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Molecular typing of *Mycoplasma pneumoniae* strains by PCR-based methods and pulsed-field gel electrophoresis. Application to French and Danish isolates

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SUMMARY

Restriction fragment length polymorphism (RFLP) analysis of the amplified P1 gene was used to type 153 strains of *Mycoplasma pneumoniae* isolated in France between 1977 and 1994, and in Denmark between 1962 and 1994, and an additional group of 28 strains isolated from Belgium and Germany between 1990 and 1993. Random amplified polymorphic DNA (RAPD) analysis was tested on French, Belgian and German strains. Both methods separated the strains into two groups corresponding to the two reference strains M129 (group I) and FH (group II), and gave concordant results. When 75 selected strains of different geographical origin were analysed by pulsed-field gel electrophoresis (PFGE), strains of group II fell into two closely related subgroups, subgroup IIa corresponding to the reference strain FH, and subgroup IIb. Most of the strains isolated in Denmark in the period 1962–86 belonged to group I. Almost all strains isolated in France and Denmark between 1987 and 1988 were from group II, the two subgroups being present. In 1991–3, almost all strains from France as well as Denmark, Germany and Belgium belonged to group I.

INTRODUCTION

Mycoplasma pneumoniae respiratory infections occur endemically with epidemic peaks at intervals of 4–7 years [1]. Genetic modification of strains may play a role in the development of outbreaks. Cell protein profiles, immunoblots and DNA restriction patterns have demonstrated the marked phenotypic and genotypic homogeneity of the species *M. pneumoniae* [2, 3]. However, some variations have been detected in the P1 gene (the gene encoding for the major adhesin of *M. pneumoniae*) by Southern blot analysis of genomic DNA [4, 5], and by sequencing the gene [6]. Clinical isolates of *M. pneumoniae* have been classified into two groups (I and II) based upon different hybridization patterns [4].

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In a preliminary study, we used restriction fragment length polymorphism (RFLP) analysis of the amplified P1 gene to type clinical strains of *M. pneumoniae*. Strains were separated into two groups. Comparable results have been found by Sasaki and colleagues [7] using our technique. Furthermore, a PCR system specific for each group has been proposed [8]. Another PCR system, nucleic acid sequence-based amplification, NASBA® technique using a set of primers chosen in the 16S rRNA, separated also the two groups of *M. pneumoniae* strains [9]. Because these methods only separated into two groups, analysis of the entire genome using random amplified polymorphic DNA (RAPD) analysis or RFLP analysis using pulsed-field gel electrophoresis (PFGE) could provide additional information useful in separating strains. However, PCR fingerprinting tested on 24

strains [10] distinguished only two groups and did not demonstrate any variation within each group.

The purposes of this study were (i) to develop a simple typing system based on genotypic differences able to differentiate between the two known groups of *M. pneumoniae* and potentially be more discriminating and (ii) to study the distribution of strains isolated in France and Denmark for 20 years and more. A total of 153 clinical strains isolated in France (1977–94), in Denmark (1962–94) and an additional group of 28 strains isolated in Germany and Belgium (1990–3), were typed by PCR–RFLP. Random amplified polymorphic DNA (RAPD) analysis was performed on the 78 French, Belgian and German strains. PFGE was used to type a panel of 75 *M. pneumoniae* strains of different geographical origin previously typed by PCR–RFLP.

MATERIALS AND METHODS

Mycoplasma strains

Two reference strains of *M. pneumoniae* were used, the M129–B25C strain, representative of group I, and the FH strain, representative of group II, provided by Dr J. G. Tully of the National Institute of Allergy and Infectious Diseases, Bethesda, MD. A second FH strain, with a low number of passages, FH-lp, was used in some experiments. A total of 181 wild strains was studied. Fifty strains were isolated from patients in France between 1977 and 1994, and 103 clinical strains were isolated in Denmark between 1962 and 1994. In addition, a panel of 28 strains isolated between 1990 and 1993 was analysed, 19 from Germany provided by Dr E. Jacobs (Freiburg) and 9 from Belgium provided by Dr H. Goossens (Antwerp). *Mycoplasma genitalium*, strain G37, was used as a control.

