Prevalence of *Chlamydia pneumoniae* in Acute Respiratory Tract Infection and Detection of Anti-*Chlamydia pneumoniae*-specific IgE in Japanese Children with Reactive Airway Disease

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Summary: We examined the prevalence of *Chlamydia pneumoniae* in acute respiratory tract infection and association of *C. pneumoniae* infection and reactive airway disease in Japanese children. Four hundred eleven children with acute respiratory tract infection were enrolled in this study, and *C. pneumoniae* was isolated from 58 (14.1%) patients by culture. Evidence of infection with *C. pneumoniae* was detected in 58 children with pneumonia (34.5%), bronchitis (41.4%) and upper respiratory tract infection (24.1%). Twenty-nine (50.0%) out of 58 patients were younger than 5 years old and 18 (31.0%) had wheezing at first visit. A logistic test for anti-*C. pneumoniae*-specific IgE showed the deference in the fluorescence unit between the patients with *C. pneumoniae* infection with and without wheezing was statistically significant ($P=0.02748$, $t_o=2.31891$). In conclusion, *C. pneumoniae* seems to be an important respiratory tract pathogen among young Japanese children, and our results support the association of *C. pneumoniae* infection and reactive airway disease.

Key words *Chlamydia pneumoniae*, children, acute respiratory tract infection, asthma, anti-*C. pneumoniae*-specific IgE

INTRODUCTION

Chlamydia pneumoniae, also known as TWAR, was first described as a respiratory tract pathogen in 1986 [1], and it is emerging as a frequent cause of community-acquired respiratory tract infections, such as pharyngitis, bronchitis and pneumonia [2]. In addition, *C. pneumoniae* has been recently implicated in atherogenesis. *C. pneumoniae* has been isolated from 15-20% in adults and children presenting with respiratory tract symptoms in the USA [3,4], however there is limited information on the prevalence of *C. pneumoniae* infection in Japan.

In 1991, Hahn et al. [5] reported an association between serologic evidence of acute *C. pneumoniae* infection and adult-onset asthma and asthmatic

bronchitis. Emre et al. [6] demonstrated an association between the presence of anti-*C. pneumoniae*-specific IgE and wheezing during infection in children with reactive airway disease. The incidence of specific IgE was 86 and 22% in patients and controls, respectively, as estimated by immunoblotting. However, previous studies were hampered by labor-intensive and time-consuming detection techniques.

The aim of this study was to assess the role of *C. pneumoniae* in acute respiratory tract infections of children in southwestern Japan, and to determine if *C. pneumoniae* infection triggers the production of anti-*C. pneumoniae*-specific IgE utilizing ELISA, thus we examined sera from children with *C. pneumoniae* infection confirmed by culture.

PATIENTS AND METHODS

Patients and specimens

Children between 1 month and 14 years of age with a diagnosis of acute respiratory tract infection, who were seen at Kurume University Hospital, Fukuoka, Japan, were prospectively examined. Kurume City is located in the southwestern part of Japan and has a population of approximately 230,000. Diagnosis of acute respiratory tract infection was based on clinical signs, symptoms and radiographic findings. Nasopharyngeal specimens were obtained from all patients using plastic-shafted dacron-tipped swabs for isolation of *C. pneumoniae*. Specimens were immediately placed in 1 ml of sucrose-phosphate-glutamine transport media at 4 °C for 1-4 hrs and stored at -70 °C. Acute phase sera were obtained from 21 patients for detection of anti-*C. pneumoniae*-specific IgE and total IgE by ELISA.

Culture

Isolation of *C. pneumoniae* was performed by cycloheximide-treated HEp-2 cells grown in 96-well microtiter plates as previously described [7]. All specimens were passed once, and isolates were identified as chlamydiae with fluorescein-conjugated antibody to the lipopolysaccharide genus antigen (Pathfinder Chlamydia Culture Confirmation System; Kallestad Diagnostic, Chaska, Minn), and confirmed using *C. pneumoniae*-specific monoclonal antibody (Washington Research Foundation, Seattle, WA). Isolates were also stained by anti-major outer membrane protein (MOMP), *Chlamydia trachomatis*-specific monoclonal antisera (Syva, Palo Alto, CA) to identify *C. trachomatis*.

