods are used most frequently to diagnose *M. pneumoniae* infection. Frequently used, widely available serologic tests for *M. pneumoniae* include complement fixation (CF), enzyme immunoassay (EIA), and particle agglutination (PA) assays. However, these ordinarily require paired sera obtained during acute and convalescent phases in order to demonstrate rises in antibody titers; fourfold increase is thought to be significant. The second sample should be obtained 7–14 days after the first, provided that *M. pneumoniae* infection still is suspected.

Although one alternative, the ImmunoCard based on an immunoglobulin M (IgM) assay, is rapid and easy to perform, reliable diagnosis of *M. pneumoniae* infection still cannot be made on the basis of single acute-phase sera; confirmation using convalescent-phase samples still is necessary, as otherwise, both false-positive and false-negative results are frequent [24, 47, 48]. Additionally, sensitivity of IgM assays tends to be low, particularly for adult patients who are known to have weak IgM responses during primary infection or reinfection [49, 50]. Neither

culture nor serologic testing can provide timely information for guiding choice of chemotherapeutic agents to use for early intervention. Serologic methods generally have only low sensitivity during the acute phase of disease.

Development of molecular methods (e.g. PCR) has lessened the importance of culture as a way to detect *M. pneumoniae* directly. Two specific targets in the P1 adhesion gene and the 16S ribosomal RNA (rRNA) gene are the main ones used in PCR assays to detect *M. pneumoniae* DNA. Various PCR methods have been developed in several laboratories. *M. pneumoniae* detection by conventional PCR or real-time PCR has been examined as an approach to early diagnosis. Many studies have described application of multiplex PCR, hybridization assays, nucleic acid sequence-based amplification (NASBA), real-time PCR using a molecular beacon probe or TaqMan probe, or cycling probe, to the identification of *M. pneumoniae* DNA [16–28]. Among these methods, real-time PCR has both high sensitivity and high specificity and can detect pathogen DNA even when damaged by empirical administration of antibiotics. Sensitivity (60–100%) and specificity (96.7–100%) of real-time PCR are both higher than those of serologic assays for *M. pneumoniae* [18, 19, 22, 24–26]. Almost all PCR-positive cases (>90%) also were confirmed serologically [22, 24]. In addition, real-time PCR using a fluorescent probe allows continuous monitoring of in vitro DNA amplification, eliminating nonspecific amplification product using fluorescence; no gel electrophoresis is needed. This makes the method suitable for clinical laboratory settings. A molecular approach including real-time PCR is useful for confirming *M. pneumoniae* and providing rapid diagnosis of CAP, thus permitting rational choice of effective antimicrobial agents.

## Mechanisms of ML resistance in *M. pneumoniae*

Intrinsically, absence of cell walls in *M. pneumoniae* confers resistance to $\beta$-lactams, and indeed to all antibiotics that inhibit synthesis of cell walls. In *M. pneumoniae* infections, 14-ML and 15-membered ring ML (15-ML) usually are considered the first-line agents. *M. pneumoniae* ordinarily are susceptible to all ML, including ketolides.

Main mechanisms of microorganism resistance to ML identified in previous studies include modification of the target sites in 23S rRNA by methylation or mutation, as well as on efflux pump [51–53]. ML act on the 50S ribosomal subunit to inhibit protein synthesis. The site of peptide bond formation on the large 50S ribosomal subunit is associated with the central loop in domain V of 23S rRNA. Nucleotides present in the central loop are necessary constituents of the ML binding site, which implies that all ML bind to the same site (Fig. 1a). A loop of hairpin 35
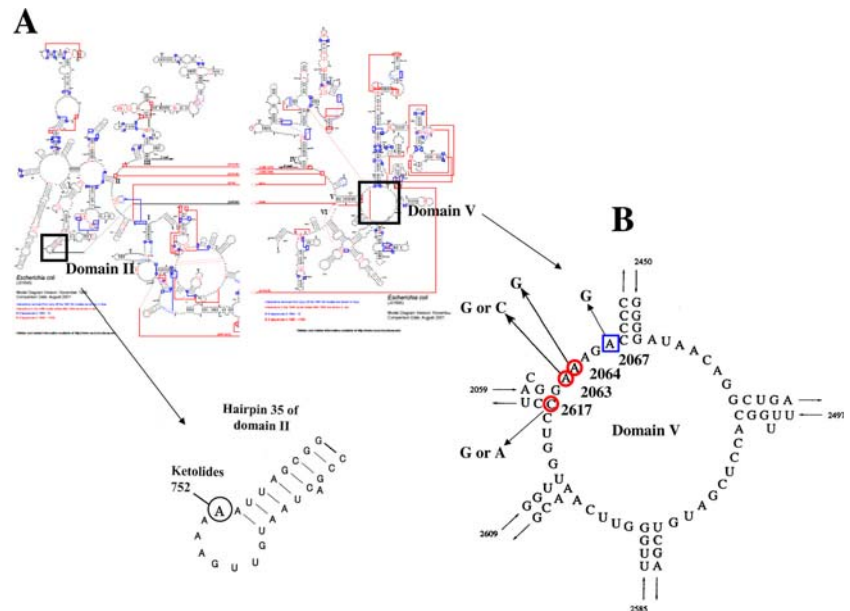
in domain II also may be part of binding site of ketolides; mutations involving ribosomal proteins L4 and L22 may contribute as well [51–54].

