Eur. J. Clin. Microbiol. Infect. Dis., May 1994, p. 401–405
0934-9723/94/05 0401-05 $3.00/0

16S rRNA Based Polymerase Chain Reaction Compared with Culture and Serological Methods for Diagnosis of *Mycoplasma pneumoniae* Infection

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The use of a 16S rRNA based polymerase chain reaction (PCR) for the detection of *Mycoplasma pneumoniae* infection was investigated. Sputum samples from 34 patients with respiratory illness and evidence of pneumonia as judged by chest X-ray were analyzed by PCR and microbiological culture. Throat swabs from 14 healthy individuals were used as controls. For serology, an enzyme immunoassay for the detection of immunoglobulin M antibodies and a complement fixation assay were performed. Evidence of *Mycoplasma pneumoniae* infection was obtained in ten patients (29 %), eight of whom were found positive by both PCR and serology. Two of the sputum samples from these eight patients were negative by culture. Of the remaining two patients positive for *Mycoplasma pneumoniae*, one was positive by PCR and culture but negative by serology, and one was found positive by serology but negative by PCR and culture. Thirteen of the 14 controls were negative by both PCR and serology. One control, however, was negative by serology but positive by PCR, which was probably due to asymptomatic carriage of *Mycoplasma pneumoniae*. The results of this study indicate the suitability of the PCR for the detection of *Mycoplasma pneumoniae* in clinical samples as well as its potential value as an additional tool for the diagnosis of infection.

Mycoplasma pneumoniae is a human pathogen that primarily causes respiratory infections. Although most of these infections are mild (1), severe bronchopneumonia and lung abscesses can occur (2). Furthermore, extrapulmonary complications such as meningitis, neuritis, myocarditis, pericarditis, and erythema multiforme have been reported, sometimes with a fatal outcome (3). A rapid and sensitive routine laboratory test is required for accurate diagnosis and adequate treatment of *Mycoplasma pneumoniae* infections.

The diagnostic methods currently used rely mainly on in vitro isolation of *Mycoplasma pneumoniae* and on serology. Culture is relatively time consuming, requires specialised media, and may be difficult to accomplish because of the fastidious nature of *Mycoplasma pneumoniae*. For serological diagnosis of *Mycoplasma pneumoniae* infection, several methods are available, but all of them have shortcomings. The cold agglutinin test is both nonspecific and insensitive (4, 5). The complement fixation assay (CFA) is not entirely specific and requires paired sera to demonstrate a rise in antibody titre (6). Paired sera are also required for the microparticle agglutination assay (7). Finally, immunoglobulin M (IgM) antibodies as detected by enzyme immunoassay (EIA) may be absent in reinfections (6, 8).

Techniques to demonstrate genomic sequences have been proposed as rapid and specific alternatives. Although hybridisation with DNA probes has been proven to be highly specific, low levels of *Mycoplasma pneumoniae* cannot be detected (9, 10, 11). The use of the polymerase chain reaction (PCR) for the detection of *Mycoplasma pneumoniae* has been tested in experimentally infected animals (12), in simulated clinical samples (13, 14), and recently also in clinical samples (15–18).

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We have developed a PCR assay with primers selected from variable regions of 16S rRNA to detect mycoplasmas at both the genus and species level (19, 20). This PCR assay has been used for the detection of experimental *Mycoplasma pulmonis* infection in rats (21), *Mycoplasma hominis* associated with a case of stillbirth (22) and mycoplasma contamination in cell cultures (23). In the present report, we describe the application of a *Mycoplasma pneumoniae*-specific 16S rRNA based PCR assay (19) for the detection of *Mycoplasma pneumoniae* infection. The value of this PCR assay was investigated by comparing the results with those obtained by microbiological culture and serology.

Materials and Methods

Patients and Clinical Samples. Clinical specimens were collected from 34 patients admitted to the Department of Infectious Diseases of the University Hospital in Uppsala. All patients suffered from respiratory illness and showed evidence of pneumonia by X-ray. Sputum and serum samples were collected about one week after the onset of symptoms. Convalescent phase sera were collected from seven patients within one to six weeks. Culture of *Mycoplasma pneumoniae* was performed both at the Department of Infectious Diseases of the University Hospital in Uppsala and at the National Veterinary Institute. For PCR analysis, the sputum samples were frozen, lyophilized, coded and sent to the Department of Medical Microbiology of the University of Nijmegen. Serological investigations were performed at the Department of Clinical Microbiology of the University Hospital in Uppsala. As controls, throat swabs and sera from 14 individuals without respiratory illness were tested.

