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Detection of *Legionella* spp. in Bronchoalveolar Lavage Fluids by DNA Amplification

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By using *Taq* polymerase, DNA amplification of a specific fragment of the macrophage infectivity potentiator (*mip*) gene from *Legionella pneumophila* was used to detect *Legionella* spp. in bronchoalveolar lavage (BAL) fluid specimens. We were able to detect DNAs from all 30 *L. pneumophila* strains tested (serogroups 1 to 14), *L. micdadei*, and *L. bozemanii* serogroup 1. DNA from bacteria of other species tested and DNA from human leukocytes were not amplified by this procedure. After optimization of the conditions for DNA extraction from BAL fluid, a 2-ml sample of BAL fluid seeded with 25 CFU/ml tested positive after DNA amplification. A total of 68 frozen BAL fluid specimens sent to the laboratory because of suspected legionellosis were tested in a retrospective study. The eight culture-positive samples were all positive after specific DNA amplification. Among 60 culture-negative samples, 7 were positive after amplification. Of these seven samples, four were from patients who had presented a typical clinical history of legionellosis; the samples had antibody titer increases of 2 dilutions. For the three remaining samples, serological diagnosis of legionellosis in the patients from whom the samples were obtained could not be documented, and although the causative agent of these pulmonary infections was not determined, the clinical features of the patients were in accordance with legionellosis.

Among members of the family *Legionellaceae*, *Legionella pneumophila* is involved in more than 95% of cases of severe atypical pneumonia (9). Isolation of the causative agent from bronchoalveolar lavage (BAL) fluid specimens is fastidious and time-consuming. Moreover, it has been shown that some *Legionella* strains may be viable but cannot be cultured (5). Because of the delayed rise in *Legionella* antibody levels with respect to the onset of illness, the serological diagnosis, which is usually determined by the immunofluorescence assay, may also be delayed. By using *Taq* polymerase, DNA amplification of *Legionella*-specific sequences (10) may thus represent an interesting tool for the detection of *Legionella* spp. from various types of samples. Hitherto, this technique was reported only for water samples artificially contaminated with *Legionella* spp., in which the target was a DNA fragment of unknown function (12), or for environmental water, in which a fragment selected from the previously sequenced macrophage infectivity potentiator gene (*mip*) from *L. pneumophila* was detected by DNA amplification (2). To our knowledge, detection of *Legionella* spp. in clinical samples has never been reported. The aim of this study was to develop a DNA amplification assay for detecting *Legionella* spp. in BAL fluid and to evaluate the feasibility of such a method in bacteriological practice.

MATERIALS AND METHODS

Bacterial strains. DNA amplification was performed by using 30 *L. pneumophila* reference strains belonging to serogroups 1 to 14 (see Table 1) and 39 other *Legionella* spp. (see Table 2). These strains were cultured on BCYE-α agar plates (8).

The specificity of the method was assessed with DNA from human leukocytes and various bacterial species isolated from the following clinical specimens: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, *Xanthomonas maltophilia*, *Pseudomonas aeruginosa*, *Fusobacterium nucleatum*, and *Bacteroides fragilis*. We tested a single strain of each bacterial species.

Patients. A retrospective study of 68 patients suspected of having legionellosis was conducted. All patients were adults with community-acquired or nosocomial pneumonia diagnosed by a temperature of 38°C or higher and pulmonary infiltrates on chest X ray. Patients with noninfectious pulmonary infiltrates were excluded.

BAL fluid specimens. The BAL fluid obtained from each of the 68 patients was homogenized, divided into two samples of 2 ml each, and kept frozen at −80°C until it was used for DNA amplification. Among these BAL fluid specimens, *L. pneumophila* was previously isolated by culture from 8 of them, whereas cultures of the 60 other BAL fluid specimens were negative.

DNA extraction from *Legionella* strains. A thermic-lysis procedure was used to release the bacterial DNA, as follows. A total of 400 μl of a *Legionella* suspension was boiled for 5 min in a 1.5-ml microcentrifuge tube and chilled on ice for 5 min; this procedure was repeated once. After 2 min of centrifugation at 1,100 × *g*, 20 μl of the supernatant was tested for specific DNA amplification.

Extraction of DNA from other bacteria. Bacteria were incubated in TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA) with 2 mg of lysozyme (Appligene, Strasbourg, France) per ml, 0.5% (wt/vol) sodium dodecyl sulfate (SDS), and 1 mg of proteinase K (Sigma) per ml for 60 min at 55°C. Nucleic acids were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (50:48:2). The DNA was then precipitated with absolute ethanol at −80°C for 30 min

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and then pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. The air-dried pellet was resuspended in TE buffer at a final concentration of 1 ng of DNA per μl and was tested for DNA amplification.

