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Detection of *Mycoplasma pneumoniae* by Using the Polymerase Chain Reaction

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The polymerase chain reaction (PCR) technique was used to detect *Mycoplasma pneumoniae*. A specific DNA sequence for *M. pneumoniae* was selected from a genomic library, and two oligonucleotides were chosen in this sequence to give an amplified fragment of 144 base pairs. We show that DNA from different *M. pneumoniae* strains can be detected by PCR, with DNA from other *Mycoplasma* species giving negative results. Analysis of biological samples (throat swabs) obtained from hamsters that were experimentally infected with *M. pneumoniae* showed that PCR was more sensitive and reliable than conventional culture techniques for the detection of *M. pneumoniae*. Initial experiments on artificially seeded human bronchoalveolar lavages showed that PCR can be used to detect 10^2 to 10^3 organisms.

Mycoplasma pneumoniae is responsible for a wide spectrum of respiratory diseases. Most of them are mild infections. However, this pathogen is an important cause of primary atypical pneumonia; thus, the development of a highly sensitive microbiological diagnostic method is worthwhile. Two methods are currently used for this purpose: serological procedures or isolation of the organism by culture (3, 5). The cultivation of *M. pneumoniae*, a fastidious microorganism, is time-consuming and may require 3 weeks for results. Serological procedures are the most widely used and require the demonstration of a rise in antibody titer. It takes too long for results of both of these diagnostic methods to be obtained to allow for the rapid application of an effective treatment.

Hybridization with DNA probes has been proposed as a rapid and specific procedure to replace the culture method for detecting *M. pneumoniae* (7, 10). Unfortunately, this procedure lacks sensitivity. Furthermore, most of the probes use radioactive labeling, a technique that is unapplicable for diagnosis on a widespread basis.

Recently, an in vitro technique was developed to amplify short segments of DNA. This is referred to as the polymerase chain reaction (PCR) (18). Two primers that flank the region to be amplified are used with this technique, which is based on repeated cycles of denaturation, annealing of primers, and primer extension by a DNA polymerase. This results in the exponential accumulation of the target sequence copies. This technique has already been used for the diagnosis of genetic diseases (17) and viral infections (16). It is also an effective tool for bacteriological diagnosis (6, 15).

We report here the enzymatic amplification by PCR of a specific DNA fragment of *M. pneumoniae*. Enzymatic amplification was performed under different conditions and was applied to several *M. pneumoniae* isolates and to dilutions of the organism in bronchoalveolar lavages (BALs). Furthermore, PCR was compared with the classical culture method for the detection of *M. pneumoniae* in experimentally infected animals.

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MATERIALS AND METHODS

Organisms and clinical specimens. *M. pneumoniae* FH and PI 1428, five *M. pneumoniae* clinical isolates, *Mycoplasma hominis* PG21, *Mycoplasma salivarium* PG20, *Mycoplasma orale* CH 19299, *Mycoplasma buccale* CH 20247, and *Acholeplasma laidlawii* PG8 were grown on the modified medium described by Hayflick (9). *Mycoplasma genitalium* G37 was grown on SP4 medium (21). BALs were obtained from hospitalized patients.

DNA extraction. A total of 250 ml of a late-exponential-phase culture of mycoplasmas was centrifuged for 30 min at 10,000 × *g*. The pellet was suspended in 5 ml of STE buffer (10 mM NaCl, 20 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) and incubated overnight at 37°C with 1% sodium dodecyl sulfate and 50 μg of proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany) per ml. DNA was extracted three times with an equal volume of phenol saturated with TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), once with phenol-chloroform-isoamylic alcohol (10:9.6:0.4; vol/vol/vol), and once with chloroform-isoamylic alcohol (9.6:0.4; vol/vol). The aqueous phase was made to 0.3 M CH_3COONa, and the DNA was precipitated with 2 volumes of absolute ethanol. The nucleic acids were suspended in TE buffer and were treated with DNase-free RNase A (50 μg/ml) at 37°C for 30 min. After another extraction with phenol and ethanolic precipitation, the DNA was suspended in TE buffer.

For experiments with *Escherichia coli*, the suspended cell pellet was incubated with 50 μg of lysozyme per ml at 37°C for 60 min and treated as described above. Human DNA was extracted from blood as described previously (11).

