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Detection of *Mycoplasma pneumoniae* by Polymerase Chain Reaction and Nonradioactive Hybridization in Microtiter Plates

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In order to improve the diagnosis of a *Mycoplasma pneumoniae* infection, we developed a polymerase chain reaction (PCR)-based assay. The gene encoding elongation factor Tu (*tuf*) was selected as the target sequence. Oligonucleotides derived from variable stretches of the *tuf* gene were able to prime the amplification of a 950-bp fragment exclusively when *M. pneumoniae* DNA was used as the template. The sensitivity of the assay was increased 10-fold when the amplification products were hybridized with an internal *M. pneumoniae*-specific oligonucleotide. The use of three to four genome copies for PCR was sufficient for obtaining a hybridization signal. In addition, we substituted radioactive filter hybridization with a microtiter plate assay. Via a biotin moiety of one PCR primer, the amplification products were immobilized on streptavidin-coated microtiter plates. Subsequent hybridization with a digoxigenin-labeled oligonucleotide resulted in the same sensitivity and specificity as those obtained by filter hybridization. Clinical application of the assay was performed on 102 throat swab specimens from patients with respiratory tract infections. Of 21 culture-positive samples, 19 were confirmed to be positive in the PCR-based assay (sensitivity, 90%). Furthermore, 14 of 19 seropositive but culture-negative samples gave a positive hybridization signal. Of 62 culture-negative and seronegative specimens, 60 gave a negative result in our assay (specificity, 97%). Of the 33 samples that were positive in our PCR-based assay, 5 samples initially gave false-negative results because of the presence of inhibitory substances in those specimens. Inhibition of *Taq* polymerase in these five cases was prevented by an additional step of phenol extraction and subsequent ethanol precipitation.

Mycoplasma pneumoniae, a pathogen of the human lower respiratory tract, is the causative agent of primary atypical pneumonia (8). An important presupposition for an effective antibiotic treatment is a rapid and specific diagnostic identification. Conventional diagnostic methods such as cultivation of the organism or serological tests do not meet the present requirements of routine diagnostic laboratories. Cultural identification may take weeks, and serodiagnosis, e.g., complement fixation by antibodies against lipid antigen (14), requires increased antibody titers which are reached only after 7 to 10 days after the onset of clinical symptoms.

To render a more rapid and specific means of detecting *M. pneumoniae*, different attempts have been made so far by using DNA probes (10), polymerase chain reaction (PCR) (13), and PCR in combination with hybridization of isotope-labeled probes (2).

Here we describe the detection of *M. pneumoniae* by PCR amplification of a 950-bp fragment of the *tuf* gene (encoding elongation factor Tu). Recently, we have described the cloning and the nucleotide sequence of a *Mycoplasma hominis* gene encoding elongation factor Tu (18). Comparison of the sequences of different bacterial *tuf* genes (12, 17, 18, 30, 31) revealed homologies in the range of 65 to 70%. Therefore, the gene coding for elongation factor Tu exhibits sufficient nonhomologous segments with sequences which are unique for a given species. Three *M. pneumoniae*-specific oligonucleotides were derived from variable stretches of the *tuf* gene. Nonradioactively labeled oligonucleotides were applied to PCR, and further analysis of the amplification products was carried out by hybridization in microtiter plates. This method circumvents the disadvantages of handling radioactive materials and improves the detection of *M. pneumoniae* with regard to sensitivity and rapidity.

MATERIALS AND METHODS

Bacterial strains. *M. pneumoniae* ATCC 15293, ATCC 15377, ATCC 15492, ATCC 15531, ATCC 29085, and ATCC 29342 were obtained from the American Type Culture Collection, Rockville, Md. *Mycoplasma genitalium* NCTC 10195, *Mycoplasma lipophilum* NCTC 10173, *Mycoplasma faucium* NCTC 10174, *Mycoplasma orale* NCTC 10112, *Mycoplasma buccale* NCTC 10136, *Mycoplasma salivarium* NCTC 10113, *Mycoplasma fermentans* NCTC 10117, and *M. hominis* NCTC 10111 were obtained from the National Collection of Type Cultures, London, United Kingdom.

