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Enzyme-Linked Immunoassay for Detection of PCR-Amplified DNA of Legionellae in Bronchoalveolar Fluid

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A nonradioactive method is described that detects 10 to 100 legionellae in 1 ml of bronchoalveolar lavage fluid. DNA is purified by a proteinase K-phenol protocol or with a commercial DNA preparation kit and amplified by PCR with amplimers specific for the 16S rRNA gene of *Legionella pneumophila*. The upstream primer is 5′ biotinylated. The amplification product is immobilized on streptavidin-coated microtiter plates. Because of the high binding capacity, no removal of nonincorporated biotin from the PCR product is required. After alkaline denaturation, the single-stranded PCR product is hybridized with a 5′ digoxigenin-labeled probing oligomer. The amplification product is then detected by using peroxidase-labeled anti-digoxigenin antibodies in a luminescence or colorimetric reaction. The assay detects as few as 10 legionellae in 1-ml bronchoalveolar lavage fluid specimens. It is specific for medically relevant *Legionella* species, including *Legionella pneumophila*, *L. bozemanii*, and *L. longbeachae*. Of over 250 clinical specimens examined, 8 were positive for legionellae by both culture and the PCR assay. Six further specimens were culture negative but PCR positive for legionellae; of these, five specimens were from patients receiving high-dose erythromycin therapy for suspected or previously diagnosed legionella pneumonia. None of the remaining 240 specimens that were culture negative for legionellae yielded a positive PCR test, although a total of over 30 different bacterial species were cultured from these specimens. The PCR assay therefore appears to exhibit high sensitivity and specificity and thus could prove suitable for use in the routine microbiological diagnostic laboratory.

Pneumonia caused by legionellae has a poor prognosis unless it is diagnosed early and treated with specific antibiotics (26). Eighty-five percent of *Legionella* pneumonias are caused by *Legionella pneumophila* (20). Cultivation of legionellae from appropriate specimens represents the definitive method for diagnosis and has a sensitivity of 50 to 90% (26). However, colonies become macroscopically visible after 3 to 4 days of culture. Therefore, methods for direct examination of specimens, in particular, the direct fluorescent-antibody (DFA) test, have been devised. The sensitivity of the DFA test is, however, rather low, and large numbers of legionellae (10^4 to 10^5/ml) must be present for the test to become positive (21). A negative result thus does not rule out infection. Immunological detection of an antigen from *L. pneumophila* serogroup I, which is responsible for 50 to 70% of legionella infections in urine, reportedly has a sensitivity of 75 to 90%. However, antigenuria commences 3 days after infection and may last up to 1 year (14).

Tests based on the use of nucleic acid probes have a specificity similar to that of the DFA test and are not sufficiently sensitive to replace culture. A ^{125}I-labeled cDNA hybridizing with rRNAs of a wide range of *Legionella* spp. has been employed to generate a detection method with virtually 100% specificity (4, 24). Clinical evaluation revealed a sensitivity of 70% (5, 19); approximately 10^3 cells per ml are required for a positive result (8).

More recently, PCR has been used to amplify legionella DNA. Amplification products are detected in stained agarose gels or by hybridization with oligonucleotides. Different DNA sequences have been selected for amplification. The first assays detected as few as 350 CFU in water (23) or 250 CFU in respiratory specimens (18). The DNA sequences of the macrophage infectivity potentiator (*mip*) and *mip*-like genes (1, 3, 6) as a determinant of pathogenicity were used for detection of *L. pneumophila* (17) and different *Legionella* spp. (12). Fifty CFU of *L. pneumophila* per ml could be detected in bronchoalveolar lavage (BAL) fluid after hybridization. However, *mip* primers could not be used to detect several relevant *Legionella* spp. (12).

Amplification of genes encoding rRNA is also feasible, and published protocols employ the 5S rRNA gene alone or combined with a *mip* gene (2). The commercial assay employing detection by reverse hybridization (22) was originally designed for environmental samples and has been successfully used to detect 3,000 legionellae per ml in BAL fluid (13) or, after hybridization, 400 CFU of *L. pneumophila* per ml (18). Recently, amplification of the 16S rRNA gene has enabled detection of 1 CFU/ml in water samples (25) or 10 CFU/ml in stimulated bronchial fluid (15). Amplification of the 16S rRNA gene has well-known advantages. In particular, a large number of 16S rRNA sequences are now available and suitable amplimers can thus be selected.

Here we describe a simple and generally applicable procedure using commercially available products. DNA was purified from BAL fluid by using an optimized protocol. A 386-bp sequence of the 16S rRNA gene was amplified, and the biotinylated product was quantitatively captured in streptavidin-coated microtiter plates without removal of the nonincorporated biotinylated primer. The amplification product was hybridized with a digoxigenin-labeled probing oligomer, and the assay was developed with the use of antibodies against digoxigenin.