Genomic library construction. Total DNA from *M. pneumoniae* FH was partially digested with *Sau*3A (Industrial Biological Laboratories Inc., Rockville, Md.) and was size fractionated by agarose gel electrophoresis. Fragments ranging in size from 0.5 to 1 kilobases were isolated from the gel and ligated to a plasmid vector (Blue-Scribe; Vector Cloning System) that was previously linearized with *Bam*HI (Appligene) and dephosphorylated with bacterial alkaline phosphatase (Appligene) following the instructions of the manufacturer. Frozen competent *E. coli* DH5α cells were used for transformation with ligation mixtures by the method of

Hanahan (8). Recombinant clones were selected on L-agar plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml, 0.5 mM isopropyl-β-D-thiogalactopyranoside, and 100 μg of ampicillin per ml.

DNA analysis and synthesis of oligonucleotides. Recombinant plasmids were prepared by the method of Birnboim and Doly (2). Plasmid DNA was digested with *Hin*dIII and *Eco*RI (Genofit), and the DNA insert was purified by 1% agarose gel electrophoresis and subcloned into bacteriophage M13 mp9 for sequencing or was used as a probe. Sequence analysis was done by the dideoxynucleotide chain-termination method (19) by using either the plasmid vector (4) or the single-stranded M13 mp9 bacteriophage (14).

The oligonucleotides MP5-1 (GAAGCTTATGGTACAG GTTGG), MP5-2 (ATTACCATCCTTGTTGTAAGG), and MP5-4 (CGTAAGCTATCAGCTACATGGAGG) were synthesized on a DNA synthesizer (model 380B; Applied Biosystems) by the methoxyphosphoramidite method (1) and purified twice by precipitation in 0.3 M sodium acetate with 3 volumes of absolute ethanol.

Dot blot hybridization. For dot blot hybridization analysis, total DNA was denatured with 0.25 M NaOH and diluted at the desired concentration in 0.125 N NaOH–0.125× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M phosphate buffer [pH 7.7], and 0.001 M disodium EDTA). Then, samples were loaded onto a nitrocellulose membrane that was presoaked in 0.5 M Tris hydrochloride (pH 7.5). After drying, the filters were baked at 80°C for 2 h. Prehybridization (1 h) was carried out at 65°C in a solution of 5× SSPE–5× Denhardt solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.], and 0.1% bovine serum albumin)–200 μg of denatured herring sperm DNA per ml. Hybridization was conducted for 16 h in the same mixture but containing nick-translated, ^{32}P-labeled probes (13). After hybridization, the filters used for dot blot hybridization were washed at 65°C as follows: twice for 15 min in 2× SSPE, once for 30 min in 2× SSPE–0.1% sodium dodecyl sulfate, and once for 10 min in 0.1× SSPE. The blots were autoradiographed with an intensifying screen.

Infection of hamsters and specimen sampling. Hamsters were inoculated intranasally with 0.2 ml of an *M. pneumoniae* PI 1428 suspension in its eighth passage on the medium described by Hayflick (9). The suspension contained 10^7 color-changing units (CCU) of *M. pneumoniae* per ml (3). Before and at various times after inoculation, two throat swab specimens were collected. One was placed in 0.5 ml of the medium described by Hayflick (9) and was used for the culture detection of *M. pneumoniae*. The other one was placed in 0.5 ml of TE buffer containing 1% sodium dodecyl sulfate and 50 μg of proteinase K per ml. After a 2-h incubation at 37°C, an equal volume of phenol was added and the tubes were kept at −80°C until analysis. Before use, each sample was centrifuged, the aqueous phase was extracted twice with chloroform, and then the solution was made to 0.1 M NaCl and DNA was precipitated with 2 volumes of ethanol.

BAL specimen analysis. BAL specimens (10 to 20 ml) were concentrated to 1 ml by centrifugation and analyzed by both the conventional culture method on the medium described by Hayflick (9) and by using the PCR.

Amplification experiments were conducted on 10 μl of these BAL samples without any extraction, with or without 10 μl of a serial dilution of an *M. pneumoniae* culture. Samples for amplification were then treated as described above.

Amplification. Amplification was performed with the thermostable *Taq* DNA polymerase (New England BioLabs, Inc., Beverly, Mass.) by the procedure described by Saiki et al. (18), with some modifications. Briefly, samples to be amplified were incubated in a 30-μl reaction volume containing 1 μM of each oligonucleotide primer; 200 μM each of dATP, dCTP, dTTP, and dGTP; 200 μg of gelatin per ml; 50 mM KCl; 2.5 mM $MgCl_2$; and 10 mM Tris hydrochloride (pH 8.4).