ML inhibit protein synthesis mainly by binding to domain V of 23S rRNA at nucleotide positions 2063 and 2064 (*M. pneumoniae* numbering); these positions appear essential for binding (Fig. 1b) [55]. Mutations at A2063 or A2064 confer the highest resistance to these antimicrobials. A lower level of antibiotic resistance is caused by muta- tions at positions A2067 and C2617, which are near A2063 and A2064 in the secondary structure, although slightly present outside the central point of ML interaction. The A2067G mutation was reported to cause highest levels of resistance only for 16-membered-ring ML (16-ML), as it interferes with formation of the covalent bond specific to 16-ML [56].

Ketolides are a new class of antimicrobial agent char- acterized by the substitution at position 3 from ʟ-cladinose to keto group. These antibiotics are active against *Strep- tococcus pneumoniae* strains resistant to ML because of interaction at both A2058/2059 (*Escherichia coli* number- ing) in domain V and A752 in domain II [51, 54, 57]. Substitutions in ribosomal proteins L4 and L22 have been found to confer resistance in *S. pneumoniae* [58, 59], but mutations in domain II of 23S rRNA and in ribosomal proteins L4 and L22 are not involved in ML resistance of clinical *M. pneumoniae* isolates [60]. Furthermore, neither plasmids nor *erm* genes mediating ribosomal modification have been described with respect to *M. pneumoniae*.

Fluoroquinolone (FQ)-resistant *M. pneumoniae* mutants may be obtained in vitro by exposure to subinhibitory concentrations of FQ [61]. These mutants possess muta- tions in *gyrA* and *gyrB* genes, which express topoisomerase

II, and in *parC* and *parE* genes, which express topoiso- merase IV; both of these enzymes are essential for bacterial DNA replication. FQ often are chosen as empiric treatment of respiratory tract infection in adults, as they are active against the main pathogens, including *S. pneumoniae*, *Haemophilus influenzae*, and *M. pneumoniae*. In contrast to in vitro findings, no FQ-resistant strains have been observed in clinical settings.

Tetracyclines inhibit protein synthesis by preventing association of aminoacyl-tRNA to the acceptor (A) site of the ribosome [62, 63]. These antibiotics bind to the ribo- somal 30S subunit involving 16S rRNA and several ribo- somal proteins. Only the *tetM* determinant associated with conjugative transposon Tn*916*, which confers resistance to the tetracyclines in *M. hominis*, has been documented so far [64]. These agents are administered for treatment of *M. pneumoniae* infection in adults and in pediatric patients >8 years, but development of resistance to tetracyclines has not yet been reported.

