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RAPID METHOD FOR QUALITATIVE DETECTION OF *MYCOPLASMA PNEUMONIAE.*

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ABSTRACT

Mycoplasma pneumoniae is a common etiological agent of atypical pneumonia responsible for 10% to 20% cases. But the organism is not given much attention due to lack of diagnostic facilities. The increased awareness among the health care professionals, timely and rapid detection of the organism could be beneficial. A real time PCR assay adopted in our study can help quicker laboratory diagnosis. The prevalence of *Mycoplasma pneumoniae* found adopting this method in a tertiary care centre of Navi Mumbai, Maharashtra was 16%.

KEY WORDS: ***Mycoasma pneumoniae*, real time PCR, prevalence, atypical pneumoniae.**

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INTRODUCTION

*Mycoplasma pneumoniae*primarily responsible for atypical pneumonia(10%-20%) can also cause extra pulmonary complications (1%-5%) involving the skin, central nervous system, cardiovascular system, gastro intestinal tract, liver, kidneys, pancreas.[1] The *Mycoplasma* infection may sometimes also occur due to the stress imposed on the immune system by the other infection.[2]*M.pneumoniae* infections occur both endemically (every 4-8 years) and epidemically worldwide, especially in children and young adults (5-20 years). More common particularly in temperate climates and can occur at any time of the year, but large outbreaks tend to occur in the late summer and fall. In developed countries it is responsible for 15% to 20% of all pneumonia cases.[3] 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 investigations.[4] Studies from India are sparse and do not provide any statistical data. This is probably due to non-availability of reliable, rapid diagnostic techniques as well as the lack of clinical awareness. Increasing awareness among health care provider countries not yet heavily affected and rapid detection methods could strengthen surveillance activities and ensure a timely diagnosis and appropriate treatment of the disease in affected patients. This study is hence an attempt to find out the prevalence of *M. pneumoniae* in this part of the country. Also an effort is made to shift from the traditional methods of detection of the organism like culture, serology and conventional PCR to a more rapid and sensitive real time PCR assay.

MATERIALS AND METHODS

2.1. *Study Group*

The present study was carried out on 100 patients admitted in Respiratory Medicine Department and ICU of a tertiary care centre. The following were the inclusion and exclusion criteria considered for the study.

Inclusion criteria

Community acquired pneumonia diagnosed on clinical, radiological basis and blood counts.

- Clinical: Cough, sputum production and fever>37.8^{0}C
- Radiological: Pulmonary infiltrate shown in chest X ray suggestive of pneumonia
- Blood Counts: Total leukocyte count (TLC) ≥ 12,000/µL

Exclusion criteria

- Hospital acquired pneumonia i.e pneumonia developed 72 hours after hospitalization or within 7 days of discharge.
- Pulmonary shadow due to a cause other than pneumonia.

2.2. Specimen collection and transport

Bronchoalveolar lavage, endotracheal tube aspirate and sputum were collected in sterile wide mouthed containers. The samples were immediately transported to the microbiology laboratory for processing. The samples were stored at 4^{0}C if any delay in processing was anticipated, but not for morue than 24 hours.

2.3. Microbiological analysis

A rapid molecular method to detect *Mycoplasma pneumoniae* in the respiratory samples of the patients was used which involves the following steps:

Controls used: DNA of *Mycoplasma pneumoniae* strains M129 (ATCC 29342) and FH (ATCC 15531).

i) Genomic DNA extraction- DNA was extracted using QIAamp DNA extraction kit (QIAGEN) which is a spin column based method. In brief 200 µL of specimen was taken in an eppendoff tube and 200 µL of lysis buffer and 20 µL of proteinaseKwas added to it. It was incubated in a water bath at 56^{0}C for 10 minutes. 200 µL of chilled ethanol was added to it after incubation. The whole content of the eppendoff was transferred to a spin column and centrifuged at

8,000 rpm for 1 minute. The collection tube was discarded and 500 µL of wash buffer 1 was added to the spin column. This was followed by a 1 minute of centrifugation at 8,000 rpm. The collection tube was discarded again and 500 µL wash buffer 2 was added, centrifuged at 12,000 rpm for 3minutes. A new collection tube was taken and 200 µL of elution buffer added to the spin column and allowed to stand for one minute. It was then centrifuged at 8000 rpm for one minute to elute the DNA in the spin column. The eluted solution obtained in the collection tube contains the extracted DNA. Extracted DNA was used immediately or stored at -80^{0}C for further use.

ii) Real Time Polymerase Chain Reaction- The real time PCR assay was performed using a 375bp fragment of the P1 cytadhesin gene. Two sets of single-stranded oligonucleotide used were forward (5 ' CCG CGA AGA GCA ATG AAA AAC TCC 3') and reverse (3' TCG AGG CGG ATC ATT TGG GGA GGT 5').[5]The amplification was performed on real time Roche Light Cycler II (LC480) using SYBRGreen. (KapaBiosystems). Each well of the PCR plate contained the following per reaction: 10 µL of 2X SYBRGreen master mix, 0.4 µL of 10pM forward primer and 0.4 µL of 10pM reverse primer, 7 µL of genomic DNA and 2.2 µL of molecular grade water to make a total volume of 20 µL. The amplification conditions were as follows: Initial holding of 2 minutes at 50^{0}C, Taq polymerase activation at 95^{0}C for 3 minutes, followed by 40 cycles of amplification (95^{0}C for 15 seconds, 64^{0}C for 20 seconds and 72^{0}C for 15 seconds) and then lastly cooling at 40^{0}C for 10 seconds. The results were analysed in FAM channel. (510-530nm)

2.4. Statistical analysis

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

RESULTS

Demographic profile

In this study 100 patients were enrolled, of which 68 were male and 32 were female. A total of 12 males and 4 females were found to be affected with *Mycoplasma pneumoniae*. But no significant association of *Mycoplasma pneumoniae* infections with the sex of the individual is found. Individuals of all the age group were taken and the infected patients did not belong to any particular age group as well.

