Original Article

Indirect Particle Agglutination Antibody Testing for Early Diagnosis of *Mycoplasma pneumoniae* pneumonia in Children

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Objectives: Outbreaks of pneumonia caused by *Mycoplasma pneumoniae* (MP) occur every 3-4 years in Korea, most recently in 2011. The aim of our study was to determine the optimal time to perform indirect particle agglutination antibody assays to improve early diagnosis of MP pneumonia in children.

Methods: A database of 206 pediatric patients treated for pneumonia at the Hanyang University Hospital from June to October 2011 was analyzed retrospectively for demographic characteristics and laboratory test results.

Results: Among the 206 patients treated for pneumonia during the study period, there were 160 children (mean age, 5.44 years) diagnosed with MP pneumonia, who were studied further. The mean age of these MP pneumonia patients was 5.44 years. Antibody titers increased with increasing time between symptom onset and the collection of serum collection: MP titers were <1:640 for sera collected after 5.44 days and titers ≥1:640 for those collected after 8.58 days; $P<0.001$. Antibody titers were considered positive when they reached ≥1:640. In 42 MP pneumonia patients in whom there was a four-fold or greater increase in titer between successive serum samples, the optimal cut-off time-point for distinguishing between the initial and second titer groups was 7.5 days after the onset of symptoms (sensitivity, 90.5%; specificity, 92.9%).

Conclusions: Negative MP antibody titers earlier than 8 days after the onset of symptoms in children with pneumonia may require repeating to confirm the diagnosis. This finding could optimize diagnosis and result in better therapeutic outcomes of MP pneumonia in children. **(Korean J Pediatr Infect Dis 2013;20:71-80)**

Key Words : *Mycoplasma pneumoniae*, Pneumonia, Diagnosis, Serology, Children

Introduction

Mycoplasma pneumoniae (MP) is a common cause of community-acquired respiratory tract infections, especially in children. It is believed to account for 7-30% of all community-acquired pneumonia cases in 3 to 15-year-olds[1, 2]. While MP pneumonia is usually mild, it can be life-threatening[3, 4]. It occurs worldwide throughout the year, and also in outbreaks at intervals of 3-7 years[5-7]. In Korea, outbreaks of MP pneumonia among children have been reported every 3-4 years[8].

Laboratory diagnosis of MP pneumonia can be made based on serology, polymerase chain reaction (PCR), or culture results. Studies have shown that MP culture from the respiratory tract and other sites requires serial blind passages, specialized and expensive growth media, and incubation periods of up to several weeks[9]. Recently, a PCR assay for early detection of MP has given better diagnostic results. However, it has also demonstrated more variable sensitivity and specificity in children and adults than serology[10, 11]. Thus, PCR alone is not sufficient for diagnosing MP infection.

At present, clinical diagnosis of MP pneumonia is based on conventional serological methods that in-

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clude complement fixation assays, enzyme immunoassays, and indirect particle agglutination assays[12]. The Serodia-Myco II® particle agglutination test is used in several countries outside the United States [13]. All the above-mentioned assays require paired serum samples for a conclusive diagnosis, which is established by seroconversion or a four-fold increase in titer[14]. However, their sensitivities for the detection of MP antibodies are known to vary[15].

The existing methods for early detection of MP pneumonia are limited because IgM antibody-based tests have low specificity, and detection rates by PCR can vary in the early stages of infection[10, 16-18]. A 4-fold or greater rise in IgG MP antibody titers is considered to indicate a current or recent infection[19], and measuring IgG antibody in paired specimens collected 7 to 10 days apart may provide the most accurate serologic diagnosis[14]. When only a single titer is available, a titer ≥1:640 is thought to be the optimal cut-off value for diagnosis[19, 20]. Diagnosis based on a four-fold increase in titer between paired sera provides only a retrospective result, and tools that enable early detection are needed in order to optimize care and facilitate decisions about antimicrobial treatment.

Using data collected during the 2011 outbreak of MP pneumonia in Korean children, we conducted a retrospective study to identify the optimal time for indirect particle agglutination testing to improve the early diagnosis of MP pneumonia.

