Research Article

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EVALUATION OF DIAGNOSTIC ACCURACY OF PCR AND SEROLOGY IN DIAGNOSING *MYCOPLASMA PNEUMONIAE* INFECTION

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Abstract: The present study is designed to evaluate the accuracy of PCR versus serology for diagnosing *M. pneumoniae* infections among the throat specimens. Samples for PCR testing were obtained from nasoopharyngeal aspirate. Serum samples were collected on the first visit and at least 2–4 weeks later. For the PCR assay, a rapid DNA extraction protocol was used. IgG antibodies were measured using the Serion Elisa Mycoplasma pneumoniae IgG kit. Overall, prevalence of *M. pneumoniae* was diagnosed in among 7.9% (23/290) of the patients. The median age of the patients positive for *M. pneumoniae* was 37 years and 38 years among negative patients which was statistically insignificant ($p>0.05$). Out of the total positive patients for *M. pneumoniae* in throat swabs, 70% (16/23) were observed to be positive by PCR and 78% (18/23) by serology. The high sensitivity (72.2%) and specificity (98.9%) of PCR was observed with positive and negative predictive value being 81.3% and 98.2% respectively. The accuracy of PCR was found to be 97.2%. The use of PCR, using either semi-nested or quantitative real-time methods, was superior to serology for diagnosing acute *Mycoplasma Pneumoniae* infections.

Keywords: Mycoplasma, Pneumoniae, PCR, Serology

INTRODUCTION

Mycoplasma pneumoniae (MP) is a small bacterium without a cell wall. It is recognized as a common cause of community-acquired pneumonia and upper respiratory tract infections, especially in children and adolescents, although all age groups may be affected. MP infections tend to occur in epidemics with a predilection for clustering in families and groups with close contacts such as military conscripts[1,2]. *M. pneumoniae* is known to be responsible for 10 to 30 per cent of community-acquired pneumonia (CAP) cases. MP infection can sometimes cause persistent respiratory symptoms, as well as a range of late-stage extra-pulmonary complications. The aetiology of these manifestations is not clear; although immune-mediated pathogenesis may be responsible, long-term MP infection could also be involved.

Correct and rapid diagnosis of *M. pneumoniae* infections is critical to initiate appropriate antibiotic treatment. Since it is impossible to diagnose this disease merely based on clinical signs and symptoms, therefore, laboratory test for detecting *M. pneumoniae* is particularly important. As it grows slowly (requiring 2 to 5 wk for colonies to become visible), culture is time-consuming[3]. Serological assays are most widely used. But sensitivity of these assays depends on whether the first serum sample is collected early or late after the onset of disease and on the availability of paired serum samples collected with an interval of 2 to 3 wk. Immunoglobulin M (IgM) assays that are more sensitive than the complement fixation (CF) test have been developed, but the IgM response may be nonspecific[4] or absent, particularly in adults[5]. Hybridization with DNA probes proposed as a rapid and specific procedure to replace culture, lacks sensitivity[6]. Nucleic acid amplification techniques (NAATs) have the potential to generate rapid, sensitive and specific results, but proper validation and standardization are often lacking, and quality control studies have revealed frequent deficiencies resulting in both false negative and false positive results. Although studies have reported the NAATs (mainly refer to PCR tests) have high sensitivity and specificity and have been widely used clinically, these may not be available in some small hospitals especially in countries and regions of limited resources (mainly in the developing countries). The serology assays are still commonly used.

Therefore, we performed this study to evaluate the accuracy of PCR and serology for diagnosing *M. pneumoniae* infections among the throat specimens in a teaching hospital of eastern Uttar Pradesh, India.

MATERIAL AND METHODS

Study site and subjects:

The study was performed in and around a teaching

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hospital situated in the eastern part of Uttar Pradesh, India. During the period an increased number of MP infections were noted in the diagnostic serological laboratory at the Department of Microbiology. The patients attending the out-patient clinic of the hospital were included. Patients with acute respiratory symptoms including cough were eligible for inclusion. No exclusion criteria were applied. A total of 290 patients were included in the study.