MATERIALS AND METHODS

Reagents. Oligonucleotides were synthesized and 5′ modified with biotin or digoxigenin by MWG-Biotech (Ebersberg, Germany).

A 10× PCR buffer, 50 mM $MgCl_2$, and *Taq* polymerase (5 U/μl) were pur-

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TABLE 1. Specificity of the 16S rRNA gene PCR-generated fragment

DNA source (serogroup)	Strain or source[a]	386 bp[b]	EIA[c]	*mip*[d]
L. pneumophila (1)	ATCC 33152	+	+	+
L. pneumophila (6)	Mz	+	+	ND[e]
L. pneumophila (7)	ATCC 33823 Chicago-8	+	+	+
L. pneumophila (8)	ATCC 35096 Concord-3	+	+	+
L. pneumophila (9)	ATCC 33289	+	ND	+
L. pneumophila (10)	ATCC 43283 Leiden-1	+	+	+
L. pneumophila (11)	ATCC 43130 797/PA/H	+	+	+
L. pneumophila (12)	ATCC 43290 570-CO-H	+	+	+
L. pneumophila (13)	RIVM 82-A-3105	+	+	+
L. pneumophila (14)	ATCC 43703 1169-MN-H	+	+	+
L. bozemanii (1)	ATCC 33217	+	+	+
L. bozemanii (2)	ATCC 35545	+	+	−
L. micdadei	ATCC 33218	+	−	+
L. longbeachae (1)	ATCC 33462	+	+	−
L. longbeachae (2)	ATCC 33484	+	+	−
L. feeleii (2)	ATCC 35849	+	(+)	−
P. acidovorum	Mz	−	ND	ND
P. aeruginosa	Mz	−	−	ND
P. putida	Mz	−	ND	ND
P. cepacia	Mz	−	ND	ND
E. coli	Mz	−	−	ND
Human T cells	Mz	−	−	ND

[a] ATCC, American Type Culture Collection; RIVM, Rijkinstituut voor Volksgezondheid en Milieuhygiene; Mz, Institute for Medical Microbiology Mainz.

[b] PCR with 16S rRNA gene primers and detection of the amplification product on an ethidium bromide-stained gel.

[c] PCR as described in footnote *b* but with detection of the amplification product by EIA.

[d] PCR with *mip* gene primers and detection of the amplification product on an ethidium bromide-stained gel as previously described (12).

[e] ND, not done.

chased from GIBCO BRL (Eggenstein, Germany). A DNA polymerization mixture was obtained from Pharmacia (Freiburg, Germany). Light mineral oil and other chemicals were from Sigma (Deisenhofen, Germany). Materials used for restriction enzyme digestion and Southern blot hybridization, including positively charged nylon membranes, blocking reagent, anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, nitroblue tetrazolium chloride, and 5-bromo-4-chloro-3-indolylphosphate, were from Boehringer GmbH (Mannheim, Germany). Streptavidin-coated microplates with high biotin-binding capacity were supplied by Labsystems (Frankfurt am Main, Germany).

Bacterial strains. If not otherwise stated, experiments were done with serogroup 1 strain *L. pneumophila* ATCC 33152. The other organisms used are listed in Table 1. Clinical isolates other than legionellae from the Institute for Medical Microbiology and Hygiene, Mainz, Germany, were identified with the API 20E, API 20C AUX, and API 20 NE systems (bioMérieux, Nürtingen, Germany). For trials with defined amounts of legionellae, bacteria grown on B.C.Y.E. alpha-agar (Biotest, Heidelberg, Germany) were resuspended in phoshate-buffered saline (PBS) and spread in a dilution series onto B.C.Y.E. alpha-agar. CFU per milliliter were calculated by counting colonies after 5 days of cultivation. After addition of 2 volumes of glycerol, the bacterial suspension was stored at −20°C.

Handling of BAL fluid specimens for culture and identification of legionellae. BAL fluid specimens used in this study stemmed mainly from patients from intensive care units or from the Hematology Department of the Mainz University Hospital. Specimens were plated on nonselective (B.C.Y.E. alpha-agar) and selective (legionella-M.W.Y. selective agar containing polymyxin B, vancomycin, and anisomycin) media. Colonies were examined under a dissecting microscope, and suspected colonies were subcultured on normal sheep blood agar. Cultures were finally identified by DFA test as *L. pneumophila* by staining with a fluorescein isothiocyanate-labeled monoclonal antibody (Fresenius, Bad Homburg, Germany) which detects an antigen common to all different serogroups of *L. pneumophila*. In some cases, serogroups were identified with a panel of monoclonal antibodies. Negative specimens were discarded after 10 days of cultivation.