M. pneumoniae culture and sample preparation for PCR

M. pneumoniae isolates were grown at 37 °C in 10 ml of Edward modified medium until mid-log phase (a red-orange colour of the phenol red pH indicator). After growth, bacteria were pelleted by centrifugation at 12000 ***g*** for 50 min and the supernatant was discarded. For PCR–RFLP studies, the pellet was resuspended in 500 μl of distilled water and stored at −20 °C. For RAPD studies, pellets of *M. pneumoniae* were suspended in 650 μl of a lysis buffer consisting of TE buffer 10:1 (10 mM Tris/HCl, 1 mM EDTA [pH 8·0]) with sodium dodecyl sulphate (1%) and proteinase K (100 μg/ml), and incubated overnight at 37 °C. Then, DNA was purified by standard phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The pellet of nucleic acids was dissolved in TE buffer 10:1, and the final concentration was determined by measuring absorbance at 260 nm [11].

Primers

For P1 gene amplification, four 20-mer primers (ADH 1, 2, 3, 4) were used to amplify the nearly full-length P1 gene in two fragments, one bracketed by ADH1–ADH2 and the second bracketed by ADH3–ADH4. They were chosen in the published sequence of the P1 gene of the strain M129 which belongs to group I [12, 13]. The sequences and the position on the gene of these primers are indicated in Table 1. The sequences of the seven primers tested for RAPD are listed in Table 2. Some of them, APϵ, HLWL 74, primer 1254 and primer 1 have been already published [14–17] for other purposes and chosen arbitrarily for these experiments, whereas PH1, X and G were randomly selected in accordance with the G+C ratio of *M. pneumoniae*.

P1 gene amplification

To amplify P1 gene sequences, the frozen *M. pneumoniae* suspension was thawed and 5 μl were denatured at 95 °C for 5 min under a drop of mineral oil and cooled on ice before adding 45 μl of PCR mixture consisting of 1·25 units of *Taq* DNA polymerase (Appligene ONCOR, Illkirch, France), 10 mM Tris/HCl (pH 9·0), 50 mM KCl, 1·5 mM $MgCl_2$, 200 μg per ml of gelatine, 200 μM of each deoxynucleoside triphosphate (Boehringer, Mannheim, Germany) and 1 μM of each primer. Thirty cycles of amplification were performed in a Perkin–Elmer Cetus thermocycler, Gene-Amp PCR System 9600 (Norwalk, CT). Each cycle consisted of 1 min of denaturation at 95 °C, 1 min of annealing at 65 °C and 2·5 min of extension at 72 °C. Negative controls, consisting of tubes containing distilled water in the place of the DNA samples were run with each amplification batch. PCR products were analysed by electrophoresis of 8 μl of the reaction mixture on an 0·8% agarose gel (SeaKem, FMC, Bioproducts,

Table 1. *DNA primers used to amplify P1 gene*

Primer	Sequence	Position on the gene (bp)	Length of amplified product (bp)	
			M129 (Group I)	FH (Group II)
ADH1	5′-CTGCCTTGTCCAAGTCCACT-3′	20–39		
ADH2	5′-AACCTTGTCGGGAAGAGCTG-3′	2261–2280	2254*	2304*
ADH3	5′-CGAGTTTGCTGCTAACGAGT-3′	2298–2317		
ADH4	5′-CTTGACTGATACCTGTGCGG-3′	4829–4848	2572†	2582†

* Fragment amplified with ADH1–ADH2.
† Fragment amplified with ADH3–ADH4.

