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Mycoplasma pneumoniae: Prevalance in Navi Mumbai, India

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Minakshi Bhattacharjee
Ph.D student (Medical Microbiology), Department of Microbiology. MGM Medical College and Hospital, Kamothe, Sector1, Navi Mumbai-410209

Dr. A.D. Urhekar
Professor and Head, Department of Microbiology. MGM Medical College and Hospital, Kamothe, Sector1, Navi Mumbai-410209

Dr. Revati Sharma
Assistant Professor, Department of Microbiology. MGM Medical College and Hospital, Kamothe, Sector1, Navi Mumbai-410209

ABSTRACT *Mycoplasma pneumoniae is a leading cause of community acquired pneumonia. They are fastidious slow growing organisms lacking a cell wall and mostly isolated from the mucosal surfaces of the respiratory tracts. There is a dearth of information regarding clinical isolates of the bacteria among Indian patients. A prevalence study on 150 patients was hence carried as an initiative to rule out the organism in this part of the country. The results of the study shed light on difficulties of Mycoplasma pneumoniae detection and the superiority of molecular techniques over culture.*

INTRODUCTION:

Mycoplasma pneumoniae is a pervasive pathogen that accounts for up to 20% of all community-acquired pneumonias.[1] Although primarily responsible for atypical pneumonia (10%-20%) it can also cause extra pulmonary complications (1%-5%) involving the skin, central nervous system, cardiovascular system, gastro intestinal tract liver, kidneys, pancreas.[2]

Most of the available data concerning the occurrence of *Mycoplasma pneumoniae* infections have come from studies performed in the United States, Europe and Japan. These studies are basically seroprevalence investigations that indicate the presence of *Mycoplasma pneumoniae* antibodies and suggest that populations in these regions have had infections due to this organism. Much of what we know about rates of the infections caused by the organism comes from population based studies relying primarily on serological measurements. [2] There is to the best of our knowledge no national surveillance system tracking *Mycoplasma pneumoniae* infections yet.

In the past, diagnosis of infection by this organism was usually based on serology, because growth in culture is slow and insensitive.[3] Therefore, nucleic acid amplification techniques have been introduced. PCR of fragments of the P1 gene or the 16S rRNA gene were shown to be considerably more sensitive than culture for the detection of *M. pneumoniae*.[4]

Developed countries like USA where *M. pneumoniae* is responsible for more than one lakh hospitalizations each year have adopted proper methods to identify the organism in routine.[1] The non-availability of reliable, rapid diagnostic techniques as well as the lack of clinical awareness is the probable reason for sparse studies from India on the organism. There are few studies from the North India and a few from the South India but these do not provide any statistical data. The primary methods adopted in the studies from the south are serological tests and culture. Also the study population are defined having certain underlying condition like HIV infection or asthma or chronic obstructive pulmonary disease.[5,6] Polymerase chain reaction has been used in a few studies from Delhi but the exact scenario of the prevalence of *M. pneumoniae* is not yet clear.[7] This study is hence an attempt to find out the prevalence of *M. pneumoniae* infections in this part of the country using culture as well as PCR as the methods of analysis.

MATERIALS AND METHODS:

Clinical samples

The present study was carried out on 150 patients in the Microbiology Department of a tertiary care centre in Navi Mumbai. Inclusion criteria of patients was community acquired pneumonia diagnosed on clinical, radiological basis and blood counts. Hospital acquired pneumonia i.e pneumonia developed 72 hours after hospitalization or within 7 days of discharge was the criterion for exclusion of patients. Respiratory samples namely bronchoalveolar lavage, endotracheal tube aspirate and sputum were taken from the patients included in the study.

Microscopy

The samples were subjected to Gram's staining to grade it as per Bartlet's grading system (BGS) in case of sputum samples, observe the type of inflammatory cells and the organisms present. If the BGS score was zero no further processing of the sample was done.

No. of Neutrophils per 10X Low-Power Field	No. of Epithelial Cells per 10X Low-Power Field	Grade
<10	-	0
10-25	-	+1
>25	-	+2
Presence of mucus	-	+1
-	10-25	-1
-	>25	-2

Table 1: Bartlet's grading System.[8]

Culture

The sample (200µL) was inoculated into 2mL PPLO broth. It was incubated at 37^{0}C in presence of 5% CO_2 in a dese-

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crator. Change in color to yellow indicates positive growth. Subcultures (100 µL) were made from positive PPLO broth on PPLO agar and incubated in CO_2 incubator at 37^{0}C. The plates were observed regularly for 3 to 4 weeks for the growth of *Mycoplasma pneumoniae*.

Control used- *Mycoplasma pneumoniae* FH strain (ATCC 15531).

