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Published in:
Journal of Clinical Microbiology

Publication date:
1994

JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1994, p. 2873–2875
0095-1137/94/$04.00+0

Vol. 32, No. 11

Typing of *Mycoplasma pneumoniae* by PCR-Mediated DNA Fingerprinting

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Received 6 June 1994/Returned for modification 1 July 1994/Accepted 24 August 1994

PCR fingerprinting was used to characterize clinical isolates of *Mycoplasma pneumoniae*. Among 24 strains tested, two types were distinguished. Nineteen strains belonged to type 1, whereas only 5 strains belonged to type 2. The majority of strains isolated since 1991 in Belgium belong to type 1. No variations in fingerprinting patterns were observed within each group, confirming the highly conserved nature of the *M. pneumoniae* genome.

Mycoplasma pneumoniae is an important respiratory tract pathogen. It is a leading cause of atypical pneumonia and other respiratory infections such as tracheobronchitis, bronchiolitis, croup, and less severe upper respiratory tract infections (8). The incidence rate varies greatly according to age and is highest among primary school children. Epidemics take place at intervals of 4 to 7 years (5). *M. pneumoniae* infections spread slowly in the family setting, with a median case-to-case interval of 23 days (4).

A 170-kDa protein, P1, has been identified as a major cytadhesin and virulence factor of *M. pneumoniae* (2). This protein and the gene coding for it have been extensively studied. On the basis of hybridization patterns with subclones of the P1 gene as probes in Southern blot analysis of genomic DNA (12), clinical isolates of *M. pneumoniae* can be classified into two types. Sequence analysis of the P1 gene revealed sequence divergence between isolates belonging to the two different types (10). This classification into two types was confirmed by restriction enzyme fingerprinting of genomic DNA and two-dimensional gel electrophoresis of total proteins (11). We report here on the use of a DNA amplification fingerprinting technique (16) for typing a collection of 24 isolates of *M. pneumoniae*.

The *M. pneumoniae* strains analyzed in this study are listed in Table 1. Fifteen *M. pneumoniae* strains were isolated from clinical samples in Antwerp, Belgium, in the course of a study of respiratory tract infections in children during two winter periods (from 1 October 1991 to 31 March 1992 and from 1 October 1992 to 31 March 1993) (6). In this study, nasopharyngeal aspirates of pediatric patients were analyzed for the presence of *M. pneumoniae* and respiratory viruses. The presence of *M. pneumoniae* was detected by culture and by a PCR that amplifies part of the P1 gene (15). The fifteen strains were isolated from 15 outpatients epidemiologically not related to each other. Besides the strains isolated in Belgium, nine other strains were analyzed: three strains isolated in the United Kingdom between 1983 and 1986 (M 15/83, M 414/86, and M 510/86) and three strains isolated in The Netherlands between 1970 and 1987 (P 635, P 71, and P 84). Finally, three *M. pneumoniae* reference strains were included in the study (FH, MAC, and PI 1428).

For cultivation, a 0.2-ml aliquot of nasopharyngeal aspirate was inoculated into 2 ml of SP4 broth (14) without thallium acetate and supplemented with amphotericin B (0.5 mg/ml) and polymyxin B (500 U/ml). Three serial 20-fold dilutions of this primary broth were made in SP4 broth. The four broths were incubated aerobically at 37°C and observed for color change over a period of 6 weeks. When color change was noticed, a 0.1-ml aliquot of the broth was subcultured on an SP4 solid medium (made by adding 8 g of agar per liter). The SP4 agar was examined weekly for the appearance of typical *M. pneumoniae* colonies, which were then subcultured in fresh SP4 broth for subsequent DNA extraction.

Lyophilized *M. pneumoniae* strains that were sent to our laboratory were cultivated accordingly.

For DNA extraction, 200 μl of culture was centrifuged at 13,000 × *g* for 40 min at room temperature. Pellets were resuspended in 150 μl of buffer containing 50 mM glucose, 25 mM Tris HCl (pH 8), and 10 mM EDTA and lysed after addition of 12.5 μl (1 mg/ml) of proteinase K and 12.5 μl of 10% (wt/vol) sodium dodecyl sulfate. Incubation took place at 50°C for 30 min. DNA was isolated by subsequent phenol and phenol-chloroform-isoamyl alcohol (25:24:1) extractions followed by ethanol precipitation (9). DNA concentrations were estimated by gel electrophoresis through 1% agarose gels in 0.09 M Tris borate–0.002 M EDTA, staining with ethidium bromide, and comparison with samples containing a known amount of DNA (9).