Table 2. *DNA primers used for RAPD typing of* M. pneumoniae *isolates*

Primer	Sequence	Reference
APϵ	5′-CTGTTGCTAC-3′	14
HLWL 74	5′-ACGTATCTGC-3′	15
1254	5′-CCGCAGCCAA-3′	16
1	5′-AACGCGCAAC-3′	17
PH1	5′-TGAGGATGCA-3′	Arbitrary
X	5′-CTCGAGTCTA-3′	Arbitrary
G	5′-GGTAACCTCG-3′	Arbitrary

Rockland, ME) in TAE buffer (40 mM Tris-acetate, pH 8·0; 1 mM EDTA, pH 8·0). The samples were electrophoresed at 100 V for 1 h. A molecular size marker (λ-DNA cleaved with *Eco*RI and *Hin*dIII, marker III, Boehringer) was used for reference. The products were visualized by ethidium bromide staining.

Restriction endonuclease digestion of amplified P1 DNA

For RFLP analysis, restriction enzymes were chosen by analysing the two fragments of the P1 gene sequence published from the two *M. pneumoniae* reference strains, M129 [12, 13] and FH [18], using appropriate analysis software (BACHREST program). The numbers and sizes of the restriction fragments generated by each of 75 different restriction endonucleases were determined in order to find an appropriate restriction treatment of the ADH1–ADH2 and ADH3–ADH4 fragments which would give a different electrophoresis pattern for each group and DNA fragments between 950 and 100 bp. The amplified products obtained by PCR were subjected to restriction endonuclease digestion overnight with *Dde*I, *Hae*III, *Hpa*II and *Mbo*I for sequences bracketed by ADH1 and ADH2 and *Hae*III and *Hpa*II for sequences bracketed by ADH3 and ADH4. Nine μl of amplified DNA were digested using 4 U of restriction enzyme in the assay buffer recommended by the manufacturer. The digested samples were analysed by polyacrylamide gel (8 %) electrophoresis. The size of fragments obtained was determined with molecular size marker V (plasmid pBR322 cleaved with *Hae*III, Boehringer).

RAPD typing

In preliminary experiments, the optimal conditions were determined using the two reference strains, M129 and FH with primer 1254 for the following parameters: (i) quantity of DNA template (0·01–1 μg); (ii) concentration of primer (0·3–1 μM); (iii) concentration and origin of *Taq* DNA polymerases. According to the results of these tests, the RAPD assays were conducted in 50 μl of a solution containing 1·25 U of *Taq* DNA polymerase (ATGC Biotechnologie, Noisy-Le-Grand, France), 5 μl of appropriate buffer (7 mM $MgCl_2$, 50 mM KCl, 50 mM Tris/HCl [pH 9·0], 0·2 mg/ml bovine serum albumin, 16 mM ammonium sulphate), 200 μM of each deoxynucleoside triphosphate, 1 μM of primer and 0·1 μg of template DNA. After a denaturation step of 4 min at 94 °C, amplification was performed with the same thermal cycler mentioned above over 36 cycles each of 1 min at 94 °C, 2 min at 36 °C and 2 min at 72 °C with a final elongation step of 10 min at 72 °C. A negative control which did not contain DNA was processed with each set of RAPD assays. The RAPD products were separated by electrophoresis on a 1·2 % agarose gel in TAE buffer with a molecular size marker (plasmid pBR328 cleaved with *Bgl*I and *Hin*fI, marker

VI, Boehringer) as reference and were visualized by ethidium bromide staining.

