RESEARCH ARTICLE

Association of *Mycoplasma pneumoniae* and asthma among Indian children

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Abstract

The role of *Mycoplasma pneumoniae* infection as a trigger for asthma exacerbations is well supported in previous studies. This study was designed to investigate the role of *M. pneumoniae* infection in acute exacerbation of asthma in children. A total of 150 patients (110 males, 40 females) were studied and immunoglobulin M (IgM) antibodies to *M. pneumoniae* were detected by enzyme-linked immunosorbent assay (ELISA), and PCR amplification was performed for the P1 gene to associate *M. pneumoniae* infection with asthma. As compared with 33 children with asthma, only two of the control subjects had positive IgM titers for *M. pneumoniae*, which was statistically significant ($P = 0.002$). A total of 15 children with asthma were positive by PCR for the P1 gene while none of the controls had a positive PCR. Of these positive cases, 24 cases were positive only by ELISA, six were positive only by PCR and nine patients were found to be positive by both ELISA and PCR. All the clinical characteristics of the patients at baseline were comparable between the moderate and the severe group of patients statistically, except for the peak expiratory flow rate. *Mycoplasma pneumoniae* infection was found to have a significant association with acute exacerbation in the moderate group of asthma patients by PCR ($P = 0.01$). These data suggest that *M. pneumoniae* infection may contribute to asthma exacerbation.

Introduction

Asthma is a syndrome characterized by bronchial hyperresponsiveness and reversible airflow obstruction, which produces recurrent respiratory symptoms in the form of shortness of breath, cough and wheeze. Several factors such as genetic, environmental, dietary change and occupational exposure are known to predispose to asthma (Kino & Oshima, 1989; Beckett, 1994; Homnick & Marks, 1998; Cengizlier & Misirlioglu, 2006; Maclennan *et al.*, 2006). Numerous viral infections trigger acute asthma exacerbations. Viral infections such as rhinovirus, coronavirus, influenza virus, adenovirus, parainfluenza virus, respiratory syncytial virus and human metapneumovirus have been found to be observed more often during severe exacerbations of asthma than during mild exacerbations (Johnston *et al.*, 1995; Reed *et al.*, 1997; Marin *et al.*, 2000). In addition to these, the role of *Mycoplasma pneumoniae* infection in the pathogenesis of asthma was speculated around 30 years ago (Berkovich *et al.*, 1970). Recently, the role of infectious agents, in particularly the atypical bacteria, *Chlamydophila pneumoniae* and *M. pneumoniae*, in asthma pathogenesis has become an active area of investigation. *Chlamydophila pneumoniae* and *M. pneumoniae* are the pathogens that primarily infect ciliated epithelial cells and alveolar macrophages (Grayston *et al.*, 1990; Dallo & Baseman, 2000). Considerable data exist implicating these organisms in the exacerbation of asthma (Huhti *et al.*, 1974; Leaver & Weinberg, 1985; Seggev *et al.*, 1986; Kondo *et al.*, 1994). Experimental infection of mice with *M. pneumoniae* induced inflammation and increased air resistance (Hardy *et al.*, 2001). These effects appear most clearly in mice previously sensitized to an allergen and subsequently infected with *M. pneumoniae* (Martin *et al.*, 2001). Even though the progression of asthma and chronic obstructive pulmonary disease has been correlated with persistent infection with

M. pneumoniae based on seroprevalence studies in children with asthma (Freymuth *et al.*, 1999; Lieberman *et al.*, 2001), the exact role and incidence of *M. pneumoniae* in acute asthma exacerbation in pediatric cases is still poorly understood. The clinical diagnosis of *M. pneumoniae* infection is frequently hindered by a lack of specific signs and symptoms, although in the majority of cases increasing cough, malaise, low-grade fever and headache have been reported. The lack of a gold standard for the diagnosis of this pathogen still hinders the current understanding of their true prevalence and role in the pathogenesis of acute and chronic respiratory infections. While molecular diagnostic techniques, such as PCR, offer improvements in sensitivity, specificity and rapidity over culture and serology, the need remains for a consistent and reproducible diagnostic technique, available to all microbiology laboratories.