Absorption of IgG

Eighty-one microliters of test serum and 0.4 mL of a 25% (v/v) suspension of recombinant protein G-agarose (Zymed Laboratories, San Francisco, CA) were mixed and shaken for one hour at room temperature, after centrifugation for 5 min at 2,000 g, the supernatant (1:5 diluted serum) was subjected to the indirect ELISA for antibody activity.

Indirect ELISA for anti-Chlamydia pneumoniae-specific IgE

One hundred microliters of 10 µg/mL of purified TW-183 strain of *C. pneumoniae* (Washington Research Foundation, Seattle, WA) in coating buffer (0.05M Na-carbonate, 0.1% NaN₃, pH 9.6) was

added to the 96-well microtiter plate (MicroFLUOR: Dynex, Alexandria, VA) and incubated at 4 °C overnight. The plate was washed with PBS Tween (phosphate-buffer saline, 0.05% Tween20, pH 7.4), and well was 0.1 mL of IgG-absorbed serum diluted 1:5 with BSA-PBS Tween (1% (w/v) bovine serum albumin) at room temperature for 3 hrs while mixing. After washing, anti human IgE- β -galactosidase (CAP RAST FEIA kit, Pharmacia, Uppsala, Sweden) diluted with BSA-PBS Tween was added and plate was incubated at 4 °C overnight. After washing, 0.1 mL of 0.3 mM 4-methylumbelliferyl- β -D-galactoside (Sigma Chemical Co., St. Louis, MO) in buffer A (0.1M MgCl₂, 0.1% BSA, 10.1% NaN₃, 0.01M Na-phosphate, pH 7.0) was added. The wells were sealed with tape, and the plates immersed in 37 °C water for 2 hrs. The enzyme reaction was stopped by adding 0.1 mL of 0.1 M glycine-NaOH, pH 10.2. The fluorescence unit (FU) of each well was measured with a Fluoroskan II (Flow Laboratories, Rockville, MD).

Tests for total IgE of serum were done with a VIDAS TOTAL IgE kit on mini VADAS (biome'rieux Vitek, MO) by enzyme linked fluorescent assay. The total IgE were calculated as prescribed by the manufacturer.

Statistical analysis

Statistical analysis for the comparison of serum anti-*C. pneumoniae*-specific IgE antibody was done using Aspin-Welch's t-test.

RESULTS

Patients

From January 1997 to December 1999, 411 children with acute respiratory tract infection were enrolled in this study. Their age ranged from 1 month to 14 years old and 196 (47.7%) children were younger than 5 years old (Fig. 1). Evidence of infection with *C. pneumoniae* was detected in 58 (14.1%) of the 411 patients by culture. Twenty (34.5%) of these 58 culture-positive children had clinical and radiographic evidence of pneumonia, while 24 (41.4%) children, who had evidence of lower respiratory tract infection on physical examination but did not have infiltrates on the chest X-ray film, were diagnosed as having bronchitis. In this study, clinical symptoms and findings of 58 patients with *C. pneumoniae* infection were as follows: 57 (98.3%) of the patients had cough, 12 complained of moderate cough for >3 weeks and 18 had wheezing at first

Fig. 1. Age distribution of 58 *Chlamydia pneumoniae* culture-positive patients with acute respiratory tract infection.

Fig. 2. Compare with fluorescence unit (FU) of anti-*Chlamydia pneumoniae*-specific IgE between the patients with *C. pneumoniae* infection with wheezing (n=11) and without wheezing (n=10). Data are shown as boxes: internal horizontal lines, medians; tops and bottoms of boxes, 25th and 75th percentiles, respectively. Upper and lower bars, 10th and 90th percentiles, respectively. *P* values comparing the mean. (Aspin-Welch's t-test, $P_o=0.02748$, $t_o=2.31891$)

visit; 29 (50.8%) patients had fever of $>37.5^\circ\text{C}$.

Indirect ELISA for anti-*Chlamydia pneumoniae*-specific IgE

We assessed 21 sera obtained from patients with *C. pneumoniae* infection with wheezing (n=11) and without wheezing (n=10). The sera of patients with wheezing elicited 267.50 ± 36.85 (mean $\pm$ SD, range 127 to 682) fluorescence unit (FU), while that of

patients without wheezing elicited 107.00 ± 90.70 FU (range 15 to 232) (Fig. 2). The logistic Aspin-Welch's t-test showed the difference in FU between patients with and without wheezing was statistically significant. ($P_o=0.02748$, $t_o=2.31891$). There were not statistical differences between two groups in the total serum IgE (data not shown).

