MYCOPLASMA PNEUMONIAE IN CHILDREN WITH PNEUMONIA AT MBAGATHI DISTRICT HOSPITAL, NAIROBI

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ABSTRACT

Objective: To determine the prevalence of *myocoplasma pneumoniae* in nasopharyngeal aspirates of children under five years of age sufferning from pneumonia.

Design: Cross-sectional survey.

Methods: Two primer sets targeting the genes coding for adhesion protein and 16S rRNA were used in PCR and *M. pneumoniae* specific antibodies were detected using commercial article agglutination kit. Microbiological investigations to isolate common acute respiratory infectious pathogens were also carried out.

Results: M. pneumoniae was detected in nasopharyngeal aspirates of 33.7% of the cases by PCR while serology was positive in only 4.1%. *M. pneumoniae* alone (single detection) was detected in 25% of the cases while 75% occurred with other acute respiratory infectious (ARI) pathogens. However, the results did not correlate with clinical outcome or the severity of pneumonia. No significant aetiology was found in 28% of the cases investigated, however microbiological investigations by culture revealed the presence of other aetiological agents as follows: *Streptococcus pneumoniae* (26%), *Klebsiella pneumoniae* (1%), *Staphylococcus aureus* (3%), *E. coli* (2%), parainfluenza viruses (5%), *Salmonella*(1%), adenovirus (4%), RSV (22%) and *Candida spp*(13%), *Mycoplasma pneumoniae* was more prevalent in children aged between six months and three years. Cases of *M. pneumoniae* PCR positive and *S. pneumoniae* exhibited similar seasonal distribution with peaks in May and September. However, there was no relationship between *M. pneumoniae* PCR positive and the severity of pneumonia.

Conclusion: More investigation is required to establish the significance of atypical pathogens in respiratory infections in Kenya.

INTRODUCTION

Mycosplasma pneumoniae as one of the causative agents of primary atypical pneumonia is responsible for other respiratory conditions such as tracheobronchitis, bronchitis and less severe upper respiratory tract infections in children(1). Trancheobronchitis and increased airway hyper-responsiveness are the most common clinical manifestations of *M. pneumoniae* infections(2). The ability of some mycoplasmas to modulate host immune responsiveness contributes to their pathogenic properties, enabling them to evade or suppress host defense mechanisms and establish a chronic, persistent infection. The ability of mycoplasmas to stimulate as well as suppress lymphocytes in a non-specific, polyclonal

manner could lead to exacerbation of acute respiratory infections (ARI) by other respiratory pathogens. Diagnosis of *M. pneumoniae* by culture is slow although isolation of the organisms is clinically significant. Other diagnostic tests such as fourfold rise in antibody titer in serum in the complement fixation tests are only suggestive because of cross-reaction with other *Mycoplasma spp*. Detection by PCR is gaininng more acceptance due to its high sensitivity and specificity. Although most cases of *mycoplasma pneumoniae* infection are mild, diagnosis of the organism is vital to avoid unnecessary administration of ineffective antibiotics. *Mycoplasma pneumoniae* is not susceptible to β-lactam antibiotics commonly used empirically to treat respiratory tract infections. Therefore, diagnosis

of these infections is important for initiation of appropriate therapy. The present study was an attempt to determine the prevalence of *M. pneumoniae* by PCR in nasopharyngeal aspirates of children under five years with pneumonia.

MATERIALS AND METHODS

Subjects: Between February and November 1998, ninety eight nasopharyngeal aspirates were obtained from children aged under five years presenting at an out-patient clinic at Mbagathi District Hospital, Nairobi. The patients were recruited based on World Health Organisation (WHO) definition of acute respiratory infections (ARI). The severity of pneumonia was categorised into three as defined by WHO criteria (i) mild (fever and cough), (ii) severe (fever, cough and chest in-drawing), (iii) with very severe (chest in-drawing with either convulsion or inability to feed)(2).

Specimen collection and microbiological investigations: Nasopharyngeal aspirates were collected for bacterial, viral and fungal detection at the Centre for Respiratory Diseases Research and Centre for Virus Research, Kenya Medical Research Institute. The aspirates were collected with a mucus extractor and delivered to the laboratory where the specimens were kept at 4°C and processed within three hours.

