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Rapid, Sensitive Detection of *Mycoplasma pneumoniae* in Simulated Clinical Specimens by DNA Amplification

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The polymerase chain reaction (PCR) was investigated as a means of diagnosing *Mycoplasma pneumoniae* infections. The target DNA sequence was a 375-bp segment of the P1 virulence protein. This DNA segment was amplified in pure cultures of five different strains of *M. pneumoniae* but not in other species of *Mycoplasma*, *Acholeplasma*, or *Ureaplasma* that were tested. Simulated clinical specimens were used to compare PCR, culture, and the gene probe. The sensitivity of PCR was between 1 and 10 organisms. The sensitivity of culture was approximately 10^3 organisms, and the gene probe detected between 10^4 and 10^5 organisms. These results indicate that PCR has significant potential as a rapid, sensitive method for detecting *M. pneumoniae* in clinical specimens.

Mycoplasma pneumoniae is a common cause of respiratory infection in humans. While the majority of these infections appear to be upper respiratory tract infections (1, 8, 12) or relatively mild cases of pneumonia (10, 11), more-severe infections, such as pneumonia requiring hospitalization (5, 10, 30) or lung abscess (23), can also occur.

A major obstacle in diagnosing infections caused by *M. pneumoniae* is the lack of a rapid and sensitive method for detecting the organism in clinical specimens (9). Cultures are relatively insensitive and generally require a week or more for recovery of the organism and thus are not practical in most laboratories (16). A DNA probe that provides rapid results has recently been marketed, but it uses iodine-125, which has a very short shelf life, and the accuracy of this test has not been clearly established (6, 13, 14, 18, 26).

Several different serological tests for diagnosing infections due to *M. pneumoniae* have been described, but these also have limitations. The cold agglutinin test has been widely used but is very insensitive and nonspecific (4, 20). Complement fixation has been available for some time (17) but is too cumbersome for routine use, and its specificity has been questioned (24). Enzyme-linked immunosorbent assays have recently been developed for immunoglobulin G (IgG), IgM, and IgA. While they seem promising (7, 19, 24, 28, 29), they have not yet gained wide acceptance, and tests for several different immunoglobulins (IgM plus either IgA or IgG) may be necessary for accurate serological diagnosis (24, 28).

Another possible approach is the polymerase chain reaction (PCR). This is a highly sensitive method for amplifying specific gene sequences (21, 22) that has recently been applied to a number of different infectious agents. The purpose of the work described here was to investigate the use of PCR for detecting *M. pneumoniae* in simulated clinical specimens and to compare this technique with conventional culture and the commercial DNA probe.

Mycoplasma cultures. All mycoplasmas were obtained from the American Type Culture Collection. The following species were used: *M. pneumoniae* (ATCC 15293, ATCC 29085, ATCC 15531, ATCC 15377, and ATCC 29342), *M. hominis* (ATCC 27545), *M. orale* (ATCC 15544), *M. genitalium* (ATCC 33530), *M. buccale* (ATCC 23636), *M. fermentans* (ATCC 15474), *M. salivarium* (ATCC 14277), *M. faucium* (ATCC 25293), *Acholeplasma laidlawii* (ATCC 29804), and *Ureaplasma urealyticum* (ATCC 27613). Stock cultures of these strains were prepared in broth media. *M. hominis* was cultured in arginine broth (Remel, Lenexa, Kans.); *U. urealyticum* was cultured in 10B broth (Remel); all other strains were cultured in SP4 broth (27) prepared in our laboratory. After growth the cultures were diluted with fresh broth and stored at −70°C. The viability and concentration of mycoplasmas were determined in these suspensions by plating on agar medium. A8 agar (Remel) was used for *M. hominis* and *U. urealyticum*, and the other isolates were cultured on SP4 agar. For plate counts, serial dilutions of the mycoplasma suspensions were made in the broth used for growth and 0.1-ml samples were plated in duplicate on agar plates. The plates were then sealed with Parafilm to prevent drying and incubated at 35°C in a humidified atmosphere of 5% CO_2 for 7 to 14 days. The colonies were visualized by examining the plates with a 4× lens objective on a standard microscope.

Simulated clinical specimens. Simulated specimens were prepared from throat swabs submitted for *M. pneumoniae* gene probe testing (Gen-Probe, San Diego, Calif.) and found negative by this analysis or from throat swabs taken from healthy volunteers. The swabs were transported in Trypticase soy broth containing 0.5% bovine serum albumin (Remel) and held at 4°C. The transport media were mixed thoroughly on a vortex mixer and pooled together. The pooled specimens were then redistributed in smaller quantities and seeded with known amounts of *M. pneumoniae*.

DNA probe. Specimens in 1.5-ml amounts were used for DNA probe analysis. The procedure recommended by the probe manufacturer (Gen-Probe) was used.