DISCUSSION

The prevalence of *C. pneumoniae* may vary according to geographic area, population density and age group examined. However, the information on the association between *C. pneumoniae* and respiratory tract infection in Japanese children is limited. A prior seroprevalence study done in Hiroshima, which involved 660 outpatients aged 7 months to 84 years, found that antibodies to *C. pneumoniae* increased rapidly between the age of 4 and 7 years. Forty-four percent of this study population had antibodies to *C. pneumoniae* by 8 to 11 years of age [8]. Numazaki et al. [9] studied 223 infants and children (aged 4 days to 14 years) with acute pneumonia utilizing MIF serology in Sapporo, in the north part of Japan. Eleven children (4.9%) had IgM antibodies to *C. pneumoniae*. Ouchi et al. [10] examined 136 children (aged 2 months to 15 years) with acute lower respiratory tract infection living in Shimonoseki, in the southwestern part of Japan, also using MIF serology. Five (3.7%) children met the serological criteria for acute *C. pneumoniae* infection. Polymerase chain reaction (PCR) was performed for throat swab specimens from these 5 children; 4 were positive.

In the present study, infection with *C. pneumoniae* was demonstrated in 58 (14.1%) of 411 children with acute respiratory tract infection by culture. Our results demonstrated that 29 (14.7%) out of 196 children under the age of 5 years had evidence of *C. pneumoniae* infection. *C. pneumoniae* seems to be an important respiratory tract pathogen among Japanese children in the Kurume area.

Previous studies have suggested that *C. pneumoniae* is a common lower respiratory tract pathogen among adults but less common among young children [11-17]. Most of these studies on the association between *C. pneumoniae* and acute lower respiratory tract infection were based on serology alone. In a study from Sudan [18], 3.6% of 110 children with acute respiratory tract infection had either culture or serologic evidence of acute *C. pneumoniae* infection. In Germany [19], 1.0% of 290 infants and children with acute respiratory tract infection were found to

have acute *C. pneumoniae* infection utilizing culture and serological techniques and PCR. In contrast, the rates of *C. pneumoniae* infection in children with acute lower respiratory tract disease have been higher in several studies from the USA which utilized culture. In Brooklyn [3], 9 (12.2%) of 74 patients less than 18 years old who presented with lower respiratory tract infection had *C. pneumoniae* infection by culture. In a nation-wide multicenter treatment study of community-acquired pneumonia, 42 (16.2%) of 260 children were demonstrated to have *C. pneumoniae* infection based on culture [4]. This is probably reflective of improved isolation techniques. In the above mentioned multicenter study, infection with *C. pneumoniae* was detected in 28% of the 260 children by both culture and serology, however, only 23% of the culture positive children met the serologic criteria for acute *C. pneumoniae* infection [20]. Emre et al. [21] isolated *C. pneumoniae* from 13 (11%) of 118 children with acute exacerbation of asthma and from 2 (4.9%) of 41 asymptomatic controls. Seven (58.3%) of 12 children with positive cultures also had no detectable antibody to *C. pneumoniae* and only 3 (25%) of the culture positive children had serologic evidence of acute infection. The antibody response in acute *C. pneumoniae* infection may take longer than 3 weeks to develop [22]. Acute infection can also occur without seroconversion, especially in children [3,20,21]. Because of the relatively long period until the development of serologic response in primary infection, the microimmunofluorescence (MIF) test may not be sufficiently sensitive in children. The MIF test is generally accepted as the golden standard for the serological diagnosis of an acute *C. pneumoniae* infection. However, interpretation of specific and non-specific fluorescence patterns requires experience and skill, and the interpretation of high titer (>512) is very difficult and subjective [23]. Moreover, cross-reactions between species of *Chlamydia* have been reported with the MIF assay [24-26]. Further prospective studies utilizing culture and PCR are required to clarify *C. pneumoniae* infection in children.

