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Rapid Detection of *Mycoplasma pneumoniae* by an Assay Based on PCR and Probe Hybridization in a Nonradioactive Microwell Plate Format

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A new molecular assay, based on a rapid DNA extraction protocol, PCR, and hybridization to a specific probe in a nonradioactive microwell plate format was used to detect *Mycoplasma pneumoniae* in bronchoalveolar fluid specimens. The sensitivity of the assay was determined to be 10 to 100 organisms with *M. pneumoniae* reference strains. Specificity testing with different bacteria capable of producing pneumonia showed no cross-reactivity. In a prospective study, bronchoalveolar lavage fluids obtained from patients with pneumonia were investigated with the PCR assay and compared to culture. Twelve positive samples were detected with the PCR assay. Seven of them were subsequently confirmed by culture. All patients with positive PCR results seroconverted. Application of the PCR assay described may lead to safe and early diagnosis of *M. pneumoniae* in patients with pneumonia.

Primary atypical pneumonia caused by *Mycoplasma pneumoniae* may reach up to 50% of the cases that occur in epidemic periods (2, 6). Recently, techniques for the detection of bacterial DNA have been developed. Introduction of DNA probes labeled either radioactively or by nonradioactive markers resulted in sensitivity levels which may not be sufficiently high for use in the clinical laboratory (3, 13). A commercially available kit employing a radioactive agent was found to be insensitive (7, 8) and was finally taken off the market (16). DNA amplification by PCR appears to be an optimal tool for enhancement of the sensitivity of detection. In earlier studies, rather complicated and time-consuming DNA extraction and hybridization protocols which mostly used radioactive agents were described (3, 15, 17). Simplified methods involving hybridization to digoxigenin-labeled probes in microtiter plates and colorimetric detection of amplified products have recently been reported (10, 18). In this study, a rapid system for the detection of *M. pneumoniae* in bronchoalveolar lavage (BAL) fluid specimens employing a rapid DNA extraction protocol, PCR, and a quick nonradioactive hybridization and detection assay in microwell plates was evaluated and tested on BAL specimens of patients suffering from pneumonia.

In a first step, the sensitivity and specificity of the rapid PCR assay were evaluated. For determination of sensitivity, *M. pneumoniae* strains (15293 and 29342) from the American Type Culture Collection (Rockville, Md.) were used. The strains were cultured with the *M. pneumoniae* culture kit Pneumofast (International Mycoplasma-DBV Production, Sanary, France) according to the manufacturer's recommendations. Color-changing unit titers of *M. pneumoniae* cultures were determined by serial 10-fold dilutions in liquid SP-4 medium as described earlier (1). One color-changing unit was estimated to be equivalent to 10 to 100 organisms (1). BAL specimens were used as matrix for seeding with the two *M. pneumoniae* ATCC strains. Specimens were obtained from patients without pneumonia and aliquoted into six equal portions each. Pneumofast culture was done on one aliquot to exclude specimens positive for *M. pneumoniae*. All results were negative. Of the remaining aliquots, one aliquot was used as a negative control and four were seeded with serial 10-fold dilutions (100 to 0.1 color-changing units) of *M. pneumoniae* ATCC 15293 or *M. pneumoniae* ATCC 29342. The lowest input of bacteria that produces positive results was taken as the sensitivity of the PCR assay. To guarantee reproducibility, the whole procedure was repeated twice. To determine specificity, agents of pneumonia, isolated in the routine bacteriological laboratory, and DNA from human leukocytes were tested.

In a second step, BAL specimens were collected from patients with pneumonia and prospectively investigated with the PCR assay. All patients were admitted to the hospital because of clinical presentation compatible with pneumonia. After confirmation of pneumonia by X-ray scan, a BAL specimen was obtained from each patient prior to therapy at a mean of 25 h (range, 14 to 56 h) after the onset of illness. Routine investigations included cultures with both blood agar and Pneumofast, serological profile, and a PCR assay for the detection of *Legionella* spp. (9). *M. pneumoniae* serology was done on both acute and convalescent sera by the complement fixation test (CFT; *M. pneumoniae* ATCC CF 1 RFC antigen/E lipid extract; Virion, Cham, Switzerland). Seroconversion was considered significant in the case of a fourfold rise in titer (convalescent versus acute) and a titer of 1:160 or higher. All patients were treated with broad-spectrum antibiotics.

A total of 116 BAL specimens were tested. Seventy-two specimens were obtained from adults (age range, 22 to 80 years), and 44 specimens were from children (age range, 1 to 16 years). The results of the PCR assay were compared with those of Pneumofast culture. If the molecular assay and/or

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culture gave a positive result, another BAL specimen was obtained on day 14 after onset of therapy and tested by both detection methods. Additionally, *M. pneumoniae* serology was repeated.

For the PCR assay, a rapid DNA extraction protocol was used. In a sterile tube, 100 μl of the BAL and 300 μl of a suspension consisting of 15% (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 in a boiling water bath. After 10 min of incubation, the tube was removed and allowed to cool to room temperature. Following complete settlement of the resin, 20 μl of the supernatant was carefully removed and used for amplification directly, without further purification.

For PCR, the upstream primer DD50B (5′-biotin-GCAAA GTTATGGAAACATAATGGAGGTT-3′ [positions 999 to 1026]) and the downstream primer DD54B (5′-biotin-GGTT AGCAACACGTTTTTAAATATT-3′ [positions 1401 to 1425]) from the published sequence of the gene encoding the small subunit rRNA (16S rRNA) of *M. pneumoniae* (GenBank accession no. M29061) were used. This set of primers, which was chosen from a region of the 16S rRNA gene which contains *M. pneumoniae*-specific sequences, allows amplification of a 427-bp fragment. PCR was done in 100-μl reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 5% glycerol, 200 μM (each) deoxynucleoside triphosphates (including dUTP instead of dTTP), 50 pmol (each) oligonucleotide primer, 20 μl of extraction supernatant, 5 U of *Taq* polymerase (AmpliTaq; Perkin-Elmer, Langen, Germany), and 2 U of uracil-*N*-glycosylase (AmpErase; Perkin-Elmer) in a Perkin-Elmer System 9600 thermocycler (Perkin-Elmer, Norwalk, Conn.). After incubation at 50°C for 2 min, two cycles consisting of 20 s at 98°C, 20 s at 62°C, and 45 s at 72°C followed by 35 cycles consisting of 20 s at 94°C, 20 s at 62°C, and 45 s at 72°C were run. After the final cycle, the tubes were incubated for an additional 10 min at 72°C. Each amplification run contained one positive and three negative controls. Negative controls consisted of BALs from patients with diseases other than (atypical) pneumonia and negative culture results. Positive controls were prepared from the reference strains described above.

Hybridization and colorimetric detection of amplified DNA were performed in microwell plates containing the immobilized oligonucleotide probe DD64 (5′-TCGCCAGCTTGTAA AAGTGAG-3′ [positions 1232 to 1252]) as described previously (20). The optical density (OD) was read on an Anthos 2001 reader (Anthos Labtec Instruments, Salzburg, Austria) at 450 nm. If all negative control values were less than 0.2 OD units, and if the positive control was greater than 2.0 OD units, the run was considered valid. Samples were classified as PCR positive or negative according to an OD cutoff value of 0.35 OD units. Samples with initial readings of between 0.2 and 0.5 OD units were considered borderline and were classified as positive or negative on the basis of retesting in duplicate.

With the PCR assay, one color-changing unit (estimated to be equivalent to 10 to 100 organisms) of *M. pneumoniae* ATCC 15293 as well as *M. pneumoniae* ATCC 29342 per 100 μl of sample was consistently detected. The amplification levels for the two different strains proved to be identical. The detection limit of the PCR assay was thus estimated to be 10 color-changing units per ml of BAL sample (i.e., 100 to 1,000 organisms). DNAs from human leukocytes as well as different pathogens including other *Mycoplasma* species and agents capable of producing pneumonia (adenovirus, *Candida albicans*, *Chlamydia psittaci*, *Chlamydia trachomatis*, human cytomegalovirus, *Escherichia coli*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, influenzavirus type B, *Klebsiella pneumoniae*, *Legionella bozemanii*, *Legionella pneumophila*, *Legionella micdadei*, *Mycobacterium tuberculosis*, *Mycoplasma genitalium*, *Mycoplasma orale*, *Mycoplasma salivarium*, parainfluenzavirus type 3, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas maltophilia*, respiratory syncytial virus, *Staphylococcus aureus*, and *Streptococcus pneumoniae*) were not detected by the PCR assay. For all samples, the whole procedure was done twice on different days, showing excellent reproducibility, and took less than 5 h.

TABLE 1. Results of patients with positive PCR *M. pneumoniae* assay

Patient no.	Age (yr)/sex[a]	Result on day[b]:					
		0			14		
		MA	CUL	CFT	MA	CUL	CFT (titer)
1	7/F	Pos	Pos	Neg	Neg	Neg	1:160
2	5/F	Pos	Neg	Neg	Neg	Neg	1:>320
3	11/F	Pos	Pos	Neg	Neg	Neg	1:>320
4	10/F	Pos	Pos	Neg	Neg	Neg	1:160
5	5/M	Pos	Neg	Neg	Neg	Neg	1:160
6	8/M	Pos	Pos	Neg	Neg	Neg	1:160
7	10/M	Pos	Pos	Neg	Neg	Neg	1:>320
8	12/M	Pos	Neg	Neg	Neg	Neg	1:160
9	3/F	Pos	Pos	Neg	Neg	Neg	1:160
10	5/F	Pos	Neg	Neg	Neg	Neg	1:160
11	35/F	Pos	Neg	Neg	Neg	Neg	1:160
12	32/F	Pos	Pos	Neg	Neg	Neg	1:>320

[a] F, female; M, male.

[b] MA, molecular assay; CUL, Pneumofast culture; CFT, complement fixation test; Pos, positive; Neg, negative.

One hundred sixteen BAL specimens from patients with pneumonia were prospectively investigated. Ten samples from children and two samples from adults were found positive with the PCR assay, and 7 of the 12 PCR positives were subsequently confirmed by culture. All sera showed a negative CFT result at day 0. The follow-up of the 12 patients who had initially been found positive with the PCR assay is shown in Table 1. Following erythromycin therapy, all BAL samples obtained on day 14 were found negative with the PCR assay as well as with culture. The CFT showed seroconversion in all 12 patients. In all other patients, the CFT remained negative on day 14. In 56 (80%) of the adults, infections produced by pathogens other than *M. pneumoniae* were diagnosed, and in 14 (20%), the pathogen remained unidentified. Evidence of bacterial infection was present in 48 patients (37 *S. pneumoniae* isolates, 9 *H. influenzae* isolates, 1 *S. aureus* isolate, and 1 *L. pneumophila* isolate), and viral infection was present in eight patients (four influenzavirus type B infections, two influenzavirus type A infections, and two respiratory syncytial virus infections). In the group of children, infections were caused by respiratory syncytial virus (in 25 children), *H. influenzae* (in 4 children), influenzavirus type B (in 3 children), and *K. pneumoniae* (in 2 children).

The PCR assay proved to be quick and reproducible. Each time the test was performed, negative controls were less than 0.2 OD units and the positive control was greater than 2.0 OD units. During the whole study, all negative control values fell within 25% of the mean value for the negative controls. Therefore, each run was valid. OD values for the positive and negative groups of specimens were widely separated, making interpretation easy. For the present study, an equivocal zone between 0.2 and 0.5 OD units was used. Only two values

(1.7%) fell within the equivocal zone. Repeat testing gave negative results in both cases.

In this study, a molecular *M. pneumoniae* assay based on a rapid DNA extraction protocol, PCR, and nonradioactive hybridization on microwell plates was evaluated with BAL specimens. The whole assay proved to be easy to use and could be carried out in less than 5 h. The assay, which included a single-round PCR, detected more positive samples than culture, which is consistent with earlier studies (11, 15, 17). Application of nested PCR, which significantly increases the risk of contamination by DNA carryover, is not necessary.

For molecular assays to be applicable in the routine diagnostic laboratory, sample preparation should be as simple as possible. The majority of earlier studies described relatively elaborate methods for DNA extraction (11, 15, 17). Because mycoplasmas have no cell walls, simple protocols should be sufficient to make the bacterial DNA accessible. Therefore, a rapid DNA extraction protocol employing Chelex 100 was used in the present study. Application of this method for successful bacterial and viral DNA extraction has recently been reported (5, 9, 20). The rapid Chelex 100 procedure is able to minimize contamination due to sample manipulation. Furthermore, it has been shown that DNA extraction using Chelex 100 may even decrease false-negative results due to PCR inhibitors (19). For *M. pneumoniae* detection, checking for inhibitors by amplification with β-globulin-specific primers prior to *M. pneumoniae* amplification has been recommended (15, 17). The discrepant rates of inhibitory samples found in those studies may be explained by the use of different clinical specimens and different methods. However, it is remarkable that, in one of those studies (17), no β-globulin-negative results were found when BAL specimens were tested. To avoid this supplementary assay which requires additional handling and time, an internal control which is introduced into each amplification reaction and is coamplified with target DNA from the clinical specimen would be helpful in the future.

Specificity is a critical factor in the clinical utility of a diagnostic assay. The primers used in the PCR assay evaluated in this study are quite specific for *M. pneumoniae*. Only DNAs from *M. pneumoniae* and *M. genitalium* are expected to be amplified efficiently.

Nucleic acid hybridization increases the sensitivity of molecular assays significantly and excludes nonspecific amplification products. Therefore, hybridization of the amplified product is a basic requirement of routine diagnostic molecular assays. The enzyme-linked immunosorbent assay-like microwell plate system for hybridization and detection used in this study is equivalent to that included in the commercially available AMPLICOR kits (4, 10, 14). It proved to be rapid and easy to perform and uses equipment that is readily available in routine diagnostic laboratories.

In summary, application of the PCR assay described proved to be useful to detect *M. pneumoniae* in BAL specimens. This assay gave more positive results than culture. Both the sufficient sensitivity and the rapid performance help clinicians to make safe and early diagnoses.

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