Materials and Methods

1. Patient population

The study included 206 patients (196 inpatients and 10 outpatients) diagnosed with pneumonia and tested for MP by measuring indirect particle agglutination (MP) antibody titers from June to October 2011 at the department of Pediatrics, Hanyang University Hospital, Seoul, Korea. Pneumonia was defined as the presence of an infiltrate on chest x-ray, and respiratory symptoms including cough, difficulty breathing, tachypnea, and/or fever. Radiographic findings that confirmed pneumonia included lobar, alveolar (bronchopneumonic), or interstitial infiltration.

Of the 206 pneumonia patients, 160 were diagnosed with MP pneumonia. Of these, 152 were inpatients, and eight were outpatients. MP pneumonia was diagnosed in 108 (67.5%) patients using single MP antibody titers, in 42 (26.3%) using paired sera, and in 10 (6.2%) patients using PCR. MP pneumonia was diagnosed in patients with clinical and radiological findings suggestive of pneumonia and positive MP antibody titers and/or a positive MP PCR. Antibody titers were considered positive when they reached ≥1:640, or when serial measurements showed a four-fold or higher increase[19, 20]. If the second titer was <1:640 but increased four-fold or higher, it was considered positive.

2. MP antibody testing

Serum specimens were titrated from 1:40 to 1:20,480 for MP antibody testing using an indirect particle agglutination assay (Serodia-Myco II®, Fujirebio, Tokyo, Japan), according to the manufacturer's instructions. The onset of symptoms to serum collection was defined as how many days past from the start of any respiratory symptoms (cough, difficulty breathing, tachypnea, and/or fever) until the serum collection. When the initial MP antibody titer

is <1:640, and MP pneumonia is suspected, antibody titer is repeated.

3. PCR for MP and respiratory viruses

Nasopharyngeal (NP) specimens were collected from patients using mucus traps and catheters within 1–2 days of admission, and were refrigerated at 4℃, or frozen at −70°C if they were not assayed within 72 hours. MP PCR was performed on 90 NP specimens using a real-time PCR kit (Accupower®, Bioneer, South Korea). PCR assays for respiratory viruses (respiratory syncytial virus, influenza A and B, parainfluenza virus, adenovirus, coronavirus, rhinovirus, and metapneumovirus) were performed on 153 NP specimens using a Seeplex RV 12 ACE Detection kit (Seegene®, South Korea).

4. Statistical analysis

Data were analyzed using SPSS (version 18.0) and SAS (version 9.2) software. Time from the onset of pneumonia symptoms to specimen collection for testing was compared between patients with positive and negative MP antibody titers, and between patients with positive and negative MP PCR, using the Wilcoxon rank sum test. The mean antibody titers and the mean times from symptom onset to specimen collection were compared between the paired titers using the Wilcoxon signed rank test. The optimal time for MP antibody testing was estimated by performing receiver operating characteristic (ROC) analysis. A P value <0.05 was considered statistically significant.

Results

Characteristics of patients with MP pneumonia was diagnosed in 160 patients. Their mean age was 5.44±3.15 years (range, 8 months to 15 years), and 86 (53.8%) patients were boys, and their age distribution is shown in Fig. 1. Other patient characteristics are summarized in Table 1.

Of the 118 patients diagnosed with pneumonia using single MP antibody titers, 108 had positive sera i.e. titers ≥1:640, and the remaining 10 patients