For PCR fingerprinting, several primers were tested. A list of the primers used is presented in Table 2. For amplification, the reaction mixtures consisted of 10 mM Tris HCl (pH 9), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 mM deoxynucleoside triphosphates. One unit of *Taq* DNA polymerase (Shaero-Q, Leiden, The Netherlands), 50 pmol of primers, and about 10 ng of DNA were used in a final reaction volume of 100 μl. The amplification time and temperature profiles that were used for primers HLW 74, HLW 85, 1254, D 14216, and D 8635 were as described previously (1, 7). With primer OJPU4, the first four cycles consisted of 5 min of denaturation at 94°C, 5 min of hybridization at 36°C, and 5 min of elongation at 72°C. For the 30 cycles that followed, the denaturation and hybridization times were reduced to 1 min, whereas the elongation time was reduced to 2 min. The final elongation step was 10 min. Amplifications were carried out in a Biomed model 60 thermocycler or a Techne model PHC-2 thermocycler. The amplification products were size fractionated on a 1% agarose gel containing 0.09 M Tris borate and 0.002 M EDTA and visualized by staining with ethidium bromide (9).

In a first series of experiments, all primers listed in Table 2

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TABLE 1. *M. pneumoniae* strains used in the study

Strain	Source[a]	Yr and source of isolation	Clinical picture	Type[b]
FH (NCTC 10119)	NCTC	1959; not specified	Pneumonia	2
M 15/83	NCTC	1983; sputum	Pneumonia	1
M414/86	NCTC	1986; sputum	Pneumonia	1
M510/86	NCTC	1986; sputum	Pneumonia	1
P635	RIVM	1970's; throat swab	Pneumonia	2
P71	RIVM	1987; BAL[c]	Pneumonia	1
P84	RIVM	1973; not specified	Pneumonia	2
M2117350	UZA	1992; NPA[d]	Acute bronchitis	1
M2107084	UZA	1992; throat swab	Acute bronchitis Erythema multiforma	1
M2117245	UZA	1992; NPA	URTI[e]	1
M2107079	UZA	1992; sputum	Acute lobar pneumonia	1
M2107374	UZA	1992; NPA	Acute lobar pneumonia	1
M2117235	UZA	1992; throat swab	Acute lobar pneumonia	1
M2117430	UZA	1992; NPA	Acute bronchitis	1
M3057031	UZA	1993; NPA	Acute bronchitis	1
M3037439	UZA	1993; NPA	Acute bronchopneumonia	1
21C86	UZA	1992; NPA	Acute bronchitis	1
21K11	UZA	1992; NPA	Acute bronchopneumonia	2
21K33	UZA	1992; NPA	Acute bronchopneumonia	1
21G93	UZA	1992; NPA	Acute bronchopneumonia	1
21I105	UZA	1992; sputum	Acute bronchopneumonia Exanthema	1
DEV	UZA	1991; NPA	Acute bronchopneumonia Erythema multiforma	1
PI1428 (ATCC 29085)	ATCC	1964; throat swab	Pneumonia	1
MAC (ATCC 15492)	ATCC	1944; lung tissue	Not specified	2

[a] Abbreviations: NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; ATCC, American Type Culture Collection, Rockville, Md.; RIVM, Rijksinstituut voor Volksgezondheid en Milieuhygiëne, Bilthoven, The Netherlands; UZA, Universitair Ziekenhuis Antwerpen, Edegem, Belgium.
[b] Typing results were obtained with PCR fingerprinting as described in this study.
[c] BAL, bronchoalveolar lavage.
[d] NPA, nasopharyngeal aspirate.
[e] URTI, upper respiratory tract infection.

were used to amplify DNA from 15 *M. pneumoniae* strains chosen randomly from the collection of strains listed in Table 1. One primer (D 8635) produced the same pattern for all strains examined. With the other primers, two patterns could be observed, the difference being most pronounced with primers OJPU4 and 1254.

In a second series of experiments, primer OJPU4 was used to amplify DNA from the 24 *M. pneumoniae* strains of the collection. Primer 1254 was used to confirm the results obtained with OJPU4.

Our collection of strains could be divided into two types on the basis of their PCR fingerprinting patterns with OJPU4 and 1254. The majority of strains (19 of 24) belonged to type 1. Only five strains were classified as type 2 because they showed a different pattern with both primers: strains MAC, FH, P 84, P 635, and 21K11. Fingerprinting patterns of representatives of the two groups with primers 1254 and OJPU4 are shown in Fig. 1. The results obtained with the PCR fingerprinting were reproducible from one run to another, except for differences in band intensities that were therefore not taken into consideration. Moreover, the method is fairly rapid, because amplification and separation of amplification products can be performed in 1 day.

Our results confirm the existence of only two types among clinical isolates of *M. pneumoniae* strains, as was demonstrated by other typing methods (10–12). Within each type, no variations in DNA or protein fingerprinting profiles were observed (11). These and our findings indicate that *M. pneumoniae* as a species has preserved overall genetic homology. Diversity of a species reflects evolutionary genetic divergence arising from random, nonlethal mutations that accumulate over time. *M. pneumoniae* has one of the smallest genomes of known self-replicating organisms, about 809 kb (17). Selective pressure

TABLE 2. DNA primers used for PCR-mediated genetic typing of *M. pneumoniae* isolates

Primer number	Primer sequence	Primer type	Reference
HLW 74	5′ ACGTATCTGC 3′	Arbitrary	7
HLW 85	5′ ACAACTGCTC 3′	Arbitrary	7
1254	5′ CCGCAGCCAA 3′	Arbitrary	1
D14216	5′ NNNAACAGCTATGACCATG 3′	Arbitrary	1
D8635	5′ GAGCGGCCAAAGGGAGCAGAC 3′	Arbitrary	1
OJPU4	5′ GGCCATGACCGCGAACTATCCG 3′	P1 gene sequence (nt 1191–1212)[a]	This study

[a] Nucleotides (nt) are numbered according to reference 13.