In India, as in other developing countries, acute respiratory tract infections are still one of the major causes of childhood morbidity and mortality. However, no epidemiological data are available on the incidence or the association of *M. pneumoniae* with asthma in India. We therefore sought to determine the role of *M. pneumoniae* in acute exacerbation among asthmatic children in an Indian population. The aim of the present clinico-microbiological study was to investigate the role of *M. pneumoniae* infection in the acute exacerbation of asthma in children aged 5–15 years with moderate or severe persistent asthma from India.

Materials and methods

Study group

The present study included 150 patients between 5 and 15 years of age, previously diagnosed as having moderate or severe persistent asthma (British Thoracic Society, 2003), and were on regular follow-up in the Pediatric Chest Clinic at the All India Institute of Medical Sciences, New Delhi, during the period November 2004–May 2006. Children were enrolled in the study after obtaining informed written consent from their parents. A detailed history of asthma symptoms, medications and recent exacerbation of symptoms were recorded in a predesigned performa.

Patients were divided into two groups according to their history of asthma symptoms and a pulmonary function test. Group-I included patients with moderate persistent asthma and group-II included children with severe persistent asthma. Clinically, children with moderate persistent asthma had daily symptoms, with the frequency of nighttime symptoms more than once a week, and required daily medication. However, children with severe persistent asthma had continuous symptoms in the form of frequent episodes of wheezing, coughing or shortness of breath requiring emergency treatment and even hospitalization. Many children with severe persistent asthma had frequent symptoms at night and only limited physical activity.

A child was considered to have controlled asthma if a visit to the emergency room was not required in the last 12 weeks, and asthma medications were either continued or stepped down after a careful assessment. A patient was considered to have uncontrolled asthma if he/she required at least one course of oral prednisolone or stepping up of the treatment after a careful assessment in the past 12 weeks. A patient was considered to have acute exacerbation if he/she showed an increase of symptoms over the past 1 week and peak expiratory flow rate (PEFR) measurement showed at least a 15% reduction from baseline PEFR. Human ethical clearance was obtained from the institute's ethics committee.

Control group

Fifty individuals with no history of asthma or other respiratory illness were included as controls in this study. A detailed history of asthma symptoms was obtained from all controls, along with a clinical evaluation. A history of present or recent symptoms of a sore throat was obtained from all controls. Control individuals having fever or other nonrespiratory illness came to the pediatric clinic of AIIMS. All controls with a history of asthma or recent respiratory symptoms were excluded.

Specimen collection

Blood was collected by a venipuncture with all aseptic precautions in a plain vial for immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) specific for *M. pneumoniae* (Serion IgM ELISA *Classic*, Germany). Throat swabs were collected from the posterior oro-pharyngeal wall from all the patients. Swabs were transported in pleuro-pneumonia-like organism (PPLO) broth medium for *M. pneumoniae* culture and PCR.

Microbiological analysis

Culture for *M. pneumoniae* was performed in PPLO broth and PPLO agar. Growth was monitored daily for 15–20 days. Even after no color change on PPLO broth, it was blindly subcultured onto a PPLO agar plate and monitored up to 1 month for the *M. pneumoniae* colonies. *Mycoplasma pneumoniae* standard strain FH-Lieu (procured from NCTC, London) was also maintained and used as a control with all the clinical samples. For the detection of other bacterial pathogens, culturing on MacConkey agar and blood agar was performed simultaneously.

Serology

IgM antibodies to *M. pneumoniae* were detected using the IgM ELISA kit (Serion IgM ELISA *Classic*). The test was performed according to the manufacturer's recommendations.

