

Prevalence of *Mycoplasma pneumoniae*: A cause for community-acquired infection among pediatric populaztion

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Abstract

Background: Atypical pneumonia caused by *Mycoplasma pneumoniae* is a leading cause of mortality among the pediatric age group.

Objectives: Our study was designed to know the prevalence of *M. pneumoniae* in children with community-acquired pneumonia and the involvement in the cytoadherence to the respiratory epithelium by *M. pneumoniae* using electron microscopy and immuno-gold labeling technique.

Materials and Methods: A total of 152 children of 1 month to 12 years of age of both sexes attending Hebei Provincial People's Hospital, Shijiazhuang, Hebei with diagnosed pneumonia were included in the study.

Results: Out of 152 children 84 (55.3%) were males, and 68 (44.7%) were females. The mean age of the patients in the control group (50 patients) was 18.5 ± 3 months with 31 (62%) males and 19 (38%) females. IgM antibodies against *M. pneumoniae* were positive in 84 (55.3%) males and 68 (44.7%) females. Out of 50 patients 9 (18%) were found to positive for IgM *M. pneumoniae* antibodies of which four (44.4%) males and 5 (55.5%) females were positive. Our study observed that the gold particles were clustered on the filamentous extension of the tip of the cells. Out of 152 serum samples subjected to particle agglutination assay 138 (90.7%) were positive 1:320 titer, 9 were >1:80 and 3 showed titer was >1:40.

Conclusion: We suggest that clinicians should consider empirical therapy of broad spectrum antibiotics therapy to cover these atypical pathogens to reduce the severity before obtaining the serological results. From our study, we also suggest electron microscopic and biochemical studies for better diagnosis of these pathogens.

Key words: Atypical, community-acquired pneumonia, electron microscope, gold labeling

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Introduction

Among children, respiratory disease like pneumonia is the heading reason of death^[1-3] with an expanding caution in recent years, chiefly in developing countries.^[4] Primary atypical pneumonia caused by *Mycoplasma pneumoniae*, which is known to cause a variety of infections, especially respiratory tract infection and also leads to complication involving central nervous system.^[5] Community-acquired pneumonia (CAP) causing bronchial asthma and chronic obstructive pulmonary

disease by *M. pneumoniae* is about 20–30%.^[6-9] In children infected with *M. pneumoniae* has a different clinical presentation appears mostly as asymptomatic conditions in the higher percentage of cases.^[10-13] *M. pneumoniae* is the most common pathogen causing CAP among children.

Mycoplasma pneumoniae a small self-replicating pathogen that is lacking a cell wall with a genome size of 800 bp. It

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has a unique terminal tip made of protein, which facilitates the organism to get adhered to respiratory cells, which in turn paves way for the establishment of infection.^[5,14-17] The cytoadherence of *M. pneumoniae* is a multifactor supported event with the presence of P1 cytoadhesion in high concentration of terminal tip organelle in a wild strain of *M. pneumoniae* present only on the surface of *Mycoplasma*.^[18-23]

In acute respiratory infection, diagnosis is essential for providing a proper treatment, especially in the selection of antibiotics if the infection is community-acquired. The particle agglutination (PA) test is an improved indirect hemagglutination test. Gelatin coated carrier particles are used rather red blood cells from animals to avoid any nonspecific reaction.^[24]

Our study was designed to know the prevalence of *M. pneumoniae* in children with community-acquired pneumonia and to study the protein MPN474 and some high molecular weight proteins involved in the cytoadherence of *M. pneumoniae* using electron microscopy and immuno-gold labeling technique. MPN474 a paralogous protein, which plays an important role in the formation of terminal tip, which in turn is involved in adherence of *M. pneumoniae* to target cells. Our study intends to throw a new insight on the pathogenicity and biology of *M. pneumoniae* with a view of providing an effective alternate key to target *Mycoplasma* infected individuals.

Materials and Methods

Study design

A total of 152 children attending Hebei Provincial People's Hospital, Shijiazhuang, Hebei with diagnosed pneumonia under the age group of 1 month to 12 years of both sexes were included in our study. All the parents/guardians were informed about the study and after obtaining their consent. The institutional ethical committee approved the study design. Fifty children with complaints of lower respiratory infection, but negative for *M. pneumoniae* by IgM Enzyme linked immunosorbent assay were included as a control group in our study.

The pediatric population included in the study was based on few inclusion criteria such as: Symptoms of any respiratory infection, fever, abnormal X-ray of the chest according to World Health Organization criteria was recorded. Those on previous antibiotic therapy were excluded from the study. All the demographic details were taken from the hospital record.