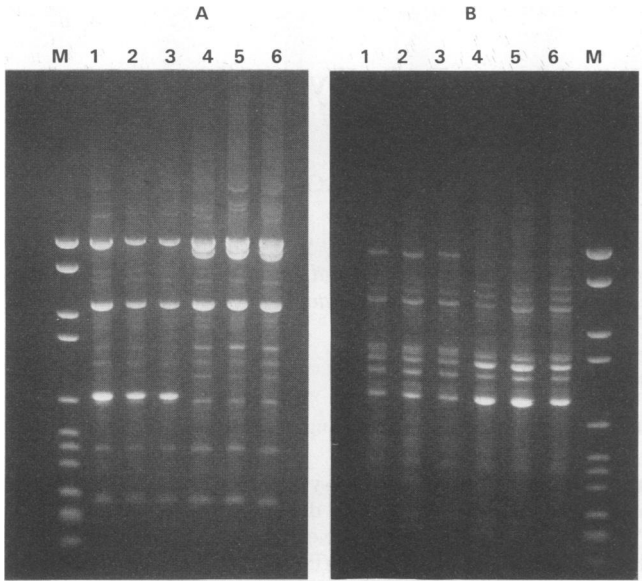

FIG. 1. PCR-mediated DNA typing of *M. pneumoniae* strains. Banding patterns obtained with *M. pneumoniae* strains with primer 1254 (A) and with primer OJPU4 (B) are shown. Lanes: 1 to 3, *M. pneumoniae* type 1 strains; 4 to 6, *M. pneumoniae* type 2 strains; M, molecular weight marker (pBR328-*Bgl*I and *Hin*fI fragments) (Boehringer Mannheim, Mannheim, Germany).

may be too high in *M. pneumoniae* because of the small size of its genome, preventing diversity of the species. Clearly, the *M. pneumoniae* population structure is highly clonal. Of course, it cannot be excluded that by performing PCR fingerprinting with other arbitrary primers or by performing sequence analysis of unknown regions of the genome, additional differences between isolates belonging to the same type will be found.

In the study by Su et al. (12), 29 *M. pneumoniae* strains were included, collected from different geographic areas (California, North and South Carolina, Texas, Washington, and France) and at different times (from 1944 to 1988). On the basis of genotyping, they found only 4 strains that belonged to type 1 out of 29 tested, whereas the great majority of their strains belonged to type 2 (25 of 29). This is in sharp contrast to our findings, since we found only 5 strains belonging to type 2 out of 24 tested. Our classification of type 2 strains is based on the type found for the reference strains FH and MAC that were classified by Su et al. (12) as type 2. The reference strain PI 1428 that we used in this study was classified by Chandler et al. (3) in the same group as strain M 129, which is classified as a type 1 strain in the study of Su et al. (12), whereas Chandler et al. also classified strains FH and MAC as type 2 in their genotypic study. Therefore, the majority of strains that were isolated in Belgium during the last 2 years belong to Su et al.'s (12) type 1 classification.

Apparently, two types of *M. pneumoniae* strains are encountered worldwide. Interestingly, the prevalence of each type seems to be related by time and geography. In the 1970s and 1980s, the majority of strains isolated in the United States and France were type 2 strains. However, the three strains isolated in the 1980s in the United Kingdom were type 1 strains. The majority of strains that have been found since 1991 in Belgium are type 1 strains. Maybe this switch in prevalent type can be explained by the immune status of the population against one type or another.

Another interesting point is whether the type of strain can be associated with the clinical picture of the patient. In this study, we could not find a relationship between the type of strain and the severity of the disease (Table 1). The majority of patients had pneumonia or bronchitis. Only one patient had an upper respiratory tract infection. This strain could not be distinguished from the others on the basis of the fingerprinting pattern.

We conclude that PCR fingerprinting is a rapid and reliable method for typing *M. pneumoniae* isolates. There is an excellent correlation with results obtained with other molecular typing methods. *M. pneumoniae* isolates can be divided into two types. The method could not distinguish isolates within each group, confirming the highly conserved nature of the *M. pneumoniae* genome and clonality of the population structure.

We thank J. Tully for providing us with very helpful information for the cultivation of *M. pneumoniae* and sending us some *M. pneumoniae* strains, R. Leach from the NCTC and A. Angulo from the RIVM for sending us their *M. pneumoniae* isolates, P. Vandamme for critical reading of the manuscript and helpful comments, and Edwin Fries for technical assistance.

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