DNA extraction

PPLO broth inoculated with the clinical sample after an incubation of 72 h was used for DNA extraction by boiling. Briefly, 200 μL of broth was placed in microcentrifuge tubes and centrifuged at 16 000 ***g*** for 15 min to pellet the cells. The cell pellet was washed twice with phosphate-buffered saline (pH 7.2). After decanting the supernatant, the sediment was resuspended in 50 μL of sterile milli-Q water. The tube was placed in a boiling water bath for 10 min and then centrifuged for 30 s at 13 400 ***g***. The supernatant was taken out in a fresh microcentrifuge tube immediately and used for DNA amplification.

PCR amplification

Mycoplasma pneumoniae-specific DNA was detected with primers, as described previously, which were able to amplify a 543-bp fragment in the gene encoding the P1 adhesion gene (Bio-Basic, Canada) (Williamson *et al*., 1992). A positive control (FH-Lieu) and a negative control were systematically run in parallel. In the negative control, 5 μL milli-Q water was added instead of template DNA.

All the samples were also investigated by PCR for the P30 gene using oligonucleotides P30-PF (5′-ATG AAG TTA CCA CCT CGA AGA AGC-3′) and P30-PR (5′-TTA GCG TTT TGG TGG AAA ACC GGG TTG-3′) and further confirmed by sequencing (data not shown). The final volume of the PCR mixture (50 μL) contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs (MBI Fermentas), 10 pM of each primer, 1 U of Taq-polymerase and 5 μL of extracted DNA. The reaction was performed in a thermocycler (MJ Research). PCR runs consisted of an initial denaturation of 94 °C for 10 min, followed by 30 cycles of amplification, each at 94 °C for 30 s, 54 °C for 45 s and 72 °C for 1 min 20 s. A final elongation step of 10 min at 72 °C was carried out to ensure that the polymerization of every amplified fragment was completed.

Analysis of amplified samples

The PCR products were analyzed on a 1.2% agarose gel. Electrophoresis was run in 0.5 × TBE buffer, followed by ethidium bromide staining.

Statistical analysis

The descriptive analysis was performed for each of the variables. Quantitative variables such as age were summarized by mean and SD. Categorical variables were presented in numbers and percentage. In univariate analysis, the χ^2 test/Fisher's exact test was performed to determine the association of categorical variables. The Student *t*-test was used for comparison of means between the moderate and the severe group. These variables (sex, family history, controlled vs. uncontrolled, and acute exacerbation vs. no exacerbation) were considered to be significant in multiple logistic regressions. Multiple logistic regressions were used to determine the effect of variables on PCR. Considering PCR as the gold standard, the sensitivity, specificity, positive predictive value and negative predictive value were obtained for ELISA. *P*-values < 0.05 were considered to be significant.

Results

A total of 150 patients were studied. The baseline characteristics of the study population are described in Table 1. The mean age of the cases was 10.30 ± 2.863 years and that of the controls was 9.92 ± 3.625 years. The male/female ratio in patients (110 : 40) and controls (30 : 20) showed a male preponderance. There were no significant differences in age ($P = 0.525$) or sex ($P = 0.109$) between patients and controls.

ELISA for IgM for *M. pneumoniae* was found to be positive in 33 (22%) cases, whereas only 15 (10%) patients showed a positive amplification with P1 PCR. Culturing was also performed for all the samples; however, none of the samples was found to be positive. Of the ELISA- and PCR-positive cases, nine patients were positive for both *M. pneumoniae*-specific IgM and PCR. Twenty-four other cases showed a positive IgM, but negative PCR results. Six cases were positive only by PCR, but negative with specific IgM for *M. pneumoniae*. For the confirmation of amplicons, all the PCR-positive samples were also confirmed by another set of PCR i.e. the P30 adhesin gene, followed by sequencing to confirm the gene (data not shown). Only two of the control subjects had positive IgM titers for *M. pneumoniae* as opposed to 33 IgM-positive asthmatic children, which is statistically significant ($P = 0.002$). Likewise, none of the control subjects was positive by PCR for the P1 gene as opposed to 15 of the asthmatic children. Taking PCR as the gold standard, the sensitivity of ELISA was 60% (32.9, 82.5), the specificity was 82.2% (74.5, 88.1), the positive predictive value was 27.3% (13.9, 45.8) and the negative predictive value was found to be 94.0% (88.7, 97.9).

