JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2007, p. 3534–3539 Vol. 45, No. 11
0095-1137/07/$08.00+0 doi:10.1128/JCM.01345-07
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First Report of Macrolide-Resistant Strains and Description of a Novel Nucleotide Sequence Variation in the P1 Adhesin Gene in *Mycoplasma pneumoniae* Clinical Strains Isolated in France over 12 Years[▽]

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Received 5 July 2007/Returned for modification 17 August 2007/Accepted 7 September 2007

***Mycoplasma pneumoniae* isolates are divided in two types based on the sequence variations in the P1 adhesin gene. The type of P1 adhesin gene of 155 clinical isolates of *M. pneumoniae* collected in France between 1994 and 2006 was determined by a PCR-restriction fragment length polymorphism method. Until 1995, all strains belonged to type 1. In 1996 and 1997, type 1 was still predominant, but type 2 increased. Finally, since 1998, both types were present in about the same proportion. In our study, a novel sequence of the P1 adhesin gene was described in one strain. This strain could not be classified into type 1 or 2 because of variability in both P1 gene repeat elements, RepMP4 and RepMP2/3. This new sequence was certainly issued from recombination with repetitive sequences localized outside of the P1 gene in the *M. pneumoniae* chromosome. Moreover, MICs of erythromycin, tetracycline, and ciprofloxacin were determined for the 155 isolates. All isolates remained susceptible to tetracycline and ciprofloxacin, but two macrolide-resistant strains, isolated from two children in 1999, were identified. They harbored an A-to-G substitution at position 2058 or 2059 (*Escherichia coli* numbering) in domain V of 23S rRNA, associated with resistance to macrolides, lincosamides, and ketolides. To our knowledge, this is the first description of macrolide-resistant isolates of *M. pneumoniae* in France, but at this time, there is no sign of recent diffusion of resistant strains.**

Mycoplasma pneumoniae is a common pathogen responsible for community-acquired respiratory tract infections, particularly in school-aged children and young adults. Epidemics occur periodically at 4- to 7-year intervals (22). The 170-kDa P1 protein is a major adhesin protein that induces a strong immunological response (1, 10, 22). Only one copy of a functional full-length P1 gene is present in the *M. pneumoniae* genome (9). This gene is composed by two repetitive regions, RepMP4 located at the 5′ end of the coding region and RepMP2/3 located at the 3′ end of the coding region (16). Eight to 10 closely related but not identical copies of both repetitive regions are dispersed through the fully sequenced genome of the *M. pneumoniae* M129 strain (16). Based on the sequence of the P1 gene, two types, 1 and 2, have been reported (3, 20). *M. pneumoniae* M129 is a type 1 prototype, while *M. pneumoniae* FH, Mac, and 1842 strains belong to type 2. Moreover, one type 1 variant and two type 2 variants that showed sequence variations in the RepMP2/3 but not the RepMP4 element of the P1 gene have been described (6, 8, 11).

Previous studies found that one or the other of the two types tended to predominate among clinical isolates in specific geographical regions and that the predominant type changed over time (3, 7, 17, 20). These changes in the P1 adhesin type may play a role in the development of outbreaks. In France, the 1987 and 1992 epidemics were due to strains belonging to type 2 and 1, respectively (3).

Macrolides are the drug of choice for the treatment of *M. pneumoniae* infections, as tetracyclines and fluoroquinolones are not recommended for children. Very few strains resistant to erythromycin were reported in the literature during the last three decades, except in Japan (2). In Japan, some recent data suggest that macrolide-resistant isolates have been spreading since 2000 (12, 13). About 15% of the *M. pneumoniae* clinical isolates reported between 2000 and 2004 in Japan were resistant to erythromycin. Most of the isolates harbored point mutations in domain V of 23S rRNA at positions 2058 or 2059 (*Escherichia coli* numbering) and were resistant to 14-, 15-, and 16-membered macrolides and lincosamides (12, 13). Substitutions at position 2611 were associated with a lower level of resistance (12). Mutations in ribosomal protein L4 and L22 genes were also reported, but their roles in erythromycin resistance were uncertain (12).

