Occurrence of macrolide-resistant *Mycoplasma pneumoniae* strains in Germany

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Abstract

In a total of 167 respiratory tract specimens from adult outpatients with confirmed *Mycoplasma pneumoniae* pneumonia, sampled between 2003 and 2008, and a further 99 isolates obtained from patients between 1991 and 2009 in Germany, *M. pneumoniae* was tested for macrolide resistance. Using PCR, real-time PCR and sequencing of the 23S rRNA gene, 1.2% of *M. pneumoniae* in the respiratory tract samples and 3.0% of the isolates were found to be resistant. The results indicate a limited but not negligible importance of macrolide-resistant *M. pneumoniae* in the population investigated, which requires the monitoring of macrolide susceptibility of isolates or the testing of respiratory samples by molecular methods.

Keywords: 23S rRNA, community-acquired pneumonia, macrolide resistance, *Mycoplasma pneumoniae*, real-time PCR
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Introduction

Mycoplasma pneumoniae is one of the most common causes of community-acquired infections of the human upper and lower respiratory tract and can lead to severe and long-lasting interstitial pneumonia [1]. Owing to the fact that mycoplasmas are cell wall-less bacteria, macrolides are in most cases the first choice for antibiotic treatment of *M. pneumoniae* infections [1]. For young children especially, the alternatives, tetracyclines and new fluoroquinolones, are not recommended. Resistance of isolated strains to macrolides is based on point mutations in domain V of the 23S rRNA gene of *M. pneumoniae*. An A to G/C transition at position 2063 or 2064 of the gene (*M. pneumoniae* numbering) resulted in a high-level macrolide resistance of the *M. pneumoniae* strain, whereas a C to G or C to A mutation at position 2617 was associated with a lower resistance to macrolide antibiotics [2,3].

Recent occurrence of macrolide resistance was shown in studies from Japan in which a significant increase in resistant *M. pneumoniae* strains of up to more than 30% in the year 2006 was reported [4,5]. Results of two current studies from China showed rates of resistant isolates of 83% and 92%, respectively [6,7]. In contrast, in an investigation of macrolide resistance among 155 *M. pneumoniae* isolates obtained from patients between 1994 and 2006 in France only two resistant isolates were found [8]. Furthermore, macrolide resistance was detected in a relatively low number of *M. pneumoniae*-positive respiratory tract samples (five/100 isolates) in the United States [9]. A more comprehensive overview of worldwide macrolide resistance is hampered by the difficulty of cultivating *M. pneumoniae* strains, which has been successful in reference laboratories only. The use of molecular methods such as real-time PCR offers the possibility of a culture-independent characterization of the circulating *M. pneumoniae* strains, including their resistance [4,9]. Furthermore, previous reports on macrolide-resistant *M. pneumoniae* have mainly been concerned with respiratory tract specimens and isolates from children who were in most cases hospitalized. Although systematic investigations are not available, differences in the occurrence of macrolide-resistant *M. pneumoniae* strains between adult and paediatric patients cannot be excluded as preliminary results have suggested [5].

This is the first study to focus on investigating respiratory tract samples from adult pneumonia outpatients using molecular methods in order to detect the actual macrolide resistance in *M. pneumoniae* found in these specimens. In addition, the investigation of 100 isolates from patients of different ages was included to obtain a more comprehensive survey of macrolide-resistant *M. pneumoniae* over time in Germany.

Materials and Methods

Respiratory tract samples (bronchoalveolar lavage fluids, nasopharyngeal or pharyngeal swabs, sputa) were a German network (Community-Aquired Pneumonia NETwork, CAPNET) established to investigate the aetiology of community-acquired pneumonia. Briefly, 12 local clinical centres distributed over different regions of Germany cooperated with a number of general practitioners to systematically collect samples from patients with symptoms of pneumonia. Between 2003 and 2008, 167 respiratory tract samples of adult pneumonia outpatients tested positive for *M. pneumoniae* [10]. Independent of the specimens sampled within the CAPNET programme, *M. pneumoniae* isolates from clinical material obtained mainly from hospitalized pneumonia patients of different ages were included in the study. Isolation and propagation of 100 *M. pneumoniae* strains isolated between 1991 and 2009 from 99 patients (two strains were isolated from different locations of the bronchial system of one patient, see below) were carried out as described [11]. DNA from patient samples and from *M. pneumoniae* strains grown in Plenrophenmonia-like organism (PPLO) broth cultures (200 μL each) was extracted using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (the protocol for blood and body fluids called for elution volumes of 50 and 200 μL, respectively). DNA from patient samples was pretested using a real-time PCR approach targeting a conserved part of the gene coding for the main P1 adhesin of *M. pneumoniae* [12]. Isolated *M. pneumoniae* strains and strains in respiratory specimens confirmed as positive were grouped into the known subtypes and variants by sequencing a variable part of the repetitive element RepMP2/3 in the P1 gene of *M. pneumoniae* as described recently [10].