## Association of ML resistance and mutation site in 23S rRNA from ML^r *M. pneumoniae*

Specific sites of the 23S rRNA mutation contribute to ML resistance in *M. pneumoniae*. In actuality, based on our results, clinical isolates with mutations at positions A2063 and A2064 show high resistance to 14-ML and 15-ML, with minimal inhibitory concentration (MIC) ≥ 32 µg/ml (Table 1). Especially, the A2063G mutation conveys the highest level of resistance to 14-ML, 15-ML, and teli- thromycin (TEL) but intermediate resistance to josamycin and rokitamycin belong to 16-ML. The A2063C and

**Table 1** Antimicrobial activity of macrolides, fluoroquinolones, and minocycline against *Mycoplasma pneumoniae* strains isolated from clinical samples

| ML$^r$ class | MIC range (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| Antimicrobial agent | Susceptible strains ($n = 423$) | A2063G ($n = 96$) | A2064G ($n = 7$) | A2063C ($n = 1$) | C2617A ($n = 1$) | C2617G ($n = 1$) |
| Erythromycin | 0.00195–0.0313 | 32 to >64 | 64 to >64 | >256 | 1 | 8 |
| Clarithromycin | 0.00049–0.0313 | 32 to >64 | 16 to >64 | >256 | 0.5 | 1 |
| Azithromycin | 0.00012–0.00195 | 16 to >64 | 16–64 | 16 | 0.0313 | 0.0313 |
| Telithromycin | 0.00024–0.0039 | 16 to >64 | 1–16 | ND | 0.0625 | ND |
| Josamycin | 0.0156–0.0625 | 0.0625–64 | 64 to >64 | 64 | 0.0625 | 0.25 |
| Midecamycin | 0.0625–0.25 | 2 to >64 | >64 | 64 | ND | 0.25 |
| Rokitamycin | 0.0039–0.0313 | 0.0156–16 | 8–16 | 4 | 0.0313 | 0.0625 |
| Levofloxacin | 0.125–1 | 0.5–1 | 0.5–1 | ND | 1 | ND |
| Moxifloxacin | 0.0625–0.125 | 0.0625–0.125 | 0.0625–0.125 | ND | 0.125 | ND |
| Sitafloxacin | 0.0313–0.0625 | 0.0313–0.0625 | 0.0313–0.0625 | ND | 0.0625 | ND |
| Garenoxacin | 0.0313–0.0625 | 0.0313–0.0625 | 0.0313–0.0625 | ND | 0.0313 | ND |
| Minocycline | 0.0313–2 | 0.0625–1 | 0.0313–1 | ND | 1 | ND |

MICs of susceptible strains and resistant strains with A2063G, A2064G, and C2617A mutations were determined in our laboratory [31, 36]

MIC data for strains with A2063C and C2617G mutations were adapted from [35, 65]

*MIC* minimal inhibitory concentration, *ML$^r$* macrolide resistance, *ND* not determined

A2064G mutations result in a high level of resistance to all ML [36, 65]. The ketolide TEL is affected by both mutations, although A2063G was associated with MICs higher than those associated with A2064G.

MICs for minocycline (MINO) and FQ in ML$^r$ strains were equivalent to those in susceptible strains. No strains having resistance to MINO and FQ have been observed among clinical isolates.

Several strains with the C2617 to G or A mutations show slight increases in MIC of ML compared with those with mutations at positions 2063 and 2064. A2063G in domain V of 23S rRNA is the most frequent mutation association with ML resistance, followed by A2064G; others (A2063C, C2617G, and C2617A) are rare.

Ribosomal protein L4 and L22 mutants with a few amino acids deleted or inserted have been described, but roles of these mutations in resistance to ML are uncertain [60]. No mutation has been detected in domain II of 23S rRNA.

### Emergence and increase of ML$^r$ strains

ML$^r$ *M. pneumoniae* isolates possessing a nucleotide mutation in 23S rRNA first were isolated from pediatric patients with CAP, as reported by Okazaki and colleagues in 2001 [34].

Together with growing use of ML in Japan, resistant strains increased rapidly year by year (Fig. 2) [36]. Along with greater overall prevalence of *M. pneumoniae* infection in 2006, ML$^r$ strains increasingly were isolated and identified in diverse regions across Japan.
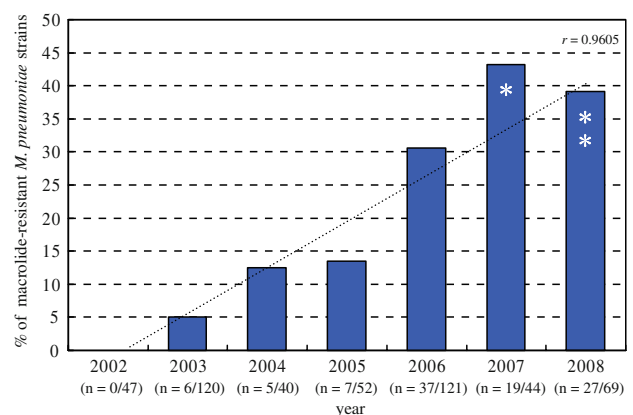


