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Rapid Detection of *Legionella* Species in Bronchoalveolar Lavage Fluids with the EnviroAmp Legionella PCR Amplification and Detection Kit

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A molecular assay based on a rapid DNA extraction protocol and the EnviroAmp Legionella Kits was used to detect *Legionella* species in bronchoalveolar fluid specimens. All *Legionella* strains isolated from tap water in hospitals could be detected distinctly. Both sensitivity and specificity were tested. In a prospective study, bronchoalveolar lavage fluids obtained from patients with atypical pneumonia were investigated. Three positive samples were detected with the molecular techniques and were subsequently confirmed by culture. Application of the system described may lead to safe and early diagnosis of Legionnaires' disease in patients with atypical pneumonia.

The relatively low prevalence of Legionnaires' disease demands a highly sensitive and specific method for detection of legionellae. Recently, techniques for detection of bacterial DNA have been developed. DNA probe hybridization was introduced for detection of *Legionella* spp. (4). A commercially available kit proved reasonably sensitive for detection of legionellae in frozen clinical respiratory tract samples (5). In a prospective study on clinical respiratory tract samples, the same kit was superior to direct immunofluorescence antibody staining of the samples (7). However, this kit employs radioactive substances. Therefore, it has not become widespread, particularly in Europe. DNA amplification by polymerase chain reaction (PCR) appears to be an optimal tool for enhancement of the sensitivity of detection. PCR combined with specific identification with probes for the genome of *Legionella* spp. was employed in environmental studies (12, 19). Recently, PCR was used for detection of *Legionella* spp. in bronchoalveolar lavage fluids (10). The primers employed were not able to distinguish between *Legionella pneumophila* and non-*L. pneumophila* strains. In addition, the procedure described, which includes a conventional DNA extraction protocol, a self-designed amplification procedure, and a hybridization technique requiring radioactive agents, proved rather complicated and time-consuming. In this study, a rapid system for detection of *Legionella* spp. in bronchoalveolar lavage fluids employing a rapid DNA extraction protocol and a commercial amplification and nonradioactive detection kit was evaluated.

In the first part of the study, the feasibility and sensitivity of a rapid molecular assay for detection of legionellae were evaluated. Thirty bronchoalveolar lavage samples were taken. Prior to the start of the study, culture and direct immunofluorescence were performed to isolate *Legionella* spp. and exclude persons with Legionnaires' disease. All results were negative. Subsequently, 20 bronchoalveolar lavage fluid samples were seeded with *Legionella* strains isolated from tap water in hospitals (14). The strains included *L. pneumophila* serogroups 1 to 14, *L. bozemanii* serogroup 1, *L. dumoffii*, *L. longbeachae*, *L. gormanii*, *L. micdadei*, and *L. jordanis*. The remaining specimens were tested with a dilution protocol to determine the sensitivity of the system. *Legionella* preparations were appropriately diluted at a 0.5 MacFarland standard. Dilutions were plated onto BCYEα medium in duplicate to obtain the initial concentration of the suspension. Each sample was divided into five equal portions. One sample was used as a negative control, and the others were seeded with 10-fold bacterial dilutions of *L. pneumophila* serogroups 1, 2, 3, 5, and 6 and *L. bozemanii* serogroup 1. One milliliter of a bacterial suspension containing from 10^9 bacteria per ml to less than 10^4 bacteria was evaluated. To guarantee reproducibility, the whole trial was repeated twice. Furthermore, frequent bacterial agents of (atypical) pneumonia, including *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Pseudomonas* spp., isolated in the routine bacteriological laboratory, and DNA from human leukocytes were tested with the system as described later.

In the second part of the study, 52 bronchoalveolar lavage fluid samples were collected from patients with atypical pneumonia and prospectively investigated with the molecular assay. The results were compared to results of routine cultures performed with both blood agar and BCYEα medium.

A rapid DNA extraction protocol was used. In a tube, 0.5 ml of the bronchoalveolar specimen and 1.5 ml of a solution consisting of 20% (wt/vol) Chelex 100 Resin (Bio-Rad Laboratories, Richmond, Calif.) in 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–0.1% sodium azide were mixed vigorously with a vortex mixer for 30 s. The tube was then placed into a boiling water bath. After 10 min of incubation, the tube was removed and allowed to cool to room temperature. A 20-μl supernatant sample was carefully removed and used for amplification directly, without further purification.

The EnviroAmp Legionella PCR Amplification Kit (Perkin Elmer Cetus, Norwalk, Conn.) was used for DNA amplification. This kit contains highly specific biotinylated primers (positions 5 to 29 and 91 to 112) complementary to conserved regions in the 5S rRNA gene to amplify genetic material from members of the genus *Legionella* (11, 20). Identification of

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L. pneumophila is achieved by biotinylated primers (positions 948 to 965 and 1092 to 1115) complementary to sequences of the macrophage infectivity potentiator (*mip*) gene (6), incorporating an internal positive control. This is a synthetic DNA sequence that is coamplified with the primers used for the *mip* gene and is included in the *Legionella* PCR mixture. The internal positive control is a strong indicator of poor amplification, like that caused by PCR inhibitors. Amplification was performed with a programmable thermal cycler (Techne PHC-2). Thirty PCR cycles consisting of 1 min at 95°C, 1.5 min at 65°C, and 15 s at 72°C were run. After the final cycle, the tubes were incubated for an additional 7 min at 72°C.