Bacterial, viral and fungal investigations: The aspirates were inoculated onto Sheep Blood Agar, Bromothymol Blue Agar (BTB), Chocolate Agar and Sabouraud's Dextrose Agar. Inoculated blood agar and chocolate plates were incubated in 5% CO_2 at 37°C, while BTB and Sabouraud's agar were incubated aerobically at 37°C. Identification of the organisms was done by methods described in Manual of Clinical Microbiology(4). Fungal identifications were done using Candida Check (Iatron Laboratories, Tokyo, Japan) and slide culture on Corn Meal Agar. Respiratory virus antigens in the specimens were screened by immunofluorescence asay (IFA), (DAKO IMAGEN™, Cambridgeshire, UK) which detects respiratory syncytial virus (RSV), influenza A and B viruses and parainfluenza virus types 1, 2, and 3). The individual virus was identified by IFA kit (DAKO IMAGEN™ for virus positive samples. After microbiological investigations, the remaining nasopharyngeal aspirates were preserved at -80°C for PCR.

PCR detection of Mycoplasma pneumoniae: Using filtered tips, 50 µl of nasopharyngeal aspirate was transferred into 200 µl of Tris EDTA pH 6.8 buffer and centrifuged at 13000xg for 15min at 4°C, 225µl of supernatant was discarded and an equal amount of sterile distilled water was added. The sample was boiled at 100°C for 10 min and centrifuged at 1000xg for 5 minutes at room temperature, 10 µl of the supernatant was used for PCR(5). Two primer sets were used, one primer set Mp-1 (5'-GAAGCTTATGGTACAGGTTGG-3') and Mp-2 (5'-ATTACCATCCTTGTAAGG-3') directed against the gene that codes for 16S rRNA with the target molecule of 349 base pairs. The second primer set P4a-P4b targets the gene coding for adhesion protein, with a target molecule of 345 base pairs. The sequence were P4-a (5-AGGCTCAGGTGAATCTGGCGTGGA-3') and P4-b (5 GGATCAAATAGATCGGTGACTGGGT-3'). The PCR was performed in a reaction mixture containing a total volume of 25µl using 'Ready-To-Go' PCR beads (Molecular Biology

Reagent, Pharmacia Blotech, St Albans UK)', µl 10µl sample and 13 µl sterile distilled water. The reaction mixture was subjected to 35cycles of amplification. The amplification products was subjected to electrophoresis in 1.5% agarose gels, and the presence of *M. pneumoniae* specific band was visualized with ultraviolet light after ethidium bromide staining. A sample was scored positive only if positive by both primers set. A negative and a positive control were included.

Contamination Precautions: Aerosol barrier pipette tips, separate areas of laboratory for master mix preparation specimen extraction, and specimen detection and the inclusion of multiple negative controls in each run were used to avoid cross contamination.

Southern blots hybridization: PCR products were subjected to electrophoresis in a 2% agarose, and transferred by capillary action to Hybond N+ nylon membranes. PCR probes were labeled by random priming with (α-^{32}P) dCTP (Ready-To-Go™) DNA Labeling Beads, Amersham Blotech Inc. Pisctaway, NJ, USA).

Sequencing: The DNA was sequenced by the dideoxy-chain termination method with the ABI prism BigDye Terminator Cycle sequencing Kit (Perkin-Elmer Applied biosystems, Norwalk, CT. USA) and an ABI prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

Serological detection: Determination of *M. pneumoniae* specific antibodies was performed using serodia-MYCO II kit (Fujirebio Tokyo, Japan). The procedures were according to the manufacture's recommendation. An antibody titre of $\geq$1:160 was considered a positive result.

RESULTS

To detect *M. pneumoniae* DNA in nasopharyngyeal aspirates from 98 patients with pneumonia, PCR was performed using 2 primer sets (Figure 1 and Table 1). The primers, P4a-P4b were specific to *M. pneumoniae* P1 adhesion gene and the other primers Mp1-Mp2 were specific to 16Sr RNA. Among 98 nasopharyngeal samples, the PCR products were amplified in 61 and 45 samples when P4a-P4b and Mp1 -Mp2 primers were used, respectively. The PCR products were confirmed by sequencing and identifying as *M. pneumoniae* by Southern blot analysis (data not shown). Thirty three samples (33.7%) were positive by both primers. These samples were therefore considered PCR positive in our investigations. A retrospective serological analysis however showed only four (4.3%) out of 71 cases with available sera had significantly high titres of $\geq$160 which is considered diagnostic for *M. pneumoniae* infection (data not shown). However, in 69 cases of which serum was available only during the acute and not convalescent stage, while in only eight cases, serum was available in both stages. In 27 cases, serum was not available. Although our study indicated that the results of PCR amplifications do not correlate with clinical *M. pneumoniae* infection, it does estimate *M. pneumoniae* exposure among the cases investigated.

