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Two Cases of *Mycoplasma pneumoniae* Pneumonia with A2063G Mutation in the 23S rRNA Gene in Siblings

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We describe 2 cases of pneumonia caused by the same macrolide-resistant *Mycoplasma pneumoniae* in siblings. *M. pneumoniae* was identified using real-time PCR. Direct sequence analysis of the 23S rRNA gene revealed a point mutation in V domain (A2063G) of the 23S rRNA gene.

Key Words: Pneumonia, Macrolide, *Mycoplasma pneumoniae*, 23S rRNA

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INTRODUCTION

Mycoplasma pneumoniae was identified as the causative of primary atypical pneumonia in the early 1960s. Since then, *M. pneumoniae* has become one of the most common pathogens of community-acquired pneumonia in children and young adults [1]. *M. pneumoniae* can be transmitted through aerosols in typical outbreak settings in which close physical contact exists (e.g., homes, schools, military barracks, and dormitories). Dorigo-Zetsma et al. [2] detected *M. pneumoniae* in 15% of the household contacts of *M. pneumoniae*-positive patients by using PCR. A significantly higher percentage of household contacts younger than 15 yr of age were *M. pneumoniae*-positive than those older than 15. The frequencies of *M. pneumoniae*-positive household contacts of patients treated with antibiotics and those not treated were similar. These data suggest transmission from effectively treated patients still occurs in a household setting and support the idea that *M. pneumoniae* transmission can be prevented only by early treatment of the patient. Therefore, proper choice of antibiotics, based on rapid and sensitive diagnostic methods, is essential for treating children with *M. pneumoniae* [3].

Historically, serologic tests were commonly used for laboratory detection of *M. pneumoniae* causing respiratory tract infections. However, antibodies against *M. pneumoniae* may not appear until 2 weeks after the onset of symptoms, and some serologic tests have low specificity. Culture of *M. pneumoniae* is not particularly sensitive, time consuming, and has no influence on treatment [3]. On the other hand, PCR may be a more sensitive and practical diagnostic tool for detecting acute *M. pneumoniae* infections [3-5].

M. pneumoniae infection is usually self-limited, but antibiotic therapy reduces the symptoms and signs of *M. pneumoniae* pneumonia. As *M. pneumoniae* has no cell wall, antibiotics such as macrolides, tetracyclines, and quinolones are effective for

treatment. In children, macrolides (erythromycin, clarithromycin, roxithromycin, and azithromycin) are the first choice of treatment for *M. pneumoniae* infection because alternative drugs (tetracycline and fluoroquinolones) have potential adverse effects [3]. However, macrolide-resistant *M. pneumoniae* has been detected in Japan, Italy, France, USA, and Denmark [6, 7], and the detection rate is 90% in China [8]. Macrolide-resistant phenotypes are defined by specific point mutations in V domain of the 23S rRNA gene of *M. pneumoniae*.

Herein, we report 2 cases of macrolide-resistant *M. pneumoniae* pneumonia with the same A2063G point mutation of 23S rRNA gene in Korean siblings.

CASE REPORT

1. Case 1

A previously healthy 9-yr-old boy with a cough and sputum since 7 days and a fever since 3 days visited the outpatient department (OPD) of Wonju Medical Center. He took cough medication for 2 days before admission but did not take any antibiotics. He was an elementary school student, and there was no recent history of sick contacts, travel, or illness.

Auscultation of the lungs revealed coarse crackles with right side rhonchi. Laboratory results showed that complete blood cell count, white blood cell differential count, electrolyte level, liver function tests, and urinalysis were normal. C-reactive protein (CRP) was 12.0 mg/L (reference range: 0-5 mg/L). The chest radiograph showed pneumonic consolidation on the right upper lung field. Sputum culture showed growth of normal flora. Sputum smears were negative for acid-fast bacilli, and a tuberculin skin test was negative. Blood culture showed no growth after 7-day incubation. The serologic test for detecting anti-mycoplasma IgM antibody (SERODIA®-MYCO II, Fujirebio, Tokyo, Japan) was negative, but the patient's cold agglutinins titer was 1:128. A PCR test of a nasopharyngeal swab specimen was positive for *M. pneumoniae*. Gene amplification of *M. pneumoniae* was performed by MyGenie 32 Thermal Block (Bioneer, Daejeon, Korea), and we used 20 μL of high fidelity PCR premix (AccuPower®Premix, Bioneer, Daejeon, Korea) as the reactive agent. PCR primers were designed for detecting known *M. pneumoniae* single point mutations (A2063G, A2064G, T2611C, and C2617A). In this case, the resistant strains displayed a point mutation in the 23S rRNA: A-to-G transition mutation at nucleotide 2063.