Electrophoresis was performed on an agarose gel (3% NuSieve–1% SeaKem GTG; FMC Corporation, Philadelphia, Pa.) in TBE buffer (17) at 100 V for 2 h. Ten microliters of the amplified sample was electrophoresed. After having been stained with ethidium bromide (5 μg/ml), the gel was photographed under UV light (300 nm).

Hybridization was performed with the EnviroAmp Legionella PCR Detection Kit in accordance with the manufacturer's advice. Biotinylated PCR products are hybridized to immobilized probes on nylon membranes. Probe sequences have 5′ poly(dT) tails which serve to attach the probe to the nylon membrane while leaving the sequence-specific probe region accessible for binding. The 5S rRNA probe hybridizes to positions 66 to 82 of the *Legionella* 5S rRNA DNA sequence, and the *mip* probe hybridizes to positions 1012 to 1036 of the *L. pneumophila mip* sequence. Additionally, positive and negative control probes are provided. The positive control probe is perfectly complementary to a sequence in the internal positive control, and the negative control probe has a 1-base mismatch. Thus, hybridization conditions have been optimized to be stringent enough to allow detection of a 1-base mismatch between a probe and a PCR product. Detection of the hybridized PCR product is performed with a streptavidin-horseradish peroxidase conjugate. Appearance of a blue dot on the nylon membrane indicates the presence of a bound PCR product.

All of the *Legionella* strains tested were detected with the extraction, amplification, and detection system described above. The amplification levels for the different species tested proved to be identical. All of the other bacteria tested, as well as human DNA, were not detected by this assay. The bacteria capable of producing (atypical) pneumonia, isolated at the author's laboratory, were as follows: *Acinetobacter calcoaceticus*, *Acinetobacter lwoffii*, *Chlamydia pneumoniae*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Proteus mirabilis*, *Proteus morganii*, *Proteus vulgaris* (Bo), *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas maltophilia* (Bo), *Pseudomonas putida*, *Serratia marcescens*, *Staphylococcus aureus* (Bo), *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. The designation Bo indicates additionally tested strains provided by N. Bornstein. For all samples, the whole procedure was done twice on different days, showing excellent reproducibility, and took less than 6 h. During the whole study, no inhibition occurred, as indicated by distinct appearance of the internal positive control. After 30 cycles of the PCR, samples seeded with 4×10^5 bacteria displayed characteristic bands. The sizes of the PCR products were 108, 135, and 168 bp for the 5S rRNA, the internal positive control, and the *mip* product, respectively. Samples containing amplified DNA of non-*L. pneumophila* legionellae lacked the 168-bp band. Application of the hybridization system strongly enhanced sensitivity: samples seeded with 3×10^4 bacteria were reproducibly detected. However, at this level the spots looked very faint. Figures 1 and 2 show typical dot blots of *L. pneumophila* and *L. bozemanii*, respectively. Because of strict precautions, no case of contamination occurred during the whole study.

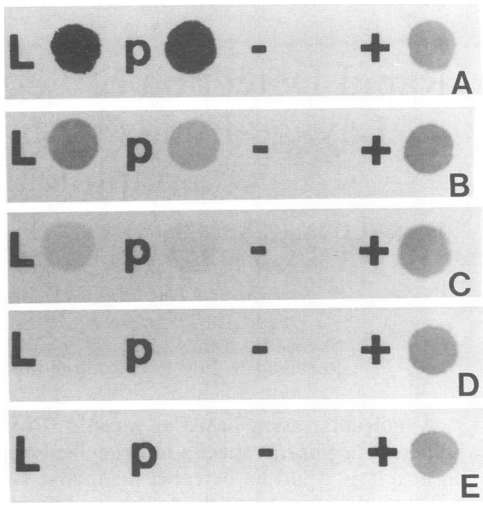

FIG. 1. Dot blots of the dilution series. Shown is amplified *L. pneumophila* DNA resulting from amplification of samples seeded with the following numbers of bacteria: 3×10^6 (A), 3×10^5 (B), 3×10^4 (C), 3×10^3 (D), and 0 (negative control; E). The letter L represents the genus *Legionella*, the letter p represents the species *L. pneumophila*, a minus sign represents the negative control, and a plus sign represents the internal positive control.