Isolates from *M. pneumoniae*-positive patients as well as isolates from guinea pigs which were experimentally infected by intranasal inoculation with *M. pneumoniae* FH (ATCC 15531) were a generous contribution of E. Jacobs, Freiburg, Federal Republic of Germany.

All *Mycoplasma* strains were grown as described elsewhere (23), and chromosomal DNAs were extracted by following the protocol of Wenzel and Herrmann (28).

The following non-*Mycoplasma* species served as controls in the present study (the strains for which no source is indicated were clinical isolates of the Institut für Medizini-

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TABLE 1. Sequences and description of the oligonucleotides used in the present study

Primer name	Sequence (5′–3′)[a]	Function	Derivation	Position
Mpn38	TACTCGTTACGACCAAATCGATAAG	PCR primer	Biotin	132–156
Mpn39	GTTCAACTGTAATCGAGGTATTG	PCR primer		1065–1087
Mpn46	TCCACGTGAGCGGAGTTAA	Probe	Digoxigenin	188–206
Msp27	AAACCACATGTCAATGTTGGTACCATTGGTCA (C)(T) (A) (A)(T)(C)	PCR primer, sequencing primer	Biotin	28–59
Msp28	CCACCTTCACGAATTGAGAACTT (G)(A)	PCR primer, sequencing primer		1120–1142

[a] Wobbles were introduced into primers Msp27 and Msp28 at the indicated positions.

sche Mikrobiologie, Hannover): *Acholeplasma laidlawii* NCTC 10116, *Pseudomonas aeruginosa* ATCC 27853, *Legionella pneumophila* ATCC 35096 and ATCC 33152, *Legionella micdadei* ATCC 33204, *Neisseria meningitidis* B1940 (received from U. Berger, Institut für Hygiene, Heidelberg, Federal Republic of Germany), *Chlamydia pneumoniae* TW183, AR39, and AR388 (Washington Research Foundation, Seattle, Wash.), *Chlamydia trachomatis* ATCC VR572, *Chlamydia psittaci* (sheep abort strain obtained from A. Wittenbrink, Tierärztliche Hochschule Hannover, Hannover, Federal Republic of Germany), *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella oxytoca*, *Escherichia coli*, *Haemophilus influenzae*, *Clostridium sporogenes*, *Streptococcus pneumoniae*, and *Streptococcus milleri*.

Clinical specimens. For evaluation of our PCR and hybridization assay, clinical specimens were investigated. A total of 102 throat swab samples from patients with pneumonia or other respiratory tract infections were investigated. The samples were collected in 1.8 ml of 2SP medium (0.2 M sucrose in 0.02 M phosphate buffer [pH 7.2] containing 500 U of penicillin G) or in 2 ml of SP4 medium (25). Aliquots of 200 μl of these suspensions were centrifuged at 13,000 × *g* for 30 min. Pellets were resuspended in 100 μl of lysis buffer (50 mM KCl, 10 mM Tris-Cl [pH 9.0], 0.1% Triton X-100, 200 μg of proteinase K per ml), and the mixture was incubated at 50°C for 2 h. After heating to 95°C for 15 min, 20-μl aliquots were subjected to PCR. Pipetting of clinical samples was generally done with cotton-plugged pipet tips. Samples that gave negative results in the microtiter plate assay but that were culture positive and/or seropositive were subjected to the following procedure. In order to detect the presence of inhibitory substances in the patient material, 20-μl aliquots were inoculated with 300 fg of purified chromosomal *M. pneumoniae* DNA and were again subjected to PCR. If no amplification of the inoculated DNA occurred, a 20-μl aliquot of lysed and proteinase K-treated sample was extracted with phenol and subsequently precipitated with ethanol before it was subjected to PCR.

Serology. The *M. pneumoniae* complement fixation test with lipid antigen was performed and the results were interpreted as described previously (26). In brief, a fourfold or greater rise in titer to ≥64 or single or standing titers of ≥512 were considered diagnostic of a current or recent *M. pneumoniae* infection. Serological results were recorded as negative only when at least two consecutive serum samples taken within an interval of more than 1 week during illness were negative.

