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Original Research

Atypical bacterial etiology of acute respiratory infections and clinical characterizations among Iranian children

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Abstract: Acute respiratory infections (ARIs) in children younger than 5 years of age are one of the leading causes of morbidity and mortality, particularly in developing countries. *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are prevalent causative agents of ARIs, worldwide. We sought *M. pneumoniae* and *C. pneumoniae* in respiratory samples from Iranian children with ARIs. From November 2014 to April 2015, respiratory samples of 150 children aged 1 month to 15 years old were screened for presence of *M. pneumoniae* and *C. pneumoniae*. Polymerase chain reaction (PCR) and culture methods were used to detect these bacteria in respiratory samples in the form of throat swabs and nasopharyngeal aspirates. A questionnaire containing demographic and clinical information has been filled up for all participants in this study. Our obtained data showed that out of 150 tested samples, 7 (4.7%) were PCR positive for *M. pneumoniae* and only one (0.7%) positive sample for *C. pneumoniae* was detected. However, none of the tested samples was detected *M. pneumoniae* using the bacterial culture method. All patients with ARIs due to *M. pneumoniae* showed up with sore throat and flu like symptoms. According to our data, PCR method is more sensitive than culture for detection of *M. pneumoniae.* With regards to our results, it appears that *M. pneumoniae* and especially *C. pneumoniae* were infrequent causative agents in our studied population.

Key words: *Mycoplasma pneumoniae*; *Chlamydophila pneumoniae*; ARIs; PCR.

Introduction

Acute respiratory infections (ARIs) are responsible for a significant number of morbidity and mortality among children worldwide (1), an overwhelming majority occurring in developing countries (2). The causative agents of these infections among children are poorly understood (3,4). The incidence of ARIs caused by atypical bacteria (*Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*) in children varies markedly around the world (5), so that account for 22% of community-acquired pneumonia and 5 to 10% of cases of tracheobronchitis, pharyngitis, laryngitis, and sinusitis (6). There are several reports which indicate the role of *M. pneumoniae* as the cause of ARIs in children and most commonly in school-aged children aged 5 to 15 years. Their Infections tend to occur in an epidemic pattern, every 3-7 years (5,7). On the other hand, *C. pneumoniae* as an obligate intracellular pathogen is associated with a number of diseases, including multiple sclerosis (8), coronary artery disease, sarcoidosis, and asthma, as well as ARIs (9,10).

Determining the etiology of these infections is very important; therefore, rapid and correct diagnosis of the causative agent is crucial to starting adequate therapy, minimizing of hospital admissions and shortening of hospitalization (4,5,11).

Laboratory methods for the diagnosis of *M. pneumoniae* and *C. pneumoniae* infection include conventional culture, serology, and nucleic acid amplification techniques such as PCR, etc (12). Recovery of *C. pneumoniae* is difficult from cell cultures, and diagnosis of *M. pneumoniae* based on culture is a time-consuming and relatively insensitive method (7). Therefore, nucleic acid-based methods as rapid and sensitive alternative tests are recommended for detection of these bacteria (5,13). As only few data on etiology of ARIs due to atypical bacteria such as *M. pneumoniae* and *C. pneumoniae* have been reported in our region, the aim of this study was to determine the frequency of the mentioned bacteria among children with ARIs using both the culture and PCR methods.

Materials and Methods

Study design

This prospective cross sectional study was performed on inpatient and outpatient children aged less than 15 years old with clinical symptoms of ARIs between November 2014 and April 2015 at Nemazee and Dastgheib Hospitals and Imam Reza Clinic in Shiraz, Southwest of Iran. The study protocol was approved by

the Ethics Committee of Shiraz University of Medical Sciences (EC=IR.SUMS.REC.1394.S109) and written informed consent was taken from the parents or legal guardians of children enrolled in the study.