All the clinical characteristics of patients at baseline were comparable between 118 (78.7%) moderate and 32 (21.3%) severe group of patients (Table 2). The proportion (21.9%) of positive PCR in the patient group with severe asthma was significantly higher ($P = 0.028$) than the patient group with moderate asthma (6.8%), whereas no such statistical

Table 1. Baseline characteristics of study population

	Cases (150)	Controls (50)	*P* value
Number	150	50	
Age (mean ± SD) years	10.30 ± 2.863	9.92 ± 3.625	0.525
Sex (male : female)	110 : 40	30 : 20	0.109
IgM ELISA	33 (22%)	2 (4%)	0.002
PCR (P1 and P30)	15	0	0.024

Table 2. Baseline characteristics between moderate and severe persistent asthma patients

		Moderate persistent asthma ($n = 118$)	Severe persistent asthma ($n = 32$)	*P*-value
Age (mean ± SD)		10.06 ± 2.95	11.19 ± 2.32	0.048
Sex	M : F	86 : 32	24 : 8	0.988
Family history (%)	Yes	27 (22.9%)	6 (18.8%)	0.810
Age of onset of illness (mean ± SD)		67.97 ± 39.667	77.84 ± 39.145	0.212
PEFR (mean ± SD)		72.52 ± 19.6	61.00 ± 16.942	0.003
Status of asthma (%)	Uncontrolled	32 (27.1%)	13 (40.6%)	0.191
Exacerbation (%)	Acute exacerbation	38 (32.2%)	12 (37.5%)	0.725
Prednisolone received (%)		5 (4.2%)	4 (12.5%)	0.098
IgM ELISA for *M. pneumoniae*		26 (22%)	7 (21.9%)	0.985
P1 PCR for *M. pneumoniae*		8 (6.8%)	7 (21.9%)	0.028

Table 3. Multiple logistic regression for the effect of variables on PCR

Variables	Coefficient (B)	Odds ratio (Exp B)	Confidence interval (CI)	*P*-value
Sex (M/F)	1.227	3.412	(1.012, 11.507)	0.048
Moderate/severe	1.393	4.027	(1.196, 13.562)	0.025
Acute exacerbation/no exacerbation	2.014	7.490	(2.120, 26.460)	0.002

Logistic regression equation: $Y = (\text{pos} = 1 \text{ vs. neg} = 0) = -4.084 + 1.227 \times \text{sex} + 1.393 \times \text{mod/sev} + 2.014 \times \text{exacerbation}$.

significance was found in *M. pneumoniae* serology ($P = 0.985$). No correlation was found between moderate/severe persistent asthma with sex, family history, age of onset of illness and status of asthma. After Bonferroni correction, only PEFR% ($P = 0.003$) were found to be significant with the moderate/severe group.

Out of 150 children, 50 had acute exacerbation of asthma. In order to determine the role of *M. pneumoniae* in acute exacerbation of asthma, we further analyzed our data and found that 19 cases (38%) were positive by IgM ELISA ($P = 0.016$) and 11 cases (22%) were positive by P1 PCR ($P = 0.003$). Of these PCR-positive cases, eight patients were positive by both IgM ELISA and PCR.

PCR results were considered as the outcome of logistic regression. The multiple logistic regression results showed the three variables (sex, moderate/severe and acute exacerbation/no exacerbation) to be significant factors as shown in Table 3. The maximum odd ratio was seen in acute exacerbation, followed by severity of asthma and sex. Patients with acute exacerbations are at a higher risk (7.49 times) of being PCR positive than patients without exacerbation. In case of severity, the risk of severe persistent asthma is 4.027 times more in *M. pneumoniae*-positive PCR than moderate persistent asthma. However. in terms of the association with sex, females have 3.412 times more risk than males.

