ORIGINAL RESEARCH ARTICLE

No detection of macrolide-resistant *Mycoplasma pneumoniae* from Swedish patients, 1996–2013

Karolina Gullsby, MSc[1,2]* and Kåre Bondeson, MD, PhD[2]

[1]Centre for Research and Development, Uppsala University/Region Gävleborg, Gävle, Sweden; [2]Department of Medical Sciences, Section of Clinical Virology, Uppsala University, Uppsala, Sweden

Background: *Mycoplasma pneumoniae* is a common cause of respiratory infections which can cause life-threatening pneumonia and serious extrapulmonary manifestations. Since the year 2000, the emergence of macrolide-resistant *M. pneumoniae* strains has increased with varying incidences across countries. In China more than 90% of the strains are resistant. *M. pneumoniae* diagnostics is mostly done with molecular methods, and in Sweden antibiotic resistance surveillance is not routinely performed. The prevalence of macrolide-resistant *M. pneumoniae* has not previously been studied in Sweden.
Material and methods: A total of 563 *M. pneumoniae*–positive respiratory samples, collected from four counties in Sweden between 1996 and 2013, were screened for mutations associated with macrolide resistance using a duplex FRET real-time PCR method. The real-time PCR targets the 23S rRNA gene, and differentiation between wild-type and resistant strains was achieved with a melting curve analysis.
Results: Of the 563 samples included, 548 were analyzed for mutations associated with macrolide resistance. No mutations were found. The detection rate of macrolide-resistant *M. pneumoniae* in this study was 0% [0.00–0.84%].
Conclusion: No macrolide-resistant *M. pneumoniae* has been detected in Sweden. However, the emergence and spread of macrolide-resistant *M. pneumoniae* strains in many countries commands continuous epidemiological surveillance.

Keywords: Mycoplasma pneumoniae; *antibiotic resistance*; *macrolide*; *treatment*; *diagnostics*

*Correspondence to: Karolina Gullsby, Department of Clinical Microbiology, Region Gävleborg, 80187 Gävle, Sweden, Email: karolina.gullsby@regiongavleborg.se

The increased usage of antibiotics in both humans and in animal production exacerbates the progression of antibiotic resistance, which is a global threat (1, 2). Diagnostics together with detection and awareness of antibiotic resistance is important to optimize the treatment of infections so that treatment failure, prolonged illness and suffering, and unnecessary use of antibiotics can be reduced.

Mycoplasma pneumoniae is a common cause of respiratory infections, which are often presented by mild, self-limiting upper respiratory infections but can also cause life-threatening pneumonia and serious extrapulmonary manifestations, such as encephalitis, pericarditis, dermatological disorders, and hemolytic anemia (3). *M. pneumoniae* is fastidious and slow growing, so cultured isolates are therefore hard to obtain. This contributes to the current limitations of antibiotic resistance surveillance. Laboratory diagnostics is mostly performed by molecular detection of the bacteria or serology, where molecular detection is advantageous since it is a fast and sensitive

method for detection of the bacteria in the acute phase of the infection (3, 4). In Sweden, clinical laboratories do not routinely perform *M. pneumoniae* culturing or antibiotic resistance surveillance.

The emergence of macrolide-resistant *M. pneumoniae* was first observed in the year 2000 in Japan (5). Since then, the incidence of infections caused by resistant *M. pneumoniae* in Asia has increased dramatically and recent reports from China describe that more than 90% of *M. pneumoniae* strains are macrolide resistant (6, 7). Macrolide resistance is an emerging problem and is detected with varying prevalence from 1 to 30% in countries, such as Australia, Denmark, France, Germany, Israel, and the United States (8–13).

The macrolide resistance in *M. pneumoniae* is caused by point mutations in the peptidyl transferase loop of domain V of the 23S rRNA gene (5, 14). The most common types of mutations are at position A2063G followed by A2064G and A2063C, which all give rise to high levels of macrolide resistance (5–7, 14). Other mutations associated with

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lower level of resistance and which are seldom encountered in clinical findings are A2067G, C2617A, C2617G, and A2063T (5, 10, 14, 15). Molecular methods for detection of mutations connected to macrolide resistance have been developed, which enable surveillance of macrolide resistance in samples from patients without the need of culturing (16–18).

Only one case of macrolide resistance, which was induced after macrolide treatment, has been described among Swedish *M. pneumoniae* patient samples (19). Previously, there have been no large studies that aim to investigate if macrolide-resistant strains of *M. pneumoniae* circulate in the Swedish community. Among the Nordic countries, Denmark has detected 1–2% resistance, and no further studies have been performed in any of the other countries in our region (9).

