Comparison of PCR, culture & serological tests for the diagnosis of *Mycoplasma pneumoniae* in community-acquired lower respiratory tract infections in children

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**Background & objectives:** *Mycoplasma pneumoniae* is known to be a major cause of lower respiratory tract infections in children. A specific diagnosis is important to institute the appropriate treatment. Information on diagnostic methods used for *M. pneumoniae* in Indian paediatric population is scarce. The study was thus conducted to compare polymerase chain reaction (PCR), culture and serology for the diagnosis of *M. pneumoniae* in community-acquired lower respiratory tract infections in children.

**Methods:** Seventy five children aged 6 months to 12 yr with signs of community-acquired lower respiratory tract infections were selected for the study. Culture of nasopharyngeal aspirates was done. The serum samples were analyzed for the detection of IgM and IgG antibodies to *M. pneumoniae*. A 543 base pairs (bp) region of P1 gene of *M. pneumoniae* was selected for amplification in PCR assay applied to nasopharyngeal aspirates.

**Results:** *M. pneumoniae* was isolated in culture from 4 (5.33%) children. Serological evidence of *M. pneumoniae* infection was observed in 16(21.3%) children. All culture positive patients were also positive by serology. Overall, PCR for *M. pneumoniae* was positive in 13 (17.3%) patients. All four culture positive patients were also positive by PCR. In 11 out of 13 (84.62%) PCR positive patients, serological evidence was there. Culture and/or serology and/or PCR positive results diagnosed *M. pneumoniae* infection in 18 (24%) of 75 patients.

**Interpretation & conclusions:** A combination of culture, serology and PCR may provide diagnostic information on the aetiology of *M. pneumoniae* community-acquired lower respiratory tract infections in paediatric population.

**Key words** Community-acquired - culture - paediatric - PCR - serology

Lower respiratory tract infections (LRTIs) are a common cause of morbidity and mortality among young children worldwide, the overwhelming majority occurring in developing countries\(^1\). *Mycoplasma pneumoniae* has been well established as a major cause of LRTIs and accounts for 15-20 per cent of all cases of pneumonia\(^2\). In the majority of cases of suspected *M. pneumoniae* pneumonia, the presumptive diagnosis is made on historic and clinical findings alone. Determining the aetiology is a challenge because some
diagnostic tests of respiratory samples that are non invasively obtained are insufficiently sensitive to identify the causative pathogens, others are flawed, cumbersome, expensive, time consuming and technically difficult. Other more reliable diagnostic procedures may place the patients at added risk of a complication or may require sophisticated methods not readily available everywhere.

A specific diagnosis is important because β-lactam antibiotics used empirically in the treatment of these infections are ineffective. Because of the fastidious nature of *M. pneumoniae*, culture methods are relatively insensitive, time consuming, expensive and labour intensive and are successful in only 30-60 per cent of the serologically diagnosed cases. The diagnosis in most cases is confirmed by serology. Serological methods are easy but generally non specific and retrospective. The commercially available enzyme linked immunosorbent assay (ELISA) is more specific and sensitive but require paired sera for diagnosis. However, the detection of antibodies in paired sera is considered the standard laboratory diagnostic method till now. Although not currently available to most clinicians, results using polymerase chain reaction (PCR) are promising with higher specificity and superior sensitivity to that of culture or single point serology.

Multifaceted approaches for detection and characterization of *M. pneumoniae* by using PCR, culture, and serology, augmented by knowledge obtained from the complete genome sequence, have been applied in epidemiological investigations; animal models of disease, evaluation of diagnostic reagents, and in clinical trials of antimicrobial agents. Despite these many advances, much is still unknown about this pathogen.

Information on comparison of various diagnostic modalities for *M. pneumoniae* as a causative agent of LRTIs in the Indian paediatric population has been scanty. Hence the aim of this study was to compare PCR, culture and serology for the diagnosis of *M. pneumoniae* in community-acquired lower respiratory tract infections in children.