The purpose of our study was to determine the type of the *M. pneumoniae* clinical isolates obtained between 1994 and 2006 in France by PCR-restriction fragment length polymorphism (RFLP) typing of the P1 gene and to verify whether one type was predominant or whether it changed over time. Susceptibility to active antimicrobials, macrolides, tetracyclines, and fluoroquinolones was also studied to determine whether resistant *M. pneumoniae* isolates are emerging in France.

MATERIALS AND METHODS

***M. pneumoniae* strains and culture.** Two *M. pneumoniae* reference strains and 155 clinical isolates were used. Strain M129 (ATCC 29342) and strain FH

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▽ Published ahead of print on 19 September 2007.

(ATCC 15531) were chosen as P1 protein type 1 and P1 protein type 2 reference strains, respectively. Sixty-one clinical isolates were obtained from respiratory specimens of patients hospitalized in Hospices Civils de Lyon (Lyon, France) during the period from 1994 and 2000, 14 isolates were obtained from patients hospitalized in Saint-Etienne Hospital (Saint-Etienne, France) between 1996 and 2001, and 80 isolates were obtained from patients hospitalized in Pellegrin Hospital (Bordeaux, France) between January 1994 and August 2006. *M. pneumoniae* isolates were grown in Hayflick modified broth medium supplemented with glucose (21).

***M. pneumoniae* P1 gene typing.** For PCR-RFLP typing, 1.5 ml of *M. pneumoniae* culture was pelleted by centrifugation at 2,000 × *g* for 40 min. The supernatant was discarded, and the pellet was resuspended in 50 μl of distilled water and stored at −20°C overnight. P1 gene PCR-RFLP typing was performed as previously described (3). Briefly, a fragment of P1 gene was amplified using primers ADH1 and ADH2 (3) and then digested with HpaII or DdeI restriction endonuclease (Promega).

DNA sequencing of the P1 gene. As the PCR-RFLP method did not allow determination of the type of one isolate (isolate Mp3896), the PCR products obtained with primers ADH1 and ADH2 and primers ADH3 and ADH4 (3) were purified with the Wizard PCR preps DNA purification system (Promega). Both strands of the PCR products were sequenced with a BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems) and a 3130 genetic analyzer (Applied Biosystems/Hitachi), according to the manufacturer's instructions, using primers ADH1, ADH2, ADH3, and ADH4 as well as internal primers. Primer pairs ADH1bis (5′-CATATTCAGGCCAGCGGTG-3′) and ADH1rev (5′-CCTGCAACGCCTTGACTTTG-3′), ADH2for (5′-GCGTACTTCGCCAA CATTGG-3′) and ADH3rev (5′-CGACTGTAAGTCGGTGTAGG-3′), and ADH32 (5′-GACCTGATTACGTGTTGCCG-3′) and ADH32rev (5′-GTGAG GGTGTTGTGGTCTTG-3′) were used to amplify and sequence the regions upstream of the ADH1-ADH2 fragment, between the ADH1-ADH2 and ADH3-ADH4 fragments, and downstream of the ADH3-ADH4 fragment, respectively.

Susceptibility testing. *M. pneumoniae* isolates were grown in Hayflick modified broth medium supplemented with glucose (21). The following agents were provided by the indicated manufacturers: erythromycin, josamycin, quinupristin, quinupristin-dalfopristin, pristinamycin, and telithromycin (Sanofi-Aventis, Paris, France); azithromycin and clindamycin (Pfizer, Orsay, France); ciprofloxacin (Bayer Pharma, Puteaux, France); and tetracycline (Sigma). The MICs of erythromycin, ciprofloxacin, and tetracycline were determined for all the isolates, as previously described, by an agar dilution method (21). The MIC was determined as the lowest concentration of antimicrobial agent that prevented a color change in the medium at the time when the drug-free growth control first showed a color change (about 5 days of incubation at 37°C). Moreover, the MICs of azithromycin, josamycin, clindamycin, quinupristin, quinupristin-dalfopristin, and telithromycin were determined for the two erythromycin-resistant isolates by the same method.