The aim of this study was to determine the prevalence of macrolide resistance of *M. pneumoniae* within a large number of patient samples in Sweden. *M. pneumoniae*–positive samples were analyzed with regard to the presence of macrolide resistance using a PCR method developed by Peuchant et al. (16). This study and future surveillance of macrolide resistance in *M. pneumoniae* will provide the basis for validation of the currently used treatment guidelines.

Material and methods

Sample preparation and extraction
In this study, we included 563 *M. pneumoniae*–positive respiratory patient samples collected from four counties in Sweden during the period 1996–2013. Four-hundred and twenty-two (75%) of the samples were from the period 2010 to 2013. The samples had previously been diagnosed as *M. pneumoniae*–positive by PCR methodology at each of the four clinical microbiological laboratories in Falun, Gävle, Karlstad, and Uppsala, and then stored at −70°C until used in this study. More than 95% of the samples consisted of oropharyngeal or nasopharyngeal swab samples and less than 5% of the samples consisted of lower respiratory samples, where sputum or bronchial alveolar samples were the most common types. Before the start of the study, the samples were anonymized and only information about the patient's age, sex, at which county and year it was taken, and if the sample was taken at a policlinic or at a hospital was mapped to the samples.

The majority of the samples were extracted using MagNA pure 96 (Roche Diagnostics, Basel, Switzerland), DNA, and viral NA small volume kits using the program pathogen universal. The starting volume was 200 µL and the elution volume 100 µL, which was then aliquoted into two vials. Samples from one of the counties were extracted with MagNA pure compact (Roche Diagnostics, Basel, Switzerland) using an external lysis program with proteinase K. After verifying the presence of *M. pneumoniae*

by real-time PCR, essentially as described previously (20), the extracted DNA samples were aliquoted and stored at −70°C until macrolide resistance screening PCR was performed.

Since the patient samples were anonymized and the results could not be connected to patient identity, ethical approval was not required for this study.

Control strains
Reference strain ATCC 29342 (M129) was used as wild-type control. Four characterized macrolide-resistant strains derived from clinical samples, kindly received from Professor Cécile Bébéar, University of Bordeaux, France, were used as positive controls. These strains had the following mutations respectively: A2063C, A2063G, A2064G, and C2617G. DNA from each control strain was extracted using MagNA pure 96 (Roche Diagnostics, Basel, Switzerland), as described above. Sterile water was used as a negative control. The controls were included in each run.

Molecular screening of macrolide resistance
Screening of six different mutations of the 23S rRNA, all associated with macrolide resistance, was performed using a duplex FRET real-time PCR method developed by Peuchant et al. (16). A wild-type strain is distinguished from mutant strains, harboring any of the following mutations: A2063C, A2063G, A2064G, A2067G, C2617A, and C2617G, by melting curve analysis showing a difference in melting temperatures (T_m) (16). The PCR analysis was performed using Cobas® z480 (Roche Diagnostics, Basel, Switzerland). Primers were obtained from Eurogentec (Liège, Belgium) and the probes from Sigma-Aldrich (St. Louis, USA). PCR mastermix included LightCycler® FastStart DNA Master HybProbe (Roche Diagnostics, Basel, Switzerland) and the composition of PCR mixture and design of PCR program was as described by Peuchant et al. with a small alteration of the melting step (16). The melting step consisted of 95°C for 30 s, 40°C for 40 s followed by a slow rise of the temperature up to 80°C with a rate at 0.1°C/s with continuous acquisition of fluorescence.

Data analysis
Melting curve analysis was performed, and melting temperature (T_m) values were determined by the software. Each melting curve and T_m was also manually revised and adjusted if necessary in order to ensure that the T_m-value represented a true melting peak.

Statistical calculations, using the modified Wald interval, were performed to establish the 95% confidence interval for the rate of macrolide resistance detected (21).

Results
Most of the patients were between 11 and 20 years of age, and the median age was 32 years (range 1–91) (Fig. 1). The age distribution is representative and shows that
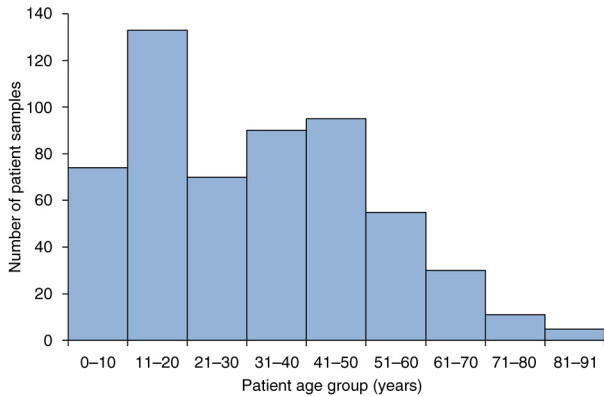
Fig. 1. Number of samples included in the study presented by patient age group.

