Increased macrolide resistance of *Mycoplasma pneumoniae* in France
directly detected in clinical specimens by real-time PCR and melting
curve analysis

O. Peuchant¹, A. Ménard², H. Renaudin¹, M. Morozumi³, K. Ubukata³, C. M. Bébéar¹
and S. Pereyre¹*

¹Laboratoire de Bactériologie EA 3671, Université Victor Segalen Bordeaux 2 and CHU de Bordeaux,
33076 Bordeaux cedex, France; ²INSERM U853, Université Victor Segalen Bordeaux 2, Laboratoire de
Bactériologie, 33076 Bordeaux cedex, France; ³Laboratory of Molecular Epidemiology for Infectious Agents,
Kitasato Institute for Life Sciences, Kitasato University, Tokyo 108-8641, Japan

Received 13 February 2009; returned 10 March 2009; revised 1 April 2009; accepted 9 April 2009

**Objectives:** *Mycoplasma pneumoniae* is a common aetiological agent of community-acquired respiratory
tract infections for which macrolides are the treatment of choice. In France, only two macrolide-resistant
isolates were reported in 1999. In contrast, several recent data reported that macrolide-resistant
*M. pneumoniae* isolates have been spreading since 2000 in Japan. Mutations A2058G (*Escherichia coli*
numbering), A2058C, A2059G, A2062G, C2611A and C2611G in domain V of the 23S rRNA gene were
associated *in vivo* or *in vitro* with this resistance. The aim of this study was to determine whether
macrolide resistance of *M. pneumoniae* is emerging in France.

**Patients and methods:** We developed a duplex real-time PCR for the detection of the six 23S rRNA
mutations associated with macrolide resistance in *M. pneumoniae* and a simplex real-time PCR for the
identification of the A2058G mutation, the most common one. Both methods rely on fluorescence reson-
cance energy transfer coupled to melting curve analysis and are directly applicable to clinical samples.
The duplex real-time PCR assay, first validated on 40 genetically characterized *M. pneumoniae*
strains, was then applied directly on 248 French respiratory tract clinical samples.

**Results:** Among *M. pneumoniae*-positive specimens collected before 2005, no macrolide-resistant
*M. pneumoniae* isolate was detected. In contrast, among 51 samples collected between 2005 and
2007, five (9.8%) yielded a resistant genotype, suggesting a recent increase in macrolide-resistant
*M. pneumoniae* isolates in France.

**Conclusions:** The epidemiological monitoring of macrolide resistance in this species has become
necessary in France and Europe, and will be made easier by using these PCR assays.

**Keywords:** target gene mutation, 23S rRNA, laboratory methods, antimicrobial resistance epidemiology, low
respiratory tract infections, LRTI

**Introduction**

*Mycoplasma pneumoniae* causes respiratory tract infections and is
responsible for up to 20% of all cases of community-acquired
pneumonia, especially among school-aged children and young
adults.¹² Owing to the lack of sensitivity and to the prolonged
time needed for *M. pneumoniae* detection by culture, molecular
techniques are currently used for the diagnosis of *M. pneumoniae*
infection. Tetracyclines, fluoroquinolones and macrolide,
lincosamide, streptogramin and ketolide antibiotics (MLSKs) can
be used for the treatment of *M. pneumoniae* infections. Macrolides
are generally considered as the treatment of choice in both children
and adults.¹³

Very few *M. pneumoniae* macrolide-resistant isolates have
been reported in the literature before 2000.³ In Japan, several
recent data reported that macrolide-resistant *M. pneumoniae*
isolates have been spreading since 2000, with a prevalence increasing
up to 30.6% according to the studies.⁴⁻⁸ In contrast, only

*Corresponding author. Tel: +33-5-57-57-16-25; Fax: +33-5-56-93-29-40; E-mail: sabine.pereyre@u-bordeaux2.fr

© The Author 2009. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved.
For Permissions, please e-mail: journals.permissions@oxfordjournals.org
two resistant isolates were reported in 1999 in France. The A2058G mutation in domain V of 23S rRNA (Escherichia coli numbering) is the most frequent substitution associated with macrolide resistance in clinical isolates, followed by the A2059G mutation, while the A2058C and C2611G mutations are rare. Moreover, we previously showed that the C2611A and A2062G mutations could be selected in vitro in the presence of different MLSKs. Current methods to detect macrolide resistance in M. pneumoniae rely on time-consuming phenotypic or genotypic methods, such as susceptibility testing, restriction fragment length polymorphism (RFLP) and 23S rRNA sequencing analysis. Recently, a real-time PCR and high-resolution melt (HRM) analysis-based technique has been developed to detect only the A2058G or A2059G mutation.

