Identification of P1 types and variants of *Mycoplasma pneumoniae* during an epidemic in Chile

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This study was conducted to determine the types of *M. pneumoniae* prevalent in adults presenting with community-acquired pneumonia during an epidemic period, and to scrutinize a variable region of the RepMP4 element for the detection of P1 variants. All 23 clinical specimens PCR-positive for *M. pneumoniae* obtained in two hospitals in Santiago, Chile, from 2005 to 2006 were typed by a multiplex PCR directly and then the RepMP4 fragment of 18 specimens was sequenced. A predominance of *M. pneumoniae* type 2 was found, 18 (78.3 %) specimens being grouped as type 2 and 5 (21.7 %) as type 1. Co-infection of *M. pneumoniae* with other respiratory pathogens was found in 10/23 (43.4 %) patients, but their frequency was not related to the *M. pneumoniae* type. Sequence analysis revealed a single nucleotide polymorphism, a transition mutation, in 50 % of amplicons belonging to type 1 and in 71.4 % of amplicons of type 2. The nucleotide changes were synonymous in each P1 variant. In conclusion, during the 2005–2006 epidemic in Santiago, both types of *M. pneumoniae* circulated. Although the analysed area in the RepMP4 was small, we detected the existence of P1 variants in the two types of this organism.

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INTRODUCTION

Mycoplasma pneumoniae is an important worldwide cause of community-acquired pneumonia (CAP), especially in children and young adults (Waites & Talkington, 2004). *Mycoplasma* infections display a cyclical pattern characterized by 4–6 year endemic periods, alternating with shorter epidemic cycles (Foy *et al.*, 1979; Sasaki *et al.*, 1996; Lind *et al.*, 1997; Layani-Milon *et al.*, 1999). During epidemics, *M. pneumoniae* is responsible for a significant proportion of CAP cases in all age groups (Layani-Milon *et al.*, 1999). The genome of *M. pneumoniae* is regarded as genetically stable (Ursi *et al.*, 1994; Dumke *et al.*, 2004; Spuesens *et al.*, 2009). Strains are classified in two main groups (types 1 and 2), which differ in the sequence of two repetitive sequences: RepMP2/3 and RepMP4 found in one copy inside the P1 gene and in eight to ten copies throughout

the chromosome (Ruland *et al.*, 1990; Su *et al.*, 1990; Ursi *et al.*, 1994; Sasaki *et al.*, 1996; Dorigo-Zetsma *et al.*, 2000). Two evolutionary lines (type 1 and 2) of *M. pneumoniae* exist, and the differences between these lines are reflected in each RepMP2/3 and RepMP4 element within the genome (Spuesens *et al.*, 2009). Studies from Asia, Europe and the USA have reported variations in the distribution of *M. pneumoniae* types over time, forming the hypothesis that epidemics may arise due to a type shift phenomenon (Ursi *et al.*, 1994; Sasaki *et al.*, 1996; Dumke *et al.*, 2004; Pereyre, *et al.*, 2007; Kenri *et al.*, 2008). Furthermore, several groups have reported the existence of P1 variants in both *M. pneumoniae* types (Ruland *et al.*, 1990; Kenri *et al.*, 1999; Dorigo-Zetsma *et al.*, 2001; Pereyre *et al.*, 2007; Spuesens *et al.*, 2009). All described P1 variants have originated by homologous recombination between the P1 RepMP2/3 or RepMP4 repetitive elements (RepMPs) and their extragenic versions (Spuesens *et al.*, 2009). Since P1 protein is a major adhesin and an immunodominant antigen, recombination may serve as a mechanism for P1 variation contributing to the evasion of the immune response of the host (Feldner *et al.*, 1982; Leith *et al.*, 1983).

Abbreviations: CAP, community-acquired pneumonia; SNP, single nucleotide polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the RepMP4 sequences of the amplicons are GQ916909–GQ916925 and GQ332741.