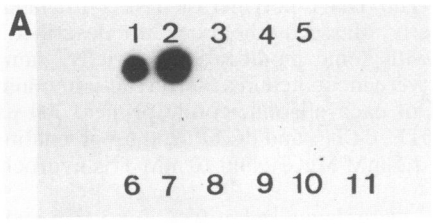
Samples were denatured at 95°C for 5 min and cooled on ice. One unit of *Taq* polymerase was added, and the tubes were centrifuged for 10 s. The reaction mixtures were then covered with 30 μl of paraffin oil, and the tubes were put in a water bath at 55°C for 2 min and then transferred to 70°C for 1 min. Subsequent rounds of amplification consisted of a 1-min denaturation step at 95°C, a 2-min annealing step at 55°C, and a 1-min extension step at 70°C. After 25 rounds of amplification, samples were incubated for an additional 5 min at 70°C. When 50 amplification cycles were to be performed, 3 μl of the 25-round reaction mixtures was diluted in 27 μl of new reaction medium, and 25 amplification cycles were performed as described above. Amplified DNA samples (10 μl) were analyzed by electrophoresis on an 8% polyacrylamide gel (13).

Analysis of amplified DNA samples. Analysis of amplified DNA samples was performed on an 8% polyacrylamide gel in Tris borate buffer (13). DNA was visualized by UV fluorescence after it was stained with ethidium bromide. Southern transfer was carried out as described previously (20). Hybridization with a nick-translated, ^{32}P-labeled DNA probe was performed as described above for the dot blot hybridization procedure. Hybridization with the 5′-end-labeled MP5-4 oligonucleotide was carried out at 50°C (13). After hybridization, filters were washed three times in 5× SSPE at 50°C for 20 min.

RESULTS

Isolation of a specific DNA sequence of *M. pneumoniae*. A genomic library of *M. pneumoniae* was constructed in the Blue-Scribe plasmid vector. A sequence from this library was demonstrated to be specific for *M. pneumoniae* by dot blot hybridization (Fig. 1A). The DNA targets were total DNA populations from *M. pneumoniae* and various other species generally found in the human respiratory tract and from *M. genitalium*, which is the closest known organism to *M. pneumoniae* (22). The MP5 clone gave a positive hybridization signal only with *M. pneumoniae* DNA. This clone was sequenced by the dideoxynucleotide chain-termination method (19). The nucleotide sequence is shown in Fig. 1B. The high A + T content was in good agreement with the base composition of *M. pneumoniae*. Two oligonucleotides (MP5-1 and MP5-2, which are underlined in the sequence in Fig. 1B) were chosen in order to contain the highest possible percentage of G + C and to give an amplified fragment of 144 base pairs (bp).

DNA amplification of purified DNA. Amplifications carried out on the MP5 plasmid, total DNA from *M. pneumoniae* FH and PI 1428, and five *M. pneumoniae* strains isolated from different patients yielded the expected 144-bp fragment. Figure 2A displays data for the MP5 plasmid (lane 1), DNA from the FH strain (lane 2), and DNA from three of five strains from hospitalized patients (lanes 8 to 10). A larger product (lane 8) was seen from time to time. This is a usual background effect which can be eliminated by lowering the magnesium concentration (data not shown) and is thought to be due to nonspecific hybridization of the primers.



B GATCTTAGCTTTGCCTTAAAGGTTTGGTACTAGGTTGA
AAAGAAGCTTATGGTACAGGTTGGTTAATTGACTGGAA
MP5-1
GGATGTTAAGCAGGACAACAAATTTACTGCTTACTTAG
CTACCAACCTCCATGTAGCTGATAGCTTACGAAATAAA
MP5-4
GACGATTACAAGCCTTACAACAAGGATGGTAATCACAA
MP5-2
GGAGTTTTTACCTGGCGATATCACCACTGAATTTTCTT

FIG. 1. Characterization of the target sequence for amplification. (A) Hybridization of the MP5 probe DNA to DNA extracted from *M. pneumoniae* (dots 1 and 2), *M. genitalium* (dots 3 and 4), *M. hominis* (dot 5), *A. laidlawii* (dot 6), *M. salivarium* (dot 7), *M. orale* (dot 8), *M. buccale* (dot 9), human leukocytes (dot 10), and *E. coli* (dot 11). Purified DNAs (10 μg [dots 1 and 3] and 100 μg [dots 2 and 4 to 11]) were spotted onto nitrocellulose membranes and hybridized with a nick-translated, ^{32}P-labeled DNA insert of clone MP5. (B) Partial DNA sequence of the 500-bp fragment isolated from the *M. pneumoniae* MP5 clone. The sequence corresponding to the synthetic oligonucleotides used as primers or as a probe for PCR are underlined. The target sequence is given in boldface, nonitalic type.

Amplifications conducted on DNA isolated from *M. genitalium*, *M. orale*, *M. salivarium*, and *E. coli* or human leukocytes did not yield any band (Fig. 2A, lanes 3 to 7).