Processing of BAL fluid specimens for DNA amplification. A 2-ml aliquot of each BAL fluid specimen obtained from each patient was mixed with an equal volume of phosphate-buffered saline and was centrifuged for 15 min at 3,500 × *g*. This wash step was repeated once. The pellet obtained was treated with 50 μg of proteinase K, 0.5% (vol/vol) Nonidet P-40 and 0.5% (vol/vol) Tween 20 in 500 μl of 10 mM Tris-HCl (pH 8)–50 mM KCl–50 mM $MgCl_2$. DNA was then purified by phenol-chloroform-isoamyl alcohol and ethanol precipitation as described above. The air-dried pellet was then resuspended in 40 μl of TE buffer and was heated for 10 min at 95°C. Half of the processed sample (20 μl) was submitted to 40 cycles of amplification in a 100-μl volume as described below. The remaining 20 μl of each sample was kept frozen in order to retain the ability to perform a new DNA amplification if trouble occurred during the amplification step. All positive samples were controlled by processing and amplifying another frozen aliquot from the same BAL fluid specimen.

Synthetic oligonucleotides and DNA amplification. Two sets of oligonucleotides, each consisting of two primers and a detection probe, were tested. The first set (LEG-1, LEG-2, and LEG-3), which was described previously by Starnbach et al. (12), was tested by using their procedures for DNA amplification as well as those for Southern blotting and hybridization.

The second set of oligonucleotides tested for DNA amplification of *Legionella*-specific sequences was chosen from the *mip* gene of *L. pneumophila* (4). The *mip* gene, which codes for a virulence protein, seemed to be a promising target for the diagnosis of legionellosis. Two 20-base oligonucleotides (Lpm-1, Lpm-2) bracketing a 600-bp DNA fragment were synthesized as primers. Lpm-1 (5′-GGTGACTG CGGCTGTTATGG-3′) was located at nucleotides 853 to 872 from the coding strand; Lpm-2 (5′-GGCCAATAGGTCCGC CAACG-3′) was located at nucleotides 1465 to 1484 complementary to the coding strand. A 25-base internal probe, Lpm-3 (5′-CAGCAATGGCTGCAACCGATGCCAC-3′), located at nucleotides 888 to 912 from the coding strand, was 5′ labeled with [γ-^{32}P]ATP (>3,000 Ci/mmol) as described by Maniatis et al. (7) and was used as a detection probe. The samples (20 μl) were submitted to 40 cycles of amplification in a 100-μl volume containing 1 U of *Taq* polymerase (Beckman), 0.1 μM (each) primer, 0.2 mM (each) the four deoxynucleotides, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mg of gelatin per ml. Reaction volumes were overlaid with 50 μl of mineral oil (Perkin-Elmer Cetus). After an initial denaturation step at 95°C for 5 min, each amplification cycle was performed as follows: 2 min for annealing at 62°C, 1.5 min for primer extension at 72°C, and 1.5 min for denaturation at 94°C.

To avoid contamination of samples, the following precautions were taken. Sample preparation, PCR amplification, and electrophoresis were performed in three different rooms. At each step, each sample was uncovered carefully and separately; gloves were changed between the handling of each sample, and DNA solutions were handled with positive-displacement pipettes (Microman; Gilson). In each DNA amplification run, three negative controls were included. The first contained the reaction mixture without any DNA, while the second contained DNA from a non-*Legionella* strain. In order to detect contamination that occurred during DNA extraction, the third control was a 2-ml aliquot of a BAL fluid specimen obtained from a patient with no infectious disease; this BAL fluid specimen was processed and amplified simultaneously with the other BAL fluid specimens.

Sensitivity of *Legionella* detection by DNA amplification. The lowest detection threshold for the method described here was investigated by using serial dilutions of purified DNA from bacterial cells. First, purified DNAs from *L. pneumophila* serogroup 1, *L. micdadei*, and *L. bozemanii* serogroup 1 were serially 10-fold diluted from 5 ng to 50 ag in TE buffer and were submitted to DNA amplification. Second, a 3-day culture of *L. pneumophila* serogroup 1 was suspended in sterile distilled water. The number of CFU was determined by plating 100 μl of serial dilutions of the culture on BCYE-α agar plates; then, a 200-μl culture sample was diluted 10-fold at 4°C in a 2-ml volume of BAL fluid containing no *Legionella* spp. and was processed as described above for amplification.