Inclusion criteria were the presence of one of the symptoms of ARIs, including cough, sore throat, upper respiratory tract symptom (rhinorrhea and sneezing), tachypnea and wheezing. Exclusion criteria included antibiotic use in the previous 10 days and lack of written informed consent.

Demographic and clinical information was recorded in standardized data collection forms, including patient's age, gender, history of disease and respiratory signs and symptoms at the time of presentation.

Sample collection

Two types of respiratory specimens (throat swabs and nasopharyngeal aspirates) were collected by trained personnel using the standard procedures, and placed in transport medium (Pleuropneumonia Like Organism = PPLO broth). The specimens were kept at 4 ˚C for a maximum of 24 h and homogenized and divided into two aliquots prior to freezing at -80 ˚C. One sample containing the transport medium was used for culture of *M. pneumoniae*, while the other part was placed onto sterile phosphate buffer saline (PBS) and used for PCR analysis.

For culture of *M. pneumoniae*, respiratory specimens using the Millipore filter (0.45 µm) were inoculated onto PPLO broth (Difco Co., USA), including 20% horse serum (Razi vaccine and serum research Institute), 25% freshly prepared yeast extract (Merck Co., Germany) supplemented with 0.025% thallium acetate (Sigma Co., USA), 10 ml glucose 10% (Merck Co., Germany), 1000 u/ml penicillin G (Sigma Co., USA) and 1 ml 0.2% phenol red (Sigma Co., USA), and then incubated aerobically at 37 °C under 5% CO_2. Broth media were subcultured on PPLO agar, when the color changed to yellow. The expected colonies (with appearance of granular or fried-egg) were observed with 40× magnification after 2-5 weeks at regular intervals (11,14).

DNA extraction and PCR assay

DNA was extracted from 2 mL PBS containing respiratory specimen with a commercial kit (QiaAmp DNA Blood Mini Kit, Qiagen) using the 'blood and body fluid' spin protocol. The extracted DNA suspension was stored at -20 °C for further analysis. The integrity of DNA was confirmed by amplification of a β-globin as housekeeping gene, using the PC04/GH20 primers (15). PCR was performed for detection of *M. pneumoniae* and *C. pneumoniae* using the specific primers for *p*1 and *omp* 2 genes, respectively (16,17). PCR amplification of DNA was carried out in a final volume of 25 µl containing 2.5 mM MgCl2, 1mM dNTP, 2.5 mM PCR-buffer 1x (Fermentas, Lithuania), 0.5U of Taq DNA polymerase (Fermentas, Lithuania), and 1 mM of each primer.

The cycling program for *M. pneumoniae* and *C. pneumoniae* was performed as described before (16,17). The amplification products were evidenced through 1 % agarose gel staining with KBC power load dye (CinnaGen Co. Iran). The bands were visualized under UV-illuminator and photographed. In all PCR runs, *M. pneumoniae* ATCC 15531 was used as positive control.

Statistical analysis

SPSS version 21.0 (SPSS Inc., Chicago, USA) was used for the statistical analysis. The association between categorical variables was assessed using the Chi-square or Fisher's exact test. Statistical significance was regarded as P value < 0.05.

Results

Population studied

The study subjects consisted of 150 patients with ARI, with a male: female ratio of 88:62 and a median age (±SD) of 3±2.5 years ranging from 1 month to 15 years old. Out of 150 participants, 59 (39.3%) and 91 (60.7%) were inpatients and outpatients, respectively. The patients' demographic and epidemiological characteristics are shown in Table 1. The respiratory samples were included 112 (74.7%) throat swabs, 36 (24%) nasopharyngeal aspirate and 2 (1.3%) sputum. Figure 1 shows the overall monthly distribution of children

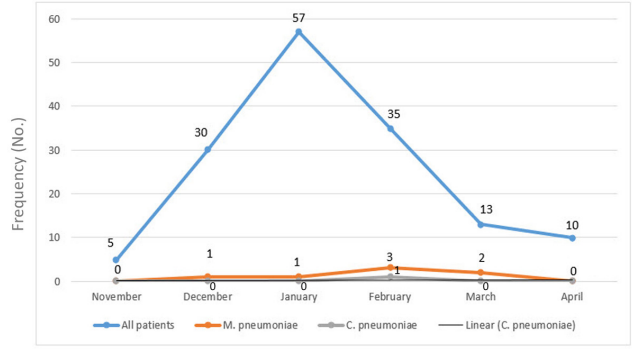

Figure 1. The overall monthly distribution of children admitted and PCR positive for each pathogen during the 5 months period.