M. pneumoniae infection can affect all ages, but is more common in adolescents, as described in the literature (3). Two-hundred and ninety-four (52%) of the patients were women and 269 (48%) were men. One-hundred and thirty-eight (24.5%) of the samples were from inpatients, 405 (72%) were from outpatients, and for 20 (3.5%) of the samples information on where the sample had been taken was missing.

Of the 563 samples, 548 could be fully analyzed with the duplex FRET PCR and all samples showed melting curve concordant with the wild-type strain. No samples contained mutations at the positions in the 23S rRNA gene associated with macrolide resistance and thus the detection rate was 0% [0.00–0.84%]. In each run, all control strains showed melting peaks at expected temperatures, confirming the capability of resistance mutation detection (Fig. 2). The mean Ct-value for the 548 samples which could be fully analyzed was 24.2, with a standard deviation of 4.0.

In addition, six samples could be only partially analyzed where results were obtained in only one of the channels in the duplex FRET PCR targeting the 262 base pair sequence, including the nucleotides at positions 2063, 2064, and 2067. A melting signal was obtained, concurrent with the wild-type for these six samples. No results were obtained from the other channel targeting the 495 base pair sequence, including the nucleotide at position 2617, and thus a potential mutation at this position could not be ruled out for these six samples. Nine samples could not be diagnosed, since no amplification or melting curves were acquired from these samples in the duplex FRET PCR. All of the samples that could not be analyzed or only partly analyzed contained low concentration of *M. pneumoniae* DNA, with Ct-values at the detection level. The mean Ct-value for the 15 samples not fully analyzed was 31.8, with a standard deviation of 1.6.

Discussions

The emergence of macrolide-resistant *M. pneumoniae* is a serious problem since macrolides are the only type of antibiotics suitable for the treatment of affected children aged <8 years (3). In this study, we investigated the proportion of macrolide-resistant *M. pneumoniae* in Swedish
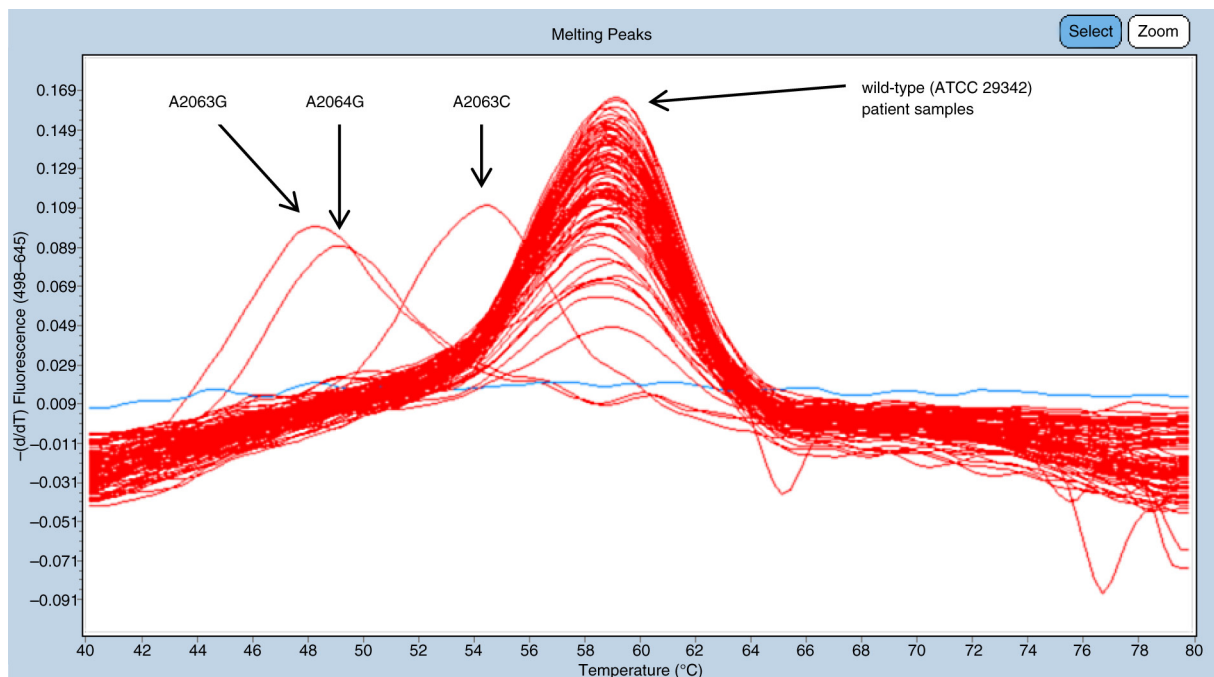