Polymerase chain reaction

i) Genomic DNA extraction- DNA was extracted using boiling method. In brief 200 µL of PPLO broth or specimen suspended in phosphate buffer saline (PBS) was centrifuged at 14,000 rpm for 15 minutes. The pellet was washed twice with PBS (pH 7.2). The pellet was re-suspended in 100 µL of sterile water and placed in boiling water bath for 10 minutes. This was followed by a 30seconds of centrifugation at 12,000 rpm. The supernatant obtained contains the extracted DNA.[7]

ii) Amplification Reaction- PCR was done using primer set specific for a 375bp fragment of P1 cytadhesion gene of *Mycoplasma pneumoniae*. P1 gene codes for P1 protein responsible for cytadhesion of *Mycoplasma pneumoniae* on the respiratory epithelial cell. Primers used were forward (5 ' CCG CGA AGA GCA ATG AAA AAC TCC 3') and reverse (5' TCG AGG CGG ATC ATT TGG GGA GGT 3').[9,10] The total PCR reaction mixture was of 25 µL which consisted of the following: 12.5 µL master mix (2X Bioline, Allied Scientific), 5.5 µL molecular grade water (Genetix), 1 µL each of 10pM forward and reverse primer (Genetix) and 5 µL of the extracted genomic DNA. Thermocycler Peqlab was used and the cycling conditions were: Initial heating of 1 minute at 94^{0}C, followed by 35 cycles of amplification each consisting of 94^{0}C for 1 minute, 64^{0}C for 1 minute, 72^{0}C for 2 minutes, lastly a cycle of final elongation of 72^{0}C for 10 minutes.

Control used- DNA of *Mycoplasma pneumoniae* FH type 2 strain (ATCC 15531)

iii) Downstreaming Process- The amplified PCR products were subjected to down streaming process using 1% agarose gel electrophoresis. The DNA bands were documented by gel documentation system. The expected 375bp band for P1 gene was compared with DNA ladder consisting of 100bp to 1000bp.

Control- PCR amplified products of *Mycoplasma pneumoniae* FH type 2 strain (ATCC 15531)

Statistical analysis

The data was analysed using SPSS version 17 software. Chi-square test was applied and $p<0.05$ was considered as significant.

RESULTS:

A total of 26 out of 150 samples (17.33%) showed the presence of *Mycoplasma pneumoniae* DNA by PCR assay. Of these 26 samples the organism could be isolated in culture in 7 samples (4.66%). The study shows a17.33% prevalence of *Mycoplasma pneumoniae* in this area by adopting a combination of culture and PCR as the detection method.

Fig 1: Change in colour of PPLO broth to yellow indicating positive *M. pneumoniae* culture.

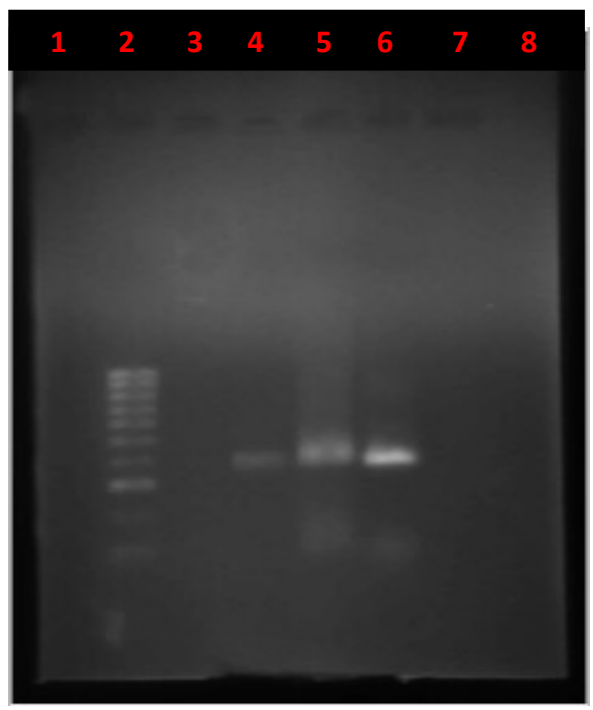

Fig 2: PCR for P1 adhesin gene-375bp product, Lane1,3,7,8: Nil, Lane 4,5,6: Positive (375bp), Lane2: DNA Ladder (100-1000bp).

The 150 patients included in the study consisted of 95 males and 55 females. A total of 16 males and 10 females were found to be infected with *Mycoplasma pneumoniae*. But no significant association of *Mycoplasma pneumoniae* infections with the sex of the individual is found (P-value is 0.861, i.e. >0.05). Individuals of all the age group were included in the study. Prevalence of *M. pneumoniae* was found to be highest in the age group 81 to 90 yrs (25%) which was followed by 71 to 80 yrs (23.07%), 61 to 70 yrs (21.73%) and 1 to 10 yrs (20%). But there was no statistically significant association seen between *M. pneumoniae* infections and age (P-value is 0.664, i.e. >0.05).