**Material & Methods**

**Study design:** This prospective, consecutive, single centered study was conducted on community-acquired pneumonia in the Department of Microbiology, Maulana Azad Medical College, in collaboration with the Department of Pediatrics, Lok Nayak Hospital (LNH) and the Institute of Cytology and Preventive Oncology, Maulana Azad Medical College Campus, New Delhi.

A total of 75 children [with prevalence rate of 15% and worst acceptable prevalence of 10 per cent at 92 per cent confidence level the sample size would be ~ 79 (4pq/d², p=prevalence, q=100-p and d=absolute error)] children aged 6 months to 12 yr with signs of community-acquired LRTIs attending the pediatrics OPD emergency at LNH were selected consecutively from February 2002 through March 2003.

A written informed consent was taken from the parent/legal guardian of the children before them being enrolled in the study. The study protocol was approved by the Ethical committee of Maulana Azad Medical College and associated hospitals, New Delhi, India.

**Selection of cases: Inclusion/exclusion criteria:**

Criteria for inclusion were (i) age between 6 months and 12 yr, (ii) presence of cough and fever with breathlessness of less than 30 days duration, (iii) increased respiratory rate (with/without features of respiratory distress) on examination, and (iv) presence of signs of consolidation/bronchospasm with/without wheeze on auscultation.

Criteria for exclusion were (i) hospital acquired pneumonia *i.e.* pneumonia that developed 72 h after hospitalization or within 7 days of discharge, (ii) severe concomitant disease, and (iii) use of antibiotics in the 48 h preceding enrollment.

**Clinical data and routine lab investigations:** Clinical data from the patients were collected using a questionnaire developed and validated by authors and a detailed examination was performed. Routine laboratory investigations were done in all cases. Convalescent phase sera were taken from the patients at 4-6 wk after enrollment.

**Collection and transport of specimens:** Nasopharyngeal aspirates were collected by standard method, homogenized and divided into two labeled aliquots, one containing the transport medium (PPLO broth) for culture and the other containing phosphate buffered saline (PBS) for PCR.

For the serological tests, 1-2 ml of venous blood sample was collected by venepuncture using all the usual sterile precautions.

**Culture:** The aliquot containing the transport medium was inoculated in a volume of 0.5 to 1.5 ml into a bottle
of diphasic medium and one or two drops were plated directly on the standard solid mycoplasma medium\(^1\) (Hi-
media laboratories, Mumbai). Each batch of media was tested for the ability to support the growth of a standard
strain of \textit{Acholeplasma laidlawaii} obtained from the Department of Veterinary Public Health, College of
Veterinary Sciences, Chaudhary Charan Singh Haryana Agricultural University, Hisar. Liquid media were
incubated at 37°C. The plates and the diphasic media were incubated at 35.5 to 36°C in a candle jar with 5 per
cent CO\(_2\) and were inspected daily for any colour change and were immediately subcultured on to a fresh plate of
solid medium if that occurred. Suspect colonies were identified by the biochemical and biological tests (Diene’s
staining, requirement for yeast extract, inhibition of growth by erythromycin 100\(\mu\)g/ml, thallium acetate 0.05
per cent w/v, methylene blue 0.01 per cent w/v and lincomycin 200 \(\mu\)g/ml, haemolysis, haemadsorption,
production of acid from glucose and ammonia from arginine and urea and tetrazolium reduction\(^1\)).

Serology: Serum was separated from the venous blood samples and stored at -20°C till assayed. Convalescent
phase sera were obtained from patients during follow up visits for which they were called back approx. 4-6
wk after enrollment. Enzyme immunoassays were performed using commercial systems (NOVUM
DIAGNOSTICA, Assar Gabrielson, Germany) for IgM and IgG antibodies. The interpretative criteria were
consistent with the recommendations of the manufacturer.

The mean absorbance value (MN) of the negative controls was determined and the cut-off value (CO) was
calculated as: \(CO = MN + 0.300\).