Culture of simulated specimens. Samples (0.1 ml) of the specimens were cultured on SP4 agar. The plates were sealed with Parafilm and incubated as described above. The plates were examined periodically over a period of 14 days by inverting them and observing the surface of the agar from the reverse side with a 4× lens objective on a standard microscope.

PCR. Samples (1 ml) were placed in 1.5-ml microcentri-

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fuge tubes, centrifuged at 16,000 × *g* for 10 min, and washed once with 0.01 M Tris-HCl. After the wash the sediment in each tube was resuspended in residual buffer (approximately 50 μl). The samples were then treated by either sonication or boiling to disrupt the mycoplasmas and remove DNA for amplification. The sonication procedure was essentially as described previously (3) except that the treatment time for both sonication and boiling was 15 min and another type of sonicator was used (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The boiling procedure was performed by simply placing the tube containing the washed specimen in a boiling water bath for 15 min and then centrifuging the tube for 30 s to sediment any particulate material. Five microliters of these preparations was added to the reaction mixture for amplification.

The target sequence for amplification was a 375-bp segment of the gene coding for the P1 cytadhesin protein (25). The primers were both 24-bp fragments (MPN-101 and MPN-102) purchased from Genemed Biotechnologies, Inc., South San Francisco, Calif. Amplification reactions were performed in a final volume of 50 μl. The final reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM (each) nucleoside triphosphates, 0.5 μM (each) primers (2.5 μl of each primer per 50 μl of final reaction mixture), and 1.25 U of *Taq* polymerase (AmpliTaq AS; Perkin-Elmer, Norwalk, Conn.). The reactions were performed in 0.5-ml tubes (Perkin Elmer type N801-0180); the reagent mixture in each tube was covered with a drop of mineral oil. The tubes were placed in a thermocycler (Perkin-Elmer model 480) and heated at 94°C for 5 min, and then 35 cycles of amplification were performed as follows: 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. An additional incubation of 72°C for 10 min was added at the end. The amplification products were analyzed on 12% polyacrylamide gels with 1× TBE buffer (89 mM Tris, 89 mM boric acid, pH 8.0). The sample volume for the gels was 20 μl per well, and the gels were run at 200 V for 30 min. After electrophoresis the gels were stained with ethidium bromide and examined by UV transillumination.

Dot blot hybridization. Five-microliter quantities of the amplification products were added to 60 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and these solutions were boiled for 10 min and then immediately cooled on ice. Each preparation (50 μl) was spotted onto a nylon membrane (S4055; Oncor, Inc., Gaithersburg, Md.). The membrane was heated at 80°C for 30 min to bind the DNA to the membrane, and the bound DNA was hybridized with a biotin-labeled DNA probe specific for the segment being amplified (MPN-301; Genemed Biotechnologies). Hybridization was carried out in a hybridization chamber (PR800; Hoefer Scientific Instruments, San Francisco, Calif.). The reagents for hybridization were purchased as a kit (Sure Blot Blue Southern; Oncor, Inc.), and the procedure was as given in the instructions accompanying the reagents. The temperature used for membrane blocking was 45°C, the temperature for hybridization was 60°C, and the high-stringency wash was performed at 55°C.

The 375-bp segment was successfully amplified from pure cultures of all five of the American Type Culture Collection strains of *M. pneumoniae* but not from the other mycoplasmas tested (data not shown); these strains represent the most likely mycoplasmas found as normal flora in human respiratory specimens. The sensitivity of detection in clinical material was tested with simulated specimens. These specimens were either actual clinical specimens submitted for DNA probe testing and found negative for *M. pneumoniae* or

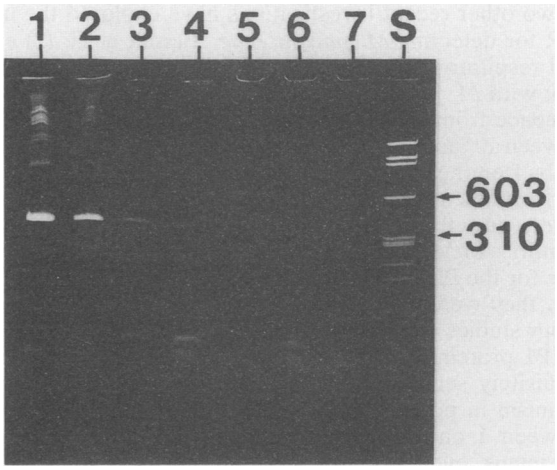

FIG. 1. Polyacrylamide gel containing amplified DNA from simulated clinical specimens with decreasing amounts of *M. pneumoniae* ATCC 15531. Five-microliter samples were amplified as described in the text, and the amplified DNA was analyzed by electrophoresis on a 12% polyacrylamide gel. Lane 1, 1,000 organisms; lane 2, 100 organisms; lane 3, 10 organisms; lane 4, 1 organism; lane 5, 0.1 organism; lane 6, 0.01 organism; lane 7, negative control consisting of simulated specimen without added mycoplasma; lane S, molecular size standard of *Hae*III-digested ϕX174 DNA. Sizes are indicated in base pairs. The 375-bp fragment was amplified in specimens containing as few as 10 organisms.