Discussion

Mycoplasma pneumoniae is a known pathogenic organism in acute respiratory tract infection and community-acquired pneumonia. The prevalence and association of *M. pneumoniae* in asthma is still not clear, especially in developing countries. To the best of our knowledge, this is the first kind of study in India. Initial studies have been conducted on *M. pneumoniae* in community-acquired pneumonia patients, acute respiratory distress syndrome, Guillian–Barre syndrome and in neurological disorders (Chaudhry *et al.*, 1998; Dey *et al.*, 2000; Pandey *et al.*, 2000; Gorthi *et al.*, 2006). Here, we investigated 150 cases of moderate and severe persistent asthma patients who visited the pediatric asthma clinic of the pediatrics department for the presence of *M. pneumoniae* infection by serology and PCR. This study confirms that infection with *M. pneumoniae* is frequently associated with acute exacerbations of childhood asthma as described earlier (Biscardi *et al.*, 2000; Daian *et al.*, 2000). Nonspecific parameters such as white blood cells, C-reactive protein and erythrocyte sedimentation rate (commonly used to measure the severity of the acute-phase response) were not correlated in this study as suggested by some studies (Isaacs, 1989; Korppi *et al.*, 1993), and they were not significantly useful for therapeutic decision making.

Mycoplasma pneumoniae can cause upper and lower respiratory tract infection in children and acute exacerbation of wheezing in asthmatic patients. Previous studies have shown a 20–50% prevalence of acute infection of *M. pneumoniae* in asthmatic children. According to a report by Kraft and colleagues, *M. pneumoniae* was detected by PCR in the airways of >55% of patients with chronic and stable asthma. There were no previous systematic evaluations of the presence of *M. pneumoniae* in the lower airways of individuals with chronic and stable asthma (Kraft *et al.*, 1998). Park *et al.* (2005) studied adults and found 11.1% *M. pneumoniae* positive using the indirect agglutination method (Serodia Myco II) as compared with 4.4% in the

control group. We found a relevant observation regarding the presence of *Mycoplasma* IgM in 22% of 150 children having asthma and only 4% of 50 controls, which is a statistically significant difference ($P = 0.002$). These data were supported by the identification of *Mycoplasma* in 15 of the asthmatic children and none of the controls by PCR, also a significant difference ($P = 0.024$). In a study of 119 children having exacerbations of known asthma, acute *M. pneumoniae* infection was found in 24 (20%) using IgM ELISA specific for *M. pneumoniae*. This study highlighted that *M. pneumoniae* may play a role in the onset of asthma in predisposed children and could be a trigger for recurrent wheezing (Biscardi *et al*., 2000). Chang *et al*. (2006) studied 126 patients with asthma exacerbation and detected a 36.5% rate of *M. pneumoniae* infection using the rapid cold agglutination test and IgM ELISA (Savyon, Ashdod, Israel). In our study, out of 50 children having acute exacerbation, 19 cases (38%) were found to be positive for *M. pneumoniae* infection compared with 14 (14%) positive cases having no exacerbation on the basis of IgM ELISA. Of these 19 cases, eight were also found to be positive by PCR, which confirms the presence of *M. pneumoniae* in these patients. We also compared IgM ELISA in the moderate and the severe group of patients; however, no such statistical significance was found in *M. pneumoniae* serology ($P = 0.985$).