DNA purification from BAL fluid specimens. Two DNA purification protocols were employed during the course of this study. Ninety specimens were processed by using a phenol-proteinase K protocol. One-milliliter samples of BAL fluid were centrifuged for 10 min at 13,000 × *g*. The pellet was mixed with 3 μl of 10-mg/ml proteinase K, resuspended in 300 μl of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and digested for 1 h at 56°C. Thereafter, 50 μl of 5 M NaCl and 40 μl of 10% cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl were added and the mixture was incubated for 10 min at 56°C. Three subsequent extractions of the aqueous phase were performed: first with chloroform, second with phenol-chloroform (1:1) including 0.1% (wt/vol) 8-OH-quinoline, and third with chloroform. The DNA was ethanol precipitated and resuspended in 5 μl of sterile distilled water. One microliter was used for PCR amplification in a 25-μl volume.

A more simplified protocol was used for a further 166 samples. DNA was purified with QIAamp Blood Kits (Quiagen GmbH, Hilden, Germany). One-milliliter specimens were centrifuged at 13,000 × *g* for 10 min, 800 μl of each supernatant volume was discarded, and the pellets was resuspended in the remaining 200 μl. DNA was purified in accordance with the manufacturer's protocol. From each eluate of the 200-μl volume, 1 and 20 μl were subjected to PCR in final reaction volumes of 20 and 40 μl, respectively. The detection limit with this column protocol was similar to that of the proteinase K-phenol protocol (data not shown).

DNA amplification. Oligomers were selected from the published full-length sequence of the *L. pneumophila* 16S rRNA gene (EMBL nucleotide sequence database release 30; accession no. M59157).

Two 20-base oligonucleotides were used as amplimers enclosing a 386-bp fragment of the 16S rRNA gene. p1.2 (5′-AGGGTTGATAGGTTAAGAGC-3′) was located at positions 451 to 470, and cp3.2 (5′-CCAACAGCTAGTTGACA TCG-3′) was complementary to positions 836 to 817. Amplimer p1.2 was 5′ biotinylated. The 5′-digoxigenin-labeled 20-mer cp2 (5′-CAACCAGTATTATC TGACCG-3′), complementary to positions 630 to 649, was used as the probing oligomer.

Amplification was performed in 1× PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 9.0])–0.04 U of *Taq* DNA polymerase per μl–each primer at 1 μM–dATP, dCTP, dGTP, and dTTP at 0.2 mM each. Initial denaturation at 95°C for 5 min was followed by 40 cycles of annealing at 57°C for 1.5 min, extension at 72°C for 1 min, and denaturation at 94°C for 1 min. Finally, incomplete PCR products were extended for 10 min at 72°C.

Gel electrophoresis. Ten microliters of the PCR product was loaded onto a 1% agarose gel in 89 mM Tris-HCl–89 mM borate–8.9 mM EDTA (pH 8.0) with 0.5 μg of ethidium bromide per ml and separated by electrophoresis. The lengths of DNA fragments were estimated by comparison with size markers (Bethesda Research Laboratories, Gaithersburg, Md.). If necessary, the amount of the PCR product was estimated by determining fluorescence intensity on an ethidium bromide-stained gel by comparison with that of DNA purified by CsCl ultracentrifugation and quantified by measurement of A_{260}.

Southern blot hybridization. DNA was blotted onto a positively charged nylon membrane by overnight capillary transfer with 0.5 N NaOH–1.5 M NaCl. The membrane was then equilibrated with 50 mM sodium phosphate buffer (pH 6.5). DNA was fixed to the air-dried membrane by 3 min of UV irradiation (Amplirad; Genetic Research Instrumentation). DNA hybridization was carried out as previously described (7) except that all temperature-sensitive steps took place at 42°C and 12 pmol of digoxigenin-labeled probe cp2 per ml was added. For detection, the membrane was incubated at 20°C for 5 min in H_2O, 5 min in maleic acid buffer (0.1 M maleic acid, 0.2 M NaCl, 0.2 M NaOH [pH 7.5]), 60 min in 2× blocking buffer (10× blocking buffer consists of 10% blocking reagent and 90% maleic acid buffer), and 60 min in blocking buffer containing 0.4 μl of anti-digoxigenin Fab fragments conjugated to alkaline phosphatase per ml. After four washes in maleic acid buffer, the membrane was equilibrated for 10 min in reaction buffer (0.1 M NaCl, 50 mM $MgCl_2$, 0.1 M Tris-HCl [pH 9.5]). The assay was developed for 2 to 3 h at 37°C in 10 ml of reaction buffer with 45 μl of 100-mg/ml nitroblue tetrazolium and 35 μl of 50-mg/ml 5-bromo-4-chloro-3-indolylphosphate. The membrane was washed once in H_2O and finally in 1 mM EDTA–10 mM Tris-HCl (pH 8).