The interpretation of the result was done as follows:
Positive: MN > 10 per cent above CO
Negative: MN > 10 per cent below CO
Grey zone: MN from 10 per cent above to 10 per cent below CO (repeat 2-4 wk later with new patient sample)

Polymerase chain reaction (PCR): The nasopharyngeal aspirates collected in PBS were centrifuged at 1957 \(\times\) g
for 10 min. Supernatant was decanted and the pellet was resuspended in 0.5 ml PBS and stored at -70°C till
further processing. The positive control consisted of relevant purified mycoplasma DNA.

DNA extraction was done using the organic method as described by Das \textit{et al}\(^5\).

For estimation of DNA concentration in these samples the intensity of individual DNA band was
compared with different band intensities of \(\lambda\) molecular weight marker.

A 543 bp section of the \(P1\) protein gene of \textit{M. pneumoniae} was selected for amplification. The primers
(Bangalore Genei Pvt. Ltd., Bangalore, India) were:

Primer 1: 5’CAAGCCAAACACGAGCTCCGGCC-3’, which is complimentary to the \(P1\) gene negative strand
residues 3666-3688, and

Primer2: 5’CCAGTGTCAGCTTGTGTCCTTTCCCCC-3’, which is complimentary to the \(P1\) gene positive
strand residues 4208-4183.

Amplification was done in 25 \(\mu\)l reaction mixture containing 100 ng of genomic DNA, 10mM tris HCl
(pH 8.0), 15 mM MgCl\(_2\), 50 mM KCl, 200 \(\mu\)M of each Dntp, 5 p mole of each oligonucleotide primer and 0.5
\(\mu\) of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) using a thermal cycler (Perkin Elmer.
Cetus, USA). DNA was initially denatured at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. In the last
cycle the extension at 72°C was allowed for 5 min.

Amplified PCR products were subjected to electrophoresis on 3 ethidium bromide stained agarose gel,
alongwith a molecular weight marker. A mixture of 2 \(\mu\)l genomic DNA, 2 \(\mu\)l of 6 X BPB and 8 \(\mu\)l of
distilled water was loaded into the 1 per cent agarose gel. The electrophoresis was carried out at a constant
voltage of 50 V for 1 h, and a band at 543 bp was taken to be a positive result.

Final positive for the diagnosis of \textit{M. pneumoniae} was defined as “positive when atleas one of the tests
was positive”.

Statistical analysis: The difference of proportion between qualitative variables was tested using the chi
square test and the Fischer exact test. \(P<0.05\) was considered as significant.

Results

Demographic profile: The age of the patients in the study group ranged from 6 months to 12 yr. The
maximum number of cases was seen in 2-5 yr of age and the male to female ratio was 1.586 with a male
preponderance but there was a slight female preponderance in 6-9 yr age group (Table I). Though 12
(26.09\%) of the total 46 males and only 4 (13.79\%) of the total 29 females were \textit{M. pneumoniae} positive,
no significant association between the sex of the patient and the incidence of \textit{M. pneumoniae} was found. There
was also no significant association between the age of
the patients and the incidence of *M. pneumoniae* infection (Table II).

**Clinical profile:** In our study population 28 (37.32%) patients had LRTIs with wheeze (audible/auscultable) and 47 (62.68%) had LRTIs without wheeze. The fact whether LRTIs due to *M. pneumoniae* was present with wheeze or not, was not found to have any statistical significance.

Majority of our patients had been unwell for < 2 wk before admission. Fever, cough and tachypnoea constituted the main criteria of LRTIs. Wheezing, rhinorrhoea, sore throat and chest pain were other predominant symptoms found in 14 (18.67%), 18 (24%), 61 (81.33%) and 23 (30.67%) patients respectively. Of the predominant symptoms, only the presence of sore throat was significantly associated with LRTIs due to *M. pneumoniae* (P<0.05). Physical examination most commonly revealed scattered rales, rhonchi, pharyngitis and cervical adenopathy in 59 (78.67%), 28 (37.33%), 38 (50.67%) and 11 (14.67%) patients respectively. Except for the presence of pharyngitis (P<0.05), none of the other signs was found to be significantly associated with the incidence of *M. pneumoniae* (Table II).

