JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1994, p. 11–16
0095-1137/94/$04.00+0

Vol. 32, No. 1

Direct PCR Enables Detection of *Mycoplasma pneumoniae* in Patients with Respiratory Tract Infections

JEROEN H. T. TJHIE,[1] FRANK J. M. VAN KUPPEVELD,[2] ROBERT ROOSENDAAL,[1] WILLEM J. G. MELCHERS,[2] ROEL GORDIJN,[3] DAVID M. MACLAREN,[1] JAN M. M. WALBOOMERS,[2] CHRIS J. L. M. MEIJER,[3] AND ADRIAAN J. C. VAN DEN BRULE[1,3]*

Departments of Clinical Microbiology[1] and Pathology,[3] Section of Molecular Pathology, Free University Hospital, 1081 HV, Amsterdam, and Department of Medical Microbiology, University of Nijmegen, 6500 HB Nijmegen,[2] The Netherlands

Received 28 June 1993/Returned for modification 12 August 1993/Accepted 27 September 1993

The sensitivities of three methods of detection of *Mycoplasma pneumoniae* by a 16S rDNA PCR were compared by using a serial dilution of *M. pneumoniae*. These methods consisted of a PCR performed directly on cells after a proteinase K pretreatment (direct PCR), a PCR after purification of nucleic acids (DNA-PCR), and a PCR with rRNA sequences as the target after reverse transcription. The direct PCR and the reverse transcription PCR had a sensitivity of 1.5 CFU (≈250 genomes). By purification, a 10-fold loss of target DNA occurred, as shown by a 10-fold decrease in sensitivity (15 CFU) of the DNA-PCR. The presence of an excess of human background DNA did not influence the sensitivity of either PCR. The direct PCR was evaluated on samples from patients with respiratory complaints. Direct PCR amplification was possible in 94.9% of the samples, which were tested by amplification of a 326-bp fragment of the β-globin gene, which was performed to test for the suitability of amplification. Nucleic acid purification was performed on the β-globin-negative samples, after which only 2% remained negative. A positive correlation between the direct *M. pneumoniae* PCR and serology, as tested by the microparticle agglutination assay (MAG assay), was found in 88.1% of the cases. A positive MAG assay result was found for samples from 10 (17%) of the patients; samples from 6 (10.2%) of these patients were also positive by PCR. Samples from three patients were found to be positive by the *M. pneumoniae* PCR and negative by the MAG assay. Persistence of *M. pneumoniae*, as detected by PCR, was observed in three patients. These results indicate that the direct PCR with 16S rDNA could prove to be useful in the detection of *M. pneumoniae* in respiratory tract samples, although more studies are needed to evaluate the correlation between clinical symptoms and a positive test result.

Mycoplasma pneumoniae is a common cause of community-acquired respiratory tract infections, especially in children and young adults. Approximately 10% of the cases of community-acquired pneumonia that occur in endemic periods and up to 50% of the cases that occur in epidemic periods are caused by *M. pneumoniae* (5). Rapid diagnosis of an *M. pneumoniae* infection is important, since *M. pneumoniae* is not sensitive to β-lactam antibiotics, which are most often used empirically in the treatment of lower respiratory infections.

In routine laboratory practice, serology is used for the diagnosis of *M. pneumoniae* infections, since culture is relatively insensitive and 3 weeks or more may be required to obtain a result. In patients with primary infections, immunoglobulin M (IgM) can be detected from 7 days after the onset of symptoms and reaches a peak within 2 to 3 weeks. In patients with reinfections, IgM is mostly absent. Therefore, paired sera are used to confirm reinfection by *M. pneumoniae*, which is demonstrated by a fourfold rise in titer in IgG antibodies (13, 17). This can be observed only when the first serum sample is taken within 10 days after the onset of disease (5). Sillis (17) showed that in the absence of IgM antibodies in patients with reinfections, IgA measurement in a single serum sample can be used for diagnosis. Thus, both in patients with primary infections and in patients with reinfections, serological diagnosis is not obtained before 1 week after the onset of symptoms.

Therefore, rapid direct tests such as antigen detection, hybridization with DNA probes, and recently, PCR have been developed. PCR seems to be the most promising direct technique because of its high sensitivity and specificity (4). Different PCRs for the detection of *M. pneumoniae* have been described. The targets are the gene coding for the P1 adhesion protein, the 16S rRNA gene, and a DNA sequence specific for *M. pneumoniae* selected from a genomic library (1, 11, 20). In different studies, the use of 16S rDNA sequences has been described for the detection of several microorganisms such as mycobacteria, *Helicobacter pylori*, and mycoplasmas (2, 10, 20). The advantage of using 16S rRNA sequences is the high degree of conservation of the target and the presence of a high copy number of rRNA molecules in the cell, which are available as templates for the PCR after reverse transcription (RT) to DNA (RT-PCR). However, a disadvantage of an RT-PCR is the need for purification of RNA, making it less suitable for routine procedures. To date, a number of laboratories have successfully performed PCR directly on clinical material, after a simple pretreatment (direct PCR), for the detection of microorganisms (19, 21).