Microbiological Culture. At the Department of Clinical Microbiology of the University Hospital in Uppsala, culture of *Mycoplasma pneumoniae* was performed according to standard procedures with SP4 broth and PPLO agar (24). At the National Veterinary Institute, culture was performed with the F medium, as described by Bölske (25). Mycoplasma isolates were identified by the indirect immunofluorescence test of unfixed colonies (26). The samples were scored positive in culture if they were found positive by either of the two methods.

Polymerase Chain Reaction. Lyophilized sputum samples were resuspended in water in their original volume. Throat swabs were suspended in 1 ml of phosphate-buffered saline solution, centrifuged for 10 min at 10,000 x *g*, and resuspended in 200 µl of water. Nucleic acids were isolated from 200 µl of sputum or throat swab sample and resuspended in 50 µl of water. For PCR analysis, 10 µl of the nucleic acid solution was used. Isolation of nucleic acid, amplification by the PCR, and analysis of the amplified samples were performed as described previously (22). In the PCR, the *Mycoplasma pneumoniae*-specific primers (forward primer, 5'-AAGGACCTGCAAGGGTTCGT-3'; reverse primer, 5'-CTCTAGCCATTACCTGCTAA-3') were used, which resulted in an amplification product of 277 bp (19). The probe GPO-1 (5'-CTCTAGCCATTACCTGCTAA-3') was used for Southern blot hybridisation (19). The thermal profile involved 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, and primer extension at 72 °C for 2 min.

Serological Methods. An IgM-EIA (MP Test IgM, Diatech Diagnostica, Israel) was used according to the manufacturer's recommendations. In addition, a CFA with commercially available *Mycoplasma pneumoniae* antigen (Behring, Germany) was performed. Serology was regarded as positive if the serum was found positive in the IgM-EIA and/or when at least a four-fold increase in antibody titre was found in paired sera tested in the CFA. In all other cases serology was regarded as negative.

Results

The results for both the patients and the control group are shown in Table 1. Evidence of *Mycoplasma pneumoniae* infection by culture, PCR or serology was found in ten of the 34 (29 %) patients with respiratory illness. The remaining 24 patients were negative by culture, PCR and IgM-EIA and had no detectable CF antibodies (data not shown). Of the 14 healthy individuals, 13 were negative by both PCR and serology, whereas one person was negative by serology but positive by PCR.

More detailed information about the ten patients positive for *Mycoplasma pneumoniae* is presented in Table 2. Convalescent phase sera were

Table 1: Detection of *Mycoplasma pneumoniae* infection by culture, PCR and serological methods in 34 patients with clinical signs of pneumonia and positive X-ray and in 14 healthy controls.

Clinical group	No. with results indicated	Culture	PCR	Serology
Patients	24	–	–	–
(n = 34)	6	+	+	+
	1	±*	+	+
	1	–	+	+
	1	+	+	–
	1	–	–	+
Healthy controls	13	ND	–	–
(n = 14)	1	ND	+	–

*Reported as suspected but unconfirmed positive by culture. Strain was lost at subculture before unequivocal identification could be achieved.
ND: not done.

Table 2: Comparison of the results of serological methods (IgM-EIA and complement fixation assay), culture and PCR for the ten patients that were positive in at least one of these tests.

Patient no.	Sex	Age (years)	Phase	Serological method		Culture	PCR
				EIA	CFA titre		
1	M	19	acute	+	1:15	±[a]	+
2	F	34	acute	–	< 1:7.5	+	+
			convalescent[b] (23 days)	–	1:60		
3	M	42	acute	+	1:60	+	+
4	M	24	acute	+	1:15	–	+
5	M	19	acute	+	< 1:7.5	+	+
6	F	16	acute	+	1:240	–	–
			convalescent[b] (7 days)	–	1:120		
7	M	30	acute	+	1:60	+	+
8	F	32	acute	+	1:15	+	+
			convalescent[b] (42 days)	+	1:120		
9	M	42	acute	+	1:60	+	+
10	M	33	acute	–	1:15	+	+

[a] Reported as suspected but unconfirmed positive by culture. Strain was lost at subculture before unequivocal identification could be achieved.
[b] Number of days between collection of acute and convalescent phase sera is given in parentheses.
CFA = complement fixation assay.

available from only three of these patients. The CF titres obtained with the acute phase sera from the seven remaining patients are also shown, because high CF antibody titres are suggestive for recent infection. Of the eight patients positive by both serology and PCR, two were negative by culture. One other patient (patient no. 10) was positive by culture and PCR, but negative by serology. In total, culture of *Mycoplasma pneumoniae* was clearly positive in seven of the nine sputum samples (78 %) that were positive by PCR. For patient no. 6, both PCR and culture were negative, but serology was positive.