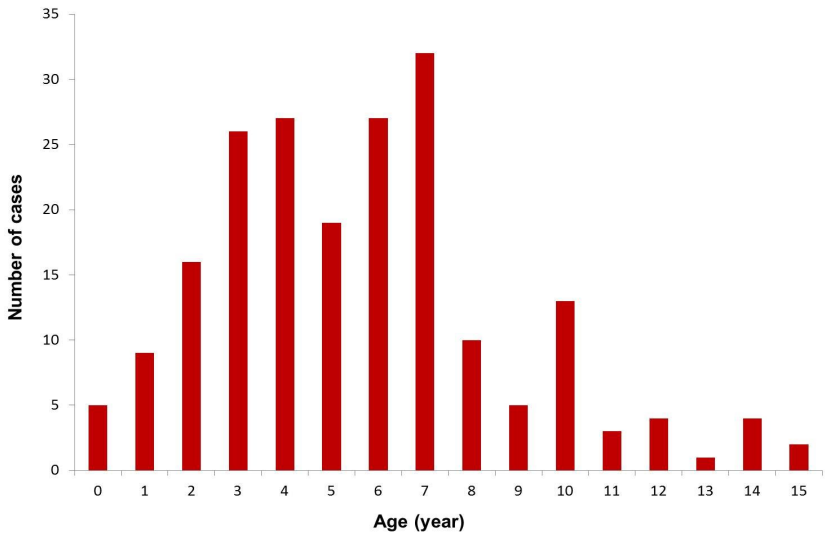

Fig. 1. Age distribution among children diagnosed with *Mycoplasma pneumoniae* pneumonia in the 2011 outbreak.

who were MP antibody-negative had positive MP PCR results. Of the 42 patients diagnosed using paired sera, all were positive (i.e. showed a four-fold or higher increase in titers). Conversely, all blood cultures (performed for all the study patients) were negative. Additionally, respiratory viruses were detected by PCR in 54 (33.8%) patients with MP pneumonia: rhinovirus in 46 patients, respiratory syncytial virus (RSV) in five patients, parainfluenza virus in five patients, adenovirus in three patients, and metapneumovirus in one patient (Table 1).

Indirect particle agglutination antibody testing and PCR

The mean (±SD) time from the onset of symptoms to serum collection for MP antibody testing was 7.03±4.10 days (range, 2-16 days; Table 1). The mean of the reciprocal MP antibody titers in the initial sera of all 160 MP pneumonia patients was 4,952 (range, 0-20,480).

The time from symptom onset to serum collection differed significantly in the antibody-positive (titers ≥1:640) and antibody-negative (titers <1:640) groups (8.58±4.22 vs. 5.44±2.06 days, P<0.001 in the Wilcoxon rank sum test; Fig. 2). Six of the patients diagnosed with MP pneumonia had negative antibody titers in the first sera, all collected more than seven days after the onset of symptoms. Three of these patients had positive PCR assays, and the remaining three showed a four-fold or greater rise in antibody titers in the second sera.

For the 108 patients diagnosed as antibody-positive using single titers, the mean of the reciprocal MP antibody titers was 7,207 (range, 640-20,480), and the mean (±SD) time from symptom onset to serum collection was 7.86±4.48 (range, 2-16) days. In the case of the 42 patients diagnosed as antibody-positive using paired sera, the antibody titers and the time from symptom onset to serum collection differed between the two measurements (P<0.001

Table 1. Selected Characteristics of the Patients with *Mycoplasma Pneumoniae* Pneumonia

	MP* pneumonia (n=160)
Age, years	5.44±3.15†
Male:female, n	86:74
Serum sample testing	
Single serum sample	118
Paired serum samples	42
Initial reciprocal MP antibody titers	4,952±7,315
Time from symptom onset to serum collection for testing, days	7.03±4.10†
WBC, per mm^3	8,325±3,720†
Eosinophils, per mm^3	170.3±171.9†
IgE, IU/mL	257.6±389.9†
CRP, mg/dL	2.7±3.3†
PCR, respiratory viruses	153‡
Negative	99 (64.7%)
Positive	54 (35.3%)

**Mycoplasma pneumoniae*
†Mean±SD
‡Rhinovirus (46 cases), RSV (5 cases), parainfluenza (5 cases), adenovirus (3 cases), metapneumovirus (1 case); 6 patients with rhinovirus had co-infections, 4 with RSV, 1 with adenovirus, and 1 with parainfluenza.