Culture of *M. pneumoniae* from patients. Throat swabs were collected on cotton-tipped swabs, which were placed into 2SP or SP4 medium by breaking the swab in a vial with medium. The specimens were transported to the laboratory within 24 h. Two agar plates with SP4 and Hayflick (9) agar were each inoculated with 50 μl of the sample and were incubated at 37°C in a moist atmosphere of 8% O_2–5% CO_2–77% N_2. Agar plates were observed twice weekly with a microscope at ×50 and ×125 magnifications. SP4 and Hayflick (9) broth media were each inoculated with 100 μl of the sample, incubated at 37°C, and observed twice weekly for pH change, and growth was subcultured onto agar after 7 days. Samples were recorded as negative if no growth was observed after 35 days. Positive cultures were identified as *M. pneumoniae* by disk growth inhibition with specific antisera on agar plates (4).

Synthesis and labeling of oligonucleotides. The oligonucleotides (Table 1) were synthesized in a Gene Assembler Plus instrument (Pharmacia LKB, Freiburg, Federal Republic of Germany) by the phosphoramidite procedure. For biotin and digoxigenin labeling, a TFA-Aminolinker (Pharmacia) was incorporated at the 5′ end; this was followed by chemical coupling with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, Deisenhofen, Federal Republic of Germany) and digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid hydroxy-succinimide ester (Boehringer, Mannheim, Federal Republic of Germany), respectively. The derived oligonucleotides were purified by reversed-phase fast-protein liquid chromatography. For Southern blot and slot blot hybridization analyses, the appropriate unmodified oligonucleotide was phosphorylated at the 5′ end with [γ-^{32}P]ATP (Amersham, Braunschweig, Federal Republic of Germany) and T4 polynucleotide kinase (Pharmacia) by standard protocols (21). The resulting specific activity was 4×10^8 cpm/μg.

PCR. Amplification reactions were performed in a total volume of 100 μl containing 10 mM Tris-Cl (pH 9), 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 μM (each) dATP, dCTP, dGTP, and dTTP. The concentration of each primer was 0.3 μM, and 20 to 50 ng of template DNA was used. Before 2.5 U of *Taq* DNA polymerase (Promega Corporation, Madison, Wis.) was added, the reaction mixture was overlaid with mineral oil (Sigma) and heated at 95°C for 5 min. Thirty-six amplification cycles were performed, each of which consisted of 90 s of denaturation at 94°C, 90 s of annealing at 60°C (primer pair Mpn38 and Mpn39) or 50°C (primer pair Msp27 and Msp28), and 120 s of extension at 72°C.

Sequencing of amplification products. Unincorporated PCR primers and deoxynucleoside triphosphates were removed with the Geneclean kit (Bio 101 Inc., La Jolla, Calif.). Sequencing was performed by the dideoxy chain-termination method (22) by using the Taquence Sequencing kit (United States Biochemical Corp., Bad Homburg, Federal Republic of Germany). A total of 0.2 pmol of template DNA was mixed with a 100-fold molar excess of sequencing primers in the appropriate buffer, denatured for 5 min at 95°C, and

cooled for 5 min on ice-alcohol. Primer annealing was performed at 50°C for 5 min. After the addition of labeled mixtures, 5 μCi of [γ-^{35}S]dATP (Amersham), and 2.5 U of *Taq* DNA polymerase (Promega), the reaction mixture was incubated at 50°C for 5 min before the polymerization was terminated by the addition of dideoxynucleotides. The termination reaction was performed at 70°C for 5 min.

Hybridization of Southern blots and slot blots. In general, 10-μl aliquots were taken from the amplification reactions for further analysis. Southern blot analysis was carried out by following standard protocols (21). Slot blot analysis was performed as described elsewhere (3). Nitrocellulose filters were hybridized for 2 h at 55°C in 5 ml of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) containing 5×10^6 cpm of ^{32}P-labeled oligonucleotide per ml. Filters were washed three times for 5 min each time at room temperature in 6× SSC and twice in 3× SSC–0.1% sodium dodecyl sulfate at 55°C, also for 5 min each time.