Table 1. The demographic and epidemiological characteristics of patients.

Age group	All patients n=150 (%)	PCR-Positive for *M. pneumoniae* n=7 (%)	PCR-Positive for *C. pneumoniae* n=1 (%)
Mean age	3±3	4±2	6±0
1 month-2 year	64 (42.7)	2 (28.6)	0
2.1 year-5 year	37 (24.7)	3 (42.8)	0
5.1 year-15 year	49 (32.6)	2 (28.6)	1 (100)
Gender			
Male	88 (58.7)	4 (57)	0
Female	62 (41.3)	3 (43)	1 (100)
Hospital admission	59 (39.3)	0	0

Table 2. Clinical diagnosis and manifestations of patients with acute respiratory disease.

Type of infection	All patients (n=150)	PCR-Positive for *M. pneumoniae* (n=7)	PCR-Positive for *C. pneumoniae* (n=1)
URI	79 (52.5)	4 (57.58)	1 (100)
Pneumonia	38 (25.3)	3 (42.4)	0
Nasopharyngitis	7 (4.6)	0	0
pharyngitis	7 (4.6)	0	0
Asthma	5 (3.3)	0	0
Others[a]	14 (9.3)	0	0
Sore throat	127 (84)	7 (100)	1 (100)
Fever	72 (48)	4 (57)	1 (100)
Cough	138 (92)	6 (85)	1 (100)
Fatigue	33 (22)	2 (28)	0
Chills	10 (6)	0	0
Dyspnea	36 (24)	1 (14)	0
Diarrhea	7 (4)	0	0
Chest pain	8 (5)	0	0
Sneezing	28 (18)	1 (14)	0
Rhinorrhea	83 (55)	1 (14)	1 (100)
Wheezing	40 (26)	2 (28)	0
Tachypnea	25 (16.6)	2 (29)	0

[a] Others: includes croup (5 patients), bronchiolitis (5 patients) and bronchitis (4 patients).

admitted and PCR positive cases for each pathogen throughout the surveillance period. More than half of patients had URTIs (upper respiratory tract infection) and less than one third of them had a principle diagnosis of pneumonia at admission (Table 2).

Relationship between culture and PCR results with clinical outcome

Seven (4.7%) patients had a PCR-positive result for *M. pneumoniae,* while culture method revealed no colony in related to this pathogen. *M. pneumoniae* occurred frequently in children older than 2 years (5 cases=71.4%) (Table 1). Indeed, only one case was positive for *C. pneumonia*e. Among the children with positive results for both bacteria, 5 (62.5%) subjects were less than 5 years of age, and 3 (37.5%) between 5 and 15 years. Moreover, all the PCR-positive cases were related to throat swab samples. The most common signs and symptoms among 150 patients were cough and fever with frequencies of 138 (92%) and 72 (48%), respectively. Despite these observations; however, there was no significant difference in terms of clinical manifestations among different groups (Table 2). Of patients with the PCR-positive results for *M. pneumoniae*, 4 (57.1%) cases had URIs and others had symptoms of pneumonia. Based on history of disease, 22 (14.7%) patients had asthma, and 10 (6.6%) cases had other diseases such as fauvism, cerebral palsy (CP) and liver or heart failure. None of these diseases were found among 8 patients with the PCR-positive results. Clinical manifestations of all patients, including PCR- positive cases are summarized in Table 2.

