Mycoplasma pneumoniae respiratory tract infections among Greek children

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Abstract
Background: M. pneumoniae is a common cause of respiratory tract infections (RTIs) of variable severity especially in children. New diagnostic techniques offered more reliable information about the epidemiology of infection by this pathogen.
Aim: The aim of this study was to investigate the prevalence and epidemiology of acute M. pneumoniae infections among Greek children hospitalized for RTIs using more advanced techniques.
Material and Methods: The study included 225 Greek children hospitalized for RTIs during a 15-month period. Throat swab specimens were tested by PCR for the detection of M. pneumoniae, while IgG and IgM antibodies were determined by ELISA and, in certain cases, also by western-blot. In parallel, specimens were tested for the presence of additional respiratory pathogens.
Results: M. pneumoniae infection was diagnosed as the only pathogen in 25 (11.1%) cases, being the second (after respiratory syncytial virus- RSV) most often detected pathogen. The proportion of cases with M. pneumoniae infection in age group 8-14 years (23.3%) was significantly higher than that in <3 years age group.
Conclusion: During our study period, M. pneumoniae was the second causative agent of RTIs after RSV. The proportion of children with M. pneumoniae RTIs increased with age, while most cases were reported during summer and autumn. Hippokratia 2011; 15 (2): 147-152

Key words: M. pneumoniae, respiratory tract infection, children, Greece

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It seems that M. pneumoniae plays a more significant role in causing respiratory tract infections (RTIs) in pediatric patients than previously thought[1]. The clinical features of M. pneumoniae infections are various, including pharyngitis, tracheobronchitis, while approximately one third of infected patients develop pneumonia[2]. However, other studies report that cases of pneumonia represent 3-10% of infections, while the majority is minor respiratory illnesses[3]. In children, M. pneumoniae causes up to 40% or more of community-acquired pneumonia, and as many as 18% of cases requiring hospitalization[4]. Epidemics of M. pneumoniae infections can occur in the community or in closed or semiclosed settings, such as military bases, hospitals, religious communities, schools, and facilities for the mentally or developmentally disabled[4,5].

Since M. pneumoniae infections cannot be diagnosed on the basis of only clinical findings, serological methods are usually performed, and combination of PCR and serology is recommended as a more reliable diagnostic approach[6,7].

The aims of the present study were to investigate the prevalence of acute M. pneumoniae infections among Greek children hospitalized for upper or lower RTIs, to examine the most frequent clinical findings and laboratory parameters associated with M. pneumoniae infections, to determine the most affected age group as well as the seasonal distribution of M. pneumoniae infections.

Materials and methods
Patients
The patients of the present prospective study were selected consecutively among children who were hospitalized in the Department of Paediatrics, in University General Hospital "AHEPA" in Thessaloniki (Northern Greece) from 1 May 2003 to 1 August 2004. The study protocol was approved by the ethnic committee of the Hospital, while informed parental consent was obtained for each patient. Children included in this study were hospitalized for symptoms of community-acquired RTIs. Only children with symptoms, signs and/or chest radiography findings consistent with atypical pneumonia were included in the study from these with pneumonia. In atypical pneumonia, X-ray show hilar adenopathy and unilateral or bilateral infiltrates, and/or patient looking worse than the symptoms suggest, and/or not showing response to treatment with b-lactam antibiotics[8-10]. Patients were excluded from the study when there was evidence of Streptococcus pneumoniae or Streptococcus pyogenes infection or chronic respiratory illness (e.g. cystic fibrosis or bronchopulmonary dysplasia), and those with other

underlying diseases that might predispose patients to pneumonia. Patients with nosocomial infection, tuberculosis or those who had received antibiotics active against M. pneumoniae, were also excluded.