**Radiological profile:** Of all patients who had a chest radiograph on admission, 5 (6.67%) had normal chest radiographs with no evidence of pneumonia. Radiological features were variable. Lower lobe involvement was more common. The most common radiographic abnormality was the presence of infiltrates seen in 26 (34.67%) patients followed by hyperinflation in 20 (26.67%), consolidation in 19 (25.33%) and bronchopneumonia in 13 (17.33%) patients. In our study, the association between *M. pneumoniae* LRTIs and the radiological abnormalities was significant for bronchopneumonia and infiltration (P<0.05) (Table II).

**Microbiological profile:** *M. pneumoniae* was isolated from the nasopharyngeal aspirates of 4 (5.33%) patients by culture. All the colonies obtained on culture were identified by microscopy using Dienes’ stain and by the biological and biochemical reactions14. Serological evidence of infection was observed in 16 (21.33%) patients. A specific IgM antibody was detected in 15 (20%) patients. Specific IgG antibody in acute phase sera was detected in 6 (8%) patients all of whom were also positive for IgM antibody. Specific IgG antibody in chronic phase sera was detected in 13 (17.33%) patients, of whom 6 (46.2%) were positive for IgG antibody in acute phase sera and 12 (92.3%) were positive for specific IgM antibody.

<table>
<thead>
<tr>
<th>Character</th>
<th>M.P. positive (%)</th>
<th>M.P. negative (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (26.09)</td>
<td>34 (73.91)</td>
<td>46 (61.33)</td>
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<tr>
<td>Female</td>
<td>4 (13.79)</td>
<td>25 (86.21)</td>
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<td>Age (yr)</td>
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<tr>
<td>&gt; 5</td>
<td>3 (25.00)</td>
<td>9 (75.00)</td>
<td>12 (16.00)</td>
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<tr>
<td>&lt;5</td>
<td>13 (20.63)</td>
<td>50 (79.37)</td>
<td>63 (84.00)</td>
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<td>Wheezing</td>
<td></td>
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<td>Rhinorrhoea</td>
<td>13 (21.31)</td>
<td>48 (78.69)</td>
<td>61 (81.33)</td>
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<tr>
<td>Sore throat*</td>
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<td>29 (90.63)</td>
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<tr>
<td>Chest pain</td>
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<td>20 (86.96)</td>
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<td>Pharyngitis*</td>
<td>4 (10.53)</td>
<td>34 (89.47)</td>
<td>38 (50.67)</td>
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<tr>
<td>Cervical adenopathy</td>
<td>2 (18.18)</td>
<td>9 (81.82)</td>
<td>11 (14.67)</td>
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<tr>
<td>LRTI with wheeze</td>
<td>6 (21.43)</td>
<td>22 (78.57)</td>
<td>28 (37.32)</td>
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<td>Consolidation</td>
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<td>14 (73.68)</td>
<td>19 (25.33)</td>
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<td>Bronchopneumonia*</td>
<td>8 (61.54)</td>
<td>5 (38.46)</td>
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<td>Hyperinflation</td>
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<td>16 (80.00)</td>
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<tr>
<td>Infiltration*</td>
<td>3 (11.54)</td>
<td>23 (88.46)</td>
<td>26 (34.67)</td>
</tr>
</tbody>
</table>

LRTI, Lower respiratory tract infection; *P<0.005

**Fig.** Detection of *M. pneumoniae* by PCR. Lanes 3,4,5,7 & 8 show amplification of *M. pneumoniae* specific 543 bp product. Lane 1: Positive control. Lanes 2 & 6: Negative for *M. pneumoniae*
Polymerase chain reaction (PCR): M. pneumoniae DNA was present in the nasopharyngeal aspirates of 13 (17.33%) patients. All culture positive samples were also positive by PCR. PCR was positive from the nasopharyngeal aspirates in 11(84.6%) of 16 patients with serologically proven M. pneumoniae infection. Two (15.4%) children positive for M. pneumoniae DNA had no serological evidence.