To investigate the most sensitive and simple PCR for the detection of *M. pneumoniae*, a direct PCR, a PCR on purified DNA (DNA-PCR), and a RT-PCR were compared with PCR with either 16S rDNA or 16S rRNA as targets (20). The optimal PCR method was compared with culture and serology in a study of 98 clinical samples collected from the

* Corresponding author. Mailing address: Department of Clinical Microbiology, Free University Hospital, de Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands. Phone: 020 (5485881/4017). Fax: 020 (5485873).

respiratory tracts of 79 patients with symptoms of respiratory tract infections.

MATERIALS AND METHODS

***Mycoplasma* strains and clinical samples.** The following *M. pneumoniae* isolates were used: P84 and P71 (Department of *Mycoplasma* Research, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Clinical specimens were obtained from 79 patients with symptoms of a respiratory tract infection. The ages of the patients ranged from 1 week to 85 years. Fifty-three subjects were inpatients of the Free University Hospital Amsterdam, and 26 patients visited the outpatient clinic. The following materials were collected from the respiratory tract: sputum ($n = 25$), throat ($n = 42$), and nasopharyngeal ($n = 4$) swabs; bronchial lavages ($n = 24$); nasopharyngeal aspirates ($n = 2$); and pleural fluid ($n = 1$). All samples were sent to the laboratory for routine culture of *M. pneumoniae*. A single serum sample was available from each of 34 patients, and at least two consecutive serum samples were available from 25 patients.

Culture. Both *M. pneumoniae* P84 and P71 were grown in SP4 broth. After a color change, the number of CFU was determined by culturing 100 μl of a 10-fold serial dilution on PPLO plates, which were subsequently incubated at 37°C in 5% CO_2 for 2 weeks. After collection, throat or nasopharyngeal swabs were placed directly into 3 ml of SP4 broth. The swabs were twirled, expressed against the side of the tube, and discarded. Of the aspirate, lavage, and sputum samples, approximately 200 μl was put into 3 ml of SP4 broth after arrival at the laboratory. Highly viscous material was first made soluble by adding an equal volume of Sputolysin (Behring Diagnostics), and the mixture was vigorously shaken for 0.5 h on an automatic shaker (Marius, Utrecht, The Netherlands). Two milliliters of the broth was incubated at 37°C, and 200 μl was subsequently cultured on PPLO (37°C, 5% CO_2) plates either after a color change or blindly after 7 and 21 days of incubation. During the 3 weeks of incubation, the plates were checked twice weekly under an inverted microscope for the presence of *Mycoplasma* colonies. Identification of the *Mycoplasma* isolates was performed by the department of *Mycoplasma* Research, National Institute of Public Health and Environmental Protection. In addition, a small amount of a number of patient samples were cultured on modified Herderschee broth and agar and incubated at 37°C (95% N_2 and 5% CO_2) by the National Institute of Public Health and Environmental Protection. Identification was done by identifying the colonies that appeared on the agar plates by indirect immunofluorescence by using specific polyclonal rabbit antibody for *M. pneumoniae* and horse anti-rabbit immunoglobulins labelled with fluorescein isothiocyanate.

Serology. A commercially available microparticle agglutination assay (MAG assay; Serodia Myco II; Fujirebio, Inc., Tokyo, Japan), which has been shown to be specific and sensitive for the diagnosis of *M. pneumoniae* infections (3), and the complement fixation test were used as serological tests. Paired serum specimens were tested by the complement fixation test and the MAG assay; single serum samples were tested by the MAG assay only. An antibody titer of ≥160 in the agglutination assay and a fourfold rise in titer in paired serum specimens in the complement fixation test and the MAG assay were regarded as positive results.