Enzyme-linked immunoassay (EIA) for detection of the PCR product. The EIA was performed as previously described (16) but with modifications. Streptavidin-coated microplates were washed, and 10 μl of the PCR product in 140 μl of PBS was pipetted into each well. After binding of the PCR product via the incorporated biotinylated primer for 45 min at ambient temperature, the wells were washed three times, the double-stranded PCR product was denatured with NaOH, and the unbiotinylated strand was discharged by three washes with 300 μl of 0.1 M Tris-HCl (pH 7.4).

The immobilized single-stranded PCR product was hybridized with 0.1 pmol of 5′ digoxigenin-labeled 20-mer primer cp2 for 2 h at 50°C. Afterwards, the wells were washed three times with 6× SSC (1× SSC is 0.15 M NaCl plus 0.15 M Na citrate [pH 7.0]), once with 3× SSC, and finally with 3× SSC–1% bovine serum albumin for 5 min at 50°C.

Anti-digoxigenin Fab fragments conjugated with horseradish peroxidase (Boehringer), diluted 1:5,000, were bound at ambient temperature for 15 min to the hybridized digoxigenin-labeled probing oligomer by incubation in 100 mM Tris-HCl (pH 7.4)–150 mM NaCl–1% bovine serum albumin. The unbound conjugate was removed by six washes with 300 μl of PBS-Tween. Assays were developed by using either 150 μl of ECL (Amersham, Braunschweig, Germany) or *o*-phenylenediamine (Dako Diagnostika GmbH, Hamburg, Germany) for determination of luminescence in a microplate luminometer (MicroLumat LB96B; EG&G Berthold, Widbad, Germany) or optical density (OD) at 492 nm in a microplate reader (Titertek Multiscan MC; Flow Laboratories, Meckenheim, Germany), respectively.

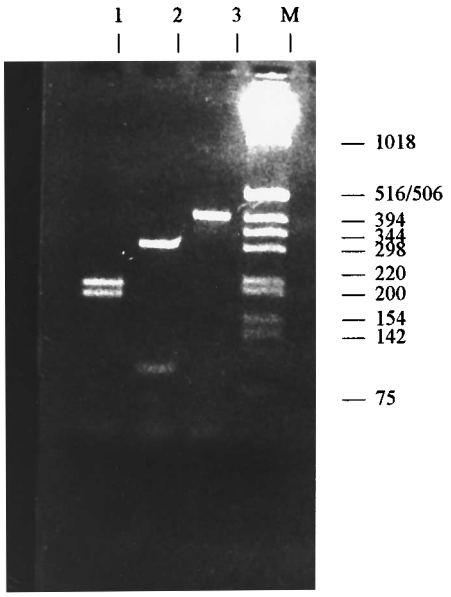

FIG. 1. Restriction fragment analysis of the 16S rRNA gene PCR product. A 20-μl volume of the PCR product was ethanol precipitated and redissolved in 60 μl of distilled H_2O. A 20-μl volume was mixed with 10 U of the respective restriction enzyme and 10× buffer as recommended by the manufacturer. Fragments separated on a 1% agarose gel are shown after digestion with *Xho*I (lane 1) or *Hae*III (lane 2) or with no enzyme (lane 3). DNA size standards (lane M) are shown for comparison, and sizes are indicated in base pairs on the right.

