Prevalence, genotyping and macrolide resistance of *Mycoplasma pneumoniae* among isolates of patients with respiratory tract infections, Central Slovenia, 2006 to 2014

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In this retrospective study we employed real-time polymerase chain reaction (PCR) to analyse the occurrence of *Mycoplasma pneumoniae* among upper and lower respiratory tract infections (RTI) in the Central Region of Slovenia between January 2006 and December 2014. We also used a culture and pyrosequencing approach to genotype strains and infer their potential macrolide resistance. Of a total 9,431 tested samples from in- and out-patient with RTI, 1,255 (13%) were found to be positive by *M. pneumoniae* PCR. The proportion of positive samples was 19% (947/5,092) among children (≤ 16 years-old) and 7% (308/4,339) among adults (>16 years-old). Overall, among those PCR tested, the highest proportions of *M. pneumoniae* infections during the study period were observed in 2010 and 2014. In these two years, 18% (218/1,237) and 25% (721/2,844) of samples were positive respectively, indicating epidemic periods. From the 1,255 *M. pneumoniae* PCR-positive samples, 783 (614 from paediatric and 169 from adult patients) were successfully cultured. Of these, 40% (312/783) were constituted of strains belonging to the P1 type II genomic group, while 60% (469/783) contained strains of the P1 type I group. Two isolates comprised both P1 type I and II strains. Results of a genotype analysis by year, showed that the dominant *M. pneumoniae* P1 type during the 2010 epidemic was P1 type II (82% of isolates; 81/99), which was replaced by P1 type I in the 2014 epidemic (75%; 384/510). This observation could indicate that the two epidemics may have been driven by a type shift phenomenon, although both types remained present in the studied population during the assessed period of time. Only 1% of strains (7/783) were found to harbour an A2063G mutation in the 23S rRNA gene, which confers macrolide resistance, suggesting that

the occurrence of *M. pneumoniae* macrolide resistance still seems to be sporadic in our geographic area.

Introduction

Mycoplasma pneumoniae is a fastidious, slow growing, cell wall-lacking bacteria that can be the causative agent of up to 40% cases of community-acquired respiratory tract infections (RTI) [1-6]. Although most cases present with mild or moderate symptoms, serious disease requiring hospitalisation occasionally occurs [7].

M. pneumoniae incidence usually increases during epidemics, which occur at intervals of four to seven years [8-10]. This pattern is presumably linked to the alternation of P1 adhesin types, which tend to change dominance over the course of time and, consequently, allow *M. pneumoniae* to elude the human host immune response [11]. According to nucleotide (nt) differences in two repetitive regions (RepMP2/3 and RepMP4) in the MPN141 gene, which codes for P1 adhesin, *M. pneumoniae* strains can be divided into two genomic groups, called P1 type I and P1 type II [12], with several subtypes [13].

Macrolide-resistant strains among *M. pneumoniae* isolates are increasingly described, with reported resistance rates among studies from European Union Member States [11,14-17], Israel [18] and the United States [19] of up to 30%. In contrast, rates of over 90% have been observed in some parts of Asia [20-25]. Low to high grade resistance against many if not all macrolides in *M. pneumoniae* has been shown to be related to nt substitutions in two regions of the V domain in the 23S rRNA gene, namely at positions 2,063, 2,064, 2,067 and 2,617 (*M. pneumoniae* numbering). Among

TABLE 1

Samples of patients with upper and lower respiratory tract infections tested by *Mycoplasma pneumoniae* real-time polymerase chain reaction and annual positivity rate, Central Slovenian region, 2006–2014 (n=9,431 samples)

Samples	Year									Period 2006–2014
	2006	2007	2008	2009	2010	2011	2012	2013	2014	
Total tested N	391	470	393	659	1,237	1,185	1,005	1,247	2,844	9,431
Positive N (%)	9 (2)	19 (4)	16 (4)	48 (7)	218 (18)	117 (10)	46 (5)	61 (5)	721 (25)	1,255 (13)

these mutations, the A2063G mutation appears to be the most common, followed by A2064G [26]. All other mutations A2063C [27], A2063T [28], A2067G, C2617G and C2617A are found much less frequently [26].

In Slovenia, *M. pneumoniae* infections are not notifiable and *M. pneumoniae* genotyping as well as antibiotic resistance testing are not yet provided as a routine service. Moreover, limited data are so far available for longitudinal studies looking into changes in the prevalence of *M. pneumoniae* infections, genetic diversity and antibiotic resistance.