In order to determine whether macrolide resistance in M. pneumoniae was emerging in France, we developed a rapid method to detect all 23S rRNA mutations associated with macrolide resistance in M. pneumoniae, directly from clinical specimens. Two real-time PCR assays using fluorescent resonance energy transfer (FRET) with melting curve analysis were developed and applied on French clinical respiratory tract specimens.

Materials and methods

Bacterial strains and susceptibility testing

Two M. pneumoniae reference strains [M129 (ATCC 29342) and FH (ATCC 15531)] and 14 susceptible clinical isolates obtained from patients hospitalized at Pellegrin Hospital (Bordeaux, France) were used. Two French and nine Japanese erythromycin-resistant clinical isolates with characterized A2058G, A2059G, A2058C or C2611G mutations in the 23S rRNA gene were used. Moreover, eight Japanese in vitro-selected mutants harbouring the A2058G or the A2059G transition and five French in vitro-selected mutants with the A2062G or the C2611A substitution were tested. MICs of erythromycin and azithromycin were determined in Hayflick modified medium by an agar dilution method as previously described. Among them, 39 samples, received between January 2005 and August 2008, yielded a M. pneumoniae isolate, for which erythromycin and azithromycin MICs were determined. These samples were collected from patients with a negative M. pneumoniae serology. Moreover, 106 respiratory tract clinical samples, collected in the same hospital, with a positive M. pneumoniae detection by PCR, were tested. Among them, 39 samples, received between January 2005 and August 2008, yielded a M. pneumoniae isolate, for which erythromycin and azithromycin MICs were determined. The 67 other specimens, only M. pneumoniae PCRs, were systematically collected between July 1998 and August 2008.

DNA was extracted from clinical specimens or from bacterial cultures with the MagNA Pure LC kit (Roche, Meylan, France) according to the manufacturer’s instructions.

Table 1. Primers and probes used for real-time PCR assays

<table>
<thead>
<tr>
<th>Primer and probe designation</th>
<th>Sequence (5′ → 3′)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duplex real-time PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1-Mpn</td>
<td>GAAGGGAGTACGGCAA</td>
<td>262</td>
</tr>
<tr>
<td>R1-Mpn</td>
<td>TTCCTCATATGATAATGTCCTG</td>
<td></td>
</tr>
<tr>
<td>anchor-probe7</td>
<td>CCGGTGAAGACACCCTGTTAGGC-fluorescein</td>
<td></td>
</tr>
<tr>
<td>sensor-probe7</td>
<td>LC-Red 640-ACGGGACGGAAAGCC-phosphate</td>
<td></td>
</tr>
<tr>
<td>F3-Mpn</td>
<td>GGATAAAGCTACTCCGGG</td>
<td>495</td>
</tr>
<tr>
<td>R4-Mpn</td>
<td>CTGCGTATTTCCTACCAAG</td>
<td></td>
</tr>
<tr>
<td>anchor-probe8</td>
<td>TTCAAACTCCTGGAGACAGGT-fluorescein</td>
<td></td>
</tr>
<tr>
<td>sensor-probe8</td>
<td>LC-Red 705-TCCCTATCTATTGTGCCCAGGA-phosphate</td>
<td></td>
</tr>
<tr>
<td><strong>Simplex real-time PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1-Mpn</td>
<td>GAAGGGAGTACGGCAA</td>
<td>262</td>
</tr>
<tr>
<td>R1-Mpn</td>
<td>TTCCTCATATGATAATGTCCTG</td>
<td></td>
</tr>
<tr>
<td>anchor-probe1</td>
<td>LC-Red 640-TCGGGCTTAACGGGGTGCTT-phosphate</td>
<td></td>
</tr>
<tr>
<td>sensor-probe1-2058G</td>
<td>TCGACGGGCTT CCTC-CC-fluorescein</td>
<td></td>
</tr>
</tbody>
</table>

