# Multilocus Variable-Number Tandem-Repeat Analysis-Confirmed Emergence of a Macrolide Resistance-Associated Mutation in Mycoplasma pneumoniae during Macrolide Therapy for Interstitial Pneumonia in an Immunocompromised Child

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CASE REPORT

# Multilocus Variable-Number Tandem-Repeat Analysis-Confirmed Emergence of a Macrolide Resistance-Associated Mutation in *Mycoplasma pneumoniae* during Macrolide Therapy for Interstitial Pneumonia in an Immunocompromised Child

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**A child with Job's syndrome was treated for pneumonia due to *Mycoplasma pneumoniae*. A mixed population of wild-type bacteria and an A2059G mutant was detected during josamycin treatment failure. The same multilocus variable-number tandem-repeat analysis (MLVA) type (MLVA type I) was isolated before and after treatment failure. The child recovered after ciprofloxacin treatment.**

## CASE REPORT

The patient, an 11-year-old boy, was managed in the Pediatric Department of Limoges University Hospital, France, for Job's syndrome or hyper-immunoglobulin E (IgE) syndrome, detected by a high total IgE level (4,963 kU/liter), staphylococcal skin infections, and recurrent lung infections. Other immunoglobulin levels were normal (IgM = 1.69 g/liter, IgG = 9.62 g/liter, and IgA = 1.47 g/liter). The diagnosis of Job's syndrome was confirmed by the detection of a heterozygous deletion of valine 463 in exon 16 of the signal transducer and activator of transcription 3 (STAT3) gene (amino acid position from the isoform NP_644805). Antibiotic prophylaxis for lung and skin infections consisted of permanent cotrimoxazole administration associated with rotating administration of pristinamycin, cefaclor, and amoxicillin-clavulanic acid. The rotating antibiotic regimens were stopped in 2009 when the boy was 9 but were resumed in 2011 because of recurrent lung infections. Despite ongoing antibiotic prophylaxis based on cotrimoxazole and cefaclor, the boy presented with fever up to 38.5°C, productive cough, and an increased respiratory rate. Laboratory tests on admission showed leukocytosis of 3,900 cells/mm$^3$ (1,480 neutrophils/mm$^3$, 80 eosinophils/mm$^3$, and 2,110 lymphocytes/mm$^3$) and a C-reactive protein (CRP) level of 24 mg/liter (reference range up to 5 mg/liter) (Table 1). Treatment with amoxicillin-clavulanic acid (100 mg/kg of body weight/day) was started pending the microbiological results. Conventional bacterial culture of sputum (<25 epithelial cells and >25 neutrophils per low-power field) showed a normal commensal flora. A parainfluenza type 3 virus was detected with the RV15 ACE kit (Seegene), while the results were negative for 14 other viral pathogens, including influenzavirus A/B, parainfluenzavirus 1/2/4, respiratory syncytial virus A/B, coronavirus 229E/NL63/OC43, bocavirus, metapneumovirus, rhinovirus A/B/C, enterovirus, and adenovirus. The boy's condition worsened on hospital day 7. His oxygen saturation was 89%, his temperature rose to 40°C, and chest radiography showed an interstitial pattern. He was transferred to an intensive care unit for monitoring and required oxygen supplementation (5 liters per minute via a facemask) to maintain his oxygen saturation at 92%. Empirical treatment with teicoplanin (20 mg/kg/day) and piperacillin-tazobactam (300 mg/kg/day) was initiated. Laboratory tests showed leukocytosis at 8,800 cells/mm$^3$ (6,870 neutrophils/mm$^3$, 90 eosinophils/mm$^3$, and 1,350 lymphocytes/mm$^3$) and a CRP level of 189 mg/liter. *Mycoplasma pneumoniae* was detected in a nasopharyngeal aspirate collected on admission to the intensive care unit, using an in-house PCR-hybridization method targeting the adhesin P1 gene. Consequently, josamycin 50 mg/kg/day in two equal doses was started for 3 weeks, followed by prophylactic roxithromycin (8.5 mg/kg/day in two equal doses) and amoxicillin-clavulanic acid (75 mg/kg/day) for 1 month, in addition to the permanent cotrimoxazole prophylaxis (26 mg/kg/day). This treatment led to a decrease in oxygen dependency. He was discharged after 40 days in hospital. Fifteen days later, while he was still receiving combination prophylaxis with cotrimoxazole, roxythromycin, and amoxicillin-clavulanic acid, he was readmitted with worsening respiratory function and recurrent fever (up to 40°C). The chest radiography was identical to the first one. Laboratory tests showed leukocytosis at 15,500 cells/mm$^3$, with 8,200 neutrophils/mm$^3$, 1,400 eosinophils/mm$^3$ and 5,270 lymphocytes/mm$^3$ (Table 1). The *M. pneumoniae* PCR was again positive on sputum. No virus was detected in this sample, which contained less than 25 epithelial cells and more than 25 neutrophils per low-power field and yielded only commensal oral bacteria on culture. The *M. pneumoniae* serology was positive, with a specific IgG titer of 40 (cutoff = 10) and a specific IgM ratio of 6.5 (cutoff = 0.9) obtained using the Platelia *M. pneumoniae* IgG and IgM TMB kits (Bio-Rad), respectively. As the emergence of a macrolide-resistant strain of *M. pneumoniae* was suspected, the antibiotic treatment was switched to ciprofloxacin (40