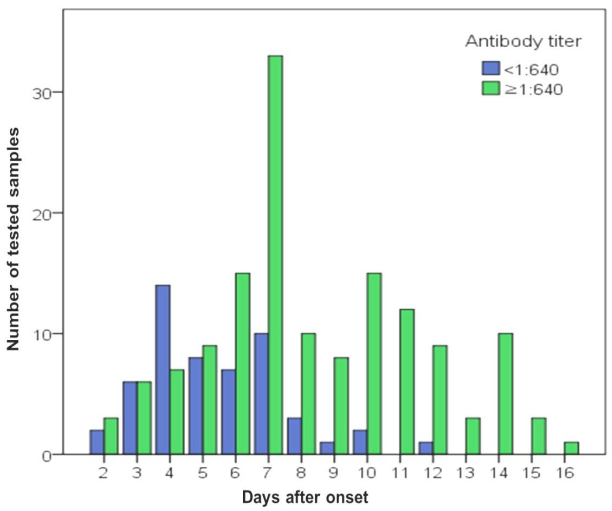

Fig. 2. Distribution of positive/negative *Mycoplasma pneumoniae* antibody titers according to time from symptom onset to serum collection.

in the Wilcoxon signed rank test; Fig. 3). Their initial sera were collected 5.24±1.71 (range, 2–9) days from the onset of symptoms, with titers of 1:125± 101. The mean time to collection of their second sera was 10.45±2.06 (range, 6–15) days from the onset of symptoms, and they had titers of 1:8,869± 8,288.

A receiver operating characteristic (ROC) curve for the time from symptom onset to serum collection is shown in Fig. 4. The area under the curve was 0.99, and the optimal cut-off value for distinguishing between the initial and second titer groups was 7.5 days after the onset of illness (sensitivity, 90.5%; specificity, 92.9%).

The results of MP PCR assays, performed on samples from 90 patients, were compared according to the MP antibody titer levels and showed that patients with titers ≥1:640 had positive PCR results in 63.6–80.0% of cases. The cumulative PCR positive rate was 68.1% (47/69) for patients with maximum MP antibody titers ≥1:640, compared with only 47.6% (10/21) for those with maximum titers ≤ 1:320 (Table 2).

Among the 12 patients showing a four-fold in-

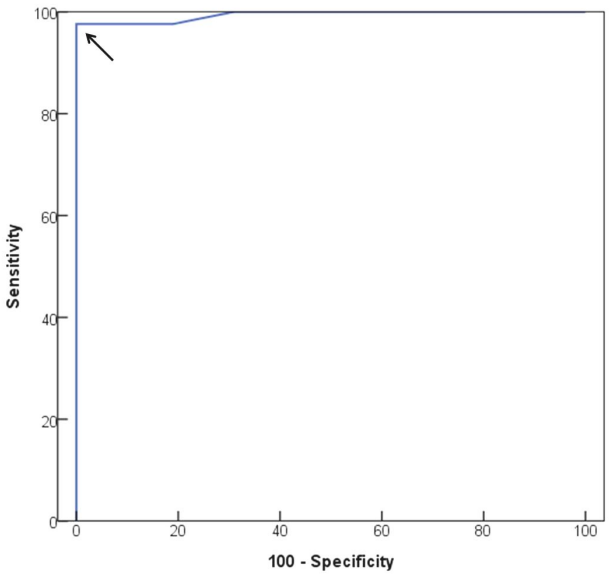

Fig. 4. Receiver operating characteristics (ROC) curve to estimate the diagnostic sensitivity and specificity of *Mycoplasma pneumoniae* (MP) antibody testing of paired sera in children with MP pneumonia based on time from onset of symptoms. Arrow points to the optimum cut-off value: 7.5 days after symptom onset (sensitivity, 90.5%; specificity, 92.9%).