Gel electrophoresis and Southern blot hybridization. Ten microliters of each sample containing amplified DNA was electrophoresed through a 1.5% agarose gel (SeaKem; FMC Products) at 120 V for 1.5 h in 0.5× TEB buffer (0.5× TEB is 44.5 mM Tris-HCl, 44.5 mM boric acid, 1.25 mM disodium EDTA [pH 8.3]). After staining with ethidium bromide (5 μg/ml), the gel was photographed under UV light.

For Southern blotting, the DNA was transferred to an Immobilon-P membrane (Millipore) by using 0.5 M NaOH and was fixed onto the membrane by heating for 2 h at 80°C. The membrane had been prehybridized for 0.5 h at 55°C in 6× SSPE buffer (7) containing 0.1% (wt/vol) SDS, 0.02% (wt/vol) bovine serum albumin, 0.02% (wt/vol) Ficoll 400, and 0.02% (wt/vol) polyvinylpyrrolidone. The filters were transferred to fresh prehybridization buffer, and 0.5 pmol of 5′-labeled probe (10^6 cpm/pmol) per ml was added. Hybridization was performed for 2 h at 60°C; this was followed by two washes at 45°C for 10 min in 2× SSPE buffer with 0.1% SDS. After air drying, membranes were exposed overnight to noninterleaved films (NIF) (3 M) X-ray film between two intensifying screens at −70°C.

RESULTS AND DISCUSSION

Using the primer set and the detection probe described by Starnbach et al., (12), we were able to detect 22 of 30 (75%) *L. pneumophila* strains (Table 1) but none of the non-*L. pneumophila* species (Table 2) tested. Interestingly, *L. pneumophila* serogroup 1 strain Pontiac, which is implicated in spontaneously resolving disease, was not detected by this assay. Moreover, only one of the four *L. pneumophila* serogroup 5 strains tested gave a positive signal; a similar phenomenon was observed with one of the two *L. pneumophila* serogroup 4 strains tested. This indicates a genotypic heterogeneity among *L. pneumophila* strains of the same serogroup, as first demonstrated by Selander et al. (11) and confirmed for *L. pneumophila* serogroup 5 by Bornstein et al. (3) by multilocus enzyme analysis.

DNA amplification of *Legionella*-specific sequences by using primers from the *mip* gene sequence permitted the detection of DNA from all the *L. pneumophila* strains of serogroups 1 to 14 that were tested; *L. micdadei* and *L. bozemanii* serogroup 1 were also detected among the non-*L. pneumophila* species (Fig. 1). DNAs from the gram-positive and gram-negative species tested and DNAs from human leukocytes were not amplified by this procedure (Fig. 1). Mahbubani et al. (6), using other primers from the *mip* gene

TABLE 1. Detection of DNA from different *L. pneumophila* strains by DNA amplification

Serogroup	Strain	Source[a]	DNA amplification by[b]: Method 1	Method 2
1	Philadelphia 1	ATCC 33152	+	+
1	Bellingham	ATCC 44311	+	+
1	Pontiac	CDC	−	+
1	Dallas 1	CDC	+	+
1	Knoxville	CDC	+	+
1	Los Angeles 2	CDC	+	+
1	Togus 2	CDC	+	+
1	Denver 5	CDC	+	+
1	Olda	CDC	+	+
1	Albuquerque	CDC	+	+
1	San Francisco 9	CDC	+	+
1	Detroit	CDC	−	+
2		ATCC 33154	+	+
3		ATCC 33155	+	+
4	Portland	CDC	+	+
4	Los Angeles 1	ATCC 33156	−	+
5	Cambridge	CDC	+	+
5	Dallas 1E	ATCC 33126	−	+
5	Pratt	CDC	−	+
5	Micu	ATCC 33735	−	+
6		ATCC 33215	+	+
7		ATCC 33823	+	+
8		ATCC 35096	+	+
9		ATCC 33289	+	+
10		ATCC 43283	+	+
11		ATCC 43130	+	+
12		ATCC 43290	+	+
13		ATCC 43736	−	+
14		ATCC 43703	+	+
Lansing 3		ATCC 35251	−	+

[a] ATCC, American Type Culture Collection, Rockville, Md.; CDC, Centers for Disease Control, Atlanta, Ga.