Procedure:

Samples for PCR testing were obtained from nasopharyngeal aspirate by a cotton-tipped swab and transferred to a tube containing either a transport medium consisting of 1mL of phosphate buffered saline supplemented with bensylpenicillin 150µg/mL, gentamicin 10µg/mL and amphotericin 2µg/mL, or a commercial transport medium (Copan Italia SPA, Brescia, Italy).

Serum samples were collected on the first visit and at least 2–4 weeks later. Subjects were asked to provide both throat samples and a blood sample for serology, The study subjects answered questionnaire concerning disease symptoms at the time of sampling. Subjects who were positive by PCR were invited to follow-up PCR testing every two weeks until two consecutive samples were negative. Three to six months after having submitted the last PCR-negative throat sample, these individuals were asked to return for another throat PCR sample in order to evaluate potential bacterial recurrence.

Methods:

PCR: For the PCR assay, a rapid DNA extraction protocol was used. In a sterile tube, 100 ml of the BAL and 300ml 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 ml of the supernatant was carefully removed and used for amplification directly, without further purification. For PCR, the upstream primer DD50B (59-biotin-GCAAA GTTATGGAAACATAATGGAGGTT-39 [positions 999 to 1026]) and the downstream primer DD54B (59-biotin-GGTT AGCAACACGTTTTTAAATATT-39 [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-ml reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3mM $MgCl_2$, 5% glycerol, 200mM (each) deoxynucleoside triphosphates (including dUTP instead of dTTP), 50pmol (each) oligonucleotide primer, 20 ml 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.

Serology: IgG antibodies were measured using the Serion Elisa *Mycoplasma pneumoniae* IgG kit (Institut Virion/serion GmbH, Würzburg, Germany) with the test cut-off 0,5×mean optical density (OD) value of the kit control serum, as indicated in the insert. A positive IgG reaction was defined as >30 AU/mL. A significant rise in IgG titre was considered to be a doubling of the OD value above the cut-off, or a sero-conversion in which the primary serum was antibody negative and the second serum had an OD at least twice the cut-off corresponding to a three-fold rise in AU/mL titre. IgM antibodies were measured using the SeroMP™ IgM kit (Savyon^{M} Diagnostics Ltd. Ashdod, Israel). A calibration line obtained by the standard sera in the kit was used to determine the IgM titre in BU/mL. A positive IgM antibody reaction was defined as >20 BU/mL according to the instruction of the manufacturer.

Diagnosis of MP infection was based on serology or PCR findings. A significant rise in MP IgG or sero-conversion in paired sera or the presence of IgM antibodies to MP were used as sufficient criteria of current MP infection. MP infection was also considered to be present by DNA detection when two independent PCR methods were positive. In cases with positive PCR results in the absence of MP antibodies, sequencing was done to confirm the presence of MP infection. Two consecutive samples negative by PCR were considered a microbiological resolution of MP infection.

Agreement of the results was calculated using the kappa index. Differences between mean values and between frequencies were tested by the Mann-Whitney U test and the chi-square test, respectively. Persistence of *M. pneumoniae* DNA was studied by survival analysis according to Kaplan-Meier. SPSS 16.0 version was used for all the analysis.

RESULTS

The mean age of the patients was 34.5 (±9.80) years and 57.2% were males (Table-1). In 157/290 (54.1 %) cases, paired sera samples were available.