Evaluation of patients and enrollment

On patient's admission, pediatricians were completing a questionnaire regarding the age of the patient, the date of onset of the disease, the clinical symptoms (malaise, fever $\geq$ 38 °C cough, sputum production, abdominal complains), the laboratory findings, and the potential presence of underlying disease. X-rays were evaluated independently by two, board-certified, pediatric radiologists for the presence or absence of pneumonia. Chest radiography was performed using standard equipment and radiographic techniques, and reviewed by the radiologists in digital format. Diagnosis of pneumonia was based on the presence of new infiltrates on chest radiography (single or multiple infiltrates, opacities or consolidations), symptoms (like chills, hoarseness, sore throat and chest pain), and physical examination findings (rales or crackles, wheezes on auscultation of bronchial breathing)[9]. Pharyngitis (includes tonsillitis, tonsillopharyngitis and nasopharyngitis) was diagnosed when symptomes of sore throat were detected. In pharyngitis the inflammation causes erythema of the pharynx, the tonsils or both structures)[11].

Tracheobronchitis was defined as acute inflammation of the lower RT associated with cough and may be with inspiratory and/or expiratory noises on auscultation but doesn't have laryngeal obstruction, audible wheeze or crackles[9,12].

All of the children of the study were hospitalized for at least two days in the Department of Pediatrics of the hospital. The stay of children with pharyngitis in the hospital was based on the severity of symptoms associated with this condition. In addition, some children with small age were hospitalized for intravenous treatments.

Specimens

Throat swabs and serum specimens were obtained on admission from the patients, whereas convalescent serum specimens were taken 9 to 24 days later mostly after discharge from the hospital. The throat specimen was collected with a viscose swab, which was placed in 2 ml of Mycoplasma transport medium[13]. All specimens were stored at -20⁰C until testing. The two serum specimens were serologically tested at the same run.

Methods
DNA extraction and PCR for M. pneumoniae.

DNA was extracted according to Waring et al[13]. A total of 500 µl of the specimen was concentrated by centrifugation at 13,000 X g for 10 minutes. A 480 µl volume of the medium was removed, and 30 µl of sterile water was added to the remaining 20 µl of medium and the pellet. The sample was then mixed well and heated to 95°C for 15 minutes. A 15µl aliquot of the sample lysate was used for PCR amplification. Primer pair P1-1 and P1-3 was used to amplify a 209-bp fragment of the P1 adhesin gene[14]. The PCR protocol was optimized in our laboratory. The final volume of the PCR mixture was 50 µl with 1 x Taq buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 2.5 U Taq DNA polymerase (Invitrogen,USA), 200 µM of each dNTP, 2.0 mM of $MgCl_2$, 50 pmol of each primer. The PCR program was as follows: one cycle of three minutes at 94°C (for denaturation), 35 cycles of amplification (one minute at 94°C [for denaturation] and 1.5 minutes at 63°C [for annealing] and two minutes at 72°C [for extension]), and one cycle of 10 minutes at 72°C (for final extension). PCR products were detected by 1.5% agarose gel electrophoresis with ethidium bromide staining.

M. pneumoniae serology

M. pneumoniae IgG antibodies were detected using ELISA (Platelia, BIO RAD, France) (cut-off 10 AU/ml), while IgM antibodies were detected using µ-capture ELISA (Platelia, BIO RAD, France) (cut-off index 1.0). For confirmation of the IgM- ELISA results, western-blot test (Virotech Rüsslsheim, Germany) was performed for 19 sera. Presence of specific IgM antibodies was confirmed when a band corresponding to P1 protein was present, together with eight or more additional bands. PCR and IgG- and IgM-ELISA were repeated, when discordant results were observed.

In addition, the first-blood specimens were tested for WBC (White Blood Cells), ESR (Erythrocyte Sedimentation Rate) by classical methods and CRP (C-Reactive Protein) by nephelometry (Image Beckmann). According to manufacturer, CRP < 0.5 mg/dl was considered normal. Normal ESR ($\leq$ 10 mm/hour) and normal ranges of WBCS in different ages were according to Camitta[15].