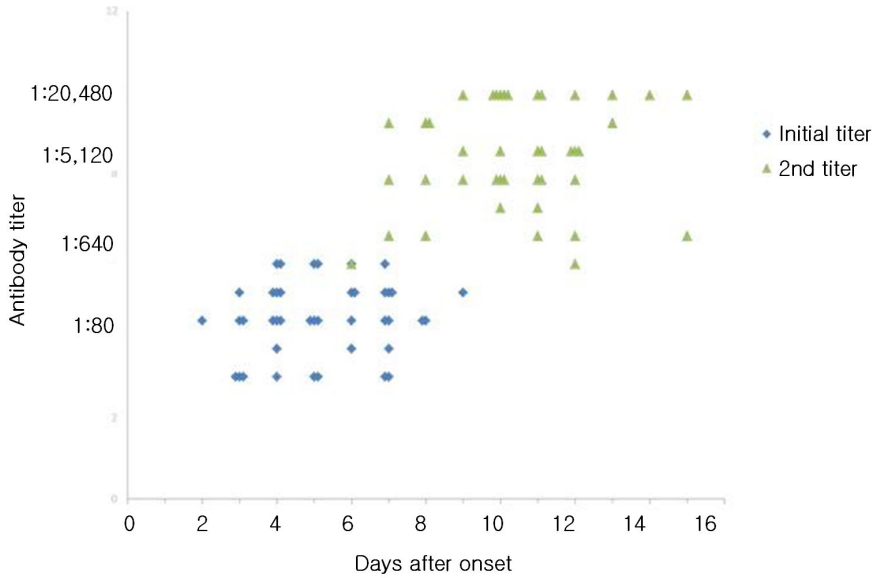

Fig. 3. *Mycoplasma pneumoniae* (MP) antibody titers in paired sera from children with MP pneumonia.

crease in antibody titers, 7 (58.3%) had positive PCR assays. Of the 21 pneumonia patients who were MP antibody-negative, 10 (47.6%) were diagnosed with MP pneumonia by MP PCR (Table 3). The mean time from symptom onset to serum collection for these 10 patients was 5.60±2.59 days.

When compared with the PCR results, the serological assay had a sensitivity of 82.5% (47 of 57) and a specificity of 33.3% (11 of 33). When compared with the serological assay, PCR had a sensitivity of 68.1% (47 of 69) and a specificity of 52.4% (11 of 21). When compared with the diagnostic definition of MP, sensitivity was 87.3% (69 of 79) for serology and 72.2% (57 of 79) for PCR. Fig. 5 shows the distribution of MP PCR results according to time from symptom onset to NP specimen collection.

Table 2. Relationship between PCR Assay Results and Maximum Antibody Titers in Children with *Mycoplasma Pneumoniae* Pneumonia

		Number (%) of PCR tests		Total
		Negative	Positive	
MP* antibody titer	<1:40	0 (0%)	1 (100.0%)	1
	1:40	2 (50.0%)	2 (50.0%)	4
	1:80	1 (50.0%)	1 (50.0%)	2
	1:160	5 (83.3%)	1 (16.7%)	6
	1:320	3 (37.5%)	5 (62.5%)	8
	1:640	3 (30.0%)	7 (70.0%)	10
	1:1,280	3 (30.0%)	7 (70.0%)	10
	1:2,560	4 (30.8%)	9 (69.2%)	13
	1:5,120	3 (30.0%)	6 (70.0%)	9
	1:10,240	1 (20.0%)	4 (80.0%)	5
	1:20,480	8 (36.4%)	14 (63.6%)	22
	Total	33 (36.7%)	57 (63.3%)	90

**Mycoplasma pneumoniae*

Table 3. Correlation between Positive Results by PCR and by Antibody Testing in Children with *Mycoplasma Pneumoniae* Pneumonia

	MP* Antibody		Total
	Positive	Negative	
MP PCR			
Positive	47	10	57
Negative	22	11	33
Total	69	21	90

**Mycoplasma pneumoniae*

Discussion

In this study we examined the clinical and laboratory findings for a group of children diagnosed with pneumonia and tested for MP antibody at presentation with fever and respiratory symptoms. We investigated the application of indirect particle agglutination antibody testing in early diagnosis of MP pneumonia in Korean children. In addition, we compared the diagnostic performance of the antibody assay with that of PCR.