PFGE

Strains were grown in 200 ml of Edward modified medium until mid-log phase. At this point, chloramphenicol was added to a final concentration of 180 mg/l. Incubation was continued for 6 h to allow DNA replication in progress to reach completion while inhibiting the initiation of new rounds of replication. The organisms were harvested by centrifugation at 12000 ***g*** for 50 min and washed three times with ice-cold Tris-buffered saline (0·5 M NaCl, 20 mM Tris/HCl [pH 7·5]). The pellets were stored at −20 °C before use. DNA was prepared in agarose blocks as follows: pelleted mycoplasmas were resuspended in a total of 500 μl of 10 mM Tris/HCl (pH 8·0), 50 mM EDTA (pH 8·0), to which an equal volume of 1% molten low-melting point agarose (SeaPlaque Agarose, FMC, Bioproducts, Rockland, ME) was added. The suspension was mixed, then dispensed in 100-μl aliquots into a plexiglass mould having multiple wells (Bio-Rad Laboratories, Richmond, CA). The hardened blocks were extruded into 0·5 M EDTA and incubated for 1 h at 37 °C. Then, cells were lysed *in situ* in 1% *N*-lauroyl sarcosine and proteins were digested with proteinase K (20 μg/ml) at 56 °C overnight. The agarose plugs were washed twice for 1 h each with 10 ml of TE buffer with gentle mixing on a rocker platform. The proteinase K was inactivated with 200 μl of phenylmethylsulphonyl fluoride (Boehringer) at 17·5 μg/ml for two 1 h washes at 37 °C and room temperature, respectively. After three additional washes in TE, blocks were equilibrated for 1 h at +4 °C in 1X restriction enzyme buffer. *Apa*I, *Ava*I, *Nci*I, *Bgl*I, *Nae*I, *Sac*II, *Sma*I, *Xho*I and *Sst*II restriction enzymes were evaluated. After an overnight incubation at +4 °C with 25 U of enzyme, digestion was conducted for 6 h at optimal temperature. PFGE was performed by the contour-clamped homogeneous field electrophoresis system (CHEF-DR III, Bio-Rad). Agarose gels were used at a concentration of 1% in 0·5× TBE (44·5 mM Tris/Borate, pH 8·0; 12·5 mM EDTA) for analysis of DNA restriction fragments. Electrophoresis was conducted at 14 °C for 18 h at 190 V, with pulse times in the range 3–20 sec. To examine the quality and size of DNA inserts, agarose gels at a concentration of 0·8% were run for 20 h at 190 V with pulse times in the range 25–75 sec. Gels were stained with ethidium bromide after completion of electrophoresis. The size of DNA fragments was determined by measuring distances of band migration compared with the DNA standards of a lambda DNA ladder (New England Biolabs, Ozyme, Montigny Le Bretonne, France) or *Saccharomyces cerevisiae* YNN295 chromosome marker (Bio-Rad).

RESULTS

P1 gene PCR-RFLP typing

P1 gene amplification of the reference strains with primers ADH1–ADH2 or with ADH3–ADH4 yielded products with the expected size (Table 1) for strain M129 (group I) and FH (group II). *M. genitalium* did not yield any band when using these primers. Whatever the amplified fragment, each enzyme produced DNA restriction patterns that discriminated between group I and group II reference strains as shown in Figure 1. Artificial mixture of the two groups was detected (data not shown).

The 78 French, Belgian and German clinical isolates were amplified with both sets of primers and the amplified fragments treated by the different restriction enzymes. All the profiles corresponded to one of those given by the two reference strains (data not shown). No mixture of the two groups was detected from the cultures obtained from clinical specimens. Since each pattern corresponded to one group, for the 103 Danish isolates, only one primer-pair, ADH1–ADH2, was used to amplify half-P1 gene, and only one restriction enzyme, *Hpa*II, was used to type strains.