Underlined bases emphasize the nucleotides at position 2055, 2059, 2062 or 2061.
nucleotide. Two hybridization probe sets were designed for duplex analysis of the two mutation regions in the 23S rRNA gene. Each couple of probes included a sensor probe, 5'-labelled with LC-Red 640 or LC-Red 705, which hybridized to the region containing the mutation sites, and an anchor probe, fluorescein 3'-labelled, which hybridized to the three or four bases upstream from the former probe (Table 1). Probes were obtained from Sigma-Aldrich (St-Quentin Fallavier, France).

The PCR and hybridization reactions were carried out in glass capillaries by using the LightCycler 1.5 thermocycler (Roche). Twenty microlitres of PCR mixture containing 2 μl of template DNA, 1.6 μl of 25 mM MgCl₂, 1 μl of the four primers (5 μM each), 2 μl of the four probe (20 μM each) and 2 μl of FastStart DNA Master Hybridization Probes (Roche) was prepared. The cycling conditions consisted of an initial denaturation cycle at 95 °C for 10 min, followed by 50 amplification cycles (with a transition rate of 20 °C/s) consisting of 95 °C for 10 s, annealing at 58 °C for 20 s and extension at 72 °C for 20 s. After amplification a melting step was performed, consisting of 95 °C for 1 s, 35 °C for 40 s and followed by a slow rise in the temperature to 85 °C at a rate of 0.1 °C/s with continuous acquisition of fluorescence, with a final cooling step for 30 s at 40 °C. Data were analysed with the LightCycler software version 1.5 (Roche). The option ‘polynomial’ was selected for the calculation method for melting curve analysis and melting temperature (T_m) values were determined using the ‘manual Tm’.

Identification of the A2058G mutation by simplex real-time PCR

An additional simplex real-time PCR assay was used to identify the A2058G mutation. Primers F1-Mpn and R1-Mpn were used with two hybridization probes, designed on the reverse strand of the M. pneumoniae 23S rRNA gene. The sensor probe, 3'-labelled with fluorescein, perfectly matched the 23S rRNA gene sequence harbouring the A-to-G transition at position 2058. The anchor probe was 5'-labelled with LC-Red 640 and 3'-phosphorylated (Table 1). PCR mixture and amplification were performed as described above.

Sequencing analysis

Domain V of the 23S rRNA gene was amplified with primers MP23S-17b and MP23S-23 from strain or specimen DNA extracts. Two fragments of interest in this domain were sequenced, one with primers MP23S-11 and MP23S-22 encompassing positions 2058, 2059 and 2062, and one with primers MP23S-9 and MP23S-23 encompassing position 2611.

Specificity and limit of detection of the assays

To assess the specificity of the assays, DNA extracts from several Mycoplasma strains (M. genitalium, M. hominis, Mycoplasma fermentans, M. penetrans, Mycoplasma palmonis, Mycoplasma amorphiforme, Mycoplasma orale, Mycoplasma salivarium, Mycoplasma pireum, Mycoplasma lipophilum and Mycoplasma faucaum), Ureaplasma strains (U. urealyticum and U. parvum) and bacterial strains from the respiratory tract (E. coli, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosus, Streptococcus anginosus, Streptococcus pneumoniae, Haemophilus influenzae, Enterococcus faecalis, Corynebacterium urealyticum, Neisseria perflava, Pseudomonas aeruginosa and Chlamydia pneumoniae) were used. In order to validate the efficiency of the extraction step, PCRs targeting the 16S rRNA genes were performed on each extract using primers F1-16S (CGTGTCGTGAGATGTTGGT) and R1-16S (GACGTCATCCCCACCTTCTCT) for non-mycoplasmal strains, and primers GP03W (GOGAGCAAAYAGGATTAGATCC) and MGSO3W (GTGAACTCCACATTRTTTCTTATA) for mycoplasmal strains. To evaluate the detection limit of both assays, M. pneumoniae M129 DNA was extracted and amplified with each of the two primer sets F1-Mpn/R1-Mpn and F3-Mpn/R4-Mpn, respectively. Each amplified product was then purified using the Wizard® PCR Preps DNA purification system (Promega, Charbonnieres, France) and subsequently cloned in the plasmid vector pGEM-®-T Easy (Promega), according to the manufacturer’s instructions. Plasmids pGEM-T MpnF1–R1 and pGEM-T MpnF3–R4 were transformed into E. coli and purified from the E. coli transformants using the Wizard® Plus SV Miniprep DNA Purification System (Promega). Purity and concentration of each plasmid DNA were determined by optical density measurements (NanoDrop 1000, Wilmington, USA). The detection limit of both PCRs assays was assessed by using 10-fold serial dilutions of plasmids pGEM-T MpnF1–R1 and MpnF3–R4.