The aims of this study were to determine the types of *M. pneumoniae* in specimens collected from adults presenting with CAP during an epidemic period and to scrutinize a variable region of the RepMP4 element for the detection of P1 variants.

METHODS

Clinical specimens. The 23 specimens characterized in this study correspond to all *M. pneumoniae*-positive specimens collected during a prospective protocol designed to determine the aetiology of CAP in adults. Patients were recruited at two hospitals in Santiago, Chile, between February 2005 and January 2008 (Martínez *et al.*, 2008). All patients had symptoms suggestive of a lower respiratory tract infection, a new infiltrate on the chest radiograph and no alternative diagnosis emerging during follow-up. All were immunocompetent, had not received immunosuppressive therapy, and had not been hospitalized in the previous 30 days. Positive specimens were obtained between April 2005 and March 2006, a period which coincided with an epidemic of *M. pneumoniae* in Santiago (Fig. 1). Patients positive for *M. pneumoniae* had a median age of 45 years (range 18–87 years), not

significantly different to the median age (65 years) of *M. pneumoniae*-negative patients ($P=0.1$) (Martínez *et al.*, 2008).

Microbiological study. The microbiological records for positive *M. pneumoniae* cases from the protocol database were reviewed. The following specimens were obtained for the study: blood, sputum, throat washes, paired sera and urine. Throat washes were processed for indirect immunofluorescent assay (IFA) and cell culture for virus detection, *Legionella* isolation and for PCR. *Streptococcus pneumoniae* was diagnosed by conventional blood and sputum cultures and by detection of urinary antigens (BinaxNOW). *Chlamydia pneumoniae* was tested by nested PCR and serological testing in paired sera (MIF; Savyon) (Martínez *et al.*, 2000). *Legionella pneumophila* was studied by culture, PCR and detection of urinary antigen (BinaxNOW) (Murdoch, 2003).

IFAs were run for the respiratory viruses influenza A and B, respiratory syncytial virus (RSV), adenovirus and parainfluenza viruses (PIV 1–3) using commercial monoclonal antibodies (Chemicon) both for detection as well as for the typing of influenza virus and PIV 1–3. Each specimen was also inoculated into Hep-2 and MDCK cell cultures as described elsewhere (Avendaño *et al.*, 2003). RSV and human metapneumovirus (hMPV) were also detected by real-time PCR assays (Avendaño *et al.*, 2003; Cote *et al.*, 2003).

P1 gene typing. The type of *M. pneumoniae* was determined by the amplification of a variable region of the RepMP4 element of the P1 gene by a multiplex PCR as described by Kenri *et al.* (2008) with two modifications. Annealing steps were carried out at 58 °C instead of 55 °C, and *Taq* DNA polymerase (GoTaq Flexi; Promega) was used in our study. DNA was extracted from throat wash specimens with the Wizard SV Genomic kit (Promega), according to the manufacturer's instructions. *M. pneumoniae* strain M129 (P1 type 1) and MAC (P1 type 2) were used as reference for comparisons. PCR products consisted of a 343 bp fragment for *M. pneumoniae* type 1 and a 560 bp fragment for *M. pneumoniae* type 2.

RepMP4 sequencing. Amplification products of the multiplex PCR of 18 specimens were purified using the QIAquick kit (Qiagen) and sequenced. Sequencing reactions were performed applying the Sequencing Terminator kit (Amersham) in the DNA sequencer ABI 310 (Applied Biosystem). Both strands were sequenced using the forward and reverse primers used in the multiplex PCRs. Sequences were analysed using the BLAST program available from the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>). The GenBank accession numbers for the RepMP4 sequences of the amplicons are GQ916909–GQ91625 and GQ332741.