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**TABLE 1** Laboratory findings, chest radiography, and antibiotic treatment of an 11-year-old boy with interstitial pneumonia

| Parameter | Day 0[a] | Day 7 | Day 55 |
|---|---|---|---|
| Rectal temp (°C) | 38.5 | 40 | 40 |
| White blood cell count (cells/mm$^3$) | 3,900 | 8,800 | 15,500 |
| Neutrophils (cells/mm$^3$) | 1,480 | 6,870 | 8,200 |
| Eosinophils (cells/mm$^3$) | 80 | 90 | 1,400 |
| Lymphocytes (cells/mm$^3$) | 2,110 | 1,350 | 5,270 |
| C-reactive protein (mg/liter) | 24 | 189 | 200 |
| Chest radiography | NE | Interstitial pattern | Interstitial pattern |
| Respiratory specimen | Sputum | Nasopharyngeal aspirate | Sputum |
| Conventional bacterial culture | Normal commensal flora | Normal commensal flora | Normal commensal flora |
| Viral PCR | Parainfluenzae type 3 virus | Negative | NE |
| Bacterial PCR | NE | *M. pneumoniae* | *M. pneumoniae* |
| Adhesin P1 subtype | NE | 1 | 1 |
| MLVA type | NE | I | I |
| Macrolide resistance-associated mutations | NE | None | Mixed population of A2059A (wild type) and A2059G |
| *M. pneumoniae* culture | NE | NE | Positive |
| *M. pneumoniae* serology | NE | NE | Specific IgG and IgM |
| Antibiotic therapy[b] | Amoxicillin-clavulanic acid | Teicoplanin and piperacillin-tazobactam then josamycin during 3 weeks then roxythromycin and amoxicillin-clavulanic acid during 1 month | Ciprofloxacin during 15 days and pristinamycin during 1 month |

[a] Days are numbered from the first hospital admission.
[b] Cotrimoxazole prophylaxis was never stopped during the course of the infection. NE, not evaluated.

mg/kg/day in two equal doses for 15 days), and pristinamycin prophylaxis (66 mg/kg/day in two equal doses for 1 month) was added to the permanent cotrimoxazole administration. The boy's condition subsequently improved. Hypoxia resolved gradually and his temperature normalized. By the end of the antibiotic treatment, he had fully recovered from the *M. pneumoniae* infection. Three weeks after the beginning of this antibiotic treatment, the *M. pneumoniae* PCR was negative on a sputum sample.