Although infants and young children are highly susceptible to respiratory pathogens such as RSV [21, 22], the clinical expression of MP infection in this age group is largely asymptomatic or mild[23, 24], and MP is considered a rare cause of community-acquired pneumonia in children under five. Older studies based

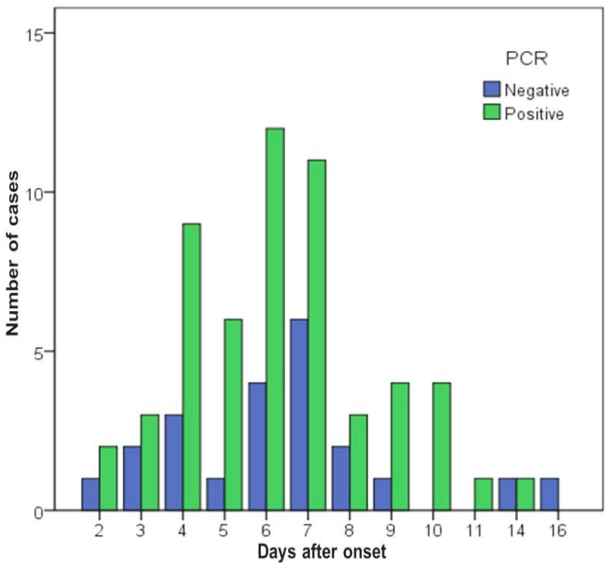

Fig. 5. Distribution of positive/negative *Mycoplasma pneumoniae* PCR results according to time from symptom onset to NP specimen collection.

on serology and culture methods reported that the frequency of MP pneumonia was highest among school-age children from 5 to 15 years of age, with a decline after adolescence and into adulthood[6, 23, 25]. Alexander et al. reported that, among children under five, the MP isolation rate of was only 1%[25].

By contrast, a French prospective study showed that MP pneumonia occurred frequently in children <4 years, and the infection rates did not differ among different age groups[26]. Similarly, Esposito et al. found that MP pneumonia was common in children under five in Italy[27], while a study in Japan reported that it most frequently affected children between 3 and 6 years old, with a peak at the age of four[19].

In Korea, one study reported that children under five accounted for 44% of all MP pneumonia cases, and the proportion of diagnosed 3-4 year-olds was significantly higher during epidemic than endemic periods[8]. Eun et al. reported that the most common age group was 5-7 years (39.1%), followed by 3-4 years (26.9%). Similarly, in our study 61.9% of all MP pneumonia patients were between the ages of 3 and 7, and the most common age group was 5-7 years (35.0% of the study population), followed by 3-4 years (26.9%). The rising numbers of positive diagnoses and the shift towards younger ages during recent MP pneumonia epidemics in Korea could be partly explained by an increase in the number of children attending day care centers.

Coinfected viruses were detected in the 54 patients (35.3%) with MP pneumonia patients. The percentage was higher than Kim et al. reported (12.5 %)[20]. Below the age of four were half of the virus coinfected MP pneumonia patients, coinfected bacteremic pneumonia was not documented among the MP pneumonia patients.

Diagnosis of MP pneumonia continues to rely on conventional serological procedures. Although the complement fixation (CF) assay is the most widely available method for detection of antibodies against MP[28], a negative CF test does not rule out MP infection. Since it predominantly measures the "early" IgM antibodies, and the IgG antibodies only to a minor extent, the diagnostic value of the CF assay may be limited to the initial stages of infection[14]. In the clinical setting, CF tests have largely been replaced by less time-consuming and labor-intensive immunoglobulin assays, including the EIA and immunofluorescence assays, and the indirect agglutination assay. A combined EIA IgG/IgM assay (Remel, Themo Fisher Scientific, Lenexa, Kansas) was reported to have a higher specificity in children than in adults, but a lower sensitivity than IgM-specific tests[29]. Serodia-Myco II® is a quantitative indirect particle agglutination assay for the detection of MP antibodies. It detects mainly IgM antibodies and some IgG antibodies[13]. In a Serodia-Myco II® assay, a four-fold or greater rise in the antibody titers and/or a single titer ≥1:640 are considered diagnostic of MP infection[19, 30].