RAPD typing

In preliminary experiments, the 2 reference strains were screened with 7 primers and 3 combinations of 2 primers (APϵ+1254, HLWL 74+1, X+G). Five primers generated RAPD banding patterns with these strains but only the two primers APϵ and 1254 generated patterns that showed discrimination between the two strains. The results were not improved when combined primers were used. Thus, the APϵ and 1254 primers were chosen to characterize the clinical strains. As shown in Figure 2, the profiles obtained for the reference strains with primer APϵ showed two or three major bands of 600–1300 bp. They differed mainly by one band of 1050 bp which was present in M129 (Fig. 2A, lane 1). With primer 1254, 4–5 bands in the range 450–2000 bp were noted. The profiles of the two reference strains differed mainly by one intensive band of 700 bp present in M129 (Fig. 2B,

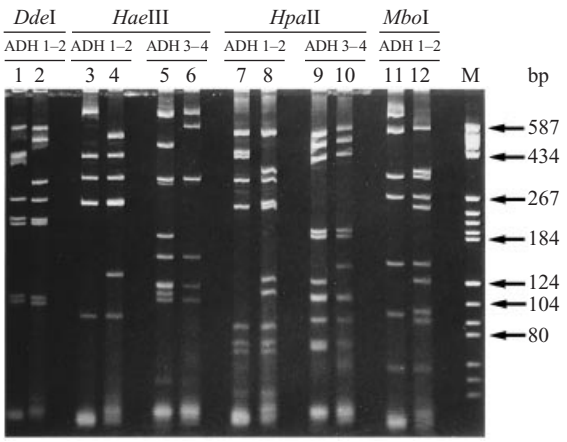

Fig. 1. RFLP of P1 gene of *M. pneumoniae* reference strains amplified with primers ADH1 and ADH2 or with primers ADH3 and ADH4 analysed on a 8% polyacrylamide gel. Reference strain FH (group II), lanes 1, 3, 5, 7, 9, 11 and reference strain M129 (group I), lanes 2, 4, 6, 8, 10, 12. Lane M, molecular weight marker.

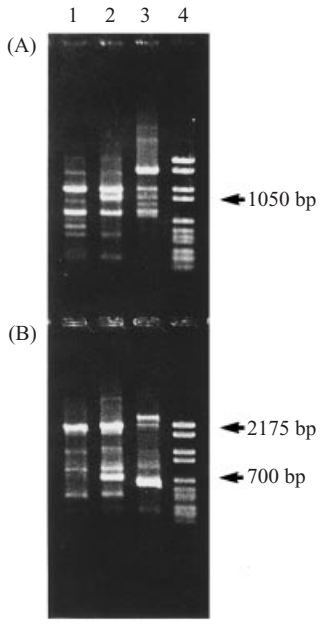

Fig. 2. RAPD profile of *M. pneumoniae* reference strains and *M. genitalium* reference strain with primer APε (A) and primer 1254 (B). Lane 1, reference strain FH (group II); lane 2, reference strain M129 (group I); lane 3, reference strain G37 of *M. genitalium*, lane 4, molecular size marker.

lane 2) but absent in FH (Fig. 2B, lane 1). RAPD profiles obtained with *M. genitalium* were different from the *M. pneumoniae* fingerprints (Fig. 2A, B, lane 3). Artificial mixture of the two reference strains could be detected whatever the primer used (data not shown).

The 78 French, Belgian and German strains were characterized using APε and 1254. The two patterns

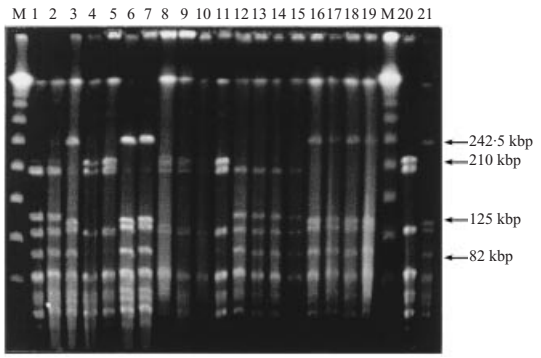

Fig. 3. PFGE of *Apa*I restriction fragments from *M. pneumoniae* reference strains and clinical isolates. Lane M, molecular size marker. Lanes 20 and 21 represent reference strains FH (group II) and M129 (group I) respectively. Lanes 3, 6, 7, 16, 17, 18, 19 represent clinical isolates belonging to group I; lanes 4, 5, 8, 9, 10, 11 clinical isolates of the subgroup IIa; lanes 1, 2, 12, 13, 14, 15 clinical isolates of the subgroup IIb; In the group IIb one band of 210 kpb is lacking and two additional bands of 82 and 125 kpb are present.

observed were identical to those obtained with the reference strains. No mixture was detected. The results obtained with this method were entirely concordant with those deduced from PCR–RFLP analysis.