Results

Detection of point mutations associated with macrolide resistance by duplex real-time PCR

A duplex real-time PCR assay was developed to detect point mutations conferring resistance to macrolides in the M. pneumoniae 23S rRNA gene. This assay, first evaluated on several genetically characterized M. pneumoniae strains, rapidly distinguished the 16 wild-type strains from those with a resistant genotype (24 strains). In the latter group, the existence of a nucleotide mismatch between the gene sequence and the hybridization probe produced a T_m lower than the T_m of the wild-type sequence. On the channel detecting the fluorescence emitted by LC-Red 640, melting curve analysis of DNA from control strains produced four different curves with T_m of 58.2 °C for the wild-type strains, 54.1 °C for mutants harbouring the A2058G or the A2062G substitution, 49.1 °C for mutants harbouring A2059G and 48.2 °C for mutants harbouring A2059G (Figure 1a). Strains harbouring the C2611G or C2611A substitutions produced the same T_m as the wild-type strains and were not detected here. On the second channel of the LightCycler, the LC-Red 705 fluorescence was measured. Two different melting curves were obtained, with T_m of 64.5 °C for the wild-type strains and 60.5 °C for mutants C2611G or C2611A (Figure 1b). As expected, strains harbouring mutations at positions 2058, 2059 or 2062 had the same T_m as the wild-type strains on this channel. It should be noted that an additional peak, weakly intense, which had a lower T_m of ~47 °C (Figure 1b), was observed in the melting curve analysis detecting substitutions at position 2611, but had no consequence on the result interpretation.

Identification of the A2058G mutation by simplex real-time PCR

Differentiating the A2058G and A2059G mutation-related T_m could be difficult during the duplex real-time PCR assay. Consequently, we developed an additional simplex real-time PCR to detect specifically the A2058G mutation, which is the most frequent one. The sensor-probe1-2058G was designed to form a mismatch T:G with the wild-type sequence during
hybridization. Therefore, the $T_m$ for the wild-type strain was lower than the one for the A2058G mutant, 60.2°C versus 65.5°C, respectively (Figure 1c). It should be noted that melting peaks obtained with the A2058C, A2059G or A2062G substitutions could not be differentiated from that of the wild-type strain (Figure 1c).

**Specificity and limit of detection of the real-time PCR assays**

With both real-time PCR assays, no amplification was observed with various mycoplasmal and non-mycoplasmal bacterial species, although all DNA extracts were amplified with the 16S rRNA PCRs. The detection limit of both the simplex and duplex PCR assays was 10 copies/μL for each probe set.

**Patient specimen testing**

The duplex real-time PCR was first validated by testing 21 DNA extracts from Japanese respiratory tract clinical specimens infected with a macrolide-resistant *M. pneumoniae* isolate, known to harbour the A2058G ($n=19$) or the A2059G ($n=2$) mutation. All DNA extracts were found to possess the expected substitution using the duplex real-time PCR assay. Moreover, the A2058G mutation was identified with the additional simplex real-time PCR in all the 19 DNA extracts known to harbour it.

---

**Figure 1.** Melting curve analysis obtained with the anchor-probe7/sensor-probe7 (a) and with the anchor-probe8/sensor-probe8 (b) by the duplex real-time PCR, and with the anchor-probe1/sensor-probe1-2058G by the simplex real-time PCR (c) for the M129 (continuous line) and FH (small dashes) wild-type strains and mutant isolates harbouring the A2058G (large and small dashes), A2058C (filled squares), A2059G (open circles) A2062G (crosses), C2611A (filled diamonds) and C2611G (large dashes) mutations. Values on the y-axis represent the ratio of the first negative derivative of the change in fluorescence (dF) to the variation in temperature.
Using the duplex real-time PCR, 142 clinical samples for which no M. pneumoniae was detected by culture or by the in-house diagnostic real-time PCR remained negative.