Fifty-two bronchoalveolar lavage fluid samples from patients with atypical pneumonia were prospectively investigated by using the technique described. Three samples were positive (Fig. 3). All positive results were subsequently confirmed by routine culture and serologic analysis. Bacterial agents were identified as *L. pneumophila* serogroup 5 in two cases and *L. pneumophila* serogroup 1 in one case. All

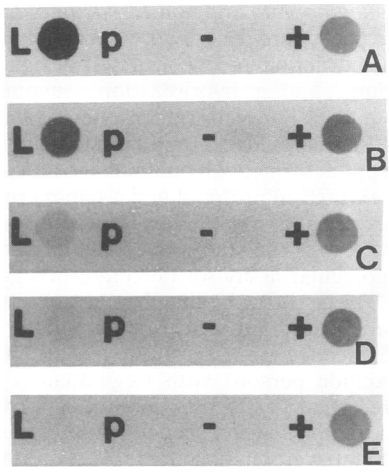

FIG. 2. Dot blots of the dilution series. Shown is amplified *L. bozemanii* DNA resulting from amplification of samples seeded with the following numbers of bacteria: 3×10^6 (A), 3×10^5 (B), 3×10^4 (C), 3×10^3 (D), and 0 (negative control; E).

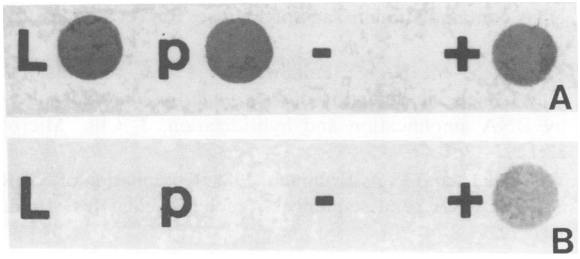

FIG. 3. Detection of *L. pneumophila* in bronchoalveolar fluid specimens. (A) Sample obtained from a 6-month-old male with atypical pneumonia. The positive result was subsequently confirmed by culture and serologic analysis. (B) Sample from a 32-year-old female giving a negative result.

negative cases were diagnosed as nonlegionella infections caused by other bacterial species.

For many years, detection of *Legionella* spp. in bronchoalveolar lavage fluids by cultivation on selective media has been considered the standard. However, culture is a time-consuming procedure, and in some environmental specimens viable bacteria could not be detected (9). Consequently, DNA probes have been introduced to detect legionellae. Amplification of target DNA sequences prior to nucleic acid hybridization offers a more sensitive alternative for detection of a small number of bacteria. Therefore, a *Legionella* sp. detection system based on amplification of target sequences, followed by nonradioactive hybridization, was tested with bronchoalveolar fluid specimens for the first time.

The whole system proved to be easy to use. Contrary to a recently reported study (9), it is possible to carry out the described procedure in less than 6 h. The rapid DNA extraction protocol employing Chelex 100, guaranteeing a high yield of DNA, seems to be especially remarkable. Use of this substrate for rapid DNA extraction from legionellae was recently reported (8), and Chelex is also included in the commercially available Perkin Elmer sample preparation kit.

In contrast to other studies (10, 19), the DNA amplification and detection system described here distinguishes clearly between *L. pneumophila* and non-*L. pneumophila* legionellae. The genus *Legionella* is identified by amplifying DNA sequences with highly specific primers complementary to conserved regions in the 5S rRNA gene (11, 20). Since the primers used in this study are not identical to those reported elsewhere (12), no cross-reactivity with *Pseudomonas* species was observed. *L. pneumophila* is identified by using primers complementary to sequences from the *mip* gene (7, 12). The *mip* gene is conserved and specific to *L. pneumophila*. However, the choice of correct *mip* primers appears critical, because *mip*-like genes are present in non-*L. pneumophila* legionellae (2). A recent study has reported the nucleotide sequence of the *L. micdadei mip* gene (1). Comparison of *mip* gene sequences with those chosen recently (10) shows that *mip* primers could be located within a DNA fragment conserved by the two genes. No cross-reactions with other frequent bacterial agents of atypical pneumonia have been observed with the primers employed.

Nucleic acid hybridization leads to increased sensitivity (15) and excludes unspecific amplification products (13, 18). In this study, as few as 3×10^4 bacteria were detected with the hybridization assay described. This kit contains probes specific for *Legionella* 5S rRNA gene sequences (11, 20) for identification of members of the genus *Legionella* and a probe for the *mip* gene for identification of *L. pneumophila* specifically. The presence of the specific 5S rRNA and *mip* PCR products is detected by using a reverse dot blot strip with immobilized probes (16). As the 5S rRNA gene is represented by multiple copies in the genome (12), the sensitivity of the 5S rRNA assay appears to be very high. The limit of detection of legionellae with the *mip* assay system is reported to be somewhat less (3).

In summary, application of the DNA amplification and hybridization system described in this report proved to be sufficiently sensitive to detect *Legionella* spp. in bronchoalveolar fluid specimens. Although there is not way to distinguish between serogroups and various non-*L. pneumophila* species, both the high sensitivity and the rapid performance of this system help clinicians to make safe and early diagnoses.

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