For partial amplification of the 23S rRNA gene in *M. pneumoniae*-positive respiratory tract samples, the primers (Biomers, Ulm, Germany) MN23SDVF and MN23SDVR (first amplification) were used as described [4]. Nested PCR with the primers MN23SDVFn (5′-GAC TGT TTA ACT AAA ACA CAA CTC TAT G-3′, position: 1777-1804) and MN23SDVRn (5′-CTA GAA GCA ACA CTC TTC AAT CTT C-3′, position: 2661-2637) was carried out according to standard procedures (annealing temperature: 60°C, 30 cycles) and resulted in an 885-bp product. The 23S rRNA gene region from DNA from patient isolates was amplified with the same primer combination. Specificity of the PCR was tested using DNA of the following reference strains: *Klebsiella pneumoniae* (ATCC 13883), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 43895), *Pseudomonas aeruginosa* (ATCC 27853), *Haemophilus influenzae* (ATCC 49247), *Chlamydophila pneumoniae* (strain TW-183), *Legionella pneumophila* (ATCC 33152), *Streptococcus pneumoniae* (ATCC 6305), *Mycoplasma genitalium* (ATCC 33530), *Mycoplasma salivarium* (ATCC 23064), *Mycoplasma orale* (ATCC 23714), *Mycoplasma hominis* (ATCC 23114), *Ureaplasma urealyticum* (ATCC 27818), *Ureaplasma parvum* (ATCC 27815) and *Mycoplasma penetrans* (ATCC 55252), respectively. Furthermore, the DNA of 50 respiratory tract samples of *M. pneumoniae*-negative pneumonia patients was included.

An aliquot (1 μL) of the amplification product of the nested PCR (respiratory tract samples) and of the first amplification (*M. pneumoniae* strains) was used as template for a pre-screening real-time PCR for the detection of mutations at positions 2063 and 2064 of the 23S rRNA gene of *M. pneumoniae*. Amplification was carried out using primers MN23SDVFn and MN23SLCR (5′-GTA GTA TTC CAC CTT TCG CAT C-3′, position: 2183-2162) and probes MN23SLCP1 (5′-GTG AAG ACA CCC GTT AGG CGC AAC-FL, position: 2032-2055) and MN23SLCP2 (5′-LC640-GGA CGG AAA GAC CCC GTG-PH, position: 2057-2074). With a LightCycler 1.5 instrument (Roche, Mannheim, Germany), real-time PCR was performed in a final volume of 20.0 μL containing 6.6 μL of water (PCR grade; Roche), 2.4 μL of $MgCl_2$ (25 mM, Roche), 2.0 μL of LightCycler Fast-Start DNA Master HybProbe mix (Roche), 2.0 μL of each primer (5 pmol), 2.0 μL of probes (2 pmol) and 1.0 μL of template. The capillaries were incubated under the following cycling conditions: pre-incubation at 95°C (10 min), 40 cycles of denaturation at 95°C (8 s), hybridization at 57°C (8 s) and elongation at 72°C (10 s). Melting curve analysis was carried out after heating from 55°C to 85°C (temperature transition rate: 0.1°C/s). Finally, the reactions were cooled to 40°C for 30 s. Data were analysed using the LightCycler software version 3.5 (Roche). Putative mutations at position 2063/2064 of the 23S rRNA gene of *M. pneumoniae*, as suggested by melting peak analysis, were confirmed by sequence analysis.

Antibiotic susceptibility to erythromycin (Sigma, St Louis, MO, USA) of *M. pneumoniae* isolates which were regarded as macrolide-resistant according to the real-time PCR and sequencing results was determined by microdilution with PPLO broth [13]. The MIC was defined as the lowest con-