When an initial antibody titer is negative, collecting paired sera could help to make a diagnosis in many infectious diseases. It is typically recommended that the second serum sample is collected within an interval of two to three weeks[31]. However, for the optimal care of patients with MP pneumonia, key treatment decisions should be made before that time. Our findings suggest that, when MP pneumonia is suspected, and the first MP antibody titer in the early phases of the disease is <1:640, the titer should be repeated 8 days after the onset of symptoms. MP pneumonia can then be diagnosed if the repeated

antibody titer is ≥1:640 or shows a four-fold or greater rise, and it can be ruled out if the titer is <1:640 or has not increased four-fold. The advantages of repeating the antibody titer were demonstrated in the 10 patients diagnosed with MP pneumonia by PCR, whose initial antibody titer (performed 5.60±2.59 days after symptom onset) was negative. In their case, a repeated assay 8 days after the onset of symptoms was able to confirm the diagnosis.

MP PCR assays can be positive earlier on in the course of the infection than serological tests, but their application in the diagnosis of MP pneumonia is limited due to their low and variable sensitivity, ranging from 29 to 78%[32]. In a Korean study, the cumulative PCR positive rate was 78.2% in patients with antibody titers ≥1:640, but only 26.7% in patients with titers ≤1:320[20]. The PCR positive rates were similar in our study (68.1% in patients with maximum antibody titers ≥1:640, but only 47.6% in patients with titers ≤1:320), and the assays were negative in 22 of the 69 patients with MP pneumonia confirmed by serology.

For one of the limitations of our study is its retrospective design, a prospective study with several cohorts, and MP antibody titers obtained at scheduled intervals could shed more light on the diagnostic performance of MP serological tests. Secondly, it is possible that the small number of patients without MP pneumonia may have contributed to the low specificity of the PCR and the MP antibody assays in our study (52.4% and 33.3%, respectively). Finally, it should be remembered that the MP antibody response may vary between epidemic and endemic settings. Consequently, the optimal time from symptom onset to serum collection identified in our study may only apply during epidemics, and not to sporadic cases of MP pneumonia[33].

In conclusion, a four-fold or greater increase in antibody titers could be detected 8 days after the onset of symptoms in MP pneumonia patients. Repeating the titer in patients whose initial titer (performed <8 days from symptom onset) was negative could help to confirm the diagnosis of MP pneumonia and lead to better therapeutic outcomes. However, the validity of our findings must be confirmed in further studies with larger patient populations before it can be applied in clinical practice.

한 글 요 약

소아에서의 마이코플라스마 폐렴의 진단을 위한 항체 검사에 관한 연구

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김진수 · 고정희 · 오성희

목 적 : 이 연구의 목적은 항체 검사를 어느 시기에 검사해야 마이코플라스마 폐렴을 가장 적절하게 진단을 내릴 수 있는지를 파악하기 위함이다.

방 법 : 2011년 6월부터 2011년 10월까지의 한양대학교병원에서 진단받은 206 명의 폐렴 환아들을 대상으로 후향적으로 분석하였다.

결 과 : 마이코플라스마 폐렴으로 진단받은 160명의 평균 연령은 5.4세이었다. 마이코플라스마 간접입자 응집항체의 측정을 위한 혈청 획득 시간은 마이코플라스마 항체가가 1:640 이상인 혈청들과(8.58일) 1:640 미만인 혈청들(5.44일) 사이에서 통계학적으로 유의한 차이가 있었다(P<0.001).

결 론 : 본 연구의 결과는 폐렴 환아에서 증상 시작일로부터 8일 전에 획득한 마이코플라스마 항체가가 음성이면 확진을 위해 반복 검사가 필요한 것으로 보였다. 이 제

안으로 마이코플라스마 폐렴에서 최적의 진단을 내릴 수 있게 도움을 줄 수 있을 것이다.

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