Discussion

Atypical bacteria such as *M. pneumoniae* and *C. pneumoniae* are important causes of ARIs in both children and young adults. They share clinical features and manifestations which are difficult to be distinguished clinically from other respiratory pathogens (5,6). In the present study, the overall frequency of these bacteria among the outpatient and inpatient children aged 1 month to 15 years old with ARIs was found as 5.3% using the PCR and conventional bacterial culture methods.

Although in our study *M. pneumoniae* has not been isolated by culture; however, through the PCR assay the bacterium has been detected in 7 (4.7%) subjects, including 4 (57%) outpatient and 3 (43%) inpatient children, which is consistent with previous studies in Iran (18,19). It has been suggested that PCR is a reliable and rapid detection method for these infections, even with colonization rate of 1-2% in each population (5). Ghotaslou and colleagues detected *M. pneumoniae* in 6.15% and 2.01% of patients by PCR and culture methods, respectively (20). In another study from Iran, *M. pneumoniae* was diagnosed in 1% children with ARIs by PCR (21). In contrast to our findings, some studies have indicated an increase in detection of *M. pneumoniae* with frequencies of 12.4%, 15% , and 20.7%, respectively (22-24). This discrepancy can be due to differences in geographical distribution, age of study population, diagnostic methods and type of sample (25). On the other hand, in the present study *C. pneumoniae* was detected by PCR only in one specimen from an outpatient child 7 years of age. This finding is similar to some other reports from Iran and other countries (18,21,26,27). Such result suggests that this agent is probably an infrequent respiratory pathogen at least in our area. Although atypical bacteria in our work were detected in males more than females, but this difference was not significant. However, these bacteria were found mostly among children less than five years of age.

Most studies have been suggested that infections due to *M. pneumoniae* and *C. pneumoniae* occur mainly among preschool and school-aged children and young adults (4,5). In our study, 3 out of 8 cases were in above

mentioned age groups; and this could be due to occurrence of more positive cases among younger population. Indeed, *M. pneumoniae* infection was more frequent in children older than 2 years (71.4%), which is in agreement with work of Liu et al. (4). On the other hand, it has been mentioned that *M. pneumoniae* epidemics occur in either summer or autumn (4). However, in the current study, this seasonal variation was not shown because sampling was performed from November 2014 to April 2015 and not throughout the entire year. In contrast with our result, Chen and co-workers (28) showed that *M. pneumoniae* was epidemic in the summer, and this difference may be due to differences in pathogen epidemiology and time of study; hence, our finding suggested that further research over longer periods is needed to confirm this pattern.

In our investigation, most of patients presented cough and fever symptoms and the rate of tachypnea and wheezing was lower than other similar studies (29-31). Also, it seems that patients with PCR positive results showed up with sore throat and more than half of them had cough and fever at the time of admission; nevertheless, there was no significant difference in clinical presentation among the patients with positive or negative results of PCR for atypical bacteria. During 5 months of our monitoring for the atypical bacteria, we found that the rate of *M. pneumoniae* infections was lower than the previously reported cases (29). This difference probably implies that our surveillance period was not during the epidemic time.

There are some limitations to our study such as difficulties in cultivation of *C. pneumoniae*, and the period limit of our study.

This study indicates that atypical bacteria are probably not common respiratory tract pathogens among the patients with ARIs in our region, and our subjects were mostly from the younger groups. Moreover, PCR showed that could be a suitable and rapid approach for diagnosis of these infections.

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Interest conflict

The authors declare that there is no conflict of interest.

Author's contribution

Study concept and design: Reza Khashei and T. Hashempour; acquisition of data and sampling: Mehrdad Halaji, G. R. Pouladfar and Farzane Ghassabi; analysis and interpretation of data: Mehrdad Halaji; drafting of the manuscript: Reza Khashei and Mehrdad Halaji; critical revision of the manuscript for important intellectual content: Reza Khashei; study supervision: Reza Khashei and T. Hashempour.

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