Discussion

In this study, the suitability of a 16S rRNA based PCR assay for the detection of *Mycoplasma pneumoniae* infection was investigated by comparison of the PCR results with those obtained by culture and serology. Evidence of *Mycoplasma pneumoniae* infection was found in ten of the 34 patients with respiratory illness. *Mycoplasma pneumoniae* was detected in sputa from nine patients by PCR. In seven of these nine patients, *Mycoplasma pneumoniae* could be cultured from sputa. The two patients who were negative in culture were, however, positive by serology, which indicates that these patients were indeed suffering from an acute infection. Culture of sputum from one of these two patients was suspected to be positive for *Mycoplasma pneumoniae*, but this could not be confirmed by typing because the isolate was lost upon subculture. Problems in culturing *Mycoplasma pneumoniae* have been reported by several authors. For instance, Skakni et al. (17) isolated *Mycoplasma pneumoniae* from only one of the 20 throat samples that were positive by PCR. Lüneberg et al. (16) detected *Mycoplasma pneumoniae* by PCR in 14 culture-negative throat swabs from serologically positive patients. In contrast to these findings, Kai et al. (15) detected *My-*

coplasma pneumoniae by PCR in only 22 of the 30 throat swabs that were positive by culture. In that particular study, however, culture was regarded as positive when a change in colour of the medium was observed and the growth of *Mycoplasma pneumoniae* was not confirmed. Therefore, the authors themselves state that their culture method may not have been specific for *Mycoplasma pneumoniae*.

The results of PCR and serology were in agreement for eight patients. Discrepancies between the results of PCR and serology were observed for two patients and one healthy control. Patient no. 10 was positive by PCR but negative by serology. Since culture was also positive for this patient, a false-positive PCR result due to DNA carry-over contamination seems very unlikely. The absence of a distinct IgM response might be due to a reinfection. Alternatively, the serum could have been collected too soon after the onset of symptoms to detect IgM antibodies. Unfortunately, no convalescent phase serum was available from this patient, which made demonstration of a rise in CFA titre impossible. The detection of *Mycoplasma pneumoniae* in the absence of an IgM response could also be due to persistence of the organism in the respiratory tract following a previous infection. Although this patient suffered from respiratory illness and showed evidence of pneumonia by X-ray, *Mycoplasma pneumoniae* may not have been the causative agent. Skakni et al. (17) and Williamson et al. (18) described the detection of *Mycoplasma pneumoniae* by PCR in patients without serological evidence of infection and in patients with a previous infection and concluded that *Mycoplasma pneumoniae* can produce persistent and asymptomatic infections. A persistent or asymptomatic carriage of *Mycoplasma pneumoniae* was also observed in this study in one of the controls who was positive by PCR but showed no clinical signs or serological evidence of infection.

A disagreement between the results of PCR and serology was also observed in patient no. 6, who was negative by PCR and culture but positive by serology. Several possible explanations can be given for this discrepancy. The number of organisms in this sputum sample may have been below the detection limit of the PCR. Alternatively, *Mycoplasma pneumoniae* may already have been eradicated before the sputum sample was taken. The serological data indicate that this patient was no longer in the acute phase of the disease; a high CF antibody titre was already detectable in the first serum sample, and serum taken one week after the first sample was negative in the IgM-EIA. Therefore, the sputum sample, which was collected on the same day as the first serum sample, may have been collected at a timepoint at which it was no longer possible to detect *Mycoplasma pneumoniae*. It is unlikely that *Mycoplasma pneumoniae* was eradicated by antibiotics, since the sputum sample was collected only one day after this patient had received erythromycin.

In conclusion, the results of this study demonstrate that culture is not always sensitive enough to detect *Mycoplasma pneumoniae*. Furthermore, culture is not rapid enough, since several weeks were sometimes required for isolation of *Mycoplasma pneumoniae*. The 16S rRNA based PCR described here is suitable for the detection of *Mycoplasma pneumoniae* in clinical samples. Thus, the PCR procedure could well replace culture and become the method of choice for the detection of *Mycoplasma pneumoniae* in clinical samples. Because it has been demonstrated that the detection of *Mycoplasma pneumoniae* in the respiratory tract does not necessarily correlate with respiratory disease, serological tests should be used in addition to the PCR to distinguish between acute and persistent infections. The PCR may be especially valuable for the detection of *Mycoplasma pneumoniae* in immunocompromised patients, as was already demonstrated by Skakni et al. (17), and for the detection of *Mycoplasma pneumoniae* in organs and tissues of patients with extrapulmonary complications to address the role of *Mycoplasma pneumoniae* in these nonrespiratory disease states.

Acknowledgements

We thank Elisabeth Wilhelmsson and Marianne Persson for skillful technical assistance and the staff at the Department of Infectious Diseases of the University Hospital in Uppsala for collecting the specimens. This work was supported financially by grants from the Swedish Council for Forestry and Agricultural Research.

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