*M. pneumoniae*-positive DNA extracts obtained from the nasopharyngeal aspirate collected on day 7 after admission and from the sputum sample collected on day 55 were examined for macrolide resistance-associated mutations in the 23S rRNA gene by using real-time PCR and melting curve analysis (16) (Table 1). The nasopharyngeal aspirate collected before josamycin administration produced a melting peak characteristic of the wild-type genotype. This result was confirmed by sequencing the corresponding fragment of the 23S rRNA gene (16). The sputum sample collected after macrolide treatment yielded two melting peaks, one with a melting temperature ($T_m$) corresponding to that of the wild-type genotype and the other with a $T_m$ corresponding to that of the gene with the A2059G mutation (*Escherichia coli* numbering, which corresponds to A2064G using *M. pneumoniae* numbering) (Fig. 1). As the $T_m$s for an A2058G mutation and an A2059G substitution vary only by 1°C, we sequenced the fragment of interest of the 23S rRNA gene. Sequencing confirmed a mixture of bases A and G at position 2059 (Fig. 1), suggesting that wild-type and mutant *M. pneumoniae* populations were simultaneously present in the sample. To confirm that the *M. pneumoniae* isolates present before and after macrolide treatment were identical, *M. pneumoniae* molecular typing methods were applied directly to both respiratory tract specimens, without culture of strains. *M. pneumoniae* was categorized as subtype 1 in both specimens, based on the subtype-specific single-nucleotide polymorphism in the adhesin P1 gene (17). Multilocus variable-number tandem-repeat analysis (MLVA) (6) was also performed. In both samples, the MLVA type of *M. pneumoniae* was 33672, also designated MLVA type I. Moreover, *M. pneumoniae* grew in Hayflick's modified broth medium (19) from the sputum sample collected on day 55 after macrolide treatment (Table 1). This isolate was also subtype 1 and MLVA type I. Unfortunately, antibiotic susceptibility testing could not be performed for this isolate because of a yeast contamination of the mycoplasma broth.

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*M. pneumoniae* is a common cause of community-acquired respiratory tract infections, accounting for 10 to 30% of cases worldwide (1). A substantial increase in the incidence of *M. pneumoniae* infections has been reported in several countries since 2010 (10). These infections can cause severe and long-lasting interstitial pneumonia in both adults and children. Although several drugs, including macrolides, tetracyclines, and fluoroquinolones, can be used to treat *M. pneumoniae* infections, macrolides remain the drug of choice for pediatric infections. In recent years, macrolide resistance has been spreading in several regions of the world. While the prevalence of resistance is below 10% in Europe and the United States, it is approximately 30% in Israel and up to 90% in Asia (2, 3, 22). The *M. pneumoniae* macrolide resistance has been associated with longer disease courses and with certain complications (3). In the case reported here, *M. pneumoniae* macrolide resistance emerged in an immunocompromised child treated with macrolides. The MLVA genotyping method confirmed that the isolate was the same before and after treatment.

Job's syndrome is a rare primary immunodeficiency characterized by high serum levels of IgE, recurrent staphylococcal skin abscesses, and pneumonia, mainly caused by *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* (9). Children with compromised immunity, including humoral immunodeficiencies, are