Diagnostic criteria for M. pneumoniae acute infection

Evidence of acute M. pneumoniae infection was defined when PCR on throat swab specimen was positive and/or IgM ELISA was positive, and/or IgG seroconversion, or significant increase of IgG titers ($\geq$twofold increase of titer measured in AU/ml) between two specimens was detected, and, at the same time, there was no evidence of infection by other pathogen(s).

Detection of other respiratory pathogens

Specimens were tested for a probable infection by Chlamydia pneumoniae; Coxiella burnetti; influenza virus types A and B; parainfluenzavirus types 1,2,3; respiratory syncytial virus (RSV); and adenovirus using ELISA (Virion/Serion, Germany) according to manufacturer's instructions. In addition, a PCR for detection of coronavirus genome was applied[16].

Serologic diagnosis of infection was established when IgM or IgA antibody titer (IgA was determined only for parainfluenzavirus) was above the cut off value provided by the manufacturer, or/and a fourfold increase in IgG antibody titers in paired serum specimens was observed.

Statistical analysis. SPSS version 12.0 for windows

(SPSS Inc, Chicago, IL, USA) was used: Fisher's exact test for comparison of the prevalence of M. pneumoniae infection between different age groups and between seasons; t-test (independent samples test) for comparison of the mean values between groups; and chi-square and Fisher's exact test for comparison of clinical and laboratory parameters between groups. A p-value <0.01 was considered significant.

Results

Our study included 225 children (124 male), with average of age of 4.4 years (range, 2 months to 14 years) from the area of Thessaloniki with sings and symptoms of community-acquired RTIs that were admitted 1 to 25 days (mean 6.9 days) after the onset of the disease. Among the 225 children, 88 (39.1%) were <3 years, 94 (41.8%) 3-7 years, and 43 (19.1%) 8-14 years. Atypical pneumonia was radiographically defined in 114/225 (50.7%), while pharyngitis and tracheobronchitis were diagnosed in 74/225 (32.9%) and 37/225 (16.4%), respectively. It may appear strange that the number of hospitalized children for pharyngitis (74) was higher than that for tracheobronchitis (37); this had resulted from the exclusion of tracheobronchitis cases with a suspected allergic origin from the present study.

Throat swabs and serum specimens were obtained on admission from all of the 225 patients, whereas convalescent serum specimens were obtained from 75 patients 9 to 24 days (mean 13.7 days) later. Although the pediatricians determined a date for the next visit to the hospital for monitoring the progress of the patients and for obtaining convalescent sera, many of the patients did not come because of the mild nature of infection and small age of the children.

PCR and/or serologic tests for M. pneumoniae were positive in a total of 34 patients: PCR was positive in 24 patients and specific IgM antibodies were detected in 27 patients, while seroconversion or significant increase of IgG antibodies in 7 patients. M. pneumoniae acute infection was diagnosed in 25 (11.1%) patients according to our diagnostic criteria, whereas 9 patients coinfected with additional pathogens were excluded: 5 with Chlamydiae pneumoniae, 4 with RSV, 2 with adenovirus and 1 with parainfluenza virus. The infection rate in males (10.5%) was close to that in females (11.9%). The distribution of M. pneumoniae infections among the three age groups is shown in Table 1. Infection of M. pneumoniae was diagnosed in 15 (13.2%) of 114 children with pneumonia, in 8 (10.8%) of 74 with pharangitis and in 2 (5.4%) of 37 with tracheobronchitis (Table 1). The age of children with M. pneumoniae infection ranged from 4 months to 12 years with the mean of 6.1 years. The mostly affected was the age group of 8-14 years (23.3%), with significantly higher number of cases than the group of children <3 years (6.8% p=0.009) and higher (not significant) than 3-7 years (9.6%, p=0.033). However, the difference between the group of <3 years and that of 3-7 years was not significant (p=0.344). While only a case of M. pneumoniae pneumonia was reported in children <3 years, statistically significant higher proportion of M. pneumoniae pneumonia cases was found in 8-14 years children (p=0.002). In addition, the frequency of M. pneumoniae pneumonia (7.5%) among 3-7 years children was higher than that among < 3 years. M. pneumoniae pharyngitis was observed in all age groups (with no significant difference among them), while M. pneumoniae tracheobronchitis was observed in two age groups (<3 years and 3-7 years) (p= 0.735).

