



Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Case Report

Molecular characterization of macrolide resistance of a *Mycoplasma pneumoniae* strain that developed during therapy of a patient with pneumoniaRoger Dumke^{a,*}, Simone Stolz^b, Enno Jacobs^a, Thomas Juretzek^c^aTU Dresden, Medizinische Fakultät Carl Gustav Carus, Institut für Medizinische Mikrobiologie und Hygiene, Fetscherstrasse 74, 01307 Dresden, Germany^bKlinik für Kinder- und Jugendmedizin, Carl-Thiem-Klinikum gGmbH, Cottbus, Germany^cInstitut für Mikrobiologie und Krankenhaushygiene, Carl-Thiem-Klinikum gGmbH, Cottbus, Germany

ARTICLE INFO

Article history:

Received 11 June 2014

Received in revised form 3 July 2014

Accepted 11 July 2014

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:

Community-acquired pneumonia

Mycoplasma pneumoniae

Typing Macrolide resistance

SUMMARY

The development of macrolide resistance that occurred during 3 days of therapy with azithromycin to treat *Mycoplasma pneumoniae* pneumonia in a paediatric patient is reported. After extended molecular characterization of strains, the parallel occurrence of clones showing the non-mutated wild-type 23S rRNA sequence as well as mutations A₂₀₆₃G and A₂₀₆₄G, which are both responsible for phenotypic resistance, was confirmed for the first time.

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1. Introduction

Mycoplasma pneumoniae is a frequent cause of respiratory tract infection in humans, resulting in disease that ranges from mild forms of tracheobronchitis to cases of severe interstitial pneumonia. Although all age groups can be infected, most cases have been reported in older children. In epidemic phases occurring every 5 to 7 years, up to 40% of all cases of community-acquired pneumonia are attributed to *M. pneumoniae*. Furthermore, extrapulmonary complications have been described, mainly involving the skin and central nervous system.¹

Mycoplasmas lack a cell wall and thus are intrinsically resistant to beta-lactam and all other antibiotics that target the cell wall. Quinolones, tetracyclines, and macrolides represent the therapeutic options. With respect to side effects, the latter antibiotics are the treatment of choice for paediatric patients. Unfortunately, since the year 2000 the emergence of resistant strains has become a growing problem. Macrolide resistance in *M. pneumoniae* is based exclusively on mutations of single-copy 23S rRNA. The A2063G/C or A2064G/C transitions are the most common point mutations

described to date, and in all strains tested they lead to a high level of resistance (minimum inhibitory concentration (MIC) >32 mg/l) to the different classes of macrolide antibiotics.² Since culture of *M. pneumoniae* is time-consuming and limited to a few laboratories worldwide, many studies have confirmed the phenotypic resistance of mutated strains with molecular methods. These have become a major tool for the detection of resistant strains. With rates of >90%, macrolide resistance is at a critical level in several Asian countries, whereas the rates of mutated strains in Europe and the USA have remained <10%.² Nevertheless, the circulation of small numbers of resistant strains in the human population requires the periodic monitoring of *M. pneumoniae*-positive respiratory samples, as well as further investigation of the mechanisms of emerging resistance.

Here, we describe the course of *M. pneumoniae* pneumonia in a boy with severe pre-existing conditions, showing the failure of macrolide therapy with special respect to the molecular characterization of the *Mycoplasma* strain.

2. Case report

A 15-year-old boy (co-morbidities: epilepsy, spastic tetraplegia, asphyxia, dystrophy necessitating percutaneous endoscopic gastrostomy (PEG), and recurrent pneumonia) was referred to hospital

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in December 2013 (defined as day 1) with symptoms of a dry cough for 7 days, persistent fever up to 40.6 °C, and crepitations on lung examination. *Candida albicans* was detected in swabs of the PEG tube. C-reactive protein (CRP) was 60.2 mg/l. Before admission to the hospital, the patient had been treated with cefuroxime axetil. He had not received any macrolides prior to admission.

The pharyngeal swab taken on the first day of hospitalization was positive for *M. pneumoniae* using real-time PCR (cycle threshold (Ct) value: 22.0). Antibiotic therapy was changed to azithromycin (11 mg/kg/day) for 3 days and oxygen was administered overnight. After 9 days of hospitalization the boy was discharged to ambulant care.

On day 19, the patient presented again because of severe multiple skin inflammations (especially in the genital region), persistent cough, and recurrence of high fever (up to 40.0 °C). CRP was 18.0 mg/ml. *M. pneumoniae* real-time PCR from the pharyngeal swab was still positive (Ct value: 23.8). Respiratory specimens were negative for *Chlamydia spp.*, respiratory syncytial virus, human metapneumovirus, and adenovirus. Swabs from the affected skin regions were taken and were positive for *Proteus mirabilis*, *Staphylococcus aureus*, *C. albicans*, and *Pseudomonas aeruginosa*, and PEG tube swabs were positive for *P. aeruginosa* and *C. albicans*. To treat these multiple infections caused by a broad spectrum of microorganisms and to minimize the risk of sepsis due to *P. aeruginosa*, intravenous therapy with tobramycin and ampicillin/sulbactam was given. All further respiratory samples taken after the antibiotic treatment were found to be negative for *M. pneumoniae*. The patient was discharged in satisfactory general condition on day 34 after the start of the first hospitalization.