Immobilization and hybridization in microtiter plates. Prior to use, streptavidin-coated microtiter plates (Bio-Products, Wrexham, United Kingdom) were washed three times with phosphate-buffered saline (PBS)–0.05% Tween (PBS is 10 mM sodium phosphate [pH 7.4], 150 mM NaCl). Generally, duplicates of 10-μl aliquots of the PCR mixtures were analyzed in the microtiter plate assay. The PCR fragments were generated with the biotinylated primer Mpn38 and the unlabeled primer Mpn39. After removal of unincorporated primers (Geneclean), 10-μl aliquots of double-stranded amplification products were diluted in 10 mM sodium phosphate (pH 7.4)–100 mM NaCl to achieve a total volume of 50 μl. All other steps were also performed in a 50-μl volume. The coupling of amplification fragments to the streptavidin-coated plates via the biotin moiety of one PCR primer was carried out for 15 min; this was followed by washing (three times) with PBS–0.05% Tween. For denaturing, 0.1 M NaOH was added for 10 min; this was followed by washing once with 0.1 M NaOH and three times with 0.1 M Tris-Cl (pH 7.5). The hybridization solution (24) contained 0.6 M NaCl, 20 mM sodium phosphate (pH 7.4), 1 mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 6 pmol of the digoxigenin-labeled oligonucleotide Mpn46 per well. The microtiter plates were sealed and incubated for 2 h in a water bath at 55°C. After hybridization, the plates were washed three times with 6× SSC at room temperature and twice for 5 min in 3× SSC at 55°C. Anti-digoxigenin antibody (Boehringer) was diluted in 1% bovine serum albumin in PBS and applied to the microtiter plates for 1 h. Antibody incubation was followed by washing (three times) with PBS–0.05% Tween. Finally, the substrate solution (1 mg of 4-methylumbelliferyl phosphate [Sigma] in 100 mM Tris-Cl [pH 9.6]–100 mM NaCl–50 mM $MgCl_2$) was added, and after 2 h, fluorescence units were determined on a microtiter plate fluorometric reader (Titertek Fluoroskan II; Flow Laboratories, Meckenheim, Federal Republic of Germany). To determine the cutoff, the mean values for the negative control wells (in which no PCR product was coupled to the microtiter plates, and all subsequent steps were carried out by the established protocol) and the standard deviation were calculated. Values greater than the means plus three standard deviations were considered positive.

RESULTS

Nucleotide sequence of the *tuf* gene of *M. pneumoniae*. A high degree of genotypic homogeneity among *M. pneumoniae* isolates has been observed by hybridization of restriction fragments of chromosomal DNA with a *tuf* gene probe (29). The question of whether the *tuf* sequences within isolates of the species *M. pneumoniae* coincide was answered by direct sequencing of PCR-generated *tuf* gene fragments of six different *M. pneumoniae* strains. For this purpose, oligonucleotides Msp27 and Msp28 (Table 1) were derived from conserved stretches of the *tuf* gene and were used to generate an 1,100-bp fragment in the PCR. After purification, these fragments were directly sequenced by using PCR primers Msp27 and Msp28 as sequencing primers. In all of the six different *M. pneumoniae* strains the determined sequences (positions 77 to 342 and positions 882 to 1099; the positions refer to the *tuf* gene sequence of *E. coli* [31]) were found to be completely homologous. However, our sequence data revealed differences from the sequence published by Yogev and coworkers (30) at positions 193, 194, and 1078, although the *tuf* gene sequence of the same strain (FH) was determined.

PCR and hybridization with a radioactively labeled probe. *M. pneumoniae*-specific oligonucleotides Mpn38 and Mpn39 as well as Mpn46 (Table 1) were derived from variable stretches of the *tuf* gene. In the PCR, Mpn38 and Mpn39 were able to prime the synthesis of a 950-bp fragment when *M. pneumoniae* DNA was used as the template. When chromosomal DNAs from patient isolates and guinea pig isolates was applied to amplification, in all cases a 950-bp fragment was visible (data not shown). We also subjected the chromosomal DNAs of numerous *Mycoplasma* species to PCR under the same conditions. In those cases, no PCR fragment was generated. The same negative results were obtained from PCRs with chromosomal DNAs from a variety of different bacterial genera, which are listed in Materials and Methods.