Pretreatment of *Mycoplasma* strains. To compare the sensitivity of the direct PCR with those of the PCRs performed on purified DNA (DNA-PCR) and RNA (RT-PCR), a 10-fold dilution series of both *M. pneumoniae* P71 and P84 ranging from 1.5×10^6 to 1.5×10^{-1} CFU/ml was made in Tris HCl (pH 7.5). The influence of background human DNA on the sensitivity of the different PCRs was tested after adding 10^5 HeLa 229 cells to each dilution. For the direct PCR, 10 μl of each dilution was incubated for 1 h at 50°C after adding a proteinase K buffer (500 μg of proteinase K per ml, 0.45% Nonidet P-40, 0.45% Tween 20, 100 mM KCl, 20 mM Tris HCl, 3 mM $MgCl_2$) to an end volume of 25 μl, and the mixture was subsequently boiled for 10 min to inactivate the proteinase K. DNA and RNA were isolated from each dilution by the method described by Gough (7). Briefly, 200 μl of cold 10 mM Tris HCl (pH 7.5)–0.15 M NaCl–1.5 mM $MgCl_2$–0.65% Nonidet P-40 was added to 200 μl of each dilution on ice. After vigorous vortexing, the nuclei were removed by centrifugation for 5 min at 800 × *g* and the cytoplasmic lysate was carefully removed and placed in a new tube containing 200 μl of 7 M urea–15% sodium dodecyl sulfate (SDS)–0.35 M NaCl–10 mM EDTA–10 mM Tris HCl (pH 7.5) plus 400 μl of phenol-chloroform-isoamylalcohol (50/50/1); the mixture was then vortexed vigorously. The deproteinized extract was separated from the phenol phase by centrifugation, and the DNA and RNA was recovered by precipitation of the aqueous phase with 1 ml of 95% ethanol and chilling at −20°C. The DNA and RNA was resuspended in 10 μl of distilled water. The RT reaction was performed in a 20-μl volume containing 50 mM Tris HCl (pH 8.3) 60 mM KCl, 3 mM $MgCl_2$, 2.5 pmol of MPn2 (antisense primer), 20 U of Rnasin (Promega), 8 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.), and 2 μl of purified nucleic acids. This mixture was incubated for 1 h at 42°C. Only one-fourth of the RT reaction mixture was used in the PCR. The DNA PCR was performed on 2 μl of diluted (four times) and purified nucleic acids, so that an equivalent amount of CFU was tested by each PCR method.

Pretreatment of clinical samples. For the direct PCR, 200 μl of the SP4 broth inoculated with the clinical sample was centrifuged for 0.5 hour at 12,000 × *g*. Subsequently, the pellet was resuspended in 40 μl of Tris HCl (10 mM). Lysis buffer containing 500 μg of proteinase K per ml, 0.45% Nonidet P-40, 0.45% Tween 20, 100 mM KCl, 20 mM Tris HCl, and 3 mM $MgCl_2$ was added to a final volume of 100 μl. This solution was incubated overnight at 37.5°C, and the samples were subsequently boiled for 10 min to inactivate the proteinase K. The direct PCR was performed on 25 μl of this product.

PCR. The nucleotide sequences of the different primers used in the *M. pneumoniae* PCR (20) and the β-globin PCR (15) are given in Table 1. The specificity of the *M. pneumoniae* PCR has been tested extensively, as described by Van Kuppeveld et al. (20). Under the conditions used, the primers amplified only *M. pneumoniae* but none of the other *Mycoplasma* species or other human pathogens (20). The *M. pneumoniae* PCR gave rise to a 277-bp fragment, and the β-globin PCR gave rise to a 329-bp fragment. The oligonucleotides were synthesized by using a Gene assembler plus apparatus (Pharmacia, Uppsala, Sweden) by the methoxy phosphoramidite method. The *M. pneumoniae* PCR and the β-globin PCR were performed separately. The PCR was performed in a total volume of 50 μl containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 25 pmol of each primer, 1 U of *Taq* DNA polymerase, and the sample. Samples were amplified for 40 cycles by using a PCR processor (Bio-med 60; Biomed, Theres, Germany), starting with a 4-min dena-

TABLE 1. Sequences of primers and probe

Target primer or probe	Nucleotide sequence	Size of PCR product (bp)
16S rDNA[a]		
M. pneumoniae 1 (sense)	5'-AAGGACCTGCAAGGGTTCGT-3'	277
M. pneumoniae 2 (antisense)	5'-CTCTAGCCATTACCTGCTAA-3'	
Probe GPO-1	5'-ACT CCT ACGGGAGGCAGCAGTA-3'	
β-Globin gene[b]		
PCO3 (sense)	5'-ACA GAACTGTGTTCACTAGC-3'	326
PCO6 (antisense)	5'-CAT CAGGAGTGGACAGATCC-3'	

[a] See reference 20.
[b] PCO3 (15) and PCO6 (this study) were described previously, as indicated.

turation step at 95°C. In the optimized PCR, each cycle consisted of the following steps: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1.5 min. After 40 cycles, a temperature delay step of 4 min at 72°C was done to complete the elongation.