In the only two published serology-based studies available from Slovenia, one from April 1996 to March 1997 [29] and the other from November 1999 to April 2001 [30], *M. pneumoniae* was frequently found as the causative agent of community-acquired pneumonia in hospitalised patients (5.7% [29] and 24.8% [30]). Based on these reports and the observation of an increased number of *M. pneumoniae* cases from routine laboratory testing all over Slovenia in 2010 and 2014 (data not shown), we decided to analyse the occurrence of *M. pneumoniae* infections in in- and out-patients with RTIs. At the end of 2005, real-time polymerase chain reaction (PCR) was introduced as the main diagnostic tool for *M. pneumoniae* infections in the Institute of Microbiology and Immunology at the University of Ljubljana, which covers the entire Central Slovenian Region. This allowed us to analyse tested samples from January 2006 to December 2014. We also used culture and molecular assays to further characterise the genotype of isolated strains during this period, and infer their potential macrolide resistance.

Methods

Patient samples and *Mycoplasma pneumoniae* testing

Our study was conducted between January 2006 and December 2014. Patients considered, were inhabitants of the Central Region of Slovenia, which on average represents 41% (843,528/2,038,281) [31] of the whole Slovenian population during the designated period of time. The distribution of patients did not have any apparent specific clustering in space and time. Thirty-two per cent (2,996/9,431) of patients were from the capital Ljubljana and 68% (6,435/9,431) from other

parts of the Central Slovenian region. Depending on the severity of the symptoms, patients were treated as in- or out-patients. During the study period, all consecutive (n=9,431) upper or lower respiratory tract specimens obtained from 5,092 (54%) paediatric (≤ 16 years of age) and 4,339 (46%) adult (> 16 years of age) patients with RTI were enrolled. Together with each sample, we received data of the patient's sex, age, address, attending physician and his/her institution, sample type, date and place of collection and basic diagnosis (RTI). An aliquot of each sample was subjected to routine laboratory testing by *M. pneumoniae* real-time PCR (Argene biosoft, France). This protocol was not changed during the time of the study. The remainder of each PCR positive sample was cultivated in order to obtain pure *M. pneumoniae* isolates.

Culture of *Mycoplasma pneumoniae* positive samples

Culture was performed by using *Mycoplasma* selective broth and agar plates (OXOID, United Kingdom (UK)) enriched with *Mycoplasma* supplement G or P (OXOID, UK) according to standard methods described elsewhere [32]. The obtained isolates were stored at -80°C until further testing.

Mycoplasma pneumoniae genotyping and macrolide resistance detection

Neither mutations in the 23S rRNA gene domain II, ribosomal proteins L4 and L22 [26], nor genomic or plasmid *erm* genes have been shown to be implicated in macrolide resistance in *M. pneumoniae* [33]. Moreover as *M. pneumoniae* harbours only one copy of the 23S rRNA gene [34], molecular tests targeting the 23S rRNA gene domain V offer valuable tools for quick identification of resistant strains. Such tests have been developed, as well as some allowing rapid and reliable genotyping, and these can even be applied directly to clinical samples [11,15,35–39]. Recent publications by Spuesens et al. [40,41] describe a pyrosequencing approach for genotyping *M. pneumoniae* and determination of macrolide resistance. Based on single nt polymorphisms (SNPs) in the MPN141 gene's constant region, and on additional information from the constant region of the MPN528a gene, the method enables quick classification of strains into P1 type I and P1 type II. Macrolide resistance implicated mutations in the two regions of the 23S rRNA gene V domain can also be detected. This

pyrosequencing approach was used for our study with some modifications which are further described.

Mycoplasma pneumoniae DNA was purified from 200 µL of liquid culture (OXOID, UK) using the MagNA Pure Compact automated DNA extraction system (Roche, Germany). A total of 5 µL of the 200 µL eluate was used in all PCR reactions.