To confirm the specificity of the PCR and to increase the sensitivity of *M. pneumoniae* detection, oligonucleotide Mpn46 was hybridized to PCR fragments. In Southern blot experiments, *M. pneumoniae* fragments exclusively gave positive hybridization signals. Since the DNAs of *Mycoplasma* species other than *M. pneumoniae* could not be amplified with the primer pair Mpn38 and Mpn39, we used primers Msp27 and Msp28 to generate PCR fragments of these species. These 1,100-bp fragments did not hybridize with Mpn46. Figure 1 shows the PCR fragments of six different *Mycoplasma* species. Some of these species are commensal organisms of the oral cavity. *M. genitalium* has also been isolated from the human respiratory tract (1), and genetic studies suggest that it is closely related to *M. pneumoniae* (5, 11). None of these 1,100-bp fragments showed a hybridization signal with Mpn46 (Fig. 1). These data demonstrate that oligonucleotides Mpn38, Mpn39, and Mpn46 are capable of highly specific detection of *M. pneumoniae*.

Determination of the sensitivity of the radioactive hybridization method. The sensitivity of the radioactive hybridization method was determined by subjecting serial dilutions of *M. pneumoniae* chromosomal DNA to PCR. In ethidium bromide-stained agarose gels, a band was visible from PCRs with 30 fg of template DNA (Fig. 2A), accounting for 30 to 40 genome copies. Slot blot hybridization with ^{32}P-labeled Mpn46 resulted in a 10-fold increase in sensitivity compared with that obtained with agarose gels (Fig. 2B). Aliquots from

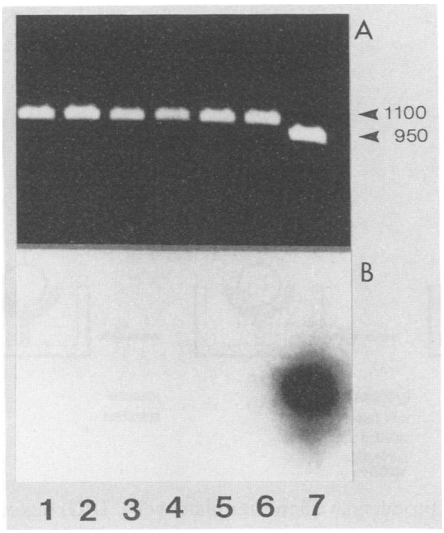

FIG. 1. Specificity of oligonucleotide Mpn46. Chromosomal DNAs of different *Mycoplasma* species were amplified with primer pair Msp27 and Msp28 (lanes 1 to 6) and primer pair Mpn38 and Mpn39 (lane 7). (A) Electrophoretic analysis of PCR fragments. (B) Hybridization analysis of the fragments in panel A. Numbers on the right indicate the length of DNA fragments (in base pairs). For Southern blot analysis, DNA was blotted onto nitrocellulose filters and hybridized with the ^{32}P-labeled Mpn46 probe at 55°C. Lanes: 1, *M. buccale*; 2, *M. orale*; 3, *M. fermentans*; 4, *M. salivarium*; 5, *M. genitalium*; 6, *M. hominis*; 7, *M. pneumoniae*.

PCR mixtures with 3 fg of template DNA gave a hybridization signal. Therefore, assuming a genome size of 800 kb (15, 28), only three to four genome copies are sufficient for specific detection of *M. pneumoniae*. However, we want to emphasize that these results were obtained with purified chromosomal DNA as the template in the PCR. Therefore, the sensitivity may not be achieved with clinical specimens.

Nonisotopic hybridization in microtiter plates. When serial dilutions of template DNA were applied to PCR with further analysis of amplification products by hybridization in microtiter plate wells, as schematically outlined in Fig. 3, the detection limit that was obtained was three to four genome copies and was thus equivalent to the sensitivity of conventional filter hybridization. Fluorescence measurement data are given in Fig. 4.