[b] Method 1, DNA amplification performed with oligonucleotides LEG-1, LEG-2, and LEG-3; method 2, DNA amplification performed with oligonucleotides Lpm-1, Lpm-2, and Lpm-3.

TABLE 2. Detection of DNA from other *Legionella* spp. by DNA amplification

Legionella spp.	Serogroup	Source[a]	DNA amplification by[b]: Method 1	Method 2
L. anisa		ATCC 35292	−	−
L. birminghamensis		ATCC 43830	−	−
L. bozemanii	1	ATCC 33217	−	+
L. bozemanii	2	ATCC 35545	−	−
L. brunensis		ATCC 43878	−	−
L. cherii		ATCC 35252	−	−
L. cincinnatiensis		ATCC 43753	−	−
L. dumofii		ATCC 33279	−	−
L. erythra		ATCC 35303	−	−
L. feeleii	1	ATCC 35072	−	−
L. feeleii	2	ATCC 35849	−	−
L. geestiae		CDC	−	−
L. gormanii		ATCC 33297	−	−
L. gratiana		ATCC 49413	−	−
L. hackeliae	1	ATCC 32250	−	−
L. hackeliae	2	ATCC 35999	−	−
L. israelensis		ATCC 43119	−	−
L. jamestowniensis		ATCC 35298	−	−
L. jordanis		ATCC 33623	−	−
L. londoniensis	1	CDC		−
L. londoniensis	2	CDC	−	−
L. longbeachae	1	ATCC 33462	−	−
L. longbeachae	2	ATCC 33484	−	−
L. maceachernii		ATCC 35300	−	−
L. micdadei		ATCC 43218	−	+
L. moravica		ATCC 43877	−	−
L. nautarum		CDC	−	−
L. oakridgensis		ATCC 33761	−	−
L. parisiensis		ATCC 35299	−	−
L. quateriensis		CDC	−	−
L. quinlivanii		ATCC 45830	−	−
L. rubrilucens		ATCC 35304	−	−
L. santhelensi		ATCC 35301	−	−
L. santicrucis		ATCC 43248	−	−
L. spiritensis		ATCC 43249	−	−
L. steigerwaltii		ATCC 35302	−	−
L. tucsonensis		ATCC 49180	−	−
L. wadsworthii		ATCC 33817	−	−
L. worsliensis		CDC	−	−

[a] ATCC, American Type Culture Collection, Rockville, Md.; CDC, Centers for Disease Control, Atlanta, Ga.

[b] Method 1, DNA amplification performed with oligonucleotides LEG-1, LEG-2, and LEG-3; method 2, DNA amplification performed with oligonucleotides Lpm-1, Lpm-2, and Lpm-3.

sequence, also reported the detection of DNAs from *L. pneumophila* serogroup 1 to 14 strains but not those from some other *Legionella* spp. This discrepancy with our results can readily be explained, since a recent study has reported the nucleotide sequence of the *L. micdadei mip* gene (1). Comparison of the two *mip* gene sequences showed that, unlike one of the primers chosen by Mahbubani et al. (6), the *mip* primers tested in this study were located within a DNA fragment conserved by the two genes. *L. micdadei*, a non-*L. pneumophila* species, is known to be responsible for legionellosis, and use of DNA amplification to detect this species in biological fluids is therefore of great interest. Considering these results, we decided to use only the primers constructed on the *mip* gene and reported here, to assess the feasibility of *Legionella* detection in BAL fluid specimens. The sensitivity of this assay was evaluated for *L. pneumophila* serogroup 1, *L. micdadei*, and *L. bozemanii* serogroup 1. The results obtained were reproducible and similar for the three species. After amplification of 500 fg of purified DNA and electrophoresis of the DNA fragments that were obtained, a 630-base DNA fragment was detected after ethidium bromide staining of the gel. Southern blot hybridization with the detection probe lowered the minimal detection threshold to 50 fg of DNA. By using cultures of *L. pneumophila* serogroup 1 serially diluted in 2 ml of BAL fluid containing no *Legionella* spp., the lowest detection threshold was reproducibly estimated to be 25 CFU/ml after Southern blot hybridization (Fig. 2). Comparison of the sensitivity obtained when amplifying purified DNA with that obtained when amplifying *Legionella* DNA contained in BAL fluid specimens showed that the DNA recovery from bacteria was about 100% and that no inhibitory effect occurred when BAL fluid specimens were processed as described above.