Fever and cough were the most common (both 84%) symptoms in patients with M. pneumoniae infection, while increased erythrocyte sedimentation rate (ESR) was the most common (96%) laboratory finding (Table 2). No significant differences in clinical and laboratory findings of the patients were observed between M. pneu-

Table 1: Distribution of cases with M. pneumoniae infection among different age groups of children with RTIs.

Age groups (years)	Children with acute RTI* n=225 (100%)			Children infected with M. pneumoniae n=25 (11.1%)			
	Total pneumonia pharyngitis tracheobronchitis No (%) No (%) No (%) No (%)			Total pneumonia pharyngitis tracheobronchitis No % No % No % No %			
<3	88 (39.1) 25 (28.4) 35 (39.8) 28 (31.8)			6 (6.8)[a] 1 (1.1)[b] 4 (4.6)[c] 1 (1.1)			
3-7	94 (41.8) 61 (64.9) 26 (27.7) 7 (7.5)			9 (9.6) 7 (7.5) 1 (1.1) 1 (1.1)			
8-14	43 (19.1) 28 (24.6) 13 (17.6) 2 (5.4)			10 (23.3) 7 (16.3) 3 (7) 0			
Total	**225 (100) 114 (50.7) 74 (32.9) 37 (16.4)**			**25 (11.1) 15 (13.2) 8 (10.8) 2 (5.4)**			

*RTI: respiratory tract infection
[a] <3 vs 3-7, p=0.344 [b] <3 vs 3-7, p=0.040 [c] <3 vs 3-7, p=0.164
 <3 vs 8-14, p=0.009 <3 vs 8-14, p=0.002 <3 vs 8-14, p=0.417
 3-7 vs 8-14, p=0.033 3-7 vs 8-14, p=0.103 3-7 vs 8-14, p=0.091

Table 2: Comparison of clinical and laboratory parameters between children with M. pneumoniae infected and other RTI.

Clinical and laboratory parameters	Children with RTI (n =225)		
	M. pneumoniae positive (n= 25) No %	M. pneumoniae negative (n=200) No %	p value[1]
Fever	21 84	174 87	0.677
Cough	21 84	180 90	0.360
Sputum production	8 32	91 45.5	0.200
Malaise	5 20	24 12	0.260
Abdominal complains[2]	3 12	17 8.5	0.388
WBC increase[3]	10 40	73 36.5	0.732
ESR increase[3]	24 96	190 95	0.827
CRP increase[4]	20 80	162 81	0.905
Symptoms' days (mean)[5]	4.7	6	0.468

[1] For all parameters measured by chi-square, except the parameter of abdominal complains measured by Fisher's exact test.[2] diarrhea and/or abdominal pain.[3] Normal ESR ($\leq$ 10 mm/hour) and normal range of white blood cell (WBC) counts in various ages were according to Camitta (15).[4] C-reactive protein (CRP) < 0.5 mg/dl was considered normal.[5] Mean time between onset of sign and symptoms and admission.
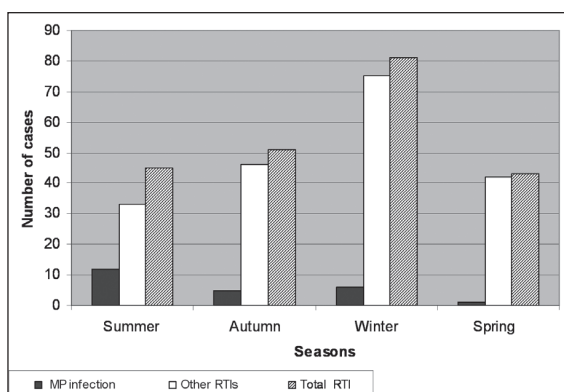
moniae and non-M. pneumoniae RTIs (Table 2). However, the frequencies of clinical and laboratory parameters (except CRP) in children with M. pneumoniae infection in both 3-7 and 8-14 years age groups were higher (not significant) than those in < 3 years age group.