Southern blot analysis of PCR products. Ten microliters of the PCR product was analyzed on a 1.5% agarose gel. DNA was transferred from the agarose gel to a nylon membrane (Gene-Screen Plus; DuPont) by diffusion blotting in 0.4 M NaOH–0.6 M NaCl overnight. The membrane was neutralized with 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and dried. Membranes were preincubated at 55°C in a hybridization solution (0.5 M sodium phosphate [pH 7.2], 7% SDS) for 2 h. Hybridization was performed overnight at 55°C in the hybridization solution containing 5'-^{32}P-end-labelled oligonucleotide probe (6×10^4 cpm/ml) (Table 1) by standard procedures (16). Subsequent washings were carried out at 55°C in 3 × SSC with 0.5% SDS. Autoradiography was performed overnight at −70°C with Kodak Royal X-Omat film and intensifying screens.

RESULTS

Comparison of direct PCR versus RT-PCR. In order to compare the sensitivities of the different PCRs, each PCR was performed on a serial dilution of *M. pneumoniae*. The PCR was performed directly on cells by using a proteinase K treatment (direct PCR), after purification of nucleic acids (DNA-PCR), or after reverse transcription of rRNA (RT-PCR). The 277-bp fragment obtained from the *M. pneumoniae* PCR was produced with both strains P71 and P84, as detected on agarose gels and after hybridization with the probe. The absolute sensitivity of the direct PCR was 1.5 CFU on agarose gels. A 10-fold decrease in sensitivity was observed by the DNA-PCR. The RT-PCR restored the initially obtained sensitivity to 1.5 CFU (Table 2). Additional hybridization did not lead to a higher sensitivity (Fig. 1). The addition of 10^5 HeLa 229 cells to each dilution did not influence the sensitivity. Therefore, the sensitivity was 250 genomes for both the direct PCR and the RT-PCR (1 CFU ≈ 160 genomes copies, according to Harris et al. [9]).

TABLE 2. Comparison of the sensitivities of direct PCR, DNA-PCR, and RT-PCR

PCR	Sample	Sensitivity (CFU)[a]	
		No extraneous DNA	10^5 HeLa cells
Direct PCR	Lysed cells	1.5	1.5
DNA-PCR	Purified DNA or RNA	15	15
RT-PCR	Purified DNA or RNA	1.5	1.5

[a] Sensitivities determined by hybridization and gel analysis were identical.

Evaluation of the PCR on clinical samples versus culture and serology. A β-globin PCR was performed to test whether the clinical samples were suitable for amplification. The β-globin PCR amplifying a 326-bp fragment was negative in five samples (5.1%) (all throat swabs). A DNA or RNA extraction was performed on these samples, and the samples were retested by the β-globin PCR and the RT-PCR for *M. pneumoniae*. Finally, two samples (2%) remained in which amplification was not possible. Fourteen (14.3%) of the 98 samples tested were found to be positive by the direct *M. pneumoniae* PCR. *M. pneumoniae* DNA was detected in seven sputum samples, one nasopharyngeal swab, and six throat swabs. These samples were collected from 10 (12.7%) of the 79 patients tested. Serological data were obtained from 59 (74.7%) patients. Table 3 summarizes the results of the comparison of serological and PCR results. Corresponding negative results were obtained for 46 (77.9%) of the patients, and corresponding positive results were obtained for 6 (10.2%) of the patients. Discrepant results were obtained for samples from 7 of the 59 patients, only 4 of which were serologically positive and 3 only of which were PCR positive. Of the six patients whose samples were positive by both PCR and serology, samples from two patients were also found to be positive by culture. Serum from one patient whose sample was positive by PCR was not available, and cultures of the sample from the patient remained negative. More detailed results for the patients whose samples were positive by any of the tests are summarized in Table 4 and are described more extensively in the Discussion section.

DISCUSSION

Nucleic acid purification, which is often used as pretreatment for PCR, is laborious and not practical for routine procedures. A PCR performed directly on clinical samples after a simple pretreatment is preferred. Therefore, the sensitivity of the direct PCR was compared with those of DNA-PCR and RT-PCR (20) on a serial dilution of *M. pneumoniae* with 16S rDNA as the target. The DNA-PCR and the RT-PCR were performed on purified nucleic acids. In contrast to other studies (1, 11, 21), no simulated clinical samples were used, since these may contain inhibitory substances. This may have a different influence on each method, making a fair comparison between the direct PCR and the DNA-PCR and RT-PCR in a model system impossible. The sensitivity of the direct PCR was identical to that