Characterization of macrolide-resistant isolates. Macrolide-resistant isolates were characterized by PCR amplification and DNA sequencing of three DNA fragments of interest in the 23S rRNA gene, one fragment in domain II (primers MP23S-17b and MP23S-24) and two fragments in domain V (primers MH23S-11 and MP23S-22 and primers MH23S-9 and MP23S-23) (15). Fragments of ribosomal proteins L4 and L22 were also amplified and sequenced with primers MPL4-1 and MPL4-2 and primers MPL22-1 and MPL22-2, respectively (15).

Nucleotide sequence accession numbers. The complete nucleotide sequence of the isolate Mp3896 P1 protein gene (4,908 nucleotides [nt]) has been assigned GenBank accession no. EF656612.

RESULTS

PCR-RFLP typing of the P1 gene. The type of P1 adhesin gene of 155 *M. pneumoniae* clinical isolates collected in France between January 1994 and August 2006 was determined by a PCR-RFLP method using primers ADH1 and ADH2, which are derived from single-copy regions flanking the RepMP4 region of the P1 gene. Thus, only the functional P1 gene was able to be amplified with this pair of primers. Fragments of approximately 2,240 bp were amplified and digested with HpaII or DdeI restriction endonuclease to generate a banding pattern characteristic for type 1 or type 2. The RFLP patterns of all isolates but one were identical to the RFLP pattern of either *M. pneumoniae* strain M129, representative of type 1, or strain FH, representative of type 2. Therefore, 88 clinical isolates were classified into type 1, and 66 clinical isolates were classified into type 2. The isolate Mp3896 could not be classified into type 1 or 2 by this method.

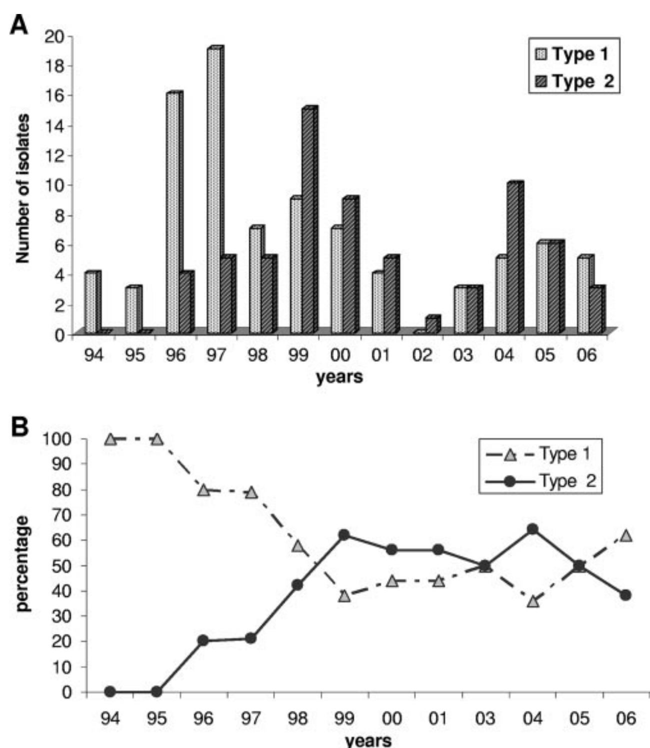

FIG. 1. Distribution of the type of P1 adhesin gene of 154 *M. pneumoniae* clinical isolates collected in France between January 1994 (94) and August 2006 (06). (A) Number of isolates of each type isolated per year. (B) Relative percentage of each type isolated per year. The year 2002 was omitted from the graph in panel B, as only one strain was isolated that year.

Figure 1 shows the distribution of the 154 *M. pneumoniae* clinical isolate types per year. Until 1995, all strains belonged to type 1. In 1996 and 1997, type 1 was still predominant but type 2 increased. Finally, from 1998 to 2006, both types were present in about the same proportion without a clear predominance of one type. When isolates from Bordeaux, France, were considered separately, the same distribution was observed with similar proportions of both types since 1998 (data not shown). For isolates from Lyon, France, a predominance of type 1 was also observed until 1997, then both types were present in equal proportions in 1998 and 1999, and finally, type 2 predominated in 2000 (data not shown).