Paired serum samples obtained within 2 weeks interval to test for the presence of IgM and respiratory sputum samples were collected for culture of *M. pneumoniae* infection.

Growth condition

The sputum samples collected were inoculated in Hayflick medium along with 1% agar with antibiotics penicillin (500 units/ml), amphotericin (5 µg/ml) Difco Laboratories Ltd., and incubated at 37°C in anaerobic condition. Further maintenance was done in tissue culture flask with Hayflick medium.^[25] Those strains with transposons were identified using isogenic mutant derivative GPM70 and they were maintained in the presence of gentamicin. Using phosphate-buffered saline (PBS) solution the attached cells are scrapped and extracted for further work.^[26]

Harvest of *Mycoplasma pneumoniae* cells

After incubation, the medium was discarded, and the attached cells were washed and scrapped in 1 ml PBS solution. This freshly harvested cell was stored in ice. Cells are also stored in glutaraldehyde and formaldehyde at a concentration of 3% and 0.25% respectively. The cells cannot be stored for > 10 days.^[26]

Labeling using immuno-gold technique

Mycoplasma pneumoniae wild strains were grown in log phase are taken. After incubation, the cells are scrapped off into Hay-flick medium prepared freshly. The cell aggregates are dispersed using acro-disc filter (Pall Life Sciences, Ann Arbor, USA) with pore size of 1.25 µm with 18-gauge needle. The cell suspensions were suspended in ultraviolet sterilized tissue culture plates, which are coated with carbon, and formvar coated 400-mesh nickel grids. The cells were incubated at 37°C for 4 h for attachment of cells to grids. After incubation, they were removed and washed with PBS gently, and later fixed.^[27]

Labeling of whole cells with immuno-gold method

Mycoplasma pneumoniae wild type strains are grown on the grid of the electron microscope and then washed thoroughly with PBS. The cells are harvested using Tris HCL 20 mM (pH 7.5) with 2% triton X-100.^[27] The grids are soaked in the water bath at 37°C for half an hour. After incubation, it is taken out and washed with PBS and fixed for a minimum of 45 min and immuno-labeled.

Scanning electron microscope

Sterile glass cover-slips are taken and wild type strain of *M. pneumoniae* are made to grow and extracted using the triton X-100 and fixed. Using liquid CO₂ and gold sputter coating the cells are dried and viewed at 20 keV of the electron microscope.^[27]

Particle agglutination assay

The test was done following the manufacturers guidelines. Agglutination assay is a semi-quantitative method using crude antigen coated gelatin particle of *M. pneumoniae* (Serodia-Myc II Fujirebio Inc., Japan). Serum samples were diluted with serum diluents up

to dilution from 1:20 to 1:320 and incubated at room temperature for 3 h. Extensive ring formations are considered as positive whereas button formation is considered as negative. A titer of >1:40 are considered as positive for the test.^[28]

Enzyme linked immunosorbent assay

The blood was allowed to clot and centrifuged at 3000 g at 4°C for 10 min in an Anke LXJ-IIB centrifuge (Anting Scientific Instrument Factory, Shanghai, China). The serum was separated and stored at -20°C till the time of use. The presence of *M. pneumoniae* IgM was determined using a *M. pneumoniae* specific immuno-gold filtration assay according to the manufacturer's instructions (Kanghua Biotech, Weifang, China).

Statistical analysis

Statistical analysis was done using SPSS software version 22.0. Mean standard deviation was calculated, and the data were analyzed using ANOVA. Statistical significance at $P > 0.01$ was used to for inference deductions.

Table 1: Demographic details of the patients included in our study

Characteristics	Children's infected with <i>M. pneumoniae</i> (n=152)	Control group (n=50)
Mean age (months)	20.4±5	18.5±3
Fever (°C) ww	39±0.2	38±0.1
Cough	4.3±1	5.2±0.4
Wheezing %	40	28.2
Tachypnea	1.5±0.5	2.4±0.3
Duration of fever	4.8±0.7	5.7±0.4
Number of days stayed in hospital	10	14
Number of male (%)	84 (55.3)	31 (62)
Number of female (%)	68 (44.7)	19 (38)