Figure 1

Results of some of the PCR detection of Mycoplasma pneumoniae using two primers
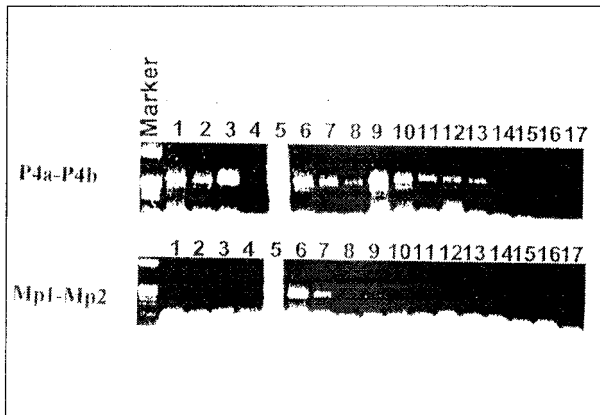
Mp1-Mp2: primer targeting 16S rRNA, P4a-P4b: primer targeting adhesion protein, lane 1-17 patient samples.

Table 1

Results of PCR detection of M. pneumoniae and serum titre levels

Detection		Positive	Negative
PCR	Primers		
	P4a-P4b	61	37
	Mp1-Mp2	45	53
	P4a-P4b/Mp1-Mp2	33*	
Serological	Titres	≥160	<160
		4	67
Others•		27	

Primer P4a-P4b targets adhesion protein, Primer Mp1-Mp2 targets 16S rRNA, * Samples positive by both primers, •Serum not available

The relationship between *M. pneumoniae* and *PCR* positive cases with *Streptococcus pneumoniae* and RSV since they were both the most frequently isolated pathogens in our routine bacterial and viral investigations from the same sample population was compared. Among 33 cases, *M. pneumoniae* PCR positive cases alone was detected in eight (25%) cases while coexistence with *S. Pneumoniae* and RSV accounted for seven and five cases, respectively (Table 2). No aetiological significance was established in 28% of the pneumonia cases investigated. However, routine microbiological investigation revealed the prevalence of other aetiological agents as follows, *Streptococcus pneumoniae* (26%), *Klebsiella pneumoniae* (1%), *Staphylococcus aureus* (3%), *E. coli* (2%), **parainfluenza**

viruses (5%), *Salmonella spp.* (1%), adenovirus (4%), RSV (22%) and *Candida spp* (13%). We analysed the severity of pneumonia based on WHO classification and *M. pneumoniae* PCR positive with other pathogens (Figure 2). There was no specific relationship between the severity of pneumonia and *M. pneumoniae* PCR positive cases or whether *M. pneumoniae* co-existed with other pathogens. Temporal distribution of PCR positive for *M. pneumoniae* samples and other pathogens is illustrated in Figure 3, cases of *M. pneumoniae* PCR positive were high in May and September. Similarly, *S. pneumoniae* had also two peaks during the same months as *M. pneumoniae* PCR positive. RSV, however, had only one peak between May and September. These results showed that *M. pneumoniae* PCR positive and *S. pneumoniae* exhibit similar seasonal distribution.

Table 2

Isolation frequency of Mycoplasma pneumoniae and other respiratory pathogens

Type of detection	Co-isolated pathogen	No. of cases (%)
Single	None	8(25)
Dual	*S. pneumoniae*	7(21.9)
	RSV	5(15.6)
	Other organisms	8(25)
Triple or more	Other organisms	5(12.5)

RSV = Respiratory syncytial virus

Figure 2

The severity of pneumonia in relation to Mycoplasma pneumoniae detection and other respiratory pathogens
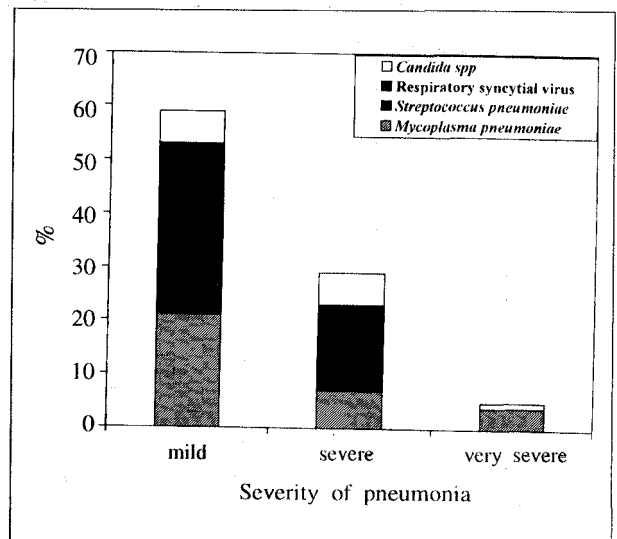
DISCUSSION