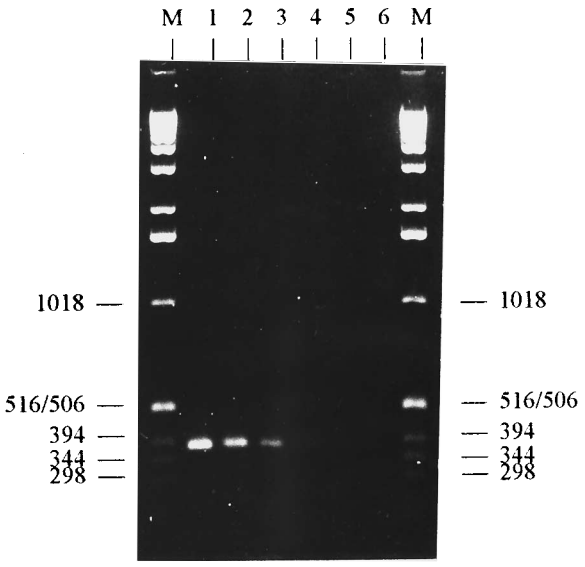

FIG. 2. Sensitivity of 16S rRNA gene PCR detecting *L. pneumophila* in BAL fluid specimens. A 1-ml volume of BAL fluid was seeded with decreasing numbers of legionellae. DNA was purified and used for PCR as described in Materials and Methods. The ethidium bromide-stained gel demonstrates 10 μl of the PCR product from BAL fluid mixed with 10^4 (lane 1), 10^3 (lane 2), 10^2 (lane 3), 10^1 (lane 4), 10^0 (lane 5), or 0 (lane 6) CFU. DNA size standards (lane M) are shown for comparison, and sizes are indicated in base pairs on both sides.

RESULTS

Specificity of the PCR. From the available 16S rRNA gene sequences of *L. pneumophila*, we chose two amplimers hybridizing in variable regions V3 and V5 (11) at positions 451 to 470 and 817 to 836, respectively, enclosing a 386-bp fragment. Screening of the EMBL database (release 34 4/93) revealed no sequences of organisms other than legionellae similar to both primers. As shown in Fig. 1, PCR resulted in a 386-bp product (lane 3) with characteristic restriction sites as predicted by the nucleic acid sequence. The PCR product yielded 201- and 185-bp fragments after digestion with *Xho*I (lane 1) or 292- and 94-bp fragments after digestion with *Hae*III (lane 2), respectively, as predicted from the published sequence.

Detection limit of 16S rRNA gene PCR. The selected amplimers enabled detection of as little as 200 fg of purified *L. pneumophila* chromosomal DNA (data not shown). To estimate the actual detection limit, 1 ml of BAL fluid was spiked with *L. pneumophila*; a typical dilution series is shown in Fig. 2. One hundred CFU of legionellae present in 1 ml of BAL fluid could be clearly detected.

Specificity of the 16S rRNA gene PCR. The specificity of the PCR was tested with a panel of bacterial strains and human DNA (Table 1). Results obtained with the different methods are shown in Table 1. In contrast to methods using the *mip* gene as a target for amplification (12, 17), the 16S rRNA gene PCR revealed the expected 386-bp product with all of the pneumophila and non-pneumophila *Legionella* spp. available in our laboratory, including *L. micdadei* and *L. feeleii*. In contrast, DNA from *E. coli* or different strains of *Pseudomonas* spp. or human DNA was not amplified. The specificity of the PCR was further underlined by the finding that clinical specimens containing a large number of different microorganisms were PCR negative (see below).

Sensitivity of hybridization assays. The 16S rRNA-based amplification product was detected after hybridization with a nonradioactively labeled probe by Southern blot hybridization and EIA.

Figure 3 demonstrates a Southern blot hybridization with digoxigenin-labeled probe cp2. No hybridization to 5 μg of the DNA size marker was observed (lane 1). However, even 2.5 ng of the PCR product could be clearly detected (lane 5), although ≥50 ng was required for visualization on the ethidium bromide-stained gel (not shown).

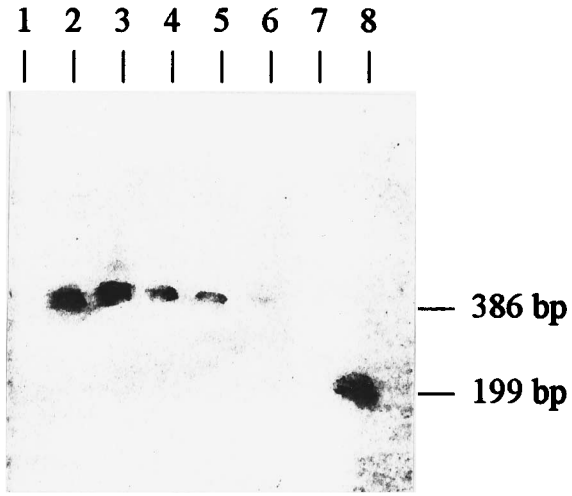

FIG. 3. Southern blot analysis of the 386-bp PCR product after hybridization with digoxigenin-labeled probing oligomer cp2. Lane 1 contained 5 μg of a 1-kb DNA ladder size marker. The next lanes contained decreasing amounts of the 386-bp product, i.e., 50 (lane 2), 25 (lane 3), 5 (lane 4), 2.5 (lane 5), 0.5 (lane 6), and 0.25 (lane 7) ng. Lane 8 contained 5 ng of a 199-bp PCR product in which cp3.2 was replaced by digoxigenin-labeled primer cp2 as a control for transfer. The amount of the PCR product was estimated on ethidium bromide-stained gels. Transfer, hybridization, and detection were done as described in Materials and Methods.