M. pneumoniae=*Mycoplasma pneumoniae*

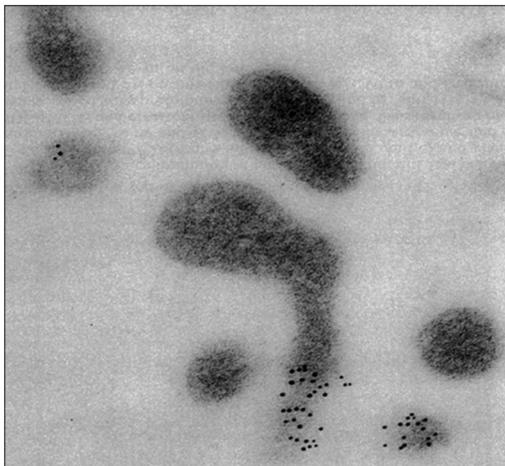


Figure 1: Micrograph of Immuno electron microscopy of *Mycoplasma pneumoniae* with immuno-gold labeling technique

Results

All the Demographic details of the patients were collected and showed in Table 1. The mean age of the patients is 20.4 ± 5 months (1 month to 12 years). Out of 152 children included in our study 84 (55.3%) were males, and 68 (44.7%) were females. Out of 50 control group patients the mean age was 18.5 ± 3 months with 31 (62%) are males, and 19 (38%) are females [Table 1].

Serological detection of *Mycoplasma pneumoniae* antibodies

IgM antibodies against *M. pneumoniae* were found to be positive in 84 (55.3%) in males and 68 (44.7%) of females. Whereas in the control group out of 50 patients only 9 (18%) were found to be positive for IgM antibodies against *M. pneumoniae*. In which 4 (44.4%) were males and 5 (55.5%) females were detected for the presence of antibodies [Graph 1]. Patients who are infected with *M. pneumoniae* 48 (31.6%) had wheezing problems compared to control group children only 14 (28%) had wheezing along with other respiratory infections. There was a statistical difference of $P < 0.01$ when analyzed. The duration of hospital stay is less in patients infected with *M. pneumoniae* than the control group patients with average hospitalization of 10 days in *M. pneumoniae* infected and 14 days in control group [Graph 2]. However, we have no morbidity reported either in study group or control group population. 5 (10%) children's in the control group developed respiratory failures and admitted in Pediatric Intensive Care Unit.

Immuno-gold labeling

The micrograph of immuno-gold labeled wild type strain of *M. pneumoniae* shows some salient features when incubated in 1:1000 dilutions [Figure 1]. In our study, we observed firstly the gold particles were clustered on the

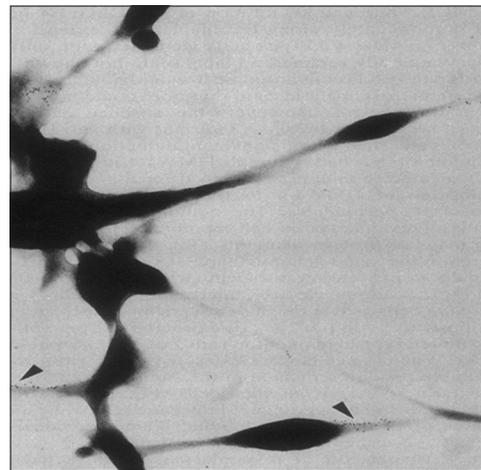


Figure 2: Immuno-gold labeling of *Mycoplasma pneumoniae* cells showing the clustered gold particles of the filamentous region (arrow mark indicated)

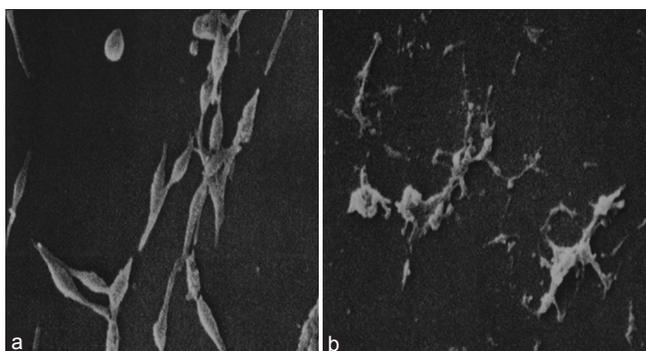
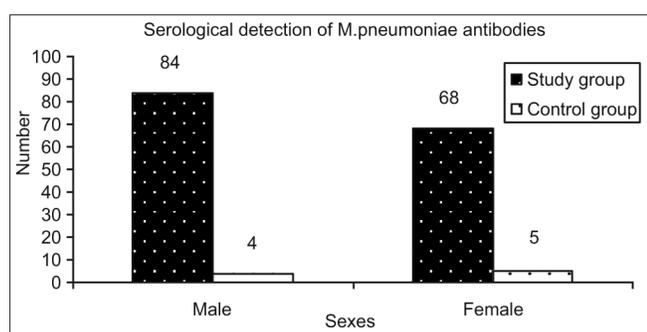
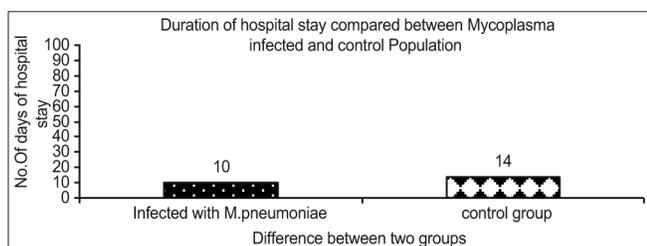