throat swabs from healthy volunteers. The specimens were pooled and inoculated with known amounts of *M. pneumoniae*. Between 1 and 10 organisms could be detected in these preparations (Fig. 1). The sensitivity of PCR was compared with that of culture and the DNA probe test by performing these tests on the same specimens. Approximately 10^3 CFU/ml could be detected by culture, and 10^4 CFU/ml could be detected by the DNA probe. Thus, the PCR was 100- to 1,000-fold more sensitive. The specificity of the PCR was examined by a dot blot hybridization procedure. DNA amplified from simulated specimens was spotted onto a nylon membrane and incubated with a biotinylated-DNA probe specific for the segment amplified (Fig. 2). The sensitivity of the dot blot procedure was similar to that of polyacrylamide gel analysis; between 1 and 10 organisms could be detected. Two or three replicates of each of these experiments were performed to confirm the results. The amplification proved to be very reproducible, with only slight variations between replicate experiments.

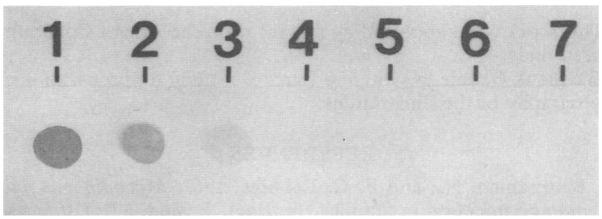

FIG. 2. Dot blot hybridization assay of amplified DNA from the same clinical specimens shown in Fig. 1. The dots (numbers 1 to 6) represent hybridization with DNA from simulated specimens containing 10-fold-decreasing amounts of *M. pneumoniae*, beginning with 10^3 CFU. Number 7 is a negative control, i.e., a simulated specimen without added mycoplasma. As in Fig. 1, between 1 and 10 organisms could be detected.

Two other recent investigations have explored the use of PCR for detecting *M. pneumoniae*. Bernet et al. (2) examined respiratory secretions from hamsters infected intranasally with *M. pneumoniae*. Using amplification of a 144-bp sequence from an unknown gene, they were able to detect between 10^2 and 10^3 organisms. Jensen et al. (15) used throat swabs from healthy subjects as simulated specimens. Material from the swabs was released by vortexing the swabs in buffer, and these suspensions were seeded with known quantities of *M. pneumoniae*. Using a 153-bp segment of the gene for the P1 virulence protein and 70 cycles of amplification, they were able to detect approximately 40 organisms. In our studies we used a primer set for a 375-bp segment of the P1 protein. Amplification of this segment provided an exquisitely sensitive and specific means for detecting this organism in pure cultures or simulated clinical specimens. Between 1 and 10 organisms could be detected in these specimens, even in the presence of human genomic DNA.

The primers used for the PCR were selected for two reasons. First, the target sequence for these primers is the P1 cytadhesin, a protein that allows *M. pneumoniae* to attach to respiratory epithelial cells (25). Thus, it appears to be a virulence factor and presumably would be present in any pathogenic strain of this organism. Secondly, the primers and a DNA probe specific for this sequence are easily available from a commercial supplier.

The major difference between the results of this and previous studies was the sensitivity of detection. Bernet et al. (2) were able to detect between 10^2 and 10^3 organisms. Jensen et al. (15) were able to detect approximately 40 mycoplasmas but required 70 cycles of amplification to reach this level of sensitivity. The reason for the difference in results is not entirely clear but is probably related to the target sequence selected for amplification. This level of sensitivity may not be necessary for a routine diagnostic test, since it is possible that significant infections with *M. pneumoniae* involve a large number of organisms. Thus, extremely sensitive detection methods may not provide any diagnostic advantage. Further work should clarify this.

Our results suggest that PCR has significant potential as a rapid, sensitive method for detecting *M. pneumoniae* in clinical specimens. It was 100- to 1,000-fold more sensitive than culture or the commercial gene probe and was highly reproducible. The method used to treat specimens to remove DNA prior to amplification is a simple, rapid procedure that could be used in any laboratory. With the addition of a more simplified procedure for detecting the amplified DNA, this test could easily be used as a rapid diagnostic test for *M. pneumoniae* infections. Additional work is under way to further evaluate the performance of this procedure by a prospective study with patient specimens.

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