Because one of the major drawbacks of RAPD typing is the lack of reproducibility of the method, 10 randomly selected strains were tested several times. The patterns obtained after amplification in different experiments were similar in our conditions.

PFGE typing

Nine restriction endonucleases were tested for their suitability for use in PFGE by using the two reference strains M129 and FH. Eight of them were not useful for PFGE, because *Nci*I, *Sst*II, *Xho*I and *Nae*I generated too many digestion products and *Ava*I, *Sac*II, *Bgl*I and *Sma*I did not discriminate between the two groups. The enzyme *Apa*I, generating at least 9 fragments, differentiated the two reference strains. Three bands, corresponding to fragments of 242·5, 120 and 82 kbp, were present in the M129 strain (Fig. 3, lane 21) but not in the FH strain (Fig. 3, lane 20). Two bands, corresponding to fragments of 210 and 189 kbp, were found in the FH strain but did not appear in M129.

PFGE was performed on 75 selected clinical isolates from different geographical origin, 19 were classified in group I and 56 in group II by PCR–RFLP. *M. pneumoniae* strains classified in group I showed in

Table 3. *Annual distribution of M. pneumoniae groups and subgroups in France and Denmark*

	Number of isolates							
					Group II			
	Total		Group I		Subgroup IIa		Subgroup IIb	
Countries …	France	Denmark	France	Denmark	France	Denmark	France	Denmark
Years *n** …	50	103	30	70	12	3	8	30
1962–4	ND	11	ND	11	ND	0	ND	0
1967	ND	2	ND	0	ND	0	ND	2
1975–6	ND	4	ND	4	ND	0	ND	0
1977	5	4	3	3	1	0	1	1
1978	0	5	0	5	0	0	0	0
1979	1	9	0	7	1	0	0	2
1980	1	3	1	3	0	0	0	0
1981	0	3	0	3	0	0	0	0
1982	0	1	0	1	0	0	0	0
1983	0	1	0	0	0	0	0	1
1984	0	0	0	0	0	0	0	0
1985	0	1	0	0	0	1	0	0
1986	2	0	1	0	0	0	1	0
1987	**5**	**8**	**0**	**2**	**3**	**0**	**2**	**6**
1988	**8**	**11**	**0**	**2**	**6**	**1**	**2**	**8**
1989	1	1	0	0	1	0	0	1
1990	1	7	1	4	0	0	0	3
1991	**4**	**16**	**4**	**11**	**0**	**1**	**0**	**4**
1992	**12**	**7**	**11**	**5**	**0**	**0**	**1**	**2**
1993	**8**	**4**	**7**	**4**	**0**	**0**	**1**	**0**
1994	2	5	2	5	0	0	0	0

* *n*, number of isolates.
ND, not done.
Bold: periods with high number of isolates.

their DNA restriction patterns obtained by PFGE, a complete similarity with the reference strain of the same group (M129) (Fig. 3, lanes 3, 6, 7, 16–19). However, *Apa*I digestion revealed differences in the strains belonging to group II. One band of 210 kbp was lacking in some group II strains whereas two additional bands of 82 and 125 kbp were present. The two varieties were designated subgroup IIa (presence of the 210 kbp fragment) and subgroup IIb (absence of the same fragment). The FH strain belonged to subgroup IIa. Figure 3 shows subgroup IIa (lanes 4, 5, 8–11) and subgroup IIb (lanes 1, 2, 12–15) profiles. The 56 isolates from group II available in this study were typed using PFGE. Sixteen belonged to subgroup IIa, 40 belonged to subgroup IIb.