For the molecular detection of *M. pneumoniae*, few studies based on PCR have been conducted. Teig *et al*. (2005) investigated nasal brush specimens and induced sputum from 38 children with stable chronic lung disease (asthma $n = 26$; chronic bronchitis $n = 12$) and 42 healthy controls for the presence of *M. pneumoniae* by nested PCR using primers for the ATPase operon gene. None of the controls, but 10.5% of the children, had a positive PCR for *M. pneumoniae*. Out of 26 asthmatic children, 7.6% and 5% of *M. pneumoniae* infection were found from nasal brush and induced sputum. Our study reveals 10% positivity by PCR using throat swabs and none of the controls were found to be positive by PCR, which correlates with the previous investigations. Freymuth *et al*. (1999) found *M. pneumoniae* to be positive in only three (2.2%) out of 132 nasal aspirates using PCR for the P1 gene in children with acute exacerbations of asthma. In our study, out of 50 children having acute exacerbation, 11 cases (22%) were found positive for *M. pneumoniae* infection compared with four positive cases (4%) with no exacerbation on the basis of PCR, that is statistically significant ($P = 0.0038$). On comparison of our PCR results in moderate and severe asthma, the proportion (21.9%) of positive PCR in the patient group with severe asthma was significantly higher ($P = 0.028$) than the patient group with moderate asthma (6.8%).

The association of *M. pneumoniae* infection with acute exacerbations of asthma was also determined in this study. Our results demonstrated the statistically significant association of *M. pneumoniae* infection with acute exacerbations by serology (0.015) as well as by PCR ($P = 0.013$) in the moderate group of patients. However, no such association was found with the severe group of patients ($P = 0.073$), but there was a trend, that could be explained by a few cases in the severe group as compared with the moderate group. Our study shows a significant association of *M. pneumoniae* infection with acute exacerbation of asthma in previously diagnosed asthma patients. The association of *M. pneumoniae* infections with asthma may play a role in the onset of asthma in predisposed children and could be a trigger for recurrent wheezing. The correlation of microbiological findings with the clinical status of the patients clearly shows the significance of *M. pneumoniae* in asthma exacerbation.

Multiple regression results were also obtained in this study, which showed three variables to be significant factors in *M. pneumoniae* infection. Patients with acute exacerbations were found to be at a higher risk of having *M. pneumoniae*-positive DNA, followed by severe persistent asthma and sex. These results clearly confirmed the association of acute exacerbation and severity with *M. pneumoniae* infection.

Although the experimental evidence has shown an association between lower airway infection with *M. pneumoniae* and chronic asthma, clinical data in this regard are still scarce. This is because of the poor diagnosis of *M. pneumoniae* infection. Culture is the gold standard for the diagnosis of *M. pneumoniae*. However, it is a very fastidious and a slow-growing organism, and takes 2–4 weeks to isolate. In this study, we did not find any culture positivity. This may be due to many reasons. First, the culture depends on the prognosis of the disease. This may lead to differences in disease diagnosis, as we collected samples from patients who were previously diagnosed as having moderate or severe persistent asthma and were on a regular follow-up at the pediatric chest clinic. Second, the patients are also likely to have received antibiotic treatment due to clinical manifestations. Third, *M. pneumoniae* DNA usually remains detectable by PCR in throat swabs even after 1–2 weeks of infection, although the culture cannot be detected due to a decline in the bacterial load. PCR is the most sensitive test available for identifying microorganisms. *Mycoplasma pneumoniae* can be detected by PCR in airways even when cultures and serological results are negative. It may also indicate the presence of an organism in the respiratory tract in lieu of negative serology (Kraft *et al*., 2002). Taking PCR as the gold standard, the sensitivity of ELISA was 60% (32.9, 82.5), the specificity was 82.2% (74.5, 88.1), the positive predictive value was 27.3% (13.9, 45.8) and the negative predictive value was 94.0% (88.7, 97.9). Therefore, both molecular and serologic diagnostic tests for *M. pneumoniae* infection with a clinical correlation can be used as a rapid marker to study its association with asthma. This study shows the importance of acute *Mycoplasma* infections

during exacerbations of asthma. The use of macrolide antibiotics should be reserved for children in whom there is a high suspicion of atypical pneumonia as well as to ameliorate acute exacerbation of asthma associated with atypical pathogens.

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