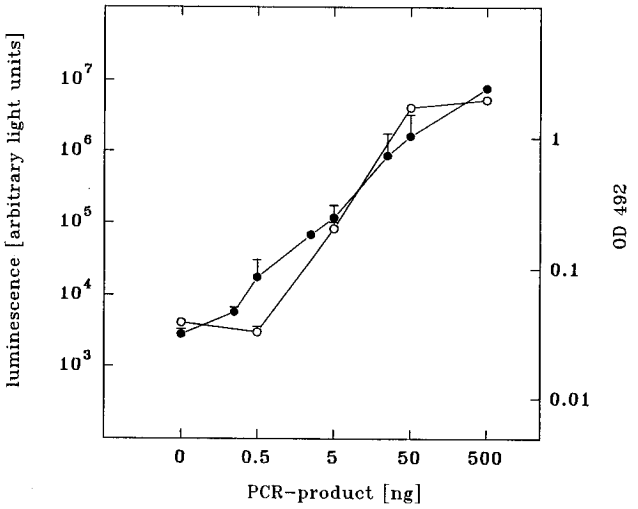

FIG. 4. Sensitivity of the EIA in detecting the 16S rRNA gene PCR product. A 10-fold serial dilution of 500 ng of the biotinylated PCR product was made. The graph compares detection by means of luminescence with ECL (●) and by measurement of OD at 492 nm with *o*-phenylenediamine (○) as the substrate. The data are means of quadruplicates (luminescence) and duplicates (OD), respectively, on a logarithmic scale.

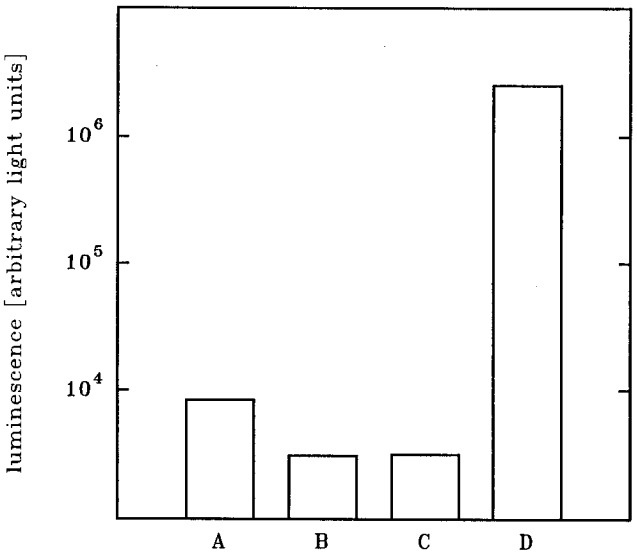

FIG. 5. Specificity of the EIA. A non-legionella biotinylated PCR product was produced. Ten nanograms of a 1-kb DNA ladder size marker was used as the template to generate an RPR with 5′-biotinylated oligonucleotide p1.2 as the sole amplimer and the low-stringency annealing temperature of 37°C for three initial cycles. The amount of the RPR was estimated on an agarose gel in comparison with the legionella-specific PCR product. The EIA was performed with duplicate samples of PBS (A), 5 ng of the RPR (B), 50 ng of the RPR (C), and 5 ng of the legionella-specific PCR product (D). The data are mean luminescence values on a logarithmic scale.

The same probing oligonucleotide was employed for detection of the 386-bp product by EIA. A 10-μl volume of the PCR product was diluted with buffer containing 10 pmol of biotinylated primer p1.2 per 10 μl; i.e., the concentration of biotin competing for streptavidin binding sites remained constant. As shown in Fig. 4, even small amounts of 0.5 to 5 ng of the amplification product could be clearly discriminated from the background signal. The background was estimated as follows. A nonspecific, randomly primed PCR product (RPR) was generated. The specific and nonspecific PCR products were applied in the EIA, and as shown in Fig. 5, hybridization with this non-legionella DNA generated no luminescence signal compared with PBS, even when employed at a 10-fold higher concentration than the legionella-specific PCR product.

Limit of *Legionella* detection in BAL by EIA. *L. pneumophila* was seeded into 1-ml aliquots of pooled BAL fluid. DNA was prepared and amplified as described in Materials and Methods. Figure 6 demonstrates that as few as 10 CFU resulted in a signal significantly above the background.