In order to evaluate the feasibility of DNA amplification for the detection of *Legionella* spp. in BAL fluid specimens, this technique was assessed in a retrospective study with 68 frozen BAL fluid specimens (2 ml) sent to the laboratory because of suspected legionellosis. After DNA amplification, all eight BAL fluid specimens from which *L. pneumophila* (serogroups 1 and 5) were previously isolated by

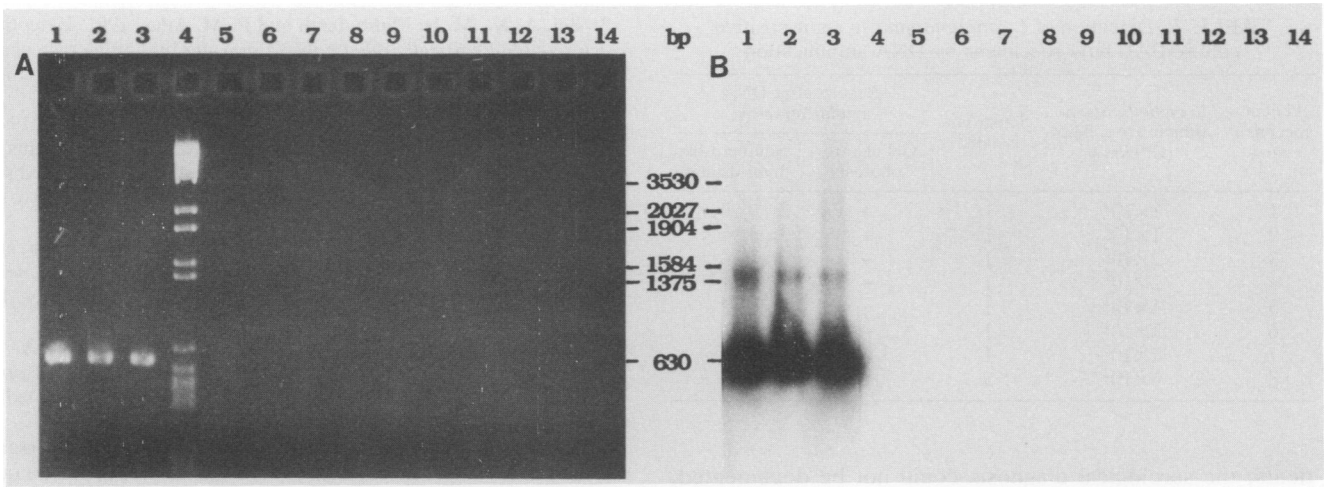

FIG. 1. Agarose gel electrophoresis (A) and Southern blot analysis (B) of amplified DNA (40 cycles) from different *Legionella* spp. and other bacterial species by using primers Lpm-1 and Lpm-2 and detection probe Lpm-3. The DNAs tested as templates were from *L. pneumophila* serogroup 1 (lane 1), *L. micdadei* (lane 2), *L. bozemanii* serogroup 1 (lane 3), *Bst*EII-digested bacteriophage lambda DNA and *Hae*III plasmid pBR322 as molecular weight markers (Appligene) (lane 4), *Staphylococcus aureus* (lane 5), *Streptococcus pneumoniae* (lane 6), *Haemophilus influenzae* (lane 7), *Moraxella catarrhalis* (lane 8), *Mycoplasma pneumoniae* (lane 9), *Chlamydia pneumoniae* (lane 10), *Pseudomonas aeruginosa* (lane 11), *Escherichia coli* (lane 12), *Fusobacterium nucleatum* (lane 13), and human leukocytes (lane 14). For each of the *Legionella* spp., 1 ng of DNA was used as the initial template; for the other templates tested, 100 ng of DNA was used.

culture (1×10^1 to 2×10^3 CFU/ml) tested positive (Table 3). The results indicate that for two of these eight BAL fluid specimens, the number of *Legionella* spp. detected (10 CFU/ml) was slightly lower than the lowest detection threshold previously determined for the DNA amplification method (25 CFU/ml). This discrepancy may reflect the inability of a percentage of viable *Legionella* spp. to be cultured. Among a second set of 60 BAL fluid specimens which were culture negative, 7 specimens were positive after DNA amplification. For each of the positive BAL fluid specimens, the second aliquot of 2 ml of BAL fluid was again processed and then amplified to confirm the results. No discrepancies were observed between the two amplification runs. These culture-negative BAL fluid specimens were not recultured, because no sample remained after the second DNA amplification. A study of the clinical records of the seven patients from whom these BAL fluid specimens were obtained revealed that four of them had presented a typical clinical history of legionellosis, with seroconversion (1/32 to 1/128), which was determined by immunofluorescence assay and formolized antigen, occurring in two patients. In the two other patients, within an interval of 1 week, an antibody titer increase of 2 dilutions (1/16 to 1/64) was observed between the two serum samples that were obtained. For the three remaining pa-