DNA sequence analysis of the functional P1 gene of isolate Mp3896. Isolate Mp3896, obtained from a nasopharyngeal aspirate of a 5-month-old girl hospitalized in February 2005 in Pellegrin Hospital, could not be classified into type 1 or 2 by PCR-RFLP, as the banding pattern generated with either HpaII or DdeI was totally different from those of the reference strains M129 and FH. In order to understand these data, the complete P1 gene sequence of 4,908 nt was determined. The 1,635-amino-acid P1 protein showed the best percentage of identity, 95%, with the type 2 P1 proteins of strain 1842 (GenBank

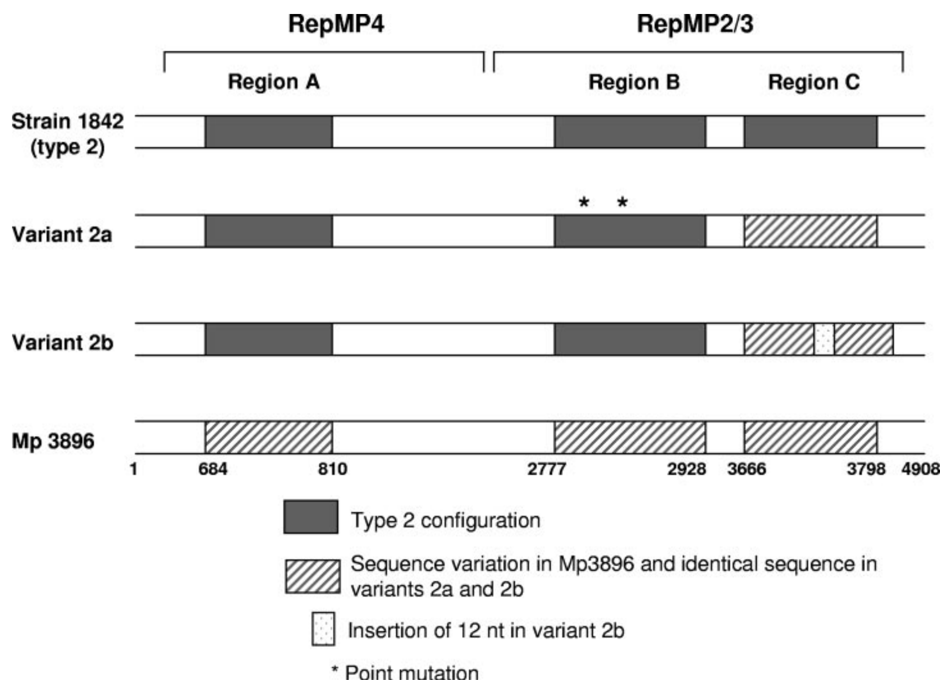

FIG. 2. Schematic representation of sequence divergence of the P1 protein gene of isolate Mp3896 and of three P1 type 2 strains, 1842 (6), variant 2a (11), and variant 2b (8). The numbers below the schematic representation indicate the nucleotide position. The figure is not drawn to scale.

accession no. AAK92040) (6) and Mac (GenBank accession no. AAK92040) (6), whereas the percentage of identity with the type 1 P1 protein of strain M129 (GenBank accession no. AAB95661) (9) was only 91.4%. In comparison with the P1 gene nucleotide sequence of the type 2 strain 1842, three variable regions were observed (Fig. 2). Region A of 127 bp between nt 684 and nt 810 was localized within the RepMP4 repeat region, and regions B and C of 151 and 133 bp and between nt 2777 and 2928 and nt 3666 and 3798, respectively, were localized within the RepMP2/3 repeat region. Although P1 protein of isolate Mp3896 presented the best percentage of identity with type 2 proteins, nucleotide variations in region A led to a different PCR-RFLP banding pattern. In the ADH1-ADH2 fragment from isolate Mp3896, a DdeI restriction site which was present at nt 681 of the gene in type 2 fragments was lacking. Conversely, a HpaII site was present at nt 683 of the gene in isolate Mp3896 but was absent in the type 2 ADH1-ADH2 fragments.