Because the size of the missing fragment in strain IIb was identical to the sum of the two bands, the difference between subgroup IIa and IIb may be explained by the presence of an additional *Apa*I site in the genome of IIb strains. To eliminate the hypothesis of a mutation due to several passages in culture, the PFGE profile of another group II reference strain, FH-lp, was compared with the profile given by the FH strain that had been subject to numerous passages in the laboratory. The FH-lp strain showed the same pattern as the high passage FH strain and belonged to subgroup IIa.

Group distribution of clinical isolates

A total of 50 strains isolated in France between 1977 and 1994 and 103 strains isolated in Denmark between 1962 and 1994 were genotyped (Table 3). These strains do not include the total number of isolates obtained in France and Denmark during these periods. Before 1987, 9 *M. pneumoniae* strains isolated in France and 44 strains isolated in Denmark were characterized. Group I was clearly predominant (5 French and 37 Danish isolates). After 1987, a total of 100 strains was characterized, 41 from France and 59

from Denmark. Table 3 shows that there were two periods with a higher number of isolates (83/100 strains). During the first period (1987–8), almost all strains belonged to group II (28/32), the two subgroups IIa and IIb being present with a predominance of subgroup IIa in France (9/13) and subgroup IIb in Denmark (14/15). During the second period (1991–3), almost all the strains belonged to group I (42/51).

A selection of 28 strains isolated from Germany and from Belgium during this last period was also characterized by our techniques. As reported previously [8, 10], almost all the strains belonged to group I (25/28). Only one strain of subgroup IIa and two strains of subgroup IIb were characterized from the Belgian and German panel respectively.

DISCUSSION

In order to generate epidemiological data, accurate and specific methods are required for *M. pneumoniae* typing. Immunological typing using two monoclonal antibodies, one reacting with all *M. pneumoniae* isolates tested, the other reacting only with group II strains, has recently been proposed [8]. However, serotyping cannot be used currently because monoclonal antibodies are not commercially available. Furthermore, no monoclonal antibody specific for group I has yet been developed.

Several systems based on DNA analysis have been proposed. Ribotyping, applied to mycoplasmas [19], has proved to be of little use for *M. pneumoniae* because of the remarkable degree of genetic homogeneity among the strains, related to the presence of a single rRNA operon in this organism. However, very recently, nucleic acid sequence-based amplification, NASBA® technique using a set of primers chosen in the 16S rRNA, was used for *M. pneumoniae* typing and separated the two groups of *M. pneumoniae* strains [9]. Restriction enzyme analysis of *M. pneumoniae* total DNA has been previously reported. DNA fingerprinting and Southern blot analysis with subclones of the P1 adhesin gene as probes allowed to classify clinical isolates in two groups [20]. However, this technique required laborious sample preparation and processing.

The first purpose of the present study was to develop a molecular typing system able to separate between the two known groups of *M. pneumoniae* and potentially be more discriminant. Since the P1 protein is the major adhesin of *M. pneumoniae* [21] and appears to be the dominant *M. pneumoniae* antigen in humans [22], analysis of the P1 gene seemed to be a convenient tool for epidemiological studies. The system combining PCR and RFLP analysis of the P1 gene, divided strains into two groups. This easy-to-use typing method requires preliminary culture of *M. pneumoniae*. Then the PCR can be performed directly on the pelleted organisms, without DNA extraction. If the method was further optimized it might even be possible to do the typing directly from clinical specimens. The two groups of *M. pneumoniae* were differentiated using a single enzyme digestion of one of the amplified fragments. Many strains can be typed simultaneously using this method. The small number of DNA bands observed enables simple differentiation of strains. Another typing system able to discriminate between the two groups using two primers sets selected on the basis of variations in the P1 gene sequences, has been recently proposed [8].