M. pneumoniae strains in the samples taken on day 1 (sample 1, first hospitalization) and day 19 (sample 2, second hospitalization) were investigated for mutations in the 23S rRNA and genotyped as described. The sequencing results of sample 1 showed a 23S rRNA sequence with no resistance-relevant mutations (Table 1), indicating a macrolide-susceptible *M. pneumoniae* strain. In contrast, in the specimen sampled after the patient had been treated with azithromycin, mutations at positions 2063 and 2064 of 23S rRNA and the wild-type sequence were suggested. To prove this, the PCR products were cloned into *Escherichia coli* using a TOPO Cloning Kit (Invitrogen, Carlsbad, CA, USA); the manufacturer's instructions were followed. Colourless colonies were picked for amplification of the region of interest of *M. pneumoniae*-specific 23S rRNA and the

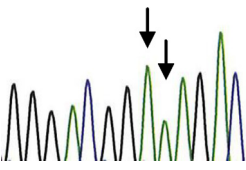
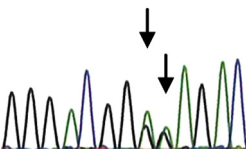
products were sequenced. In contrast to sample 1 (wild-type 23S rRNA sequence), results from sample 2 confirmed a mixture of genotypes, showing the wild-type sequence and clones with two mutations typical for a macrolide-resistant strain. Furthermore, the strains in both samples were investigated with different methods for typing of *M. pneumoniae*. Genotyping revealed identical results for the two specimens, suggesting that the macrolide-resistant mutants were selected from wild-type Mycoplasma. P1 and VNTR types of strain were found both in macrolide-resistant and susceptible mycoplasmas, confirming the absence of particular clones showing macrolide resistance.^{1,2}

3. Discussion

The development of macrolide resistance in *M. pneumoniae* after adequate antibiotic treatment appears to be a rare event. In a recent Swedish study, resistance of the strain after treatment with macrolides was demonstrated in only one of 22 patients.³ In most cases, persistent symptoms after antibiotic therapy are the reason for additional diagnostic procedures. Interestingly, 9 days after the end of therapy, macrolide-susceptible as well as macrolide-resistant strains were detected in the patient presented here. It might be assumed that the treatment was not able to induce resistance in all mycoplasmas colonizing the host. Subtyping of strains in both samples showed no evidence of an independent second infection. Although the co-occurrence of mutated and non-mutated clones in one sample has already been described,⁴ this is the first time that a mixture of genotypes showing different mutations that cause macrolide resistance in *M. pneumoniae* has been detected. From a diagnostic point of view, the results of real-time PCR with melting point analysis for the detection of macrolide-resistant strains, as well as sequencing results to confirm a resistant *M. pneumoniae* strain, should be interpreted with caution. The demonstrated transitions led to comparable levels of resistance against azithromycin.²

In conclusion, the use of macrolides in paediatric patients with severe pneumonia can generally be recommended in countries with a low rate of resistant *M. pneumoniae* strains, such as those of Europe and the USA.^{1,2} Paediatric patients with confirmed *M. pneumoniae* infection, an unsatisfactory response to macrolide treatment, and severe co-morbidities like the patient presented here should undergo additional diagnostic investigations and a

Table 1
Results of molecular characterization of *Mycoplasma pneumoniae* in respiratory tract samples taken from the patient at different time-points

Sample (description)	Typing of <i>M. pneumoniae</i> strain			Mutation in 23S rRNA	Result of cloning (%)	
	(arrows: position 2063/2064)	VNTR type	VNTR P1 type			
1 (Day 1; before treatment with azithromycin)	Subtype 1	4/4/5/7/2	7	None	GGGACGGAAAGAC 2057 2067 	No mutation (100)
2 (Day 19; 16 days after the end of treatment with azithromycin)	Subtype 1	4/4/5/7/2	7	Two	GGGACGGAAAGAC 2057 2067 	A ₂₀₆₃ G (46) A ₂₀₆₄ G (28) No mutation (26)

change of antibiotics. Studies have reported that the diseases caused by macrolide-resistant *M. pneumoniae* strains are characterized by prolonged respiratory symptoms and extended hospital stays compared with infections due to macrolide-susceptible mycoplasmas.^{1,2} The decision to use alternative antibiotics should be assessed carefully and will depend mainly on the severity of the clinical symptoms.

Acknowledgement

The authors thank S. Gäbler for excellent technical assistance.

Conflict of interest: The authors declare that they have no conflict of interest.

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