All primers, reaction conditions and master mix compositions used for the amplification of *M. pneumoniae* MPN141, MPN528a and two parts of the 23S rRNA domain V (designated as Assay 1 – region encompassing macrolide resistance important positions 2,063, 2,064, 2,067 and Assay 2 – region encompassing macrolide resistance important position 2,617) were as described by Spuesens et al. [40], with several modifications. Briefly, for all PCR assays the number of cycles was increased to 50, with the denaturation step prolonged to 15 s and the extension step shortened to 15 s. The annealing step was 30 s with the temperature set to 55 °C for all PCR assays. The master mix was as previously published [40]. The resulting biotinylated amplification products were checked for expected sizes and suitable amounts by 1.5% agarose gel electrophoresis stained with 1X SYBR Safe (Invitrogen, Germany).

All remaining amplified fragments of MPN141, MPN528a, 23S rRNA Assay 1 and Assay 2 were pyrosequenced on a PyroMarkID instrument (Biotage, Sweden) with PyroMark Gold Q96 SQA Reagents (Qiagen, Germany) using previously designed sequencing primers (TIB MOLBIOL, Germany) [40]. For the detection of P1 type specific SNP, we adopted the dispensation order developed earlier [40], but we constructed our own specific nt dispensation orders that cover all so far detected mutations linked with macrolide resistance in respective portions of the *M. pneumoniae* 23S rRNA gene domain V. Moreover, the dispensation order for the *M. pneumoniae* 23S rRNA pyrosequencing Assay 1 was extended to produce 79 bases long pyrosequencing products, which were later used to additionally check the specificity of the PCR assay, by National Center for Biotechnology Information (NCBI) basic local alignment tool (BLAST) search.

Genotype and possible macrolide resistance designations were attributed using IdentityFire software (Biotage, Sweden) loaded with custom built libraries.

***Mycoplasma pneumoniae* 23S rRNA polymerase chain reaction Assay 1 and Assay 2 specificity**

NCBI BLAST search of the primers [40] used in the 23S rRNA domain V Assay 1 and Assay 2 showed a possible nonspecific annealing with *Mycoplasma genitalium*. Although *M. genitalium* is not a commonly recognised pneumonia agent and despite the fact that RTI isolates were used as starting material, we decided to additionally check the specificity of the primers. Both *M. pneumoniae* 23S rRNA assays were therefore performed

with DNA from *Chlamydia pneumoniae*, *C. psittaci*, *C. trachomatis*, *Corynebacterium* spp., *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Neisseria gonorrhoeae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Ureaplasma* spp.

Results

Patient samples and *Mycoplasma pneumoniae* testing

From 9,431 patient samples, 1,255 (13%) were found to be *M. pneumoniae* positive by PCR. Of the latter, 51% (n=640) were from males and 49% (n=615) from females. The median age of patients infected by *M. pneumoniae* was 8 years (range: 43 days to 85 years).

While *M. pneumoniae* was found among 19% (947/5,092) of children samples, 7% (308/4,339) of adult samples tested positive. The highest proportion of positive samples were found in the age group comprising individuals between six and 16 years-old (26%; 565/2,162), followed by preschool children ≤5 years-old (13%; 382/2,930), adults between 17 and 65 years-old (8%; 288/3,253) and elderly persons >65 years-old (2%; 20/1,086).

A closer look at the distribution over time of positive patient samples revealed that the majority were found in 2010, 2011 and 2014, when 218/1,237 (18%), 117/1,185 (10%) and 721/2,844 (25%) of patient samples contained detectable amounts of *M. pneumoniae* DNA, respectively (Table 1).

An even more detailed month to month analysis shows that the rate of detected *M. pneumoniae* infections was overall low from 2006 to 2009 (between 2% and 7%), with seasonal peaks between June and August. However, in December 2009 the rate of *M. pneumoniae* infections quickly rose to 21% (15/70) and remained high through to November 2011, when 11% (13/115) of patients with RTIs tested *M. pneumoniae* positive. Only later did the rate of *M. pneumoniae* infections return below the 2006 to 2014 average (9%) and remained low, with seasonal peaks during the summer, similarly to the time before 2010. Another unusual increase in the rate of *M. pneumoniae* positive samples occurred after the typical summer peak in 2013, during winter 2013 and spring 2014, until November 2014, when 37% (214/580) of patients were positive by *M. pneumoniae* PCR (Figure). It was also found that 15% (436/2,996) of patients in Ljubljana and 13% (819/6,435) in other parts of the Central Slovenian region had *M. pneumoniae* acute infections, which seems to indicate that *M. pneumoniae* was overall similarly present in the geographical area included in the study. Additionally, the rise in the number of *M. pneumoniae* positive patients during 2010 and 2014 occurred almost evenly throughout the whole studied area. We also did not observe

Rates of samples testing positive for *Mycoplasma pneumoniae* among samples from patients with upper and lower respiratory tract infections, Central Slovenian region, January 2006–December 2014 (n=9,431 tested samples)

any specific clustering that could indicate a merely localised outbreak during the time of the study.