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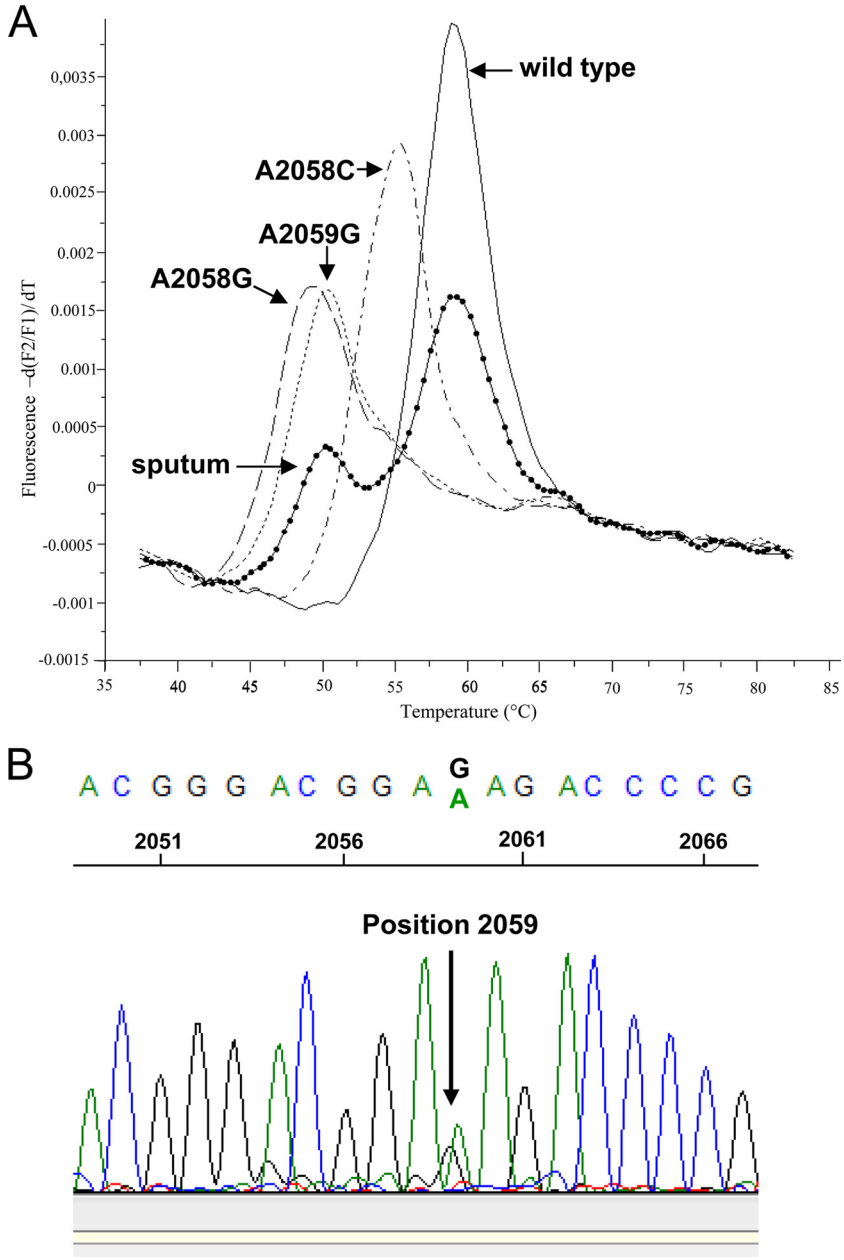


**FIG 1** Melting curve analysis and sequencing analysis of the sputum collected on day 55 from the first hospital admission. (A) Melting curve analysis obtained with the anchor-probe7/sensor-probe 7 by the duplex real-time PCR (16) for the sputum collected on day 55 (filled circles), wild-type *M. pneumoniae* reference strain M129 (continuous line), and genetically characterized isolates of *M. pneumoniae* harboring substitutions A2058G (large dashes), A2059G (small dashes), and A2058C (large and small dashes) in the 23S rRNA gene. (B) Chromatogram presenting the mixed population of susceptible (2059A) and resistant (2059G) *M. pneumoniae* in the sputum (*E. coli* numbering).

also at a higher risk of developing respiratory tract infections due to *M. pneumoniae* (20). Consequently, although never previously reported in the literature, the association between *M. pneumoniae* infection and the Job's syndrome in this child is not surprising. *M. pneumoniae* was recently reported to produce a community-acquired respiratory distress syndrome (CARDS) toxin that induces pulmonary eosinophilic and lymphocytic inflammation in mice (11). Variation of CARDS toxin production among *M. pneumoniae* strains may be associated with the severity of pulmonary disease in mice (18). To date, the CARDS toxin-coding gene has been present in every isolate of *M. pneumoniae* and no relationship has been found in humans between the concentration of CARDS toxin and the severity or prolonged course of the illness. Interestingly, eosinophilia and lymphocytosis were reported on day 55 in this child. Although the eosinophilia may be associated with the Job's syndrome and the lymphocytosis may be linked to viral infections that were not tested for on day 55, evaluation of the CARDS toxin production from this strain and comparison with CARDS toxin production from *M. pneumoniae* strains isolated from patients with milder syndromes would be interesting to consider for further investigations.