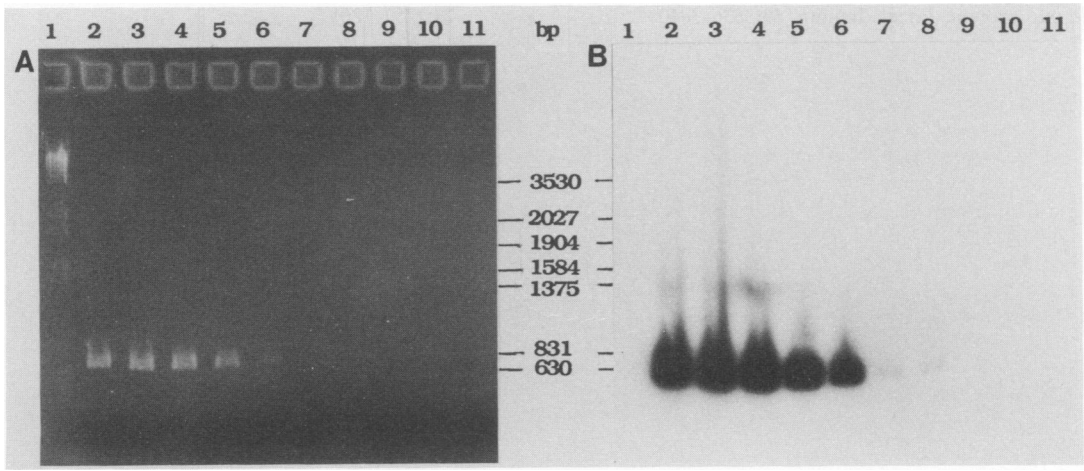

FIG. 2. Sensitivity of the DNA amplification method applied to the detection of *Legionella* spp. in BAL fluid specimens. Tenfold serial dilutions of the culture of the strain *L. pneumophila* serogroup 1 ATCC 33152 were seeded in 2-ml BAL fluid samples and submitted to amplification by using primers Lpm-1 and Lpm-2. (A) Analysis of the polymerase chain reaction products by gel electrophoresis and ethidium bromide staining after 40 amplification cycles. (B) Southern blot analysis after hybridization with the detection probe Lpm-3. Lane 1, *Bst*EII-digested bacteriophage lambda DNA and *Hae*III plasmid pBR322 as DNA molecular weight markers (Appligene); lane 2, 1 ng of purified DNA of *L. pneumophila* serogroup 1. In the other lanes, the number of CFU in each sample was 2.5×10^6/ml (lane 3), 2.5×10^5/ml (lane 4), 2.5×10^4/ml (lane 5), 2.5×10^3/ml (lane 6), 2.5×10^2/ml (lane 7), 2.5×10^1/ml (lane 8), 2.5/ml (lane 9), and 2.5×10^{-1}/ml (lane 10). No bacterium was included in lane 11.

TABLE 3. Detection of *L. pneumophila* in eight positive culture-BAL fluid specimens by DNA amplification

Patient specimen no.	*Legionella* organisms in BAL fluid (CFU/ml)	Serogroup isolated	Results after DNA amplification by:	
			Gel electrophoresis	Southern blot hybridization
1	1×10^3	1	+	+
2	1×10^2	1	+	+
3	2×10^2	1	+	+
4	2×10^1	1	−	+
5	1×10^1	1	−	+
6	1×10^1	1	−	+
7	2×10^3	1	+	+
8	6×10^2	5	+	+

tients, the serological diagnosis could not be documented, since only one serum sample, which was negative by immunofluorescence assay, was obtained from each patient at the onset of the illness. The causative agent of these pulmonary infections could not be determined, but the clinical features were in accordance with legionellosis. Other usual causes of infection were excluded, since the cultures performed on usual bacteriological media were negative and the serological diagnoses performed for *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, cytomegalovirus, and respiratory syncytial virus were negative.

The primers that we described here allowed the detection of the *Legionella* spp. most frequently implicated in respiratory tract infections. DNA amplification appears to be a promising tool for the diagnosis of legionellosis.

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