Discussion

Community-acquired LRTIs in childhood remains a major cause of morbidity worldwide, and a significant cause of death in developing countries. We studied children with community-acquired LRTIs to compare the diagnosis of M. pneumoniae infection by two rapid methods, PCR and serology, with diagnosis by the more traditional method of culture. About half 56 per cent cases belonged to age group 2-5 yr and this age group also had a maximum number of positive cases which is in agreement with the epidemiological data based on serological studies according to which M. pneumoniae infections are found predominantly in children above the age of two years. Although the percentage of males was higher than the percentage of females who were positive for M. pneumoniae, we could not establish any statistical significance of this association. According to Clyde also, gender was not an important consideration. We found that except for the presence of sore throat and pharyngitis, none of the signs, symptoms or laboratory parameters were sufficient to distinguish precisely one infection from another to be useful in therapeutic decision. Our findings support those of previous studies that have cast doubts on the specificity of clinical and laboratory features in predicting the microbial cause of LRTIs. Segmental consolidation with perihilar infiltration is the classical radiographic appearance. In our series the presence of bronchopneumonia and infiltration was statistically significant. Ali et al noted that either of the upper lobes was involved in 24 per cent (20% in our study) and one or both lower lobes in 50 per cent (60% in our study).

Diagnosis of M. pneumoniae infection is challenging due to the fastidious nature of the pathogen, the considerable seroprevalence, and the possibility of transient asymptomatic carriage. Laboratory diagnosis is greatly hampered by the lack of standardized, sensitive, and specific methods for the detection of these atypical respiratory pathogens. A second major barrier is the difficulty in sampling the lower respiratory tract in representative populations of patients. Serologic tests for the presence of antibodies against these pathogens have been most commonly used as a diagnostic approach. The predominant role of serology in the routine diagnosis is partly due to the ease of specimen collection and the widespread availability of serological tests. Fifteen (20%) children with LRTIs enrolled had IgM antibodies to M. pneumoniae, data in agreement with another study from India. In order to rule out the possibility that the elevated antibodies are due to past infection; serology has to be combined with direct pathogen detection. Emphasis has been placed on the rapid diagnosis of M. pneumoniae infection by PCR. The main advantage of PCR is its superior sensitivity. PCR testing can detect both organisms rendered nonviable during transport and organisms that are noncultivable in persistent infection; however, this attribute also limits the clinical utility of PCR, as it cannot distinguish between viable and nonviable organisms after antibacterial treatment. It may be possible to overcome this by using reverse transcriptase-PCR, which can identify metabolic activity via the detection of messenger RNA; however, this new method is not yet well validated. In our study PCR findings went well with culture, as all culture positive patients were also positive by PCR. In 2 children PCR was positive without serological evidence. Positive PCR results without serologically proven infection with M. pneumoniae could represent either false positive PCR results (carry over contamination), the detection of a carrier state not manifesting systemic antibody response or false negative serology despite active infection. Despite advanced in vitro technology, PCR alone is not always sufficient for the diagnosis of respiratory infections by M. pneumoniae. Several studies have revealed a poor correlation between antibody response and positive PCR results in patients with M. pneumoniae. Comparable observations have been made when culture techniques have been applied. Although PCR is widely used for the rapid diagnosis of M. pneumoniae in research settings, methods are typically laboratory-specific and may use a variety of clinical source materials (e.g., peripheral blood or respiratory secretions) and hence serology offers a cheaper and better alternative for routine diagnosis.

Though studies on a larger sample size are required to get a better evaluation of the diagnostic modalities, our study suggests that perhaps a combination of culture, serology and PCR may provide diagnostic information on the aetiology of M. pneumoniae community-acquired LRTI in paediatric population.
References


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