**Fig. 2** Increases in numbers of macrolide-resistant *Mycoplasma pneumoniae* strains from 2002 to 2008 in Japan according to our analyses. *Asterisks* indicate macrolide-resistant strains isolated from adult patients. The *dotted line* can be expressed by the equation $y = 7x - 8.62$ ($r = 0.9605$)

In Japanese adult CAP patients, ML$^r$ strains first were isolated in 2007; since then, occasional isolations have continued [66]. An increase in ML$^r$ strains among adult patients, las in pediatric patients, may occur in the future. However, FQ are recommended for CAP treatment in adults, and no FQ-resistant strains have been observed among isolates from adult patients with CAP.

Emergence of ML$^r$ isolates having either an A2063G or an A2064G mutation has been reported not only in Japan but also in other countries, including France, the USA, Denmark, and China [37–41]. A notably high isolation rate (92%) of ML$^r$ strains was reported in China [38, 40].

The emergence and increase of ML$^r$ *M. pneumoniae* has attracted the attention of microbiologists worldwide. Several recent studies demonstrated direct detection of ML$^r$ isolates using real-time PCR [37, 41, 67].

DNA profiles of ML$^r$- and ML-susceptible (ML$^s$) strains obtained by pulsed-field gel electrophoresis (PFGE) have suggested classification into two groups [36, 68]. Significant differences in these DNA patterns were not recognized between strains, geographic locations, or years.

PCR-based restriction fragment length polymorphism (RFLP) types of the P1 adhesin gene also were used to divide strains into two types. ML$^r$ isolates were present in both types [38, 39, 69, 70], showing no predominance.

## Clinical aspects of ML$^r$ *M. pneumoniae* infection

The clinical course of patients with ML$^r$ *M. pneumoniae* infection appears to be prolonged [36, 66, 71, 72]. Patients infected with ML$^r$ strains mostly had been treated with ML previously [36, 40]. Among CAP patients infected with ML$^r$, treatment frequently was changed from ML to MINO or levofloxacin (LVFX) because of either persistent symptoms (i.e., fever and cough) or unresolved or worsening chest radiographic abnormalities. In our observations, treatment was changed from ML to MINO or LVFX in 8.2% of patients with ML$^s$ strains and in 39.7% of patients with ML$^r$ strains, representing a significant difference between the two patient groups ($P < 0.01$). ML also was more frequently discontinued in favor of MINO among ML$^r$ patients than ML$^s$ patients in a report by Suzuki et al. [71]. Neither LVFX nor MINO are recommended for pediatric patients. However, when ML are ineffective against *M. pneumoniae* infection, pediatricians have little choice of antimicrobials except for MINO. In adult inpatients infected with ML$^r$ strains, antibiotic agents were changed from CAM to intravenous pazufloxacin when symptoms did not improve [66]. Orally administered respiratory FQ such as moxifloxacin, sitafloxacin, and garenoxacin may be selected for adult outpatients with ML$^r$ strains.

Among clinical features of ML$^r$ *M. pneumoniae* infection, fever duration has been significantly longer than for ML$^s$-strain infection. In our data, fever persisted after ML initiation for $1.6 \pm 0.8$ days in ML$^s$ *M. pneumoniae* infection and for $4.1 \pm 2.3$ days in ML$^r$-strain infection, showing a significant difference ($P < 0.01$). In other studies, febrile days during ML administration also were significantly greater in ML$^r$-infected patients than in ML$^s$-infected patients (3.5–4.0 days vs. 1.0–1.5 days) [71, 72]. Furthermore, the mean duration of persistent cough after ML administration was 7.0 days in ML$^s$-infected patients and 11.4 days in ML$^r$-infected patients [72]. Finally,

the efficacy rate of ML therapy was 91.5% and 22.7% in ML$^s$- and ML$^r$-strain infections, respectively [72].