Southern analysis with ^{32}P-labeled MP5 cloned DNA was used to control the specificity of the amplified fragment (Fig. 2B). A hybridization signal was observed with the 144-bp fragment that was obtained with DNA from MP5 and *M. pneumoniae* strains (Fig. 2B, lanes 1, 2, and 8 to 10). DNAs from other species did not yield any signal (Fig. 2B, lanes 3 to 7). Further control experiments were performed under the same conditions with DNA from a variety of microorganisms that are likely to be found in the human respiratory tract. These microorganisms included *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptococcus faecalis*, *Lactobacillus* species, *Peptostreptococcus anaerobius*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. No signals were observed (data not shown).

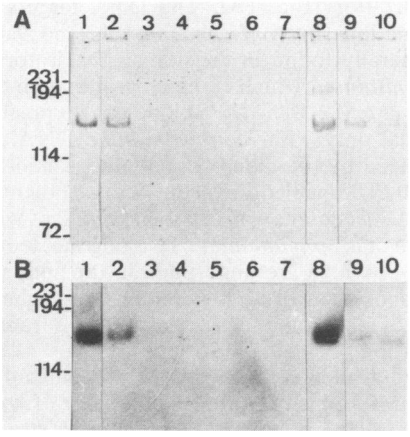


FIG. 2. Specificity of PCR amplification. (A) Electrophoretic analysis of the PCR products obtained with 35 ng of DNA from the MP5 clone (lane 1) and with 100 ng of DNA from *M. pneumoniae* (lane 2), *M. genitalium* (lane 3), *M. orale* (lane 4), *M. salivarium* (lane 5), *E. coli* (lane 6), human leukocytes (lane 7), and three *M. pneumoniae* strains isolated from hospitalized patients (lanes 8 to 10). A total of 25 rounds of amplification were performed. (B) Southern analysis with ^{32}P-labeled MP5 cloned DNA of the polyacrylamide gel shown in panel A. Numbers next to the gels are in base pairs.

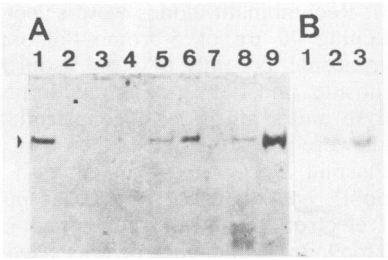


FIG. 3. Analysis of throat swab samples from experimentally infected hamsters. (A) Electrophoretic analysis of the PCR products obtained with DNA extracted from *M. pneumoniae* (lane 1) and throat swab samples from hamsters. Amplifications were performed with extracts from hamster H3 on days 0, 2, 4, 8, and 14 after *M. pneumoniae* infection (lanes 2 to 6, respectively) and from hamster H5 on days 1, 4, and 7 after infection (lanes 7 to 9, respectively). DNA samples were amplified for 50 rounds. (B) Southern blot showing the specific amplified product obtained by amplification of extract from hamster H5 on day 4 after infection (lane 2) and hamster H3 on day 8 after infection (lane 3). Lane 1, Negative control. Hybridization was performed with ^{32}P-labeled MP5 clone DNA. The arrowhead indicates the position of the 144-bp amplified sequence.

DNA amplification of biological samples. Throat swabs from hamsters that were experimentally infected with *M. pneumoniae* PI 1428 were analyzed by both the conventional culture method and PCR. Samples obtained before infection or *n* days after infection were amplified, and the products of amplification were analyzed on polyacrylamide gels. Results of a typical experiment are shown in Fig. 3A for two hamsters (H3 and H5 in lanes 2 to 6 and 7 to 9, respectively) and for DNA from MP5 plasmid DNA (lane 1). The specificity was confirmed (Fig. 3B) for hamster H5 on day 4 after infection (lane 2) and hamster H3 on day 8 after infection (lane 3) by Southern analysis with the ^{32}P-labeled MP5 DNA sequence. Results of all such experiments are shown in

TABLE 1. Comparison of detections of *M. pneumoniae* in hamster throat specimens by culture and PCR[a]

Day of sampling	Result for hamster:									
	H1		H2		H3		H4		H5	
	C	PCR	C	PCR	C	PCR	C	PCR	C	PCR
0	−	−	−	−	−	−	−	−	ND	ND
1	−	−	+	+	−	−	−	+	+	+
2	−	+	−	+	−	−	−	+	ND	ND
3	−	ND	+	+	−	ND	+	ND	ND	ND
4	−	+	−	+	−	−	−	+	CT	+
6	+	+	+	+	+	ND	+	+	ND	ND
8	−	ND	−	+	+	+	+	ND	CT	+
14	−	+	−	+	+	+	+	ND	CT	ND

[a] C, Culture; ND, not determined; CT, contaminated.