Among 39 specimens for which both the diagnostic real-time PCR and culture were positive for M. pneumoniae, eight failed to amplify and 28 produced a melting peak characteristic of the wild-type genotype. These samples yielded M. pneumoniae isolates susceptible to erythromycin and azithromycin, as determined by MIC studies (data not shown). Three clinical specimens, named Mpn-3655, Mpn-3927 and Mpn-4276, collected in 2005–06, were positive for resistance with the duplex real-time PCR. An A-to-G substitution, at position 2058 or 2059, was detected for the Mpn-3655 and Mpn-3927 specimens, according to the melting curve analysis (Table 2). Then, using the simplex real-time PCR, the A2058G mutation was identified in the Mpn-3655 clinical sample and confirmed by both sequence analysis and macrolide MIC values. With the simplex real-time PCR, the Mpn-3927 specimen produced a Tm lower than that of the A2058G control strain, suggesting that the A2058G transition was not present in this sample. The A2059G transition was identified by sequencing analysis and confirmed by MIC studies for the corresponding isolate MP-3996 (Table 2). Concerning the Mpn-4276 clinical sample, two melting peaks were observed with the duplex real-time PCR, one with a Tm corresponding to a wild-type strain and one with a Tm corresponding to an A2058G or A2059G mutation (Table 2). With the simplex real-time PCR, two melting peaks were also observed, one showing the presence of the A2058G mutation and the other showing a lower Tm. Analysis of the 23S rRNA sequence of the corresponding isolate MP-4391 showed a mixture of bases A and G at position 2058, suggesting that wild-type and mutated M. pneumoniae populations could be present in the same sample. Eight subcultured clones obtained from the MP-4391 isolate only harboured the A2058G mutation.

Among 67 clinical specimens, for which only the diagnostic real-time PCR was positive for M. pneumoniae, 21 failed to amplify with the duplex real-time PCR while 44 showed a susceptible genotype. Two clinical specimens, Mpn-4293 and Mpn-4294, harboured a macrolide-resistant genotype. A mutation at position 2611 was found for the Mpn-4293 bronchial aspirate specimen (Table 2). The sequencing analysis from this sample identified the C2611G transition. An A-to-G mutation, either at position 2058 or 2059, was detected for the Mpn-4294 clinical specimen with the duplex real-time PCR and the A2058G transition was identified using the simplex real-time PCR.

To summarize, among 106 M. pneumoniae PCR-positive clinical samples collected over the last decade, 77 were amplified with the duplex real-time PCR, showing that the sensitivity of our assay was 72.6% (77/106). Among them, a resistant genotype was detected for five specimens, that is to say a frequency of 6.5% (5/77). These five specimens were collected between 2005 and 2007 and represented 9.8% (5/51) of the 51 M. pneumoniae-positive clinical samples amplified with our duplex real-time PCR during this period.

### Discussion

Respiratory tract infections caused by M. pneumoniae are empirically treated with macrolides. However, recent reports indicate that macrolide-resistant M. pneumoniae isolates are}

### Table 2. Information about respiratory tract clinical samples with macrolide-resistant M. pneumoniae

<table>
<thead>
<tr>
<th>Patient’s age (years)</th>
<th>Year of collection</th>
<th>Clinical specimen designation</th>
<th>Corresponding isolate designation</th>
<th>MIC (μg/L)</th>
<th>Antimicrobial treatment</th>
<th>First prescription</th>
<th>Second prescription</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2005</td>
<td>Mpn-3655</td>
<td>MP-3655</td>
<td>0.0013</td>
<td>ERY, AZM, SPI, JOS, SPI, JOS</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>3.5</td>
<td>2005</td>
<td>Mpn-3927</td>
<td>MP-3927</td>
<td>0.0013</td>
<td>ERY, AZM, SPI, JOS, SPI, JOS</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>11</td>
<td>2006</td>
<td>Mpn-4276</td>
<td>MP-4276</td>
<td>0.0013</td>
<td>ERY, AZM, SPI, JOS, SPI, JOS</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>11</td>
<td>2007</td>
<td>Mpn-4293</td>
<td>MP-4293</td>
<td>0.0013</td>
<td>ERY, AZM, SPI, JOS, SPI, JOS</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>2007</td>
<td>Mpn-4294</td>
<td>MP-4294</td>
<td>0.0013</td>
<td>ERY, AZM, SPI, JOS, SPI, JOS</td>
<td>8</td>
<td>64</td>
</tr>
</tbody>
</table>