To confirm the specificity of hybridization in the microtiter plate, we biotinylated oligonucleotide Msp27. Chromosomal DNAs of different *Mycoplasma* species were amplified by PCR by using primers Msp27-biotin and Msp28. The resulting 1,100-bp fragments were immobilized on streptavidin-coated microtiter plates, and after denaturation, they were hybridized with Mpn46-digoxigenin. As expected, none of the PCR fragments obtained from non-*M. pneumoniae* DNA gave a hybridization signal.

Detection of *M. pneumoniae* in clinical specimens. A total of 102 clinical specimens were investigated for the presence of *M. pneumoniae* DNA. Twenty-one of the 102 samples were confirmed to be positive by culture and serology, 19 samples were seropositive but culture negative, and 62 samples were negative by serology and culture. We subjected the samples to PCR and further analyzed the PCR products by hybridization in microtiter plates. Nineteen of 21 culturally and serologically positive specimens gave a positive signal in our PCR assay, but 4 of the specimens tested positive only after phenol extraction. In addition, 14 of 19 serologically positive but culture-negative samples revealed a positive result (1 of the specimens tested positive only after phenol extraction). It should be pointed out that in 12 of the 40 culture-positive and/or seropositive samples, no hybridization signal was initially detected. These 12 samples were therefore inoculated with purified chromosomal *M. pneumoniae* DNA and were again subjected to PCR to examine the presence of inhibitory substances in the patient material. In 7 of the 12 samples, no PCR amplification of the inoculated DNA was observed. Therefore, these proteinase K-treated specimens were phenol extracted and ethanol precipitated before they were again subjected to PCR. In five of the seven samples, phenol extraction led to removal of inhibitory substances, and subsequent PCR amplification and hybridization resulted in a positive signal. Two samples from the culture-positive and seropositive group remained repeatedly negative, as did five samples from the seropositive but culture-negative group. Sixty of 62 culture and serologically negative samples also gave negative signals in the microtiter plate hybridization of PCR amplicons. Two of the samples gave reproducibly positive results. It is not clear whether this result is due to false-positive amplification or to a failure in cultivation and serology. In summary, the clinical sensitivity of the assay was 90% compared with that of culture (19 PCR-positive specimens of 21 culture-positive and seropositive specimens). When positive serology alone was used to define the positive group, the sensitivity was 83% (33 PCR positive of a total of 40 seropositive). The specificity was 97% (60 PCR negative of 62 seronegative).

DISCUSSION

We developed a specific and sensitive PCR-based method of detecting *M. pneumoniae* DNA. The gene encoding elongation factor Tu (*tuf*) served as the target sequence for amplification. Even though the *tuf* gene is present as a single-copy gene in *Mycoplasma* species (18, 29), it exhibits some advantageous features. Elongation factor Tu plays an essential role in protein synthesis, and because of this function, the corresponding gene shows a high degree of conservation during evolution (6, 7). Highly conserved

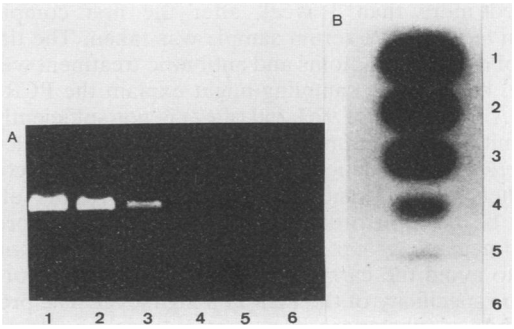

FIG. 2. Sensitivity of PCR and slot blot hybridization. Serial dilutions of chromosomal *M. pneumoniae* DNA were subjected to PCR. A total of 10 μl of amplification reaction mixtures was analyzed. (A) Electrophoretic analysis of 950-bp amplification products. (B) Slot blot analysis of amplification products. Hybridization was performed with the ^{32}P-labeled Mpn46 oligonucleotide at 55°C. Lanes: 1, 30 pg of template; 2, 3 pg of template; 3, 300 fg of template; 4, 30 fg of template; 5, 3 fg of template (corresponding to three to four genome copies); 6, 0.3 fg of template.