Table.1: Demographic distribution of the patients

Demographic parameters	n=290
Age in years, mean±sd	34.5±9.80
Male sex, no. (%)	166 (57.2)
Weight in kg, mean±sd	37.12±19.87

Overall, prevalence of *M. pneumoniae* was diagnosed in among 7.9% (23/290) of the patients. The median age of the patients positive for *M. pneumoniae* was 37 years and 38 years among negative patients which was statistically insignificant (p>0.05). Out of the total positive patients for *M. pneumoniae* in throat swabs, 70% (16/23) were observed to be positive by PCR and 78% (18/23) by serology. The high sensitivity (72.2%) and specificity (98.9%) of PCR was observed with positive and negative predictive value being 81.3% and 98.2% respectively. The accuracy of PCR was found to be 97.2% (Table-2).

Table.2: *M. pneumoniae* detection by PCR and serology

PCR results	Serology results		Total
	Positive	Negative	
Positive	13	3	**16**
Negative	5	269	**274**
Total	**18**	**272**	**290**

Sensitivity=72.2%, Specificity=98.9%, Positive predictive value=81.3%, Negative predictive value=98.2%, Accuracy=97.2%

For 91 patients with paired sera and throat swabs, the agreement between serology and PCR was κ = 0.92. Two PCR-positive patients had unexpectedly late serological responses. Their first serum samples were obtained during the 3rd week of illness when they had low IgG titers and undetectable IgM. *M. pneumoniae* infection was serologically confirmed by IgG titre rise in convalescent phase samples obtained on days 21, 30, and 40, respectively. Patients who presented during the 2nd week of illness were all positive by PCR and serology. The sensitivity of PCR declined in the small number of patients who had their first test later than three weeks after onset of symptoms. Quantitative PCR could be carried out in 9 of the 23 patients who were positive by semi-nested PCR. One sample contained insufficient material for analysis but the infection was confirmed by IgG titer rise. qPCR confirmed *M. pneumoniae* infection in 3/9 patients while two tested negative by this method. These two patients were further analysed by DNA sequencing and found to belong to genotype 2 of *M. pneumoniae,* thus, confirming MP infection.

DISCUSSION

During the early phase of *M. pneumoniae* infection, serological methods have low sensitivity and can often only provide a retrospective diagnosis. In contrast, PCR tests on respiratory secretions may provide an early diagnosis for MP infection and could be a useful diagnostic alternative. In this study, we compared PCR from throat swabs and serology in symptomatic patients tested and found that PCR on throat swabs detected 70% of patients who later developed an antibody response to *M. pneumoniae*. The superior sensitivity of PCR was noted in patients tested during the first three weeks after onset of illness. These findings are in agreement with other studies[7-9] and suggest that PCR testing should be considered as the method of choice for diagnosis of MP infection during the early stages of illness.

There are several limitations of the present study. The patients were mainly recruited at a hospital department and only few MP cases were detected among patients attending general practitioners. Therefore patients with more severe symptoms may have been overrepresented. The patients are also more likely to have received antibiotic treatment due to the clinical manifestations.

The PCR results were reproduced by two independent methods and in addition most patients were positive in two or more samples. Positive PCR results were also corroborated by serological results in all but two cases. These two cases had repeated samples positive by both semi nested PCR and qPCR and seem to represent true *M. pneumoniae* infections. False positive serological results may also occur, in particular regarding IgM[7].

The sensitivity of PCR testing depends on the type of sample tested. We used throat secretions obtained in the nasopharyngeal. Besides, sputum production is often minimal in patients with *M. pneumoniae* infection. Previous reports have found that sputum samples give the highest rate of positive findings, followed by nasopharyngeal swabs as the second best, with throat swabs appearing to be less efficient. Räty et al.,[10] reported a sensitivity of sputum samples of 69%, nasopharyngeal swabs of 50% and throat swabs 37.5%.

We used throat swabs in our study, and found that the clinical sensitivity was high, since only 3 of 23 samples from patients with positive MP serology were PCR negative. It is possible that the yield was improved by the fact that our samples were obtained by swabbing the posterior wall of the oropharynx and not the tonsil area.

CONCLUSION

The use of PCR, using either semi-nested or quantitative real-time methods, was superior to serology for diagnosing acute *Mycoplasma Pneumoniae* infections.

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