Recently, two type 2 variants (variants 2a and 2b) of *M. pneumoniae* that showed sequence variations in the RepMP2/3, but not in the RepMP4, repeat element were described (8, 11). In contrast, P1 protein of isolate Mp3896 differed from type 2 proteins in the RepMP4 repeat element, as among the 43-amino-acid region A of isolate Mp3896, the identity with the type 2 P1 protein of strain 1842 was only of 48.8% (21/43 identical amino acids). The P1 protein gene of isolate Mp3896 was compared to the published fragments of both P1 protein variants in the RepMP2/3 repeat element (Fig. 2). Region B of isolate Mp3896 differed from both variants 2a and 2b (GenBank accession no. BAA83588 and ABD36416, respectively) (8, 11). Region C of isolate Mp3896 was identical to the variant 2a region but differed from the variant 2b region.

Since intragenomic recombination with other repetitive regions outside of the P1 gene may have occurred, sequences homologous to regions A, B, and C of isolate Mp3896 were looked for in the complete genome of *M. pneumoniae* M129 strain (9). Three sequences homologous to region A were found in the M129 genome at nt 609612 to 609738 (100% identity), nt 130876 to 131002 (98% identity, two mismatches), and nt 248969 to 249095 (97% identity, three mismatches). One sequence homologous to region B was found at nt 341980 to 342130 (99% identity, one mismatch). For region C, only a fragment of 75 nt from isolate Mp3896 was found to be identical (100% identity) to a fragment of M129 genome from nt 128796 to 128870.

Susceptibility testing of *M. pneumoniae* clinical isolates. The MICs of erythromycin, ciprofloxacin, and tetracycline for the 155 *M. pneumoniae* clinical isolates are presented in Table 1. Ciprofloxacin MICs ranged between 0.5 and 2 μg/ml (MIC_{90}, 2 μg/ml), and tetracycline MICs ranged between 0.12 and 1 μg/ml (MIC_{90}, 0.5 μg/ml). The MIC_{90}s were similar to the MICs of ciprofloxacin (1 μg/ml) and tetracycline (0.25 μg/ml) for both susceptible reference strains, M129 and FH. The

TABLE 1. MICs of erythromycin, ciprofloxacin, and tetracycline for 155 *M. pneumoniae* clinical isolates collected between January 1994 and August 2006 in France

Drug	MIC (μg/ml)		
	Range	MIC_{50}	MIC_{90}
Erythromycin	0.008–256	0.016	0.032
Ciprofloxacin	0.5–2	1	2
Tetracycline	0.12–1	0.25	0.5

TABLE 2. MICs of macrolides, lincosamides, streptogramin combinations, and ketolide for the susceptible reference strain M129 and the two macrolide-resistant clinical isolates MPL1 and MPL2

M. pneumoniae strain	MIC (μg/ml)[a]								Nucleotide change (amino acid change) in:		
	ERY	AZM	JOS	CLI	QUI	PRI	Q-D	TEL	23S rRNA[b]	L4 gene[c]	L22 gene[c]
M129	0.008	≤0.0017	0.03	2	1	0.06	0.06	≤0.0017			
MPL1	256	256	128	8	1	0.12	0.06	1	A2059G	C162A[d] A430G (M144V)	T279C[d]
MPL2	256	64	8	128	1	0.06	0.06	128	A2058G	C162A[d] A430G (M144V)	T279C[d]

[a] ERY, erythromycin A; AZM, azithromycin; JOS, josamycin; CLI, clindamycin; QUI, quinupristin; PRI, pristinamycin; Q-D, quinupristin-dalfopristin; TEL, telithromycin.
[b] *E. coli* numbering.
[c] *M. pneumoniae* numbering.
[d] Silent mutation.

MICs of erythromycin for 153 clinical isolates and reference strains M129 and FH ranged between 0.008 and 0.032 μg/ml. Two isolates, MPL1 and MPL2, were found resistant to erythromycin (MICs, 256 μg/ml). MPL1 and MPL2 are P1 type 2 isolates that were isolated in Hospices Civils de Lyon in 1999 from a sputum specimen from an 8-year-old boy and a bronchoalveolar fluid specimen from a 12-year-old girl, respectively. Both children had been hospitalized for febrile pneumopathy, which was associated with pleural effusion in the second case. The MICs of macrolides, lincosamides, streptogramin combinations, and ketolide were determined for both isolates and are presented in Table 2, in comparison with those of the reference susceptible strain M129. Both isolates presented a macrolide-lincosamide-ketolide resistance phenotype but remained susceptible to streptogramin combinations, pristinamycin, and quinupristin-dalfopristin. For isolate MPL1, the MICs of the macrolides azithromycin and josamycin were 256 and 128 μg/ml, respectively, while they were 4- and 16-fold lower, respectively, for isolate MPL2. In contrast, the MICs of clindamycin and telithromycin were higher for MPL2 (128 μg/ml) than for MPL1 (8 and 1 μg/ml, respectively) (Table 2).