ERY, erythromycin; AZM, azithromycin; SPI, spiramycin; JOS, josamycin; SPI, spiramycin; PRI, pristinamycin; TEL, telithromycin; AMX, amoxicillin; ND, not determined.
spreading, which could modify the therapeutic management of *M. pneumoniae* infections.\(^6,13\) In *M. pneumoniae*, macrolide resistance is associated in vivo with point mutations in domain V of the 23S rRNA gene at positions 2058, 2059 and 2611.\(^5\)

Currently, except sequencing analysis, few technologies have been developed to detect resistance-associated mutations. Concerning *M. pneumoniae*, only a RFLP method\(^4\) and, recently, a real-time PCR followed by HRM curve analysis were reported to detect A-to-G substitution at position 2058 or 2059.\(^11\)

However, sequencing the 23S rRNA gene remained necessary to identify the substitution and only those two mutations were reported to be detected with this technology. In our study, we describe a duplex real-time PCR for the detection of at least six point mutations associated in vivo or in vitro with macrolide resistance in *M. pneumoniae*. This method was applied directly on clinical samples. Two probe sets were designed for multiplex analysis of the two mutation regions in the *M. pneumoniae* 23S rRNA gene. As the nucleotide environment of 2058, 2059 and 2062 positions was very rich in guanine and cytosine bases and as mutations were situated in a region with many high-binding secondary structures, it was difficult to design probes compatible with a FRET technology. Consequently, the design of the probes was handmade as software usually used to design real-time PCR, using the same technology, to accurately identify this transition in clinical specimens.

Our assay allowed the detection of the A2058G and A2059G transitions, but their distinction was difficult because of the close proximity of their respective Tm\(_{\text{m}}\)s. As the A2058G mutation is involved in ~90% of cases of macrolide-resistance in *M. pneumoniae*,\(^4-6\) we developed an additional simplex real-time PCR, using the same technology, to accurately identify this transition in clinical specimens.

It should be noted that our assays were less sensitive than the in-house diagnostic real-time PCR performed at the Pellegrin Hospital laboratory for *M. pneumoniae* detection in clinical specimens. Indeed, among the 106 samples with a positive in-house diagnostic real-time PCR, 29 (27.4%) failed to amplify. This could be due to a difference in the target gene between the two techniques. The present methods target the 23S rRNA gene, which occurs in only one copy in the *M. pneumoniae* genome.\(^14\) while the in-house diagnostic real-time PCR targets the P1 adhesin gene, which is present in 8–10 copies.\(^7\) The HRM technology also reported a limited sensitivity to detect point mutations associated with macrolide resistance in *M. pneumoniae*, as seven patient specimens failed to amplify among 30 PCR- and serology-confirmed *M. pneumoniae* cases.\(^11\) Thus, our real-time PCR assays should be used secondarily, on clinical samples for which the diagnostic real-time PCR would be positive.

Before 2000, very few macrolide-resistant *M. pneumoniae* isolates had been reported worldwide.\(^3\) Resistance to MLSKs was reported in 2000 and spread rapidly in Japan, with 30.6% (37/121 strains) resistant isolates described in 2006.\(^6\) In a recent *M. pneumoniae* outbreak in the USA, 27% (3/11) of isolates harboured a macrolide-resistant genotype.\(^11\) In France, only two macrolide-resistant isolates were reported in 1999 among 155 collected between 1994 and 2006.\(^9\) In our study, among 51 specimens amplified with our duplex real-time PCR and collected between 2005 and 2007, 9.8% (5/51) yielded a *M. pneumoniae* resistant genotype, suggesting a recent increase of macrolide-resistant *M. pneumoniae*. This could also be due to a better sensitivity of our genotype-based technique over phenotype-based methods, which require strain isolation by culture before *in vitro* susceptibility testing. Indeed, a *M. pneumoniae* isolate was obtained from three of the five macrolide-resistant clinical samples (Table 2). MLSK susceptibility testing was more fastidious for these three isolates than for the macrolide-susceptible strains. In agar medium, these isolates grew more slowly than the control strain, yielding tiny and uncharacteristic colonies (data not shown). These isolates were wrongly considered as susceptible to MLSKs when the routine susceptibility testing was first realized.