The PCR products of different strains were used for detection by EIA, as listed in Table 1. All serogroups of *L. pneumophila*, *L. longbeachae*, and *L. bozemanii* could be detected. *L. feeleii* could be detected, but this required the use of at least 50 ng of the PCR product. *L. micdadei* could not be detected by EIA, although all of the species used yielded similar amounts of the 386-bp amplification product. This was probably due to several mismatches between the sequence of the probing oligomer and the corresponding sequence of the 16S rRNA gene, as shown in Table 2. The respective sequence of *L. feeleii* was not available.

To define the cutoff absorbance value, BAL fluids, including specimens proved positive by culture, were analyzed by EIA. All culture-positive samples were clearly identified. The majority of specimens without growth of *Legionella* spp. after 10 days remained negative in the EIA as well. There were 13 culture-negative samples left with an absorbance value slightly over a cutoff value calculated as the mean OD plus 3 standard deviations. To clarify the ambiguities, these samples were investigated by Southern hybridization. A 386-bp hybridization product could not be detected in any case. From these data, we therefore calculated the cutoff by adding 0.12 OD unit to the mean of the blank OD plus 3 standard deviations.

Two hundred fifty-six BAL fluid samples, including eight that were culture positive for *L. pneumophila*, were analyzed by

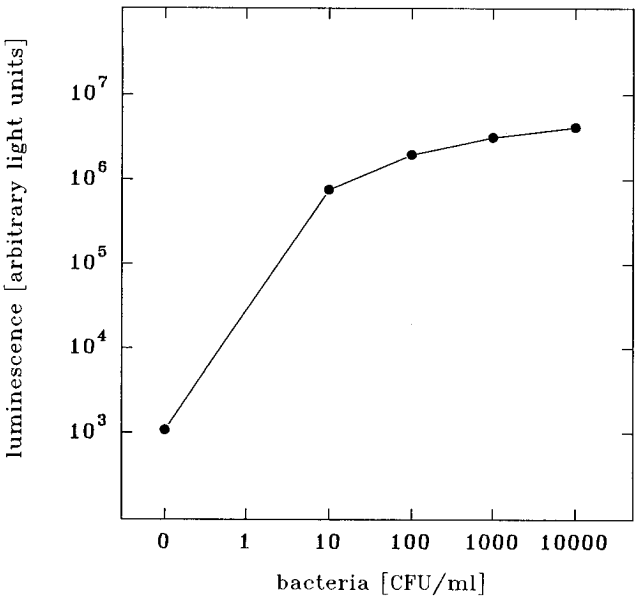

FIG. 6. Sensitivity of 16S rRNA gene PCR from BAL fluid as detected by EIA. A 1-ml volume of BAL fluid was seeded with decreasing amounts of *L. pneumophila*. DNA was purified and used for PCR as described in Materials and Methods. A 10-μl volume of the PCR product was used in duplicates for the EIA, and luminescence was detected. The data are mean luminescence values on a logarithmic scale.

TABLE 2. Aligned 16S rRNA gene sequences hybridizing with chosen oligomers from *L. pneumophila*, *L. bozemanii*, *L. longbeachae*, and *L. micdadei*[a]

Bacterium	p1.2	cp2	cp3.2
L. pneumophila	AGGGTTGATAGGTTAAGAGC	CGGTCAGATAATACTGGTTG	CGATGTCAACTAGCTGTTGG
L. bozemanii	---T-Y-C-----N------	A--------N-----N--NN	----------N---R----
L. longbeachae	-----Y-------GN-----	A--------G----------	---------------R----
L. micdadei	--N-R---------------	T----------G----TGA-	-------------------

[a] Differences with respect to the sequence of *L. pneumophila* are underlined, questionable differences are not underlined, and identical bases are represented by dashes. The data are from reference 9.

PCR. Each assay was run with one positive and three negative controls. All eight culture-positive samples were clearly identified. In addition, six culture-negative BAL fluid samples from four different patients were PCR positive. Of these, five were from patients receiving high-dose erythromycin therapy for suspected or proven *Legionella* pneumonia.

In two patients, *L. pneumophila* infection had been proven by positive culture from earlier samples. Interestingly, the two other patients negative for legionellae by culture but positive by PCR were hospitalized at the same time in the same intensive care unit. No microorganisms other than the following were isolated from these cultures: the fungi *Aspergillus* spp., *Geotrichum* spp., and *Candida* spp.; the gram-positive bacteria *Corynebacterium* spp. *Lactobacillus* spp., *Peptostreptococcus* spp., *Propionibacterium* spp., viridans streptococci, beta-hemolytic streptococci, *Streptococcus pneumoniae*, *Enterococcus* spp., *Staphylococcus aureus*, and coagulase-negative staphylococci; and the gram-negative bacteria *Acinetobacter anitratus*, *Bacteroides* spp., *Capnocytophaga* spp., *Eikenella corrodens*, *Enterobacter* spp., *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Haemophilus influenzae*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Moraxella catarrhalis*, *Neisseria* spp., *Proteus* spp., *Pseudomonas acidovorans*, *Pseudomonas aeruginosa*, *Veillonella* spp., and *Xanthomonas maltophilia*. The other 242 specimens without growth of *Legionella* spp. after 10 days remained negative by the PCR assay. The microorganisms listed above were cultured from these PCR-negative samples, and the results clearly underlined the high specificity of the PCR assay.