Characterization of two clinical isolates of *M. pneumoniae* resistant to macrolides. DNA sequence analysis of three fragments of interest in the 23S rRNA gene, one in domain II and two in domain V, revealed an A-to-G substitution at position 2059 (*E. coli* numbering) in MPL1 isolate and at position 2058 in MPL2 isolate (Table 2). No mutation was found in domain II of 23S rRNA. Both isolates were also examined for any change in conserved fragments of the ribosomal protein L4 and L22 genes. Both isolates harbored two point mutations in the ribosomal protein L4 gene, a C-to-A substitution at nucleotide 162 and an A-to-G substitution at position 430. The latter mutation led only to an amino acid change from methionine to valine at position 144 (*M. pneumoniae* numbering). Moreover, a T-to-C substitution was found at nucleotide 279 in the ribosomal protein L22 gene with no amino acid change.

DISCUSSION

Clinical isolates of *M. pneumoniae* have been grouped in two types based on the sequence of the P1 adhesin gene with different techniques, including PCR-RFLP, random amplified polymorphic DNA, nucleic acid sequence-based amplification, and pulsed-field gel electrophoresis. Previous studies showed the dominance of one type followed by an increase in the other type during the next years (3, 7, 17, 20). This alternation may participate in the cyclic pattern of *M. pneumoniae* infections. A type-specific host immune response could develop and protect the population against the dominant type, then the following outbreak can occur with the other type, which enables *M. pneumoniae* to escape from the host immune response. The switch of the dominant type could be linked to recombination between the functional P1 gene and repetitive sequences RepMP dispersed through the genome that serve as a reservoir to generate antigenic variations. A total of 8 copies of the RepMP4 sequence, including the P1 gene one, and 10 copies of the RepMP2/3 sequence are dispersed in the genome (9). However, in discrepancy with this theory, clear shifts between the *M. pneumoniae* types were observed in Japan between 1979 and 1995 but did not appear to be correlated with *M. pneumoniae* epidemic cycles (17).

In France, after a peak of type 2 isolates in 1987 followed by a peak of type 1 isolates in 1992 (3), we observed that type 1 was still predominant between 1994 and 1997. Among German *M. pneumoniae* clinical isolates, a majority of type 1 was also noted before 1998 followed by an increase in type 2 strains in 1998 and a return to a large majority of type 1 in 1999 (7). Unexpectedly, in our study, since 1998, both types were present in about the same proportion without a clear dominance of one type. As we have collected ≤16 isolates per year since 2000 (Fig. 1), this result could not be significant. However, a study of DNA samples from specimens collected in Germany and Switzerland also showed almost equal distribution of types 1 and 2 for 3 years between 2003 and 2005 (8). The reason for the recent lack of switch in the dominant type in several countries remains unknown.

Among the 155 clinical isolates studied, the type of one, isolate Mp3896, could not be determined by PCR-RFLP on the ADH1-ADH2 fragment with either DdeI or HpaII enzyme. With this method, five subtypes were previously discriminated among type 1 strains and three subtypes were discriminated among type 2 strains (5). However, the general banding pattern of each type was still recognizable, as only minor differences occur in the banding pattern.

Beside these subtypes, three variants of the *M. pneumoniae* P1 gene were described, one type 1 variant (1a) and two type 2 variants (2a and 2b) (6, 8, 11). These variants showed sequence variations mainly in the RepMP2/3 element of the P1 gene, but the sequence of the RepMP4 element remained characteristic of either type 1 or type 2 strains. For isolate Mp3896, we described a novel sequence of the P1 adhesin gene that differs from those of type 1 and 2 in the RepMP2/3 repeat

element and in the RepMP4 repeat element. The RepMP4 element is the region where most of the nucleotide differences between the P1 gene sequences from types 1 and 2 are localized. In isolate Mp3896, the 127-nt region of RepMP4 (region A) was found to be different from those of both type 1 and 2. This new sequence was certainly a result of recombination with repetitive sequences localized elsewhere in the *M. pneumoniae* chromosome, as three sequences identical or nearly identical to region A could be found in the complete sequence genome of *M. pneumoniae* M129. Moreover, it should be noted that region C of isolate Mp3896, localized in the RepMP2/3 repeat element, is strictly identical to the variant 2a region (11). However, the P1 sequence of this type 2 variant differs from that of isolate Mp3896 in both regions A and B.