Fever may have resolved spontaneously in some patients with continuing ML treatment for ML$^r$ infections; *M. pneumoniae* infection is associated with occasional spontaneous symptomatic recovery. According to previous reports, fever duration was 1 week in symptomatically treated *M. pneumoniae* infection [73, 74]. In another study, mean fever duration in hospitalized patients receiving AZM or EM was 2.1 days, whereas fever persisted for about 1 week in patients not receiving ML [75]. Similarly, mean cough duration was reported as 8.5 days [76]. Although treatment failure or serious illness has not yet been attributed to ML$^r$-strain infection, the clinical course of patients infected with ML$^r$ strains may be prolonged.

A brief consideration of the host response is helpful here. *M. pneumoniae* resides on the surface of ciliated human respiratory epithelial cell. Several membrane proteins in *M. pneumoniae* have high affinity for various surface receptors on host cells [77]. Through interaction with recognition receptors, including toll-like receptors 2 and 6, mycoplasma membrane lipoproteins are able to induce host immune responses [77, 78]. Adherence of *M. pneumoniae* to host cells in the respiratory tract is mediated by the P1 adhesin protein and accessory proteins [79–82]. Following adherence, macrophages become activated and release cytokines, and a mononuclear cell inflammatory response is established. Human lung epithelial cells infected with *M. pneumoniae* induce expression of interleukin (IL)-8, which is both a potent chemoattractant and an activator of neutrophils, monocytes, and T lymphocytes [83–85]. In particular, significant production of IL-8 and IL-18, a cytokine that induces interferon gamma (IFN-$\gamma$) production and promotes a type 1 cytokine response, is associated with the severity of *M. pneumoniae* pneumonia in children [86–88] and adults [89].

As previously described, the inflammatory response to *M. pneumoniae* infection is considered to play a crucial role in the pathogenesis of the ensuing clinical disease. ML possess antimicrobial activity against *M. pneumoniae*, and also have anti-inflammatory effects. For example, CAM is able to suppress IL-8 induction, even in ML$^r$ *M. pneumoniae* infection [78, 86, 90]. Thus, clinical efficacy of ML in treating *M. pneumoniae* infection may reflect not only direct antimicrobial activity but also anti-inflammatory effects of inhibition of production of cytokines, including IL-8.

Symptoms and severity of illness due to *M. pneumoniae* were similar in younger and older patients, and the mortality rate was low, even in the elderly [13, 91, 92]. This disease ordinarily is mild, and reinfection may occur in adults who already experienced *M. pneumoniae* infection, as protective immunity usually is not established following initial infection [93]. However, symptoms appear to be

prolonged in certain clinical situations regardless of anti-microbial resistance [15, 94]. Takahashi and colleagues reported *M. pneumoniae* DNA in sputum after 2 weeks of MINO therapy. Elderly persons with *M. pneumoniae* pneumonia may have a longer clinical course than younger persons. Further observations from various patients infected with *M. pneumoniae* are necessary.

In the future, alternative treatment strategies, such as promptly initiated steroid therapy, might be considered for patients with ML^r strains as a symptomatic measure [95–97].

## Conclusions

In pediatric patients with CAP, ML^r *M. pneumoniae* possessing a 23S-rRNA mutation was first isolated in 2000; numbers of these isolates have increased rapidly year by year in Japan. In other countries, ML^r *M. pneumoniae* also is emerging. Symptoms often are relatively prolonged in patients with ML^r *M. pneumoniae* infection. ML^r *M. pneumoniae* should be considered an emerging issue for not only pediatric patients but also adult patients with CAP.

No FQ-resistant *M. pneumoniae* strain has been observed thus far among specimens from adult patients with CAP. However, emergence of infections with FQ-resistant *M. pneumoniae* might occur, considering the increasing FQ prescription rate among adult patients. Further studies are needed to clarify the prevalence of ML^r *M. pneumoniae* in pediatric and adult patients with RTI and to establish clinical guidelines regarding the most appropriate antimicrobial agents to use against these strains. Interventions to prevent emergence and increases of novel antibiotic-resistant strains are needed, including rapid identification of pathogens by methods such as real-time PCR, as well as antimicrobial treatments capable of eliminating the organisms.

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