Individuals	Samples	Positive	Percentage (%)
Male	68	12	17.6%
Female	32	04	12.5%
Total	100	16	16%

Table1
Prevalence of Mycoplasma pneumoniae in male and female patients attending the tertiary care centre.

Microbiological profile

A total of 16 sputum samples showed the presence of *Mycoplasma pneumoniae* DNA by real time PCR assay. This indicates a16% prevalence.

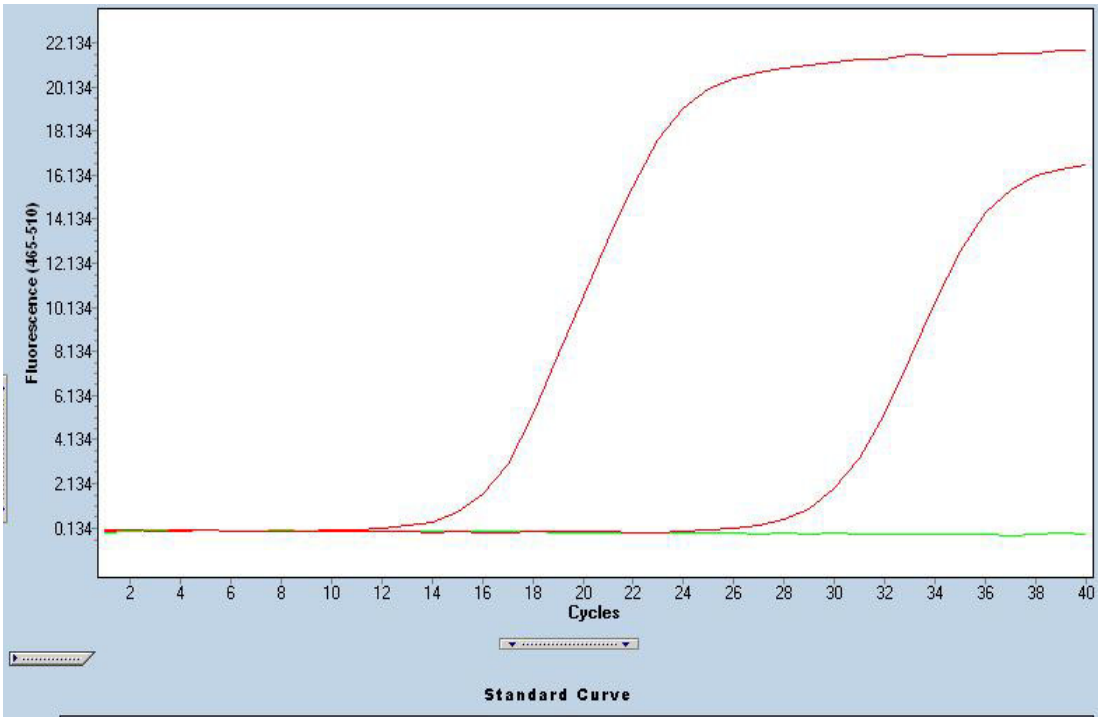

Figure 1
Amplification curves for positive control (Cp value:16.12), test sample (Cp value:29.80) and negative control as seen in Roche real time PCR, light cycler II (LC 480).

4. DISCUSSION AND CONCLUSION

The above study is an attempt to find out the prevalence of *Mycoplasma pneumoniae* in a tertiary care centre of Navi Mumbai. The method used for the prevalence study is real time PCR assay which is a rapid, more sensitive and less laborious method. The main aim behind this assay was to standardize the real time PCR so that a shift to a rapid method from the traditional culture, serology and conventional PCR method could take place. In this study we have found a 16% prevalence of *Mycoplasma pneumoniae* among the patients. This matches the results of Kashyap *et al.* who reports a 17.33% prevalence using PCR as a method of analysis.[6]Chaudhry *et al.* in their study used qRT-PCR assay and reported 19.4% prevalence.[7] The use of real time PCR assay has an advantage over the conventional PCR due to the higher sensitivity of the technique. Chaudhry *et al.* also reported that in comparison to conventional PCR the qRT-PCR detected an additional positive case.[7] Tempelton *et al.* reported 12 (11%) positive results by qRT-PCR assay for *M. pneumoniae* infection in a group of 106 patients. [8] Other studies have shown 20-21 per cent positivity by qRT-PCR assay.[9,10] These results are close to our study giving us 16% prevalence of *M. pneumoniae.* The total time consumed by the entire real time PCR assay in our study is one hour and 35 minutes. This includes 20 minutes for DNA extraction and 1hour and 15 minutes for DNA amplification.The time required for *Mycoplasma pneumoniae* identification is relatively very less as compared to the conventional PCR method. Also the conventional PCR requires additional down-streaming process of the amplified products. In addition to this the real time PCR assay is less labour intensive. Real time PCR assay can detect *M. pneumoniae* infection early in the course of disease.The method can be used to rule out infection with *M. pneumoniae* when the samples are tested for new emerging respiratory pathogens.The assay may as well as quantitate bacterial load and help to initiate

specific antibiotic therapy Supplementary studies in this area to estimate the bacterial load in a sample is promising and worth undertaking.

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