DISCUSSION

Since early diagnosis can decide the outcome of *Legionella* pneumonia, several approaches have been devised for direct detection of these bacteria or their DNAs in BAL fluid. The DFA test has the disadvantage that large numbers of cells are required (21). Radioactively labeled nucleic acid probes (5, 19) are hazardous, decay rapidly, and also require the presence of relatively large numbers of cells in the BAL fluid samples. PCR therefore represents the method of choice, and different target sequences have been proposed. The gene encoding the macrophage infectivity potentiator of *L. pneumophila* (*mip*) (17) and the *mip*-like gene of other *Legionella* spp. (12) have been used. However, application of these target sequences does not permit detection of several clinically relevant *Legionella* spp. Selection of a 16S rRNA gene sequence, on the other hand, harbors many advantages. A large database of 16S rRNA genes is available, facilitating selection of appropriate sequences. Large amplification products are generated that can easily be identified by agarose gel electrophoresis even after digestion with restriction endonucleases.

Subsequent to gel electrophoresis, most researchers propose Southern blot analysis with ^{32}P-labeled oligomers, which increases the sensitivity 10- to 100-fold. We have chosen a nonradioactive method instead, to avoid the many disadvantages associated with the use of radioactive probes. By employing digoxigenin-labeled probes for Southern blots, we increased sensitivity by 1 order of magnitude. To simplify the technique and shorten the time for routine usage, an immunoassay was developed without loss of sensitivity. Quantitative immobilization of PCR products labeled with biotin requires high biotin-binding capacity of the microtiter plates, since 10 μl of the PCR reaction mixture contains 6×10^{12} molecules of biotinylated primers, of which most are not incorporated. The use of high-capacity streptavidin-coated microtiter plates met this demand and abrogated the need for first separating free from incorporated biotinylated primers. Hybrids between single-stranded amplification products and the digoxigenin-labeled probe could be detected immunologically with horseradish peroxidase-labeled anti-digoxigenin Fab fragments. Horseradish peroxidase was chosen since the immunoassay could then be developed colorimetrically or by using enhanced chemiluminescence. ECL chemiluminescence can be read within a few minutes, and this technique has the advantage of somewhat higher sensitivity.

A commercially available kit employs the technique of reverse hybridization (22); i.e., the biotin-labeled PCR product is hybridized and captured by a probing oligonucleotide which is immobilized via a dT homopolymer on a solid support. Originally introduced for detection of legionellae in environmental samples (2), it has been employed for clinical specimens with a detection limit varying between 10^4 (13) and 400 (18) bacteria. Apart from its lower detection sensitivity, the applicability of this rather expensive method is limited. In particular, probing oligomers hybridizing to different targets must first be tailed with a dT homopolymer and then immobilized in a controlled manner. The use of streptavidin-coated microtiter plates is much more flexible. This technique has been proposed for immobilization of biotin-labeled PCR products of *Mycoplasma pneumoniae* and subsequent fluorometric detection (16). In that assay, the biotin-binding capacity of the microtiter plates was low and prior removal of unincorporated biotin was required. We have therefore exploited the advantage of high-biotin-binding streptavidin-coated plates as recently described for detection of viruses (10).

Over 250 BAL fluid specimens were screened in parallel by culture on agar and by PCR. *L. pneumophila* was detected by PCR in 14 specimens, of which 8 were also culture positive. Six further PCR-positive specimens from four patients remained culture negative. In two patients, *L. pneumophila* infection had been proven by positive culture from earlier specimens. The two other patients negative for legionellae by culture but positive by PCR were hospitalized at the same time in the same intensive care unit. Not a single specimen that was PCR negative yielded a positive culture. A large number of bacterial species other than legionellae were cultured from the PCR-negative specimens; this finding strengthened the contention

that the PCR is highly specific. In sum, this is the first description of a sensitive technique for identification of legionellae in BAL fluid by hybridization of a specific PCR product with a nonradioactive probe in microtiter plates with high biotin-binding capacity. This method should eventually also be transferable to other infectious agents by employment of appropriate primers.

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