Infections with viruses, such as respiratory syncytial virus, parainfluenza virus and rhinovirus, have been identified in 40%-60% of children with acute exacerbation of asthma [6]. Although several viral and atypical pathogens have been linked with possible asthma initiation and promotion, the association of *C. pneumoniae* infection with asthma is the most extensively documented. Evidence of the role of *C.*

pneumoniae in the pathogenesis of asthma has been obtained by culture isolation, PCR, serologically and by preliminary treatment trial. Most of the evidence for *C. pneumoniae* infection in adult-onset asthma has been based on serology, since successful culture isolation of *C. pneumoniae* from the upper airway of adults with stable chronic asthma has been only infrequently reported [27]. In our study, 18 (31%) out of 58 children positive for *C. pneumoniae* by culture, had wheezing.

In 1996, Hahn et al. [27] reported that patients with symptomatic asthma had significantly more often IgA antibodies present in the serum than controls. Recently, Cook et al. [28] found that elevated IgG antibody levels to *C. pneumoniae* were significantly associated with severe chronic asthma, and this association was not modified by potentially confounding factors. In 1999, Hertzen et al. [29] reported the results of a logistic regression analysis that controlled for age, sex and smoking showing that asthma was significantly associated with elevated IgG antibody levels to *C. pneumoniae* (odds ratio 3.3). A number of mechanisms have been proposed to explain airway hyperresponsiveness following infection, including the development of organism-specific IgE antibody [6].

Emre et al. [21] investigated the prevalence of *C. pneumoniae* infection in children with acute exacerbation of reactive airway disease, and found that *C. pneumoniae* was isolated from 11% of children with acute episodes of wheezing compared with 4.9% of age- and sex-matched healthy controls. Subsequently, they demonstrated that anti-*C. pneumoniae*-specific IgE was associated with asthma in culture-positive children [6]. Specific IgE was detected in 86 and 22% of patients and controls, respectively, assessed by immunoblotting. In contrast to this study of children with asthma-like symptoms, Larsen et al. [30] examined adults diagnosed as having bronchial asthma and detected anti-*C. pneumoniae*-specific IgE in the patients as well as in the control group without differences in the pattern between the two groups. The prevalence of anti-*C. pneumoniae*-specific IgE was approximately 69% in both groups. In the present study, to further investigate the mechanism of *C. pneumoniae*-associated reactive airway disease, we examined sera from the children with *C. pneumoniae* infection with and without wheezing for the presence of anti-*C. pneumoniae*-specific IgE. Our results showed that anti-*C. pneumoniae*-specific IgE was detected significantly more frequently in the sera from the patients with wheezing than without wheez-

ing, utilizing ELISA. Whereas, the total serum IgE level in patients with *C. pneumoniae* infection was not significant differences between the patients with wheezing and without wheezing. *C. pneumoniae* infection may be related to the development of chemical mediators leading to airway reactivity. Although the size of the population in this study was relatively small, our results support our clinical observations on the association of *C. pneumoniae* infection and reactive airway disease. The role of host factor as well as other immune mechanisms in the pathogenesis of *C. pneumoniae* infections needs to be further investigated.