Fig. 2. A representative example of a typical result from one of the channels (645 nm) in the duplex FRET real-time PCR showing distinct T_m-differences of patient samples, aligned with the wild-type control strain ATCC 29342 and three control strains, respectively. The blue line without melting point corresponds to a negative sample with no amplification of *M. pneumoniae* DNA.

specimens by screening 563 *M. pneumoniae*–positive samples for mutations associated with macrolide resistance by using a FRET real-time PCR. Macrolide resistance was not detected in any of the samples.

Previously, only one case of macrolide-resistant *M. pneumoniae* has been reported in Sweden, which was induced after treatment of erythromycin (19). There have been other reports where *M. pneumoniae* with macrolide resistance has been induced by treatment (12, 22, 23). Treatment status for the patients included in this study is unknown, but the majority of the samples were taken from policlinics and therefore the sampling is assumed to have taken place before antibiotic treatment was initiated. According to Swedish treatment guidelines for respiratory infections within outpatient care, antibiotics should only be used under strict conditions and bronchitis caused by *M. pneumoniae* is not an indication for antibiotic treatment (24). For adults, doxycycline is the treatment of choice for pneumonia caused by *M. pneumoniae* at policlinics, whereas doxycycline or erythromycin is the treatment of choice according to guidelines for hospital-treated *M. pneumoniae* (24, 25). Erythromycin is the treatment of choice for children aged <8 years (25). The majority of the samples included in this study was collected around the year of the epidemic peak of 2011, where there was a 25% rise in the prescription of tetracycline and macrolides when compared with the same period in 2009 (26). There was a higher antibiotic burden in the population at that point, but macrolide resistance was not detected in any of the included samples.

Detection of macrolide resistance among specimens within the closely related species *M. genitalium*, which has the same mechanism of macrolide resistance, was recently performed in samples from two of the four counties included in this study (27). *M. genitalium*–positive specimens from 2006 to 2011 were analyzed for mutations associated with macrolide resistance, and no mutations were detected from specimens collected in the period 2006–2007. However, afterward, an increase of specimens with mutations was detected each year with up to 21% of the specimens in 2011 (27).

The treatment strategy for *M. genitalium* differs from that of *M. pneumoniae* and should, according to Swedish guidelines, always be treated with antibiotics when detected (28). The treatment of choice for *M. genitalium* is azithromycin, and the previous recommendation of a single-dose treatment with 1 g azithromycin has been associated with the selection of macrolide-resistant *M. genitalium* strains, which could possibly explain the development of resistance in *M. genitalium* when compared with *M. pneumoniae* (27). Further, doxycycline is a common choice of antibiotic for treatment of respiratory tract infections, including infections caused by *M. pneumoniae*, and thus the selective pressures of macrolides are probably lower for that species (29). Compared

with other European countries, Sweden has a generally low consumption of macrolides (30). The use of macrolides and in particular azithromycin is avoided because of the indication that it favors the selection of resistant strains of, for example, *Streptococcus pneumoniae*, probably due to the long half-life of the substance (31, 32).

Undetected macrolide-resistant *M. pneumoniae* could lead to exposure to prolonged illness for the patient. Studies have shown that hospitalized children infected with macrolide-resistant, as compared with macrolide sensitive, *M. pneumoniae* strains have a longer duration of fever, cough, and hospital stays (33, 34). Zhou et al. also saw a higher prevalence of extrapulmonary complications in children infected with macrolide-resistant *M. pneumoniae* (34). The high incidence of macrolide-resistant *M. pneumoniae* could result in the need to change treatment guidelines.

The method of resistance detection applied in this study enables antibiotic resistance surveillance screening with a high sensitivity, where 97% of the samples were successfully analyzed. This study showed no detection of macrolide-resistant *M. pneumoniae* in Sweden; whether this reflects a prudent use of macrolides remains to be investigated. Despite the situation in Sweden, the progress of antibiotic resistance in many countries and the dramatic spread of macrolide-resistant *M. pneumoniae* strains in Asia is a threat of great concern. This commands continuous epidemiological surveillance of macrolide resistance, and global strategies to minimize overuse of antibiotics must be a priority.

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