Figure 3: (a) Micrograph of scanning electron microscope showing the wild type *Mycoplasma pneumoniae* cells before extraction with Triton X-100 (b) after extraction with triton X-100 shows the dissolution of the membrane



Graph 1: Serological detection of *M.pneumoniae* antibodies



Graph 2: Duration of hospital stay compared between *Mycoplasma* infected and control group

Table 2: Positive titre for *M. pneumoniae* antibody by particle agglutination assay

Total number of samples (n=152)	Particle agglutination assay
138	>320
9	>80
3	>40

M. pneumoniae=*Mycoplasma pneumoniae*

filamentous extension of the cells. Secondly, they appear as small spherical cells sections according to the cell orientation at the time of sectioning. We also observed most of the gold particles appeared interior of the cells and only few were seen on the periphery. Few drawbacks are labeling and is limited to only a particular number of cells in a given field.

Figure 2 shows gold particles clustered mostly on the surface of filamentous extensions. Few labeled cells were seen on the surface of the cells. The cells of *M. pneumoniae* grown on grid of electron microscope, which are extracted using triton X-100, are examined using immuno-electron microscopy also shows presence of clustered gold particles along the extension of the filaments in cells [Figure 3a]. The cells that are extracted with Triton X-100 showed more exposed epitopes based on the intensity of labeling [Figure 3b].

Particle agglutination assay

Out of 152 serum samples subjected to PA assay 138 (90.8%) were positive with titer > 1:320. In 9 patients the titer of > 1:80 were observed and 3 patients the titer was > 1:40. No false positive results were obtained. The test was done in duplicates with controls. All controls were found satisfactory [Table 2].

Discussion

Atypical pathogens are the important cause for increased mortality in children affected with pneumonia.^[11,29] Other studies show a prevalence of 6–40% of pneumonia in children. In a recent study on atypical pathogens, 48% of the children’s were infected with *M. pneumoniae*.^[11] In our study, we reported 44.7% of *M. pneumoniae* in children. Our study also reports 40% of children’s infected with *M. pneumoniae* has wheezing compared to Prapphal et al.^[11] study which reported 38% of children has wheezing infected with *M. pneumoniae*, which is slightly less, compared to our study.

Labeling of whole cells of *M. pneumoniae* with antibody mostly seen clustering to filamentous extensions of the cells especially, near the tip region *M. pneumoniae* cells has filamentous tip which are proved by many morphological studies one of them is the study of the protein MPN474 which plays an important role in cytoadherence of the cells during infection.^[30,31] From our study Figure, 2 also supports the previous studies that immuno-gold labeling shows that gold particles are attached mostly to the filamentous tail.^[30,31] Cytoskeletons like structure are drawn between *M. pneumoniae* and eukaryotic cells especially the cells that are extracted with triton X-100.^[32-34]

The PA assay has best specificity and sensitivity. A PA titre of >1:160 has been reported as greatest diagnostic power among children’s.^[35] Our study we have reported a PA titer of 1:320 in 138 (90.7%) *M. pneumoniae* patients. Choi et al.^[36] had evaluated antibody titer for *Mycoplasma* with titer >320 which supports our study. We used Serodia MycoII, which have sensitivity of 90.7% were found to be positive with 1:40 as cutoff by the kit manufacturers.^[36] MPN474 was found essential for cytoadherence, even the mutant strain GPM70 was able to grow and adheres to both

glass and plastic like the wild-type strains of *M. pneumoniae* under laboratory conditions, which plays a role in adhesion of the bacterium which differs from other studies where MPN474 was not considered essential for adhesion.^[37]

Conclusion

Our study, suggest that antibiotics treatment should be started at the earliest to reduce the morbidity rate and to decrease the duration of the respiratory symptoms. We suggest the clinicians to start an empirical therapy of a broad spectrum to cover these atypical pathogens to reduce the severity before obtaining the serological results. From our study, it is clear that more electron microscopic studies and biochemical studies are required for better understanding of these pathogens.

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