As suggested for other bacterial species, in *M. pneumoniae* a fitness cost could be connected with macrolide resistance-associated mutations in the 23S rRNA gene\(^15\) and could explain these data. Indeed, it was reported that clarithromycin-resistant *H. pylori* isolates, harbouring the A2058G or the A2059G mutation, had a decreased fitness compared with wild-type isolates.\(^16\) This emphasizes the importance of developing genotypic techniques to identify *M. pneumoniae* resistant isolates.

In our study, among the five patients infected with a macrolide-resistant *M. pneumoniae* isolate, four of them received a macrolide as the first antimicrobial treatment (Table 2). For all of them, the initially prescribed treatment was changed to another MLSK antibiotic, spiramycin, telithromycin or pristina-mycin. In agreement with these observations, it was reported that patients infected with macrolide-resistant *M. pneumoniae* isolates were shown to have the initially prescribed macrolide treatment more frequently changed to another antimicrobial agent, e.g. minocycline or levofloxacin.\(^6,13\) In our study, neither tetracyclines nor fluoroquinolones were prescribed when antimicrobial change occurred as these antibiotics are not recommended in children. It should be noted that the five patients ultimately recovered.

In *M. genitalium*, an urogenital pathogen phylogenetically close to *M. pneumoniae*, macrolide-resistant isolates harbouring mutations at position 2058 or 2059 were associated with treatment failure.\(^17\) In the majority of cases, drug-resistant mutants were selected during macrolide treatment. In our study, as clinical samples collected before treatment were not available for the five patients infected with macrolide-resistant *M. pneumoniae*, it was not possible to test this hypothesis.

*M. pneumoniae* infections commonly occur in children and young adults. In our series, 17 clinical samples were collected from adults (16%). Among them, one macrolide-resistant *M. pneumoniae* was detected in a 33-year-old man. To our knowledge this is the first description of a macrolide-resistant *M. pneumoniae* in an adult. Although the majority of studies were conducted in paediatric specimens,\(^4,5\) it was reported that no macrolide-resistant *M. pneumoniae* had been observed among 30 isolates from adult patients with community-acquired pneumonia.\(^6\) Therefore, it would be of interest to include adults in a global surveillance for the occurrence of macrolide-resistant *M. pneumoniae*.

In conclusion, we have developed two real-time PCR assays: a duplex real-time PCR for the detection of the 23S rRNA point mutations associated with clinical macrolide resistance in *M. pneumoniae* and a simplex real-time PCR for the identification of the A2058G mutation, the most common mutation, both directly
applicable to clinical samples. The system of anchor and sensor probes used in these assays is able to discriminate all the genotypes in one reaction, contributing to the rapidity of the method. According to the results of these assays on clinical specimens, we have observed that macrolide resistance of *M. pneumoniae* is increasing in France, as 9.8% of resistant genotypes were detected between 2005 and 2007. Thus, epidemiological monitoring of *M. pneumoniae* macrolide-resistant genotypes in clinical specimens has become necessary in Europe, in children as well as in adults. In addition, the rapid determination of the *M. pneumoniae* genotypic resistance profile to MLSKs could allow the prompt prescription of an alternative antimicrobial treatment if a macrolide-resistant strain is detected.

**Acknowledgements**

We are greatly indebted to T. Sasaki for providing the *M. pneumoniae* macrolide-resistant isolates from Japan and thank D. Ayache from the Sigma-Aldrich company (Paris, France) and D. Papi from the TibMolBiol company (Berlin, Germany) for their assistance in designing probes.

**Funding**

This study was supported from internal funding.

**Transparency declarations**

None to declare.

**References**