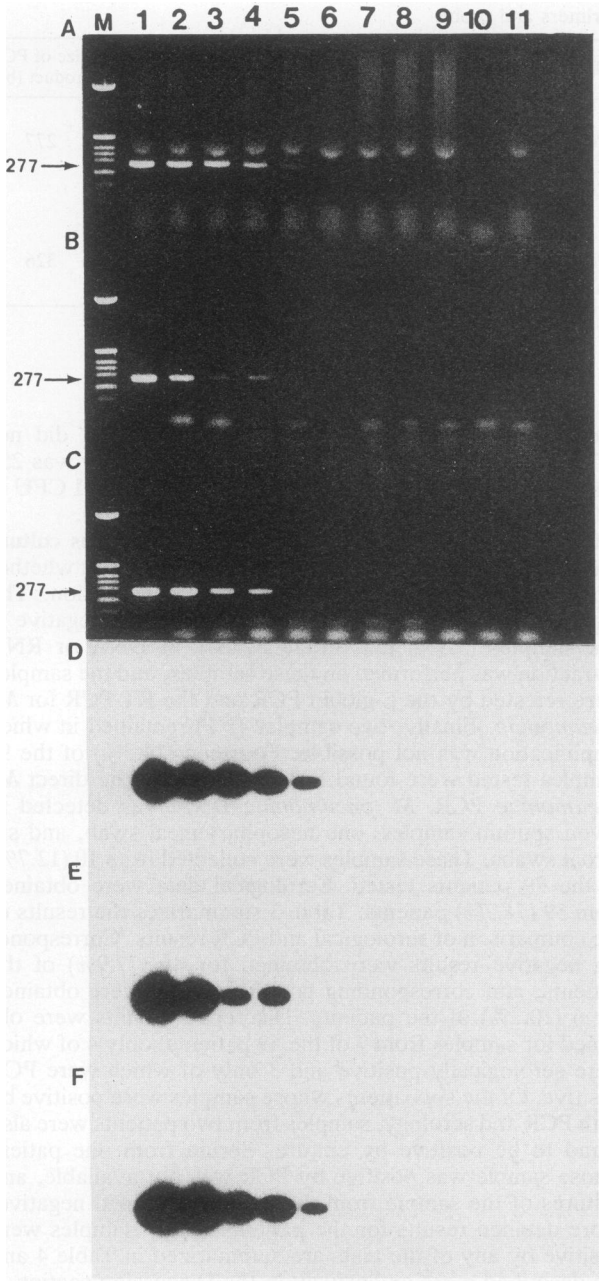

FIG. 1. Sensitivity of the direct PCR (A and D), DNA-PCR (B and E), and RT-PCR (C and F) as tested on a dilution line of *M. pneumoniae* with 10^5 HeLa 229 cells added to each dilution. Ten microliters of the PCR product was analyzed on a 1.5% agarose gel stained with ethidium bromide (A, B, and C), and additional Southern blot analysis (D, E, and F) was performed with 5′ [^{32}P]ATP-labeled oligonucleotide GPO-1. Lanes: 1, 1.5×10^4 CFU; 2, 1.5×10^3 CFU; 3, 1.5×10^2 CFU; 4, 1.5×10^2 CFU; 5, 1.5×10^1 CFU; 6, 1.5 CFU; 7, 1.5×10^{-1} CFU; 8, 1.5×10^{-2} CFU; 9, 1.5×10^{-3} CFU; 10, PCR mixture; 11, negative controle; M, molecular length marker (*Hin*fI-digested plasmid pBR322; in base pairs).

of the RT-PCR and was not influenced by an excess of background human DNA. A sensitivity greater than 1.5 CFU (≈250 genomes) achieved by the direct PCR is probably not possible, since mycoplasmas tend to grow in small colonies, making suspensions of single cells impossible (9). Most likely for similar reasons, no improvement in sensitivity after hybridization was found, since this sensitivity was already reached at the gel level. A loss of DNA occurs during the purification step, as was shown by a 10-fold decrease in the sensitivity of the DNA-PCR compared with that of the direct PCR. This is in agreement with a study by Hammar et al. (8), who showed a decrease in sensitivity after each subsequent step in a purification procedure. Therefore, a purification method with a minimum of extraction and centrifugation steps, like the method of Gough (7), which has proved to be fast and reliable in our laboratory, is preferred. In conclusion, the results determined on an *M. pneumoniae* dilution series favor the direct PCR as the method of detecting *M. pneumoniae* in clinical samples because of its high sensitivity and relative simplicity.