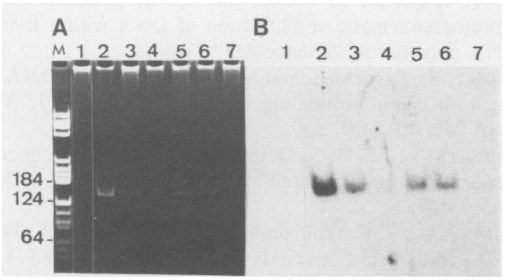


FIG. 4. Electrophoretic analysis of the PCR products obtained with BAL specimens. (A) Ethidium bromide-stained gel. Molecular weight standards (lane M); amplification on a BAL sample with no addition (lane 1); and amplification with the addition of 1,000 CCU (lanes 2 and 5), 100 CCU (lanes 3 and 6), and 10 CCU (lanes 4 and 7) of *M. pneumoniae* is shown. A total of 25 rounds of amplification were performed. Numbers next to the gel are in base pairs. (B) Southern transfer of the polyacrylamide gel shown in panel A and hybridization with the ^{32}P-labeled MP5-4 probe.

Table 1. For a given infected hamster, cultures gave erratic results. For example, hamster H2 was positive on days 1, 3, and 6 and negative on days 2, 4, 8, and 14. On the contrary, detection of the presence of *M. pneumoniae* by amplification gave totally homogeneous results. Moreover, the analysis by culture for hamster H5 was uninterpretable because of a contamination of the samples. On the contrary, the analysis of the corresponding samples by PCR gave clear results. Only a slight background was observed (Fig. 3, lanes 7 to 9).

Amplification on BAL specimens. Amplifications that were first conducted on a series of 20 BAL specimens gave negative results, in agreement with results of the conventional culture method (data not shown). To check the sensitivity of PCR on these samples, we added 10 CCU (Fig. 4A, lanes 4 and 7), 100 CCU (Fig. 4A, lanes 3 and 6), and 1,000 CCU (Fig. 4A, lanes 2 and 5) of *M. pneumoniae* cells to one of the samples before amplification (1 CCU was estimated to be equivalent to 10 to 100 *Mycoplasma* particles). On the ethidium bromide-stained gel, a 144-bp DNA band was visualized for 100 and 1,000 CCU (Fig. 4A, lanes 2, 3, 5, and 6). Southern analysis of these products showed (Fig. 4B) that this band hybridized with the MP5-4 oligonucleotide which was complementary to part of the amplified sequence, confirming the specificity of the amplification. Furthermore, the same band was also detected in the amplification product of the 10-CCU sample (Fig. 4B, lanes 4 and 7).

DISCUSSION

With the aim of improving the diagnosis of *M. pneumoniae* infection, we applied the PCR technique to this study. We first randomly cloned small *M. pneumoniae* genomic sequences. One of them was found by dot blot hybridization analysis to hybridize specifically to *M. pneumoniae* DNA. Then, two oligonucleotides complementary to two flanking sequences of a 144-nucleotide-long fragment were synthesized and used as primers for PCR.

We showed that DNA amplification allows for the detection of *M. pneumoniae* FH DNA. DNA from a set of *M. pneumoniae* strains (PI 1428 and clinical isolates) also gave consistently positive signals. Human DNA, DNA from species generally found in the respiratory tract, and *M. genitalium* DNA gave negative results. Altogether, these data show that our PCR assay allows for the specific and sensitive detection of *M. pneumoniae* DNA.

The validity of the assay was then checked by using hamsters as an animal model. In this case, experimental infection could be controlled by the classical culture method. This also allowed a comparison of PCR and the culture method as detection tools. We found PCR to be more sensitive and more reproducible than the culture method, and the PCR was completely unaffected by bacterial contamination.

BAL is an accepted technique for diagnosing pulmonary diseases (12). In preliminary experiments with 25 cycles of amplification, we were able to consistently and specifically detect 10 CCU of *M. pneumoniae* (i.e., 10^2 to 10^3 organisms), in comparison with 10^3 to 10^5 organisms, which is the limit of detection with radioisotopically labeled DNA probes (7, 10).

Thus, with our assay we were able to detect with a high sensitivity and specificity all of the *M. pneumoniae* strains that we tested. Moreover, the assay was convenient and fast enough to be used on a routine basis and can be made more sensitive, if necessary, by increasing the number of cycles.

We are currently validating the assay on a large number of clinical samples.

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