Initial treatment with flomoxef was given intravenously (70 mg/kg/day, divided into 3 doses). The result of PCR for *M. pneumoniae* was positive, and roxithromycin (7 mg/kg/day, divided into 2 doses) was added on the 2nd day of hospitalization. On the 5th day of hospitalization, neither respiratory symptoms nor follow-up chest radiography findings improved. Treatment was, therefore, switched from roxithromycin to azithromycin (10 mg/kg/day, once a day for 3 days). On the 9th day of hospitalization, clinical symptoms and chest radiography findings improved, so the patient was discharged. Four days later, he was symptom-free with no detectable complications on OPD follow-up. The *M. pneumoniae* isolated from this patient carried an A2063G mutation.

2. Case 2

The second patient is the younger brother of the patient described in case 1. This patient was 5-yr-old boy who presented with cough and sputum for 10 days without fever. He visited a local pediatric clinic and also took roxithromycin for 7 days before the visit to our OPD. He had attended preschool and had been relatively healthy. On physical examination, his breath sounds were coarse with fine crackles, but chest radiography findings were normal. Roxithromycin (7 mg/kg/day, divided into 2 doses) was administered. However, 10 days later, the patient revisited the OPD with aggravated cough and newly developed fever. The chest radiography revealed pneumonic consolidation on the right upper lung field, similar to that seen on the chest radiographs of his elder brother. Although the respiratory symptoms of the siblings started at around the same time, fever with changes in chest radiography findings in case 2 (younger brother) developed 14 days later than those in case 1. The patient was admitted to the hospital, and azithromycin treatment was started immediately (10 mg/kg/day for 3 days). Laboratory results, including urinalysis, complete blood count, electrolyte level, and liver function tests, were all normal. CRP was 10 mg/L. The titer of anti-mycoplasma IgM antibody (SERODIA®-MYCO II) was 1:1,280 and the cold agglutinins titer was 1:256. Real-time PCR for *M. pneumoniae* was also positive and 23S rRNA sequencing showed A2063G mutation in the 23S rRNA gene, which was also present in *M. pneumoniae* isolated from this patient's brother. He underwent conservative management in hospital for 7 days, and then he was discharged.

METHODS

1. DNA preparation

M. pneumoniae DNA was extracted using an Exiprep Genomic DNA Kit (Bioneer, Daejeon, Korea). Extraction was performed

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according to the manufacturer's instructions.

2. Real-time PCR

AccuPower® MP Real-time PCR Kit (Bioneer) was used for detection of *M. pneumoniae* according to the manufacturer's instructions. Briefly, *M. pneumoniae*-specific DNA regions were detected with fluorescence of specific hydrolysis probes (5′-fluorescein carboxylic acid [FAM]; 3′-Black Hole Quencher-1 [BHQ1]) during thermal cycling (Exicycler™ 96 Real-Time Quantitative Thermal Block, Bioneer) [9]. PCR was carried out using a PCR premix kit for detection of *M. pneumoniae* (Bioneer). Five microliters of extracted DNA, positive control DNA, or distilled water (negative control) were added into individual premix tubes. The reaction mixture was then subjected to denaturation for 10 min at 95˚C, then 40 PCR cycles, each of 20 sec at 95˚C and 30 sec at 55˚C, followed by final cooling step of 1 min at 25˚C.

3. Analysis of macrolide resistance gene

A PCR assay followed by direct amplicon sequencing was developed to detect point mutations conferring resistance to macrolides in domain V of the *M. pneumoniae* 23S rRNA gene. After we obtained the DNA sequence of *M. pneumoniae* from GenBank® (NIH, Bethesda, MD, USA), we designed primers specific for polymorphism. Forward primer (Target gene: 23s rRNA 1851-2675 bp): 5′-GAA GGT TAA AGA AGG AGG TTA GCG CAA-3′. Reverse primer: 5′-TCG GTC CTC TCG TAC TAG AAG CAA CA-3′. Amplification of the 23S rRNA gene of *M. pneumoniae* was performed using a MyGenie 32 Thermal Block (Bioneer) thermal cycler. PCR was carried out in 20 μL of high fidelity PCR premix (Bioneer) using the following profile: denaturation for 5 min at 94˚C and 35 cycles of 30 sec at 94˚C, 30 sec at 55˚C, and 1 min at 72˚C. Final extension (10 min at 72˚C) was followed by cooling at 4˚C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized under ultraviolet light. The amplicons were purified using gel purification (Bioneer), and were directly sequenced on an ABI Prism 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA).