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Macrolide resistance first emerged in France in 2005, with a prevalence of almost 10% (16). The most frequently reported mutations are A2058G and A2059G in the 23S rRNA gene, with the A2059G substitution representing between 20 and 30% of all macrolide resistance-associated mutations in France (13, 16). This percentage is similar to the percentage of A2059G mutations reported in other parts of the world, such as Japan, the United States, Germany, and Italy (5, 8, 12, 21). As josamycin is not available in many of these countries, the occurrence of this less-frequent A2059G mutation in this child does not seem to be related to the use of josamycin. In our patient, a mixed population of resistant (A2059G) and susceptible (A2059A) *M. pneumoniae* bacteria was detected after macrolide treatment failure. A mixed population of wild-type *M. pneumoniae* and mutants with mutations at the adjacent position 2058 has already been associated with high macrolide MICs (16) and, recently, with treatment failure (2). In the latter study, emergence of macrolide resistance during treatment was strongly suspected but could not be confirmed because of the lack of *M. pneumoniae* typing. Cardinale et al. (4) detected the A2058G mutation in an *M. pneumoniae* variant 2a strain during treatment. As the prevalence of variant 2a circulating in the European population was recently reported to be 19% (7), it was not possible to demonstrate that the strain was the same throughout the disease course. In contrast, we were able to show that the same MLVA type I *M. pneumoniae* isolate was present in our patient before and after treatment. MLVA typing is a discriminatory method that can identify over 30 distinct MLVA types. MLVA type I is rare, as it was only detected once in a collection of 212 French isolates (6). This prompted us to confirm that our patient had not been reinfected by another *M. pneumoniae* isolate and that the A2059G mutation had indeed emerged within the same clone during macrolide treatment. We cannot exclude the possibility that the mutation was present at a very low level before treatment, as the real-time PCR and conventional sequencing methods used here to detect mutations may not have perfect sensitivity. However, we have previously shown that macrolide resistance-associated mutations can be selected *in vitro* from a susceptible reference strain on exposure to all macrolides and related antibiotics (15). Thus, emergence of the mutation during treatment is the most plausible hypothesis. Further studies are required to determine whether a specific macrolide molecule might be associated with fewer emergences of resistance during treatment.

Given this child's immunodeficiency, an antibiotic combination was chosen for second-line treatment, consisting of ciprofloxacin and prophylactic pristinamycin administration. *M. pneumoniae* is intrinsically susceptible to ciprofloxacin, and fluoroquinolone resistance has never been reported in a clinical isolate (3). Although contraindicated in children in this indication, ciprofloxacin has previously been used with success to treat macrolide-resistant *M. pneumoniae* infections in children (2, 4). Pristinamycin is a streptogramin combination that can be used in children. It may have had potent activity against this macrolide-resistant *M. pneumoniae* isolate, as it has been shown that A2059G isolates resistant to macrolides remain susceptible *in vitro* to streptogramin combinations, such as quinupristin-dalfopristin and pristinamycin (3, 14).

In conclusion, we show for the first time that a mixed population of the wild type and an A2059G mutant of *M. pneumoniae* can be associated with macrolide treatment failure. Moreover, by using MLVA typing, we show that macrolide resistance can emerge in the same *M. pneumoniae* isolate during macrolide treatment. Thus, rapid detection of resistance-associated mutations is necessary in the case of persistent or recurrent *M. pneumoniae* infection in order to enable prompt prescription of an alternative antimicrobial regimen.

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