DISCUSSION:

In the current study two different methods namely culture and PCR are used for the detection of *Mycoplasma pneumoniae* in the clinical samples. On comparing the detection methods PCR was found to be more sensitive and specific. The prevalence percentage found by culture was 4.66% (7 cases) whereas; by PCR was 17.33% i.e. 26 cases which is statistically significant (p-value 0.000373, i.e. <0.05).

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Low isolation rate of *M. pneumoniae* in culture could be due to prior antibiotic treatment received by the patients elsewhere before coming to our hospital. It can prevent the growth of *M. pneumoniae* in the culture medium.

The current study is in concordance with Kashyap B et al.[11] who also found the prevalence of *Mycoplasma pneumoniae* by culture to be 5.33% (4 out of 75 cases) and by PCR to be 17.3% (13 out of 75 cases). Hence, PCR was seen to be more sensitive as found in this study.

Basil MV et al.[12] found the seroprevalance of *M. pneumoniae* in 16 out 100 cases (16%) by ELISA. In none of the cases the organism could be isolated in culture. Sahoo R et al.[6] reported 17% prevalence of *M. pneumoniae* by culture and 37% by ELISA. 15% cases showed both culture and ELISA positive, 2% cases showed only culture positivity and 22% only ELISA positivity. These results are comparable with the present study as we also found culture to be less sensitive.

Ieven M et al.[4] in their study detected *M. pneumoniae* in 8 out of 371 samples (2.15%) by culture and 13 out of 371 samples (3.50%) by PCR. Sensitivity of culture compared to PCR was to be 61.5%. Varshney AK et al.[13] found 15 of 150 (10%) cases to be infected with *M. pneumoniae* by PCR but, the organism could not be isolated in culture in any of the cases. Dorigo-Zetsma JW et al.[14] studied 92 patients and 74 controls. They found 7 patients to have *M. pneumoniae* infections by PCR (8%) 6 patients by culture (7%). None of the control cases showed the presence of the organism. Buck GE et al.[15] from their study concluded that the much more sensitive PCR could detect 1 to 10 organisms, whereas detection by culture requires 10^3 CFU/mL organisms. The findings of all these workers are in accordance of the current study wherein PCR was found to be more sensitive than culture.

Difference in the prevalence of *M. pneumoniae* infections among various workers in India and other countries could be due to various reasons as follows- the methodology of laboratory procedures, patient study group, age and sex distribution, and the predisposing factors. The spread of *M. pneumoniae* infections in the community is by means of droplet infections and formites. Viability and spread of *M. pneumoniae* from one patient to another may depend on environmental and climatic conditions like temperature, humidity and season. Lastly the health education of the patients regarding prevention of the disease and implementation of aseptic procedures by the health care workers and doctors may also be responsible for the variation in the prevalence.

The prevalence percentage of *M. pneumoniae* in males is 16.84% and 18.18% in females. But there was no statistically significant (p-value 0.861) association found between gender and *M. pneumoniae* positivity. Chaudhry R et al.[7] in their study found 27 of 92 (69%) males and 16 of 42 (38%) females to be infected with *M. pneumoniae*. Kashyap B et al.[11] reported 12 out of 46 males (26.09%) and 4 out of 29 females (13.79%) to have *M. pneumoniae* infection. No statistically significant association between sex and *M. pneumoniae* infections were found in the above studies, which concords the current findings.

On distributing the patients according to the age group it was found that *M. pneumoniae* infections were more in the age group 1 to 10 years (20%) which was followed by 61 to 70 years (21.73%), 71 to 80 years (23.07%) and 81 to 90 years (25%). But there was no statistically significant (p-value 0.664) association found between *M. pneumoniae* infection and age distribution.

Chaudhry R et al.[7] reported 6 of 37 (16%) paediatric (0-15 years) and 37 of 97 (38%) adult (16-90 years) cases with *M. pneumoniae* infections. In the current study it was also found that 2 of 11 (18.18%) belonged to the age group 0-15 years and 24 of 139 (17.26%) belonged to the age group16-90 years.

Kashyap B et al.[11] found that culture or serology or PCR diagnosed 18 of 75 cases (24%) with *M. pneumoniae* infection in the age group 6 months to 12 yrs. The present study found that 2 of 8 cases (25%) belonged to the age group 6 months to 12 years. These studies also did not find any statistically significant association between age and *M. pneumoniae* infections and hence, complement the current study.

The laboratory tests for diagnosis of *M. pneumoniae* infections were not available in this hospital as well as in the public hospitals of this area. As a result no data is available regarding regular diagnosis and treatment of *M. pneumoniae* infections. For the same reason, very few studies have been done in India on *M. pneumoniae*. Hence, this study gives an idea of the current scenario of *M. pneumoniae* infections in this part of the country. The laboratory tests set up during this research work will help the diagnosis of *M. pneumoniae* infections in our hospital for proper treatment and management. This will help to reduce the morbidity and mortality due to *M. pneumoniae* infections.

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