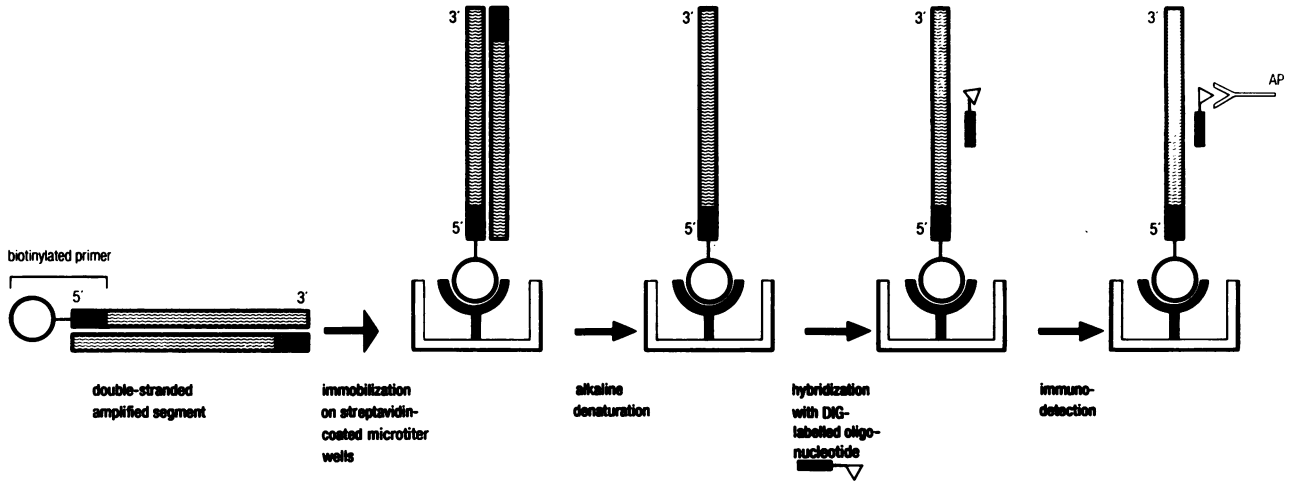

FIG. 3. Schematic diagram of immobilization and hybridization of amplification products in microtiter plate wells. DIG, digoxigenin; AP, alkaline phosphatase.

stretches allow the derivation of oligonucleotides for amplification of numerous bacterial *tuf* genes, whereas variable regions offer the feasibility of defining species-specific sequences. It should therefore be possible to identify any *Mycoplasma* species on the basis of amplification of the *tuf* gene. In the present study, *M. pneumoniae*-specific oligonucleotides were used as amplification primers and as the hybridization probe. By this means we were able to detect as few as three to four genome copies of purified *M. pneumoniae* DNA. Clinical application of the assay described here for the diagnosis of *M. pneumoniae* infection was performed on 102 throat swab specimens. A clinical sensitivity of 90% (19 of 21) for the PCR assay compared with the culture method was obtained. This is similar to the sensitivity of the complement fixation test when compared with that of culture, as recently evaluated by Kenny et al. (14). Two of the 21 samples remained repeatedly negative, which may have been due to a very low number of *M. pneumoniae* cells in the clinical sample, thus being beyond the detection limit of the assay. Fourteen of 19 samples of the seropositive but culture-negative collection revealed a positive result. However, four of the five repeatedly PCR-negative samples were obtained more than 1 week after the first complement fixation test-positive serum sample was taken. The times of onset of clinical symptoms and antibiotic treatment were not known, but the late sampling might explain the PCR negativity. A specificity of 97% (60 of 62) is not sufficiently high to permit screening of populations with a low prevalence of *M. pneumoniae* infections. However, further work comparing other highly sensitive tests with the results of PCR should be done in order to establish a larger, more well defined negative group. All available precautions should be taken to avoid the carryover of amplified DNA in order to keep the specificity of the PCR at a high level. The presence of inhibitory substances in patient material should also be considered. The inclusion of an internal positive control in each clinical sample would be valuable for avoiding false-negative PCR results.