DISCUSSION

To our knowledge, this is the first report of pneumonia caused by macrolide-resistant *M. pneumoniae* with the A2063G point mutation in the 23S rRNA gene in siblings from a Korean family. Foy et al. [10] reported that *M. pneumoniae* infection spreads slowly in the family settings, with a median case-to-case interval of 23 days. *M. pneumoniae* infection may precede and intensify subsequent infections caused by respiratory viruses and bacteria [11]. Consequently, rapid and accurate diagnosis of *M. pneumoniae* infection is essential. Until now, a combination of real-time PCR and serologic test has been the most reliable approach for identification of *M. pneumoniae* because the sensitivity of PCR is usually affected by the type and quality of the respiratory specimen [12-14].

In 1970, Niitu et al. [15] reported the first clinical case of erythromycin-resistant *M. pneumoniae* in a girl with pneumonia. Lucier et al. [16] reported macrolide-resistant *M. pneumoniae* isolates with A-to-G transitions at position 2063 (A2063G) or 2064 (A2064G) in the V domain of the 23S rRNA gene after exposure of susceptible strains to erythromycin *in vitro*. In Japan, the clinical cases of macrolide-resistant *M. pneumoniae* increased rapidly since 2002 [6]. The prevalence of macrolide-resistant *M. pneumoniae* is variable, depending on the country [17], macrolide use [12], and patient's age [7]. Macrolide resistance is associated with mutations in the V domain of the 23S rRNA, or in the ribosomal proteins L4 and L22. A2063G is the most common mutation, followed by A2064G. Other mutations such as C2617A in the V domain of the 23S rRNA and mutations in the ribosomal proteins L4 and L22 have rarely been reported [6, 12, 18]. A2063 or A2064 mutations confer the highest resistance to macrolides, including resistance against erythromycin, clarithromycin, azithromycin, telithromycin, and josamycin, whereas a lower level of macrolide resistance is associated with mutations at nucleotide positions 2067 and 2617 in the V domain of the 23S rRNA [12]. The A2067G mutation was reported to cause the highest levels of resistance only for 16-membered-ring macrolides [19]. *M. pneumoniae* harboring the A2063G point mutation was sensitive only to rokitamycin [6].

Patients infected with macrolide-resistant *M. pneumoniae* required significantly longer durations of antibiotic therapy and presented with fever for longer durations [12, 17, 20]. However, our cases of macrolide-resistant *M. pneumoniae* pneumonia with the A2063G point mutation in the 23S rRNA gene were effectively treated with azithromycin. There are several possible explanations for this observation. First, the minimum inhibitory concentration $(MIC)_{50}$ and MIC_{90} for azithromycin are lower than those of clarithromycin or erythromycin for resistant *M. pneumoniae* [8]. Second, the inflammatory response to *M. pneumoniae* infection plays a crucial role in the pathogenesis of clinical disease [12]. Macrolides possess antimicrobial activity against *M. pneumoniae*, and have anti-inflammatory effects in macrolide-resistant *M. pneumoniae* infections [21-23]. Thus, clinical efficacy of macrolides for *M. pneumoniae* infection may reflect

not only direct antimicrobial activity but also the anti-inflammatory effect of cytokine inhibition, including inhibition of interleukin (IL)-8 [12, 21-23]. Third, fever may have resolved spontaneously in some patients with continuous macrolide treatment for macrolide-resistant infections; *M. pneumoniae* infection is associated with occasional spontaneous symptomatic recovery [12].

In conclusion, we described 2 cases of siblings infected with macrolide-resistant *M. pneumoniae*. Patients who do not respond to empirical antibiotics need to be screened for *M. pneumoniae* infection, and an accurate and rapid test is needed. In addition, if the clinical course of patients with *M. pneumoniae* pneumonia appears to be prolonged, macrolide-resistant *M. pneumoniae* should be carefully considered. The multi-center epidemiologic monitoring of macrolide resistance of *M. pneumoniae* is necessary in Korea, as is further research into treatment guidelines of *M. pneumoniae* pneumonia.

Authors' Disclosures of Potential Conflicts of Interest

This study was supported by Bioneer, Korea. No other potential conflicts of interest relevant to this article were reported.

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