RESULTS AND DISCUSSION

M. pneumoniae types

All 23 specimens positive for *M. pneumoniae* could be unequivocally assigned to a type. Five specimens (21.7%) were grouped as type 1 and 18 specimens (78.3%) as type 2 (Fig. 1a). *M. pneumoniae* infections are historically characterized by a cyclical pattern of epidemics. The distribution of *M. pneumoniae* types during epidemics has shown that in some outbreaks there was a clear predominance of one type over the other, while in other outbreaks, as in our case, both types co-circulated in the population (Sasaki *et al.*, 1996; Dumke *et al.*, 2006; Pereyre *et al.*, 2007; Kenri *et al.*, 2008). By the time that this study was conducted, a prolonged co-circulation of both *M. pneumoniae* types was observed in Europe, while in Japan most isolates belonged to type 1

Fig. 1. Distribution of types and variants of the *M. pneumoniae* P1 gene in clinical specimens collected in Santiago, Chile, between March 2005 and March 2006. (a) Number of specimens of each type detected per month. □, Type 1; ■, type 2. (b) Number of P1 variants detected per month. □, Type 1; ■, type 2.

(Dumke *et al.*, 2006; Pereyre *et al.*, 2007; Kenri *et al.*, 2008). Kenri *et al.* (2008) have suggested that the type shift phenomenon and the spread in the population of a dominant type may be causally linked to epidemic outbreaks. The humoral immune response of patients infected with *M. pneumoniae*, like that of experimentally infected guinea pigs, shows evidence of a protective status directed to the *M. pneumoniae* type (Jacobs *et al.*, 1996; Dumke *et al.*, 2004). This is not surprising given the key role of the P1 adhesin in the adherence and colonization of the organism in the respiratory tract (Feldner *et al.*, 1982; Leith *et al.*, 1983). Thus, the type shift phenomenon could allow the organism to escape the host immune response, giving rise to an accumulation of susceptible individuals in the population.

Mixed infection was found in 10/23 (43.4%) patients positive for *M. pneumoniae*, and presented with a similar frequency in CAP cases caused by type 1 and type 2 organisms. Thus, 2/5 (40%) co-infections were seen with *M. pneumoniae* type 1 and 8/18 (44.4%) co-infections occurred with *M. pneumoniae* type 2. The agents detected were hMPV ($n=4$), RSV ($n=3$), *C. pneumoniae*, *S. pneumoniae* and *L. pneumophila*, one in each case. Respiratory viruses are common in adults with pneumonia (Johnstone *et al.*, 2008). Nevertheless, it is difficult to establish the contribution of hMPV and RSV to *M. pneumoniae* pneumonia cases in our study since they were diagnosed in non-invasive respiratory samples. The use of serological methods may help to clarify a possible role as co-pathogens. hMPV cases clustered in a month during 2006, coincidental with a hMPV epidemic in the city, while RSV infections were scattered throughout the *M. pneumoniae* circulation period. In six out of seven co-infections with respiratory viruses, IgM, IgG or IgM/IgG seroconversions to *M. pneumoniae* were established. Thus, *M. pneumoniae* pneumonia probably predisposed to a viral infection, especially when hMPV peaked. Viral infections affected both outpatients ($n=4$) and hospitalized patients ($n=3$). *C. pneumoniae* infection was documented by PCR, and by a decrease in two dilutions of the IgG titres in the convalescent serum taken 6 weeks apart, while the *M. pneumoniae* aetiological role is supported by IgG seroconversion. Thus, in this case, *C. pneumoniae* infection probably preceded and predisposed to a *M. pneumoniae* infection. In the three other mixed infections (*S. pneumoniae*, *L. pneumophila* and hMPV), the participation of *M. pneumoniae* was documented only by PCR, as no IgM was detected in the acute phase serum, and no convalescent serum was available. Since *S. pneumoniae* was isolated from the blood cultures, there is no doubt about its involvement, but *L. pneumophila* was detected only by PCR, which suggests the possibility of a transient patient colonization or a specimen contamination.