Because the P1 gene based typing systems have a limited power of discrimination, the study of the total chromosomal DNA could be more useful. RAPD is a simple technique proposed for whole genomic DNA analysis. Using a combination of primers including primer 1254, it can be used to separate the two biovars of another mycoplasma species, *Ureaplasma urealyticum* [17]. In the study reported here on 78 strains of *M. pneumoniae*, and the study of Ursi and colleagues [10] on 24 strains, only 2 patterns were found using 2 arbitrary primers separately. Primer 1254 was used in both studies. A second primer APϵ was used here. Contrary to PCR–RFLP analysis, RAPD testing requires an extra step of DNA extraction and quantification. Its reproducibility may be affected by several factors such as *Taq* DNA polymerase and the type of thermal cyclers used [23]. In our experience, differences were found between the results obtained with different *Taq* DNA polymerases affecting both the number and relative amounts of amplified products. The high concentration of $MgCl_2$ in the reaction buffer of ATGC *Taq* DNA polymerase could explain the improved results observed with this enzyme. In this study, the results obtained with APϵ and 1254 primers were reproducible, except for differences in band intensities. For *M. pneumoniae* typing, Ursi and colleagues used primer 1254 but their amplification conditions were different and the amplitypes were different. Nonetheless, the nine strains tested by both laboratories gave similar results.

PFGE was able to differentiate strains into two groups, as PCR–RFLP and RAPD. Restriction

enzyme *Apa*I treatment seems to be well adapted to PFGE because it generates a limited number of fragments. Thirteen restriction sites have been identified on the physical map of the M129 strain [24, 25]. Due to the presence of doublets or triplets, after *Apa*I treatment, PFGE detected at least nine bands. Restriction enzyme fingerprinting obtained after *Apa*I treatment and reverse field electrophoresis, previously described [26], distinguished both groups. In the present study, group II could be subdivided into two subgroups by PFGE, contrary to Su and colleagues, who did not reveal any heterogeneity inside group II, possibly because of the very small number of strains characterized. The PFGE group II subgroups found in this study are closely related based on the criteria proposed for epidemiological analysis [27]. Therefore, at our present state of knowledge, the variation found in group II strains in this study may not be useful for epidemiological purposes. However, among all strains studied, a predominance of subgroup IIb was observed in Denmark (30/33 strains of group II) compared to a more balanced distribution of the two subgroups in France (8 subgroup IIb and 12 subgroup IIa). Further studies, including clinical data, would be necessary to look for a possible significance of this discrimination. With the publication of the complete *M. pneumoniae* genome sequence [28] it may also be possible to locate the additional *Apa*I site of group IIb and to develop a PCR for subtyping group II. Using a PCR based approach in contrast to PFGE would simplify the analysis significantly.

Concerning epidemiological data, a shift from group II to group I was observed in France and Denmark between the two periods 1987–8 and 1991–3. Two epidemic peaks were observed in Scotland at the same periods, one in 1987, the second one in 1991 on the basis of serological results [29], while the peak of an epidemic outbreak was observed in Germany in 1992 [8]. During the second period, a similar distribution of the strains was observed in France, in Denmark and the neighbouring countries, Germany and Belgium. *M. pneumoniae* epidemics were identified by serological methods in Denmark [30]. Our data differ from those observed in Japan at similar periods. In the country, after a predominance of group II in 1979–80, an epidemic occurred in 1984 and was marked by a shift to group I that remained predominant until 1992 [7]. Most of the strains isolated in 1994–5 belonged to group II.

An important point would be to know if there are any differences between the pathogenic pattern of the different strains. In this study, no relationship between the group and the severity of the disease could be determined. Recently, it has been shown [8] that patients with *M. pneumoniae* group I infections developed adherence inhibiting antibodies more frequently than did patients infected with group II strains. A better knowledge of *M. pneumoniae* strains would permit more discriminating epidemiological studies and perhaps a better approach to understanding their pathogenic role.

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