In order to become a routine procedure, the detection of PCR amplification products needs to be simplified with regard to saving time and processing large numbers of samples as well as avoiding the use of radioactively labeled compounds. So far, very promising approaches have been published. Those approaches take advantage of the biotin-streptavidin interaction for immobilization of amplification products either on beads (20, 24, 27) or on microtiter plates (16, 19). Landgraf et al. (16) and Rimstad et al. (20) detected the immobilized amplification products by using a label attached to the second PCR primer, thus lacking the advantage of an additional means of confirming the specificity of the test by hybridization. Syvänen et al. (24) reported hybridization with a ^{32}P-labeled probe, whereas Urdea and coworkers (27) investigated the detection limits of different labels attached to oligonucleotide probes. Enzymes directly

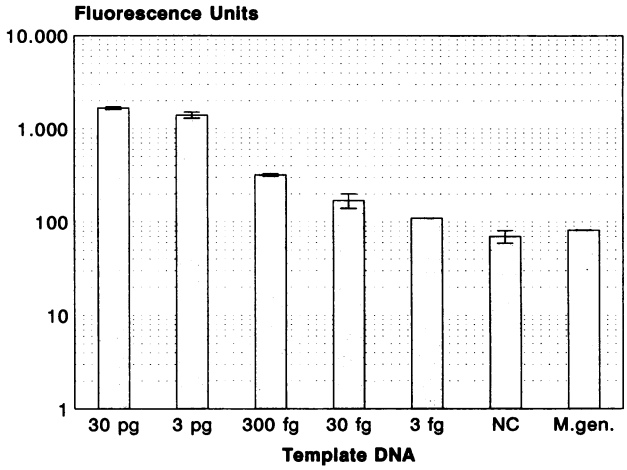

FIG. 4. Sensitivity and specificity of hybridization of amplification products in microtiter plates. The data presented here are the means of duplicate values. Serial dilutions of *M. pneumoniae* DNA were applied to PCR, and duplicate 10-μl aliquots of the amplification products were analyzed in microtiter plates. A total of 3 fg of template DNA corresponds to three to four genome copies. NC, background level determined without DNA immobilized on the microtiter plate, but all subsequent steps were performed as with the samples subjected to amplification. The specificity of the assay is represented by the data obtained with *M. genitalium* (M. gen.) DNA as the template in the PCR by using primer pair Msp27-biotin and Msp28. A total of 100 ng of *M. genitalium* amplification products was applied to the microtiter plate.

coupled to oligonucleotides revealed a detection limit comparable to the sensitivity obtained with ^{32}P-labeled probes. Mantero et al. (19) bound a biotin-labeled oligonucleotide probe to a streptavidin-coated microtiter plate and hybridized the probe with the single-stranded amplification product. In that study, detection was carried out with an anti-DNA antibody, which binds exclusively to double-stranded DNA. Although the results that were obtained revealed the same sensitivity as that which was obtained by radioactive Southern blot hybridization, the method might be able to detect not only hybridized double-stranded DNA but also double strands originating from secondary structure formation of single-stranded DNA.

In the present study we immobilized PCR amplification products on streptavidin-coated microtiter wells using the biotin moiety of one PCR primer. Subsequent hybridization was carried out with an internal digoxigenin-labeled oligonucleotide. The detection limit was found to be identical to the sensitivity of filter hybridization with the ^{32}P-labeled oligonucleotide probe. The quality of the streptavidin-coated microtiter plates that are used is a crucial determinant with regard to the reliability of the assay. Initial experiments were carried out with avidin and streptavidin coupled to microtiter plates by passive adsorption. Those plates proved to be unsuitable for the assay described here, because treatment with NaOH led to removal of streptavidin from the plates. We also found that commercially available microtiter plates with covalently bound streptavidin exhibit considerable plate-to-plate variations. It is therefore absolutely necessary that positive and negative controls be included on every microtiter plate for the discovery and exclusion of low-quality plates.

Our assay combines the advantage of simple and rapid processing of PCR amplification products with the opportunity of using oligonucleotides that can be easily synthesized and labeled on a large scale at acceptable expense. For this reason, the method described here can be applied in a routine diagnostic laboratory for the PCR-based detection of numerous bacterial, viral, or parasitic infections. Moreover, the detection of *Mycoplasma* contamination in cell cultures is conceivable on the basis of genus-specific PCR amplification and species-specific hybridization, since the versatility of the microtiter plate assay allows the application of different PCR amplicons and hybridization probes in a single assay.

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