M. pneumoniae infection was more frequent (26.7%) during summer, where the number of observed cases was significantly higher than that in spring (5%; p=0.005) and winter (7%; p=0.004), and higher, but not significantly, than in autumn (9.8%, 0.029) (Fig. 1).

Apart from M. pneumoniae, other detected respiratory pathogens were: Chlamydia pneumoniae in 21 (9.3%) cases, Coxiella burnetti in 2 (0.9%), influenza virus type A in 5 (2.2%), influenza virus type B in 4 (1.8), parainfluenzavirus in 5 (2.2%), RSV in 54 (24%), adenovirus in 18 (8%), and coronavirus in 5 (2.2%).

In total, co-infections were observed in 21 (9.3%) cases; M. pneumoniae was involved in 9 of them. All pathogens involved in co-infections are shown in Table 3.

The clinical history of the patients showed that 45

children (20%) had received antibiotic therapy prior to admission, and had no response to treatment.

Discussion

The present study was carried out to provide new information about M. pneumoniae infection in children in Greece using more specific techniques. During the 15-month study period, M. pneumoniae acute infection was definitely diagnosed in 25 (11.1%) children hospitalized with community-acquired RTIs. It is often difficult to know which pathogen in a mixed infection is more important cause of symptoms of the disease. Therefore 9 out of 34 children with positive results for M. pneumoniae were not definitely diagnosed having M. pneumoniae infection because they had mixed infection. In our study, 15

Table 3. Pathogens detected in respiratory tract co-infections.

Pathogens	No. (%) of patients
M. pneumoniae + C. pneumoniae	3 (11.5)
M. pneumoniae + RSV	2 (7.8)
M. pneumoniae + adenovirus	1 (3.8)
M. pneumoniae + C. pneumoniae + RSV	1 (3.8)
M. pneumoniae + parainfluenza virus	1 (3.8)
M. pneumoniae + C. pneumoniae + RSV+ adenovirus	1 (3.8)
Influenza virus A + adenovirus	2 (7.8)
Parainfluenzavirus + RSV	1 (3.8)
C. pneumoniae + RSV	2 (7.8)
C. pneumoniae + RSV + adenovirus	1 (3.8)
C. pneumoniae + adenovirus	2 (7.8)
C. pneumoniae + parainfluenza virus	1 (3.8)
Coronavirus + RSV	1 (3.8)
C. pneumoniae + influenza virus A + RSV	2 (7.8)
No of co-infections (% of 225 cases)	21 (9.3)

Figure 1: Seasonal Distribution of M. pneumoniae (MP) infection.

pneumonia cases were caused by M. pneumoniae, which accounted for 6.7% of all RTIs (15/225). In a Finnish study based also on serology and PCR, M. pneumoniae infection was diagnosed in 24/278 (8.6%) pediatric patients with pneumonia[17]. In another study from Asia (12 Asian medical centers), 61/448 (13.6%) children, 2-15 years old, had M. pneumoniae pneumonia[18]. In children with wheezing, M. pneumoniae infection was diagnosed by PCR or serology in 22.5%[19]. Recently, M. pneumoniae was detected in 19.7% among 127 children with acute pharyngitis[20]. Differences between studies may be attributed to community outbreaks during the study periods, and inter-country differences due to diversity of climate, geography, and human activities. Different applied methods and rigidity of diagnostic criteria also play a role in prevalence determination differences.

Diagnosis of M. pneumoniae infection is based mainly on serology. In the present study, we applied a combination of serological and molecular methods to increase sensitivity. In a previous study[6], we found that the combination of PCR (sensitivity alone 75%) and μ-capture IgM ELISA (sensitivity alone during acute phase 66.7%) is the most appropriate approach for the laboratory diagnosis of acute M. pneumoniae infection in children. Dorigo-Zetsma et al.[7] found high PCR specificity (100%) and predictive value positive (100%) for diagnosis of acute M. pneumoniae infection in children, while combination of IgM-ELISA and PCR had been reported to present a sensitivity of 95-100%[6,17].