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REFERENCES

- Grayston JT, Kuo CC, Wang SP, and Altman J. A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infection. *N Engl J Med* 1986; 315:161-168.
- Grayston JT. Infections caused by *Chlamydia pneumoniae* strain TWAR. *Clin Infect Dis* 1992; 15:757-763.
- Chirgwin K, Roblin PM, Gelling M, Hammerschlag MR, and Schachter J. Infection with *Chlamydia pneumoniae* in Brooklyn. *J Infect Dis* 1991; 163:757-761.
- Roblin PM, Montalban G, and Hammerschlag MR. Susceptibility to clarithromycin and erythromycin of isolates of *Chlamydia pneumoniae* from children with pneumonia. *Antimicrob Agents Chemother* 1994; 38:1588-1589.
- Hahn DL, Dodge RW, and Golubjatnikov R. Association of *Chlamydia pneumoniae* (Stain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. *JAMA* 1991; 266:225-230.
- Emre U, Sokolovskaya N, Roblin PM, Schachter J, and Hammerschlag MR. Detection of Anti-*Chlamydia pneumoniae* IgE in children with reactive airway disease. *J Infect Dis* 1995; 172:265-267.
- Roblin PM, Dumornay W, and Hammerschlag MR. Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. *J Clin Microbiol* 1992; 30:1968-1971.
- Kanamoto Y, Ouchi K, Mizui M, Ushio M, and Usui T. Prevalence of antibody to *Chlamydia pneumoniae* TWAR in Japan. *J Clin Microbiol* 1991; 29:816-818.
- Numazaki K, Chiba S, and Umetsu M. Detection of IgM antibodies to *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci* from Japanese infants and children with pneumonia. *In Vivo* 1992; 6:601-604.
- Ouchi K, Nakazawa T, Karita M, and Kanehara Y. Prevalence of *Chlamydia pneumoniae* in acute lower respiratory infection in pediatric population in Japan. *Acta Paediatr Jpn* 1994; 36:256-260.
- Saikku P, Ruutu P, Leinonen M, Panelius J, Tupasi TE et al. Acute lower-respiratory-tract infection associated with chlamydial TWAR in Filipino children. *J Infect Dis* 1988; 158:1095-1097.
- Grayston JT, Diwan VK, Cooney M, and Wang SP. Community- and hospital-acquired pneumonia associated with *Chlamydia* TWAR infection demonstrated serology. *Arch Inter Med* 1989; 149:169-173.
- Wang SP, and Grayston JT. Population prevalence antibody to *Chlamydia pneumoniae*. In: *Chlamydia Infections: Proceeding of the Seventh International Symposium on Human Chlamydia Infections*, ed. Bowie et al., Cambridge University Press, Cambridge, England, pp 402-405, 1990.
- Aldous MB, Grayston JT, Wang SP, and Foy HM. Seroepidemiology of *Chlamydia pneumoniae* TWAR infection in Seattle families, 1966-1979. *J Infect Dis* 1992; 166:646-649.
- Carballal G, Mahony JB, Videla C, Cerqueiro C, and Chernesky M. Chlamydial antibodies in children with lower respiratory disease. *Pediatr Infect Dis J* 1992; 11:68-71.
- Yeung SM, McLeod K, Wang SP, Grayston JT, and Wang EEL. Lack of evidence of *Chlamydia pneumoniae* infection in infants with acute lower respiratory tract disease. *Eur J Clin Microbiol Infect Dis* 1993; 12:850-853.
- Grayston JT. *Chlamydia pneumoniae* (TWAR) infections in children. *Pediatr Infect Dis J* 1994; 13:675-685.
- Herrmann B, Salih MAM, Yousif BE, Abdelwahab O, and Mårdh P-A. Chlamydial etiology of acute lower respiratory tract infections in children in the Sudan. *Acta Paediatr* 1994; 83:169-172.
- Jantos CA, Wienpahl B, Schiefer HG, Wagner F, and Hegemann JH. Infection with *Chlamydia pneumoniae* in infants and children with acute lower respiratory tract disease. *Pediatr Infect Dis J* 1995; 14:117-122.
- Block S, Hedrick J, Hammerschlag MR, Cassell GH, and Craft JC. *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in pediatric community-acquired pneumonia: comparative efficacy and safety of clarithromycin vs. erythromycin ethylsuccinate. *Pediatr Infect Dis J* 1995; 14:471-477.
- Emre U, Roblin PM, Gelling M, Dumornay W, Hammerschlag MR et al. The association of *Chlamydia pneumoniae* infection and reactive airway disease in children. *Arch Pediatr Adolesc Med* 1994; 148:727-732.
- Grayston JT, Campbell LA, Kuo CC, Mordhort CH, Saikku P et al. A new respiratory pathogen: *Chlamydia*

- pneumoniae* strain TWAR. J Infect Dis 1990; 161:618-625.
23. Verkooyen RP, Van Lent NA, Mousavi Joulandan SA, Snijner RJ, Van Den Bosch JM et al. Diagnosis of *Chlamydia pneumoniae* infection in patients with chronic obstructive pulmonary disease by micro-immunofluorescence and ELISA. J Med Microbiol 1997; 46:959-964.
 24. Kern DG, Neill MA, and Schacter J. A seroepidemiologic study of *Chlamydia pneumoniae* in Rhode Island. Evidence of serologic cross-reactivity. Chest 1993; 104:208-213.
 25. Moss TR, Darougar S, Woodland RM, Nathan M, Dines DJ et al. Antibodies to chlamydia species in patients attending