The maximum detection of aetiological agents in acute respiratory tract infection is about 80% which means 20% of the infections in children remains undiagnosed(5). In the present study, the aetiology of pneumonia in 28% of the cases was undiagnosed. Although these may be attributed to previous administration of antibiotics, the pneumonia could be due to other agents not isolated in routine microbiological procedures.

The global incidence of *M. pneumoniae* pneumonia in paediatric population has been reported to be between 3.5% and 11%, but differs considerably with age and clinical conditions. A prevalence rate of 29.2% had been reported in Belgium and 30-40% in Munich(5,6). It is reported to be the third most commonly detected agent after RSV and influenza virus(7-9). In Kenya, the prevalence of *M. pneumoniae* and its significance in respiratory infection has not been previously investigated. In the current study, a prevalence of 33.7% in our study population was detected by PCR. Higher sensitivity and clearly visible bands were found by PCR detection when Mp1-Mp2 primers were used than when 16S rRNA primer was used. This is probably due to multiple copies of the P1 sequence in the genome(5).

Figure 3

Temporal distribution of Mycoplasma pneumoniae (a) and other Pathogens (b) by month between February and November 1998
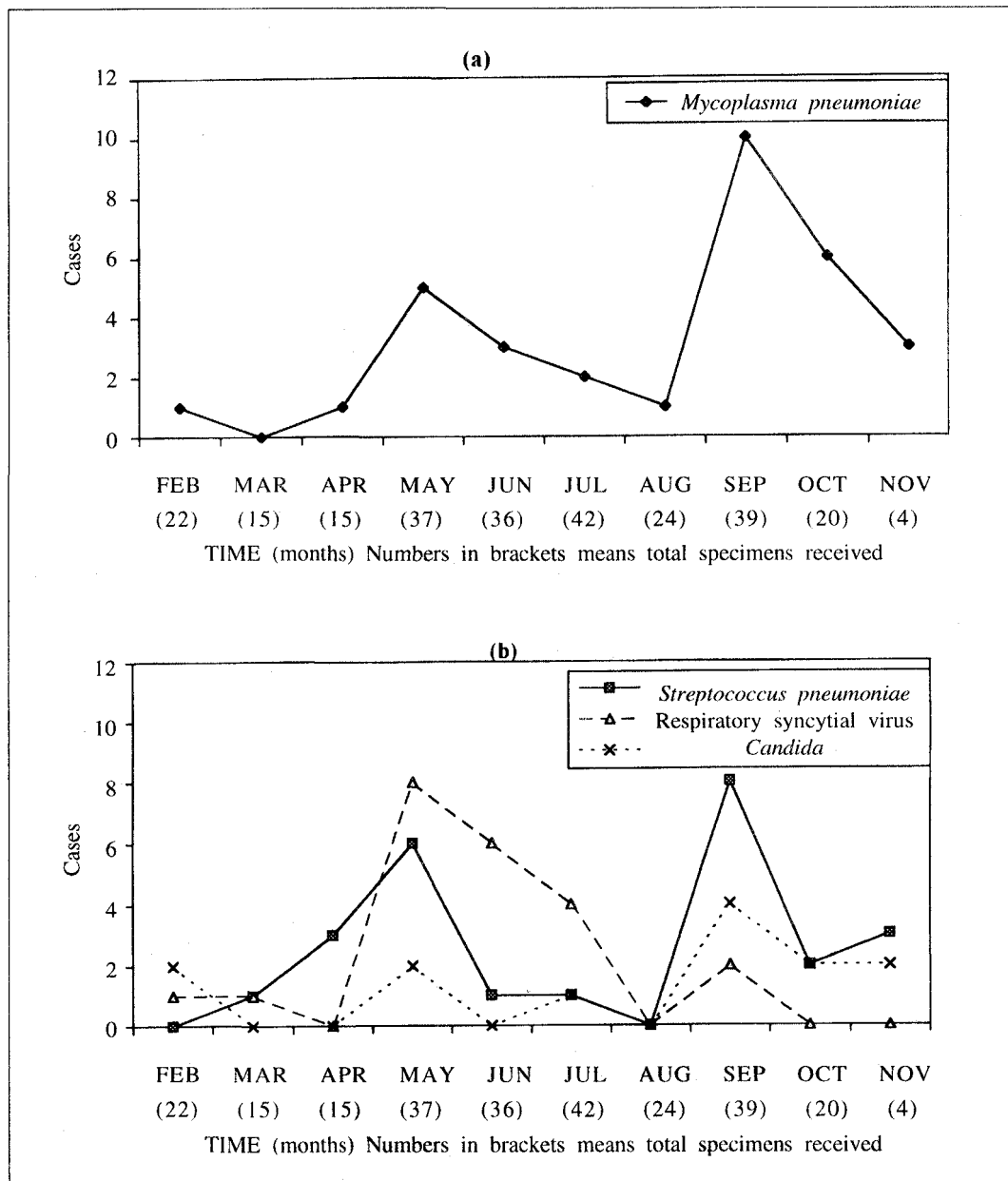
Results of serological detection did not correlate with PCR detection probably because PCR is not only sensitive but can detect the presence of DNA as a result of past infection, while serology detect very recent and present infections which manifest clinically. In our study serology detection was also disadvantaged due to unavailability of paired serum taken at 2-3 weeks intervals. Although PCR results did not correlate with serology or clinical manifestation, it does indicate the presence of and high exposure to *M. pneumoniae* in children below five years in Nairobi. Although a carrier state could be suggested in this case, work done by Tjhie *et al* (10) and Foy(11) has shown that only 2% possible carrier-state by PCR detection which means 30% PCR positive cases in our study is unaccounted for apart from a recent exposure. On the other hand, the inability of PCR to detect. *M. pneumoniae* in patients with good clinical resolution of initial infection or after appropriate therapy suggests that the organism is cleared from the oropharynxes and a carrier state is therefore unlikely(12). In a Kenyan situation where culture of *M. pneumoniae* is not practical and serum availability is not feasible, PCR detection method is an alternative as DNA amplification of throat and nasopharyngeal specimens has previously been used for the diagnosis of *M. pneumoniae* (12,13).