In the present study, M. pneumoniae DNA was detected in 6 children with mild infections (pharyngitis or tracheobronchitis) in whom there was no serologic evidence of acute infection. There have been studies suggesting that PCR can detect mild cases of infections lacking detectable immune response[21,22]. Conversely, IgM antibodies were detected in the first serum specimen in most (11/14) of the M. pneumoniae pneumonia cases. An additional factor might be the immaturity of the immune system and in our case, 5 of the 6 patients with no detectable antibodies were less than 2 years old. There are also some individuals who do not mount an antibody response against M. pneumoniae, probably due to genetic differences[23].

Concerning the age distribution, the mostly (23.3%) affected group was 8-14 years, where the infection rate in this group was significantly higher than that in the age group of < 3 years (6.8%, p=0.009) and higher, but not significant (p=0.033), compared to the group of 3-7 years (9.6%). In addition, the number of pneumonia cases caused by M. pneumoniae was increasing with age (Table 1), with the highest rate (16.3%) in the group of 8-14 years. M. pneumoniae lacks cell wall, and it is very sensitive to environmental conditions. Consequently, transfer of infection requires close contact, which is usually more frequent in older children, who acquire the infection in school or during playing with other children. Korppi et al.[24] found that the prevalence of M. pneumoniae infection in children tends to increase according to age.

A previous study[1] found that diagnosis of M. pneumoniae RTIs cannot be made on the basis of clinical and common laboratory parameters. Similar findings were found in the present study. However, we found that M. pneumoniae causes more signs and symptoms in older children. Most likely the symptoms and signs reflect the immune response to the pathogen, which is more mature in older children.

A seasonal clustering of acute RTIs due to M. pneumoniae was observed in summer and autumn (Fig 1). The same seasonal distribution was reported in another study[25], although Nadal et al[26] did not observe such seasonal distribution.

In total, causative atypical pathogens were detected in 118/225 (52.4%) children requiring admission to hospital for RTIs. The most common pathogen was RSV, followed by M. pneumoniae. Woodhead reviewed 26 prospective studies from 10 European countries (5,961 patients in total) and found that C. pneumoniae was the most frequent (≈17%) causative pathogen of community-acquired pneumonia[27].

Co-infections were observed in 21/225 (9.3%) cases; M. pneumoniae was involved in 9 of them (Table 3). C. pneumoniae was detected in 5 cases together with M. pneumoniae. Mixed mycoplasmal-chlamydial infections have been reported previously, and it is not known whether one pathogen facilitates the penetration of the other in the host cells, or whether the combination of both pathogens causes more severe clinical illness[28].

Convalescent serum specimens were available for only 75 of the 225 studied patients; this happens usually because parents do not like to visit again the hospital when their child is not ill any more. This problem has been previously reported in a large scale study[29]. However, it is not expected to have a strong effect on the results of the present study with respect to M. pneumoniae, since we applied PCR for the detection of the pathogen in throat swab specimens, which is recorded to have the highest sensitivity during the early phase of the infection[6,7]. Furthermore, it's not uncommon to find antibodies against M. pneumoniae in the first week of the disease, due to the long incubation period (2-3 weeks) of the pathogen[30].

In conclusion, during our study period, M. pneumoniae was the second causative agent of RTIs after RSV. Most of these children (60%) had pneumonia, however M. pneumoniae infection must be taken into account when pharyngitis (32%) or thracheobronchitis (8%) are diagnosed. The proportion of children with M. pneumoniae RTIs increased with age, while most cases were reported during summer and autumn. M. pneumoniae infection cannot be predicted on the basis of clinical manifestations and routine laboratory testing, thus combination of PCR on throat swab specimens and detection of serum IgM antibodies is of great diagnostic help in the early phase of the disease.

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