The susceptibility of the 155 clinical isolates was studied by determination of erythromycin, tetracycline, and ciprofloxacin MICs. All isolates harbored tetracycline and ciprofloxacin MICs included within the ranges described for wild-type *M. pneumoniae* isolates (2, 22). This result is in agreement with the absence of reports of clinical isolates having acquired tetracycline or fluoroquinolone resistance in this species (2, 22). Concerning erythromycin susceptibility, two macrolide-resistant clinical isolates were identified. Except in Japan where macrolide-resistant *M. pneumoniae* isolates were first found in 1968 (14) and have been spreading since 2000 (12, 13), very few strains resistant to erythromycin were reported in the literature elsewhere in the world. Three erythromycin-resistant clinical isolates were reported in Israel during the 1980s (18), and two resistant isolates from the United States and Finland were collected between 1995 and 1999 (4). With only two resistant isolates found in Hospices Civils de Lyon in 1999, macrolide-resistant *M. pneumoniae* isolates remain very rare in France. Unlike the situation in Japan, no sign of recent diffusion of resistant strains was observed in France, as no resistant *M. pneumoniae* isolates have been obtained in our study after 1999.

For the two children infected with the macrolide-resistant strains, antibiotic treatments and clinical courses during the hospitalization were unknown. However, it is interesting to note that the girl infected with the MPL2 strain had a fever for 8 days before hospitalization, despite administration of roxythromycin, a 14-membered macrolide. In agreement with this observation, Suzuki et al. reported that the total number of febrile days and the number of febrile days during macrolide administration were longer in patients infected with macrolide-resistant isolates than in those infected with macrolide-susceptible isolates (19).

Resistant MPL1 and MPL2 isolates harbored an A-to-G substitution at position 2059 and 2058, respectively, in domain V of 23S rRNA, associated with a macrolide-lincosamide-ketolide phenotype of resistance. Those mutations, especially the A2058G substitution, are the most frequently reported in the erythromycin-resistant isolates of *M. pneumoniae* genetically characterized (2). As previously described, the isolate with the A2058G substitution harbored lower MICs of 16-membered macrolides but higher MICs of lincosamides and ketolides than the isolate with the A2059G mutation (2). Moreover, three point mutations were observed in ribosomal proteins L4 and L22 that led to a single M144V amino acid change. Those substitutions were previously reported in clinical isolates and reference strains FH and Mac that belong to type 2, whereas they were absent in clinical isolates and reference strain M129 that belong to type 1 (12). As *M. pneumoniae* FH and Mac strains are both susceptible to macrolides, those three substitutions are certainly not involved in the macrolide resistance observed in MPL1 and MPL2 isolates but are probably linked to their type 2 P1 protein gene.

To summarize, no clear shift between the *M. pneumoniae* types has been observed in France since 1998. A novel sequence of the adhesin P1 gene was described that could not be classified into type 1 or type 2 because of variability in the RepMP4 repeat element. Moreover, two macrolide-resistant clinical isolates were detected in 1999, but at this time, there is no sign of recent diffusion of resistant strains in France. However, the recent apparition and diffusion of macrolide-resistant strains in Japan show that epidemiological monitoring of the susceptibility of *M. pneumoniae* clinical isolates seems to be necessary, especially in case of therapeutic failure.

ACKNOWLEDGMENTS

We thank M. P. Milon and F. Grattard for providing *M. pneumoniae* clinical isolates from the Hospices Civils de Lyon and from St. Etienne Hospital, respectively. We thank M. Clerc and B. de Barbeyrac for assistance typing isolates from Hospices Civils de Lyon and St. Etienne Hospital.

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