Mycoplasma pneumoniae has been documented as the third most common agent of pneumonia after *S. pneumoniae* and RSV. However, in the present study, it was the most prevalent followed by *S. pneumoniae* and RSV respectively. The sensitivity of PCR used to detect *M. pneumonie* cannot be compared with culture and immunoflouresce technique used to detect *S. pneumoniae* and RSV respectively.

In the present study, there was a significant difference between detection of *M. pneumoniae* alone (mono-detection) compared with co-existance of *M. pneumoniae* with other respiratory pathogens (P<0.05). Staugas and Martin(13) have reported secondary bacterial infections in children with proven *M. pneumoniae*. Therefore, there is a possibility that *M. pneumoniae* predisposes to other bacterial or viral infections that causes severe pneumonia. *M. pneumoniae* penetrates the mucocillliary blanket, causing epithelial cell damage and also reduce mucocilliary clearance leading to airway inflammation hence susceptibility to secondary infections. The detection of *M. pneumoniae* in the very severe cases, unlike *S. pneumoniae* and RSV, could also be incriminating as an agent predisposing to secondary bacterial infections. Although most cases of *M. pneumoniae* are mild, subtle pulmonary function abnormalities have been noted several years after *Mycoplasma* infection(14). We could not address this in our study because most of the cases of pneumonia were mild. Future investigations will focus on more severe cases. Most of the *M. pneumoniae* positive cases were aged between six months and three years. However,

Ieven *et al* (5) demonstrated that *M. pneumoniae* is more prevalent in children above two years. Although according to Paroth *et al* (7), there is no seasonality for *M. pneumoniae* infection. In the present study high prevalence of *M. pneumoniae* and *S. pneumoniae* was noted in May and September. These months represent the cold and wet season in Nairobi, which could have influenced the distribution. Temporal distribution was also reflected on the high number of pneumonia patients recruited during the same period from the same patient catchment area (data not included). Although several authors have reported carriage of *M. pneumoniae* after asymptomatic infection, others report it as a resistant harmless organism(11).

The results from our investigation reveal a high exposure rate to *M. pneumoniae* among children below five years in Mbagathi Hospital. In a developing country like Kenya where severity and outcome of pneumonia is complicated by malnutrition, malaria and recently HIV, the role of *M. pneumoniae* in exacerbation of respiratory infections requires further investigation.

ACKNOWLEDGEMENTS

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