RepMP4 polymorphism

A fragment of the RepMP4 of four (80%) specimens belonging to *M. pneumoniae* type 1 and 14 (77.8%)

specimens belonging to type 2 was reamplified and sequenced. The specimens were randomly selected for sequencing; approximately 80% of each P1 type specimens were sequenced. Sequence analysis revealed a single nucleotide polymorphism (SNP) in 50% of amplicons belonging to P1 type 1 and in 71.4% amplicons of P1 type 2. The nucleotide changes corresponded to silent mutations: the transition 573G→A for type 1 specimens and the transition 891G→A for type 2 specimens. Only one amplicon, corresponding to a type 2 specimen (accession no. GQ916910), presented a non-silent mutation (304G→T), and resulted in the amino acid change R→L (Table 1). Fig. 1(b) shows the temporal pattern of distribution of P1 variants. Type 2 variants predominated and were distributed over time, while type 1 variants appeared only twice in a 3 month period. We could not determine differences in the distribution of types and variants by site of collection, since most positive specimens were obtained in one hospital (University Hospital).

Repeated sequences constitute about 8% of the *M. pneumoniae* genome and as in other bacteria provide DNA for homologous recombination. The RepMP4 element is found in the 5' part of the P1 gene and also in other seven versions throughout the genome of *M. pneumoniae*. Most nucleotide variation, represented by deletion and insertion events and SNPs, is found in the middle part of the RepMP4 element located in the P1 gene, and allows the differentiation of *M. pneumoniae* in types, but also the recognition of type-independent variants (Spuesens *et al.*, 2009). The SNPs found in this study were similarly located. Since we sequenced only a short extension of the RepMP4, we cannot determine the degree of sequence variation of RepMP4 in our strains.

The identification of SNPs is of interest for the recognition of particular phenotypes that may confer adaptive advantages, e.g. increased virulence, and also for the identification of strains in the community. The SNPs identified in this study will contribute to the knowledge of the epidemiology of *M. pneumoniae* in our country, since

Table 1. Nucleotide changes found in the RepMP4 sequence of the P1 gene of 18 specimens positive for *M. pneumoniae*

Nucleotide changes indicate the variation found in our specimens as compared to reference *M. pneumoniae* strain M129 (P1 type 1) and MAC (P1 type 2) in GenBank.

P1 adhesin type	No. of specimens with nucleotide changes/ number of specimens (% with changes)	Position of nucleotide change	Amino acid change
1	2/4 (50)	573G→A	Silent
2	10/14 (71.4)	891G→A	Silent
2	1/14 (7.1)	304G→T	Arg→Leu

they will be used as genetic markers to distinguish circulating strains.

One of the limitations of this study is that we did not use a proofreading enzyme, and it is known that conventional DNA *Taq* polymerase can introduce mutations on amplicons. Furthermore, the genotyping procedure that we used included several rounds of amplifications, increasing the risk of the introduction of mutations. Nevertheless, the finding of identical mutations in several strains in repeated sequencing cycles performed in the variants supports the existence of SNPs in our specimens. Moreover, with a unique exception, transitions involved purines and not pyrimidines as is favoured by DNA *Taq* polymerase.

To our knowledge, this is the first study that genotypes strains of *M. pneumoniae* in Latin America. Traditional methods of *M. pneumoniae* typing required the isolation of the micro-organism, a procedure which is difficult and not sensitive. Later procedures, like those used in our study, allow the typing of the organism directly from clinical specimens by conventional or nested PCR (Dumke *et al.*, 2006; Kenri *et al.*, 2008). However, recently Schwartz *et al.* (2009a, b) described a combination of real-time PCR and high-resolution analysis of melting curves (HRM) to distinguish between P1 types and to identify variants in isolates of *M. pneumoniae*. In comparison with the procedure used in our study, HRM analysis is faster and easier to perform, and since it does not need further amplicon manipulation, has a lower potential for cross contamination.

In conclusion, during the 2005–2006 epidemic in Santiago, both types of *M. pneumoniae* circulated in the population. Each type exhibited an SNP in RepMP4, a transition mutation that was found in several specimens.

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