Of the 1,255 PCR positive samples, *M. pneumoniae* was successfully cultured from 62% (n=783). The distribution of the obtained isolates according to sex was 50% (390/783) men, 50% (393/783) women, and overall 78% (614/783) of cultured samples were from children and 22% (169/783) from adults.

Mycoplasma pneumoniae genotyping and macrolide resistance detection

For all 783 *M. pneumoniae* cultured isolates, PCR products were successfully obtained for all targets, namely MPN141, MPN528a and both parts of 23S rRNA gene domain V (Assay 1 and Assay 2). All assays produced good quality pyrosequencing products of 7, 10, 79 and 16 nt for MPN141, MPN528a, 23S rRNA domain V Assay 1 and Assay 2, respectively. During the assessed time period, *M. pneumoniae* P1 type I and P1 type II strains were found in 60% (469/783) and 40% (312/783) of cultured isolates, respectively. We also detected two isolates with both P1 type I and P1 type II *M. pneumoniae* DNA.

A more detailed analysis on a year-to-year basis showed that although both P1 types remained present during the whole study period, the most observed *M. pneumoniae* P1 type during the 2010 epidemic was P1 type II (82%; 81/99), while in the 2014 epidemic P1 type I dominated (75%; 384/510) (Table 2). Moreover it seems that between these two epidemic years, the proportion of isolates with P1 type II decreased in favour of P1 type I (Table 2).

Seven samples (1%; 7/783), all from inpatients, were found to contain an A2063G mutation (Table 2). Six mutated strains were cultured from respiratory samples of paediatric patients and one from an adult patient. Three of the presumed macrolide resistant strains belonged to the P1 type I and four to the P1 type II. All other isolated *M. pneumoniae* strains had no mutation in the two sequenced areas of the 23S rRNA gene and were therefore presumed to be macrolide sensitive.

Mycoplasma pneumoniae 23S rRNA polymerase chain reaction Assay 1 and Assay 2 specificity

As predicted by the NCBI BLAST search, no tested organism except *M. genitalium* produced a positive PCR result in either *M. pneumoniae* 23S rRNA Assay 1 or Assay 2. However, after pyrosequencing the PCR product from Assay 1, the sequence of *M. pneumoniae* had six differences (73/79; 92% identity) distinguishing it from *M. genitalium*.

Discussion

From our data, we observed that in 2006, 2007, 2008, 2009, 2012 and 2013 the number of *M. pneumoniae* positive cases usually rose during the summer but quickly returned to low values during the autumn,

TABLE 2

Annual number of *Mycoplasma pneumoniae* isolates, P1 type distribution, percentage of inferred macrolide-resistant strains and detected 23S rRNA mutations, Central Slovenian region, 2006–2014

Year	Number of isolates	P1 type I N (%)	P1 type II N (%)	P1 type I+II N (%)	Mutated strains N (%)	23S rRNA mutation
2006	4	2 (50)	2 (50)	0 (0)	0 (0)	None
2007	19	9 (47)	10 (53)	0 (0)	0 (0)	None
2008	7	2 (29)	5 (71)	0 (0)	0 (0)	None
2009	33	12 (36)	21 (64)	0 (0)	1 (3)	A2063G
2010	99	17 (17)	81 (82)	1 (1)	2 (2)	A2063G
2011	46	12 (26)	33 (72)	1 (2)	0 (0)	None
2012	29	8 (28)	21 (72)	0 (0)	0 (0)	None
2013	36	23 (64)	13 (36)	0 (0)	1 (3)	A2063G
2014	510	384 (75)	126 (25)	0 (0)	3 (1)	A2063G
Total	783	469 (60)	312 (40)	2 (0)	7 (1)	–

winter and spring, suggesting that in these years the occurrence of *M. pneumoniae* infections was most likely endemic. On the other hand, a different pattern with unusual increases in *M. pneumoniae* infections was seen from November 2009 to December 2011 and from May 2014 to December 2014. Based on these results, we suggest that a *M. pneumoniae* epidemic took place in Slovenia from November 2009 to December 2011 similarly to other European countries [42]. Interestingly, our results also seem to show that another, even more extensive, outbreak of *M. pneumoniae* infections began in May 2014, reached its peak in November 2014, when 37% (214/580) of patients were positive for *M. pneumoniae* DNA and persisted at least until December 2014, when 29% (171/598) of patients were infected. Comparable results from other countries are not yet available, so we cannot say whether such a scenario is limited to Slovenia or correlates with a new European-wide epidemic.

We do not believe that the rises in the number of *M. pneumoniae* positive patients were due to increased awareness or testing availability, since no changes were made in the healthcare system in Slovenia during the time of the study. Additionally, the same test was used throughout and increased numbers of positives cannot therefore be ascribed to methodological changes in sensitivity. Moreover, a similar increase in the number of *M. pneumoniae* cases was also observed by other laboratories in Slovenia (data not shown). Finally, the number of tested patients did increase from 2006 to 2014 but most likely as a result of more patients seeking help at healthcare facilities during epidemics.

Analysis of *M. pneumoniae* P1 type distribution in Central Slovenia from 2006 to 2014 shows that both P1 types were present in the studied population during the whole period. No complete type shift phenomenon, as described in a Japanese study [43], could be observed in our population but it would seem that the

2010/11 epidemic was caused mostly by *M. pneumoniae* P1 type II and the 2014 epidemic by *M. pneumoniae* P1 type I. Although we observed a large increase in *M. pneumoniae* cases in 2014, we cannot be sure that the reason for the new outbreak was an increase in the number of P1 type I strains. Further studies using multilocus variable-number tandem repeat analysis (MLVA) [44] are needed to assess whether the 2010/11 and 2014 epidemics were caused by a specific MLVA type or multiple types, as seen in a recent study from France and Israel [45]. On the basis of P1 typing, however, it might be concluded that the prevalence of the two major *M. pneumoniae* genetic types in Slovenia does oscillate during time.

The results of our study suggest that macrolide-resistant *M. pneumoniae* strains, although represented by small numbers of isolates, are already present in Slovenian patients, which seems to be in concordance with most European studies, which also show low level of macrolide resistance in *M. pneumoniae* [11,14,15,17]. An exception is a study from 2011, in which macrolide resistance was detected in 26% of children presenting with *M. pneumoniae* pneumonia or bronchitis in an Italian city [16].

Our low prevalence of *M. pneumoniae* macrolide resistant strains, however, is not unexpected, since a national antibiotic usage survey shows that macrolides are being carefully used in Slovenia with Daily Doses (DD) per 1,000 inhabitants of 2.14, 2.43, 2.22, 2.11, 1.85, 1.75, 1.84, 1.67 and 1.65 in 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013 and 2014, respectively [46,47]. Interestingly, *M. pneumoniae* macrolide-resistant strains were first detected in 2009, and then in 2010, 2013 and 2014, when macrolide antibiotic DDs per 1,000 inhabitants were constantly declining.

We noted that all mutated strains harbour an A2063G substitution, which is recognised as the most prevalent and high level macrolide resistance mediating mutation [27]. Although all strains harboured the same type of mutation, we did not find any connection among the patients from whom these were derived. The patients were from different geographical parts of Central Slovenia and the dates of sample collection were several months or even years apart. Moreover, basic molecular typing studies showed that three presumed macrolide-resistant strains belonged to P1 type I and four to P1 type II subgroup, which additionally supports a lack of connection between the strains. Studies using more accurate genetic methods (MLVA) could shed further light on the phylogeny of these strains.

To summarise, we observed two epidemics of *M. pneumoniae* infections. The first was, similarly to some other European countries, in 2010/11. The second epidemic seems to have started in Slovenia in 2014. We also found that *M. pneumoniae* P1 type II may have been the main cause of the 2010 epidemic while P1 type I may have been responsible for the new 2014

outbreak. Additionally, in concordance with the situation in Europe, we documented the presence of macrolide-resistant *M. pneumoniae* strains in children and in adults at very low level.

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Conflict of interest

None declared.

Authors' contributions

RK: execution of experiments, analysis and interpretation of results, manuscript writing; DK: project leader, analysis and interpretation of results, manuscript writing, TM and MP: sample collection, patient data, pre-submission manuscript reviewing.

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