TABLE 3. Results of *M. pneumoniae* PCR compared with that of serology by the MAG assay in patients with respiratory complaints

PCR result	Serology result (no. [%] of samples)		
	+	−	Total
+	6 (10.2)	3 (5.1)	9 (15.3)
−	4 (6.8)	46 (77.9)	50 (84.7)
Total	10 (17)	49 (83)	59

To ensure that clinical samples were suitable for amplification, all were first tested by performing a PCR by amplifying a 326-bp fragment of the β-globin gene. It is important that the β-globin PCR amplifies a fragment comparable in length to the fragment amplified in the specific PCR, since smaller fragments have been found to be more efficiently amplified and less sensitive to the degradation of DNA (unpublished data). Negative β-globin results were found in only 5.1% (n = 5) of the samples by direct PCR and in 2% (n = 2) of the samples after purification of nucleic acids; this is in contrast to the 25% failure of amplification of β-globin found by Skakni et al. (18). These findings might be explained by several differences between the studies, such as the use of different clinical samples, a β-globin PCR with other primers, and a different pretreatment. All β-globin-negative samples in the direct PCR were throat swabs. A negative PCR result could be the result of the small amount of DNA available in throat swab samples or inhibition. Amplification was successful in three of the five throat swab samples after purification of nucleic acids, indicating the removal of inhibitory factors.

The direct PCR for the detection of *M. pneumoniae* was evaluated on clinical samples by comparing the results obtained by the *M. pneumoniae* PCR with those obtained by culture and serology. The MAG assay and direct *M. pneumoniae* PCR yielded concordant results for samples from 88.1% of the patients (Table 3). To exclude false-negative results obtained by the direct *M. pneumoniae* PCR, the RT-PCR was performed on a substantial part of samples negative by the direct *M. pneumoniae* PCR (data not shown). No additional positive samples were found in this group, which included a sample from a serologically positive patient. Although a good correlation was found between serology and PCR results, samples from seven patients gave discrepant results, as shown in Table 4. A positive serological test with a negative PCR result was found for samples from four patients. *M. pneumoniae* was probably eradicated from two patients (patients 4 and 12), since samples for the

TABLE 4. Results for patients with respiratory complaints and whose samples were positive for *M. pneumoniae* by any of the diagnostic tests[a]

Patient no.	Age[b]	Clinical status	Sample	PCR result	Culture result	Titer by:			
						CFT		Agglutination	
						Acute phase	Convalescent phase	Acute phase	Convalescent phase
1	2	Myeloid leukemia	TH	+ (4 m)	−	ND	32 (3 m)	ND	160 (4 m)
		Prolonged rhinitis and Otitis media	TH	+ (6 m)	−				
2	22	Pneumonia	SP	− (10 d)	−	128 (20 d)	ND	160 (20 d)	ND
3	22	Hyper IgE syndrome	TH	+ (11 d)	−	512 (11 d)	512 (11 d)	>320 (11 d)	>320 (25 d)
		Pneumonia	SP	+ (11 d)	−				
			SP	+ (21 d)	−				
4	31	Sarcoidosis	BR	− (2 m)	−	64 (10 d)	32 (1 m)	40 (10 d)	>320 (1 m)
		Upper respiratory infection							
4	29	Pneumonia	TH	+ (2 d)	−	64 (1 d)	64 (1 d)	<40 (1 d)	<40 (11 d)
6	9	Asthma	SP	+ (8 d)	−	128 (5 d)	ND	>320 (5 d)	ND
		Pneumonia	NP	+ (18 d)	+				
7	7	Upper respiratory infection	TH	+ (19 d)	−	4,112 (17 d)	ND	>320 (1 d)	ND
8	4	Pneumonia	TH	− (13 d)	−	2,056 (16 d)	ND	>320 (16 d)	ND
9	6	Pneumonia	SP	+ (5 d)	−	4,112 (10 d)	ND	>640 (10 d)	ND
10	8	Pneumonia	TH	+ (9 d)	+	2,056 (9 d)	ND	>320 (9 d)	ND
11	25	Pneumonia	SP	+ (4 d)	−	ND	ND	ND	ND
12	5	Pneumonia	TH	− (3 m)	−	1,024 (15 d)	1,024 (3 m) 512 (4 m)	>640 (15 d)	>320 (3 m) >320 (4 m)
13	14[c]	IRDS	NPA	− (8 d)	−	<4 (20 d)	ND	<40 (20 d)	ND
14	21	Leukemia Streptococcal sepsis ARDS	SP	+ (1 d)	−	64 (5 d)	128 (12 d)	<40 (5 d)	<40 (12 d) <40 (1 m)

[a] Abbreviations: CFT, complement fixation test; IRDS, infant respiratory stress syndrome; ARDS, adult respiratory distress syndrome; TH, throat swab; SP, sputum; NP, nasopharyngeal swab; BR, bronchial lavage; NPA, nasopharyngeal aspiration; ND, not done; d and m, time in days and months, respectively, after the initial symptoms that the sample was taken.
[b] Units are years unless indicated otherwise.
[c] Age is in days.

PCR were taken after prolonged treatment with either erythromycin or tetracycline and the disappearance of symptoms. A sample error because of the small volume (50 μl) tested in the PCR might explain the remaining two negative PCR results (samples from patients 2 and 8), since inhibition was excluded by a positive β-globin PCR. A negative serological test with a positive PCR result was obtained for samples from three patients. Serological samples were probably taken too early in the disease from one patient (patient 5). The two other patients (patients 13 and 14) might represent (transient) carriers, since the PCR result did not correlate with the clinical and other laboratory findings. Kai et al. (12) tested a selection of culture positive-samples and samples from healthy volunteers. *M. pneumoniae* DNA from 22 of 30 culture-positive throat samples was amplified, although these results are difficult to interpret since identification was done by the observance of a color change in the diphasic medium, which is not a proper identification technique for *M. pneumoniae*. Skakni et al. (18) found, in contrast to what we found in our study, a large number of *M. pneumoniae* PCR-positive samples from serologically negative patients. However, this was explained by the lack of antibody responses in samples taken too early in the disease and in samples derived from immunocompromised patients and patients less then 12 months old. Williamson et al. (21) compared the results of PCR with those of an antigen capture assay and serology, in which current, recent, and past infections were defined by serological criteria. Their results indicated that a laboratory diagnosis cannot rely on direct tests such as the PCR or antigen capture assay alone, but should also include serology. This conclusion cannot be drawn from the results of our study. A point of interest in the study by Williamson et al. (21) was the finding of more positive samples by a 16S rDNA PCR than by a PCR with the P1 adhesion gene. In conclusion, there was good agreement between the serological results obtained by the MAG assay and the direct PCR in our study. The direct PCR is the method of choice, although the RT-PCR could prove useful when purification of nucleic acids is indicated because of the presence of inhibitory substances. The loss of nucleic acids is then compensated for by using the multiple copies of cDNA sequences generated by the RT reaction.

Different studies have shown that *M. pneumoniae* can be detected in healthy people (6, 18, 21). Therefore, it is possible that detection of *M. pneumoniae* does not always represent a pathological state. This is confirmed by the study of Gnarpe et al. (6), in which they found the presence of *M. pneumoniae* by culture in 4.6% of healthy volunteers; the value rose to 13.5% during a peak period in the incidence of *M. pneumoniae*. However, the fact that these findings reflect the very early phase (incubation phase) of a subsequent clinical infection cannot be excluded. Skakni et al. (18) and Williamson et al. (21) showed in their studies that PCR is capable of detecting *M. pneumoniae* in serologically negative patients and in patients with a past infection. The latter patients represented those in a convalescent-phase carrier

state, such as three patients (patients 1, 3, and 6) in the present study in whom *M. pneumoniae* DNA was detected 3 weeks to 6 months after the start of the illness. *M. pneumoniae* DNA was also detected in two patients (patients 13 and 14) who clearly had other causes for their respiratory problems. Probably because of its high sensitivity, PCR is capable of detecting *M. pneumoniae* in circumstances other than acute infections. Therefore, PCR could be an attractive test for studying this persistence. On the other hand, it will prove difficult to correlate a positive PCR result with clinical symptoms if this persistence or carriage in healthy people is much more common then was presumed.

The PCR could also prove useful in the diagnosis of extrapulmonary *M. pneumoniae* infections. Narita et al. (14) showed that *M. pneumoniae* DNA can be detected by PCR in the sera and cerebrospinal fluid of patients with clinical central nervous system infections. *M. pneumoniae* DNA in cerebrospinal fluid could be detected by the direct PCR described in the present study in a 9-year-old patient with high serological titers who died of encephalitis (data not shown). This patient and other serologically positive patients with symptoms of an central nervous system infection will be more extensively described in a future report.

In conclusion, the *M. pneumoniae* PCR directed at the 16S rRNA gene from respiratory tract samples after a simple pretreatment was found to be at least as sensitive and more practical than an RT-PCR. The RT-PCR was useful after nucleic acid purification, as indicated by the presence of PCR inhibition, since the improved sensitivity compensated for the loss of nucleic acids. Inhibition or loss of nucleic acids was encountered in only a small percentage of samples after a simple pretreatment protocol. Therefore, the direct 16S rRNA PCR proved to be valuable as a technique for detecting *M. pneumoniae* in respiratory tract samples, although future studies must be performed to evaluate the correlation between a positive PCR result and clinical symptoms. These investigations will be performed on samples from children with community-acquired respiratory infections.

ACKNOWLEDGMENT

We thank A. F. Angulo for providing the different *Mycoplasma* strains, typing of the isolates, and critical reading of the manuscript.

REFERENCES

1. **Bernet, C., M. Garret, B. de-Barbeyrac, C. Bebear, and J. Bonnet.** 1989. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. J. Clin. Microbiol. **27**:2492–2496.
2. **Bödinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28**:1751–1759.
3. **Echevarria, J. M., P. Leon, P. Balfagon, J. A. Lopez, and M. V. Fernandez.** 1990. Diagnosis of *Mycoplasma pneumoniae* infection by microparticle agglutination and immuno-capture enzyme-immunoassay. Eur. J. Clin. Microbiol. Infect. Dis. **9**:217–220.
4. **Eisenstein, B. I.** 1990. New molecular techniques for microbial epidemiology and the diagnosis of infectious diseases. J. Infect. Dis. **161**:595–602.
5. **Foy, H. M., G. E. Kenny, M. K. Cooney, and I. D. Allan.** 1979. Long term epidemiology of infections with *Mycoplasma pneumoniae*. J. Infect. Dis. **139**:681–687.
6. **Gnarpe, J., A. Lundbäck, B. Sundelöf, and H. Gnarpe.** 1992. Prevalence of *Mycoplasma pneumoniae* in subjectively healthy individuals. Scand. J. Infect. Dis. **24**:161–164.
7. **Gough, N. M.** 1988. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Anal. Biochem. **173**:93–95.
8. **Hammar, M., T. Tyszkiewicz, T. Wadstrom, and P. W. O'Toole.** 1992. Rapid detection of *Helicobacter pylori* in gastric biopsy material by polymerase chain reaction. J. Clin. Microbiol. **30**:54–58.
9. **Harris, R., B. P. Marmion, G. Varkansis, T. W. Kok, B. Lunn, and J. Martin.** 1988. Laboratory diagnosis of *Mycoplasma pneumoniae* infection. 2. Comparison of methods for the direct detection of specific antigen or nucleic acid sequences in respiratory exudates. Epidemiol. Infect. **101**:685–694.
10. **Ho, S.-A., J. A. Hoyle, F. A. Lewis, A. D. Secker, D. Cross, N. P. Mapstone, M. F. Dixon, J. I. Wyatt, D. S. Tompkins, G. R. Taylor, and P. Quirke.** 1991. Direct polymerase chain reaction for detection of *Helicobacter pylori* in humans and animals. J. Clin. Microbiol. **29**:2543–2549.
11. **Jensen, J. S., J. Sondergard-Andersen, S. A. Uldum, and K. Lind.** 1989. Detection of *Mycoplasma pneumoniae* in simulated clinical samples by polymerase chain reaction. Brief report. APMIS **97**:1046–1048.
12. **Kai, M., S. Kamiya, H. Yabe, I. Takakura, K. Shiozawa, and A. Ozawa.** 1993. Rapid detection of *Mycoplasma pneumoniae* in clinical samples by the polymerase chain reaction. J. Med. Microbiol. **38**:166–170.
13. **Moule, J. H., E. O. Caul, and T. G. Wreghitt.** 1987. The specific IgM response to *Mycoplasma pneumoniae* infection: interpretation and application to early diagnosis. Epidemiol. Infect. **99**:519–537.
14. **Narita, M., Y. Matsuzono, T. Togashi, and N. Kajii.** 1992. DNA diagnosis of central nervous system infection by *M. pneumoniae*. Pediatrics **90**:250–253.
15. **Saiki, R. K., D. H. Gelfland, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
16. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Sillis, M.** 1990. The limitations of IgM assays in the serological diagnosis of *Mycoplasma pneumoniae* infections. J. Med. Microbiol. **33**:253–258.
18. **Skakni, L., A. Sardet, J. Just, J. Landman-Parker, J. Costil, N. Moniot-Ville, F. Bricout, and A. Garbarg-Chenon.** 1992. Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by polymerase chain reaction. J. Clin. Microbiol. **30**:2638–2643.
19. **Van den Brule, A. J. C., C. J. L. M. Meijer, V. Bakels, P. Kenemans, and J. M. M. Walboomers.** 1990. Rapid human papillomavirus detection in cervical scrapes by combined general primer-mediated and type-specific polymerase chain reaction. J. Clin. Microbiol. **28**:2739–2743.
20. **van Kuppeveld, F. J. M., J. T. M. van der Logt, A. F. Angulo, M. J. van Zoest, W. G. V. Quint, H. G. M. Niesters, J. M. D. Galama, and W. G. J. Melchers.** 1992. Genus and species-specific identification of mycoplasmas by 16S rRNA amplification. Appl. Environ. Microbiol. **58**:2606–2615.
21. **Williamson, J., B. P. Marmion, D. A. Worsick, T. W. Kok, G. Tannock, R. Herd, and R. J. Harris.** 1992. Laboratory diagnosis of *Mycoplasma pneumoniae* infection. 4. Antigen capture and PCR-gene amplification for detection of the *Mycoplasma*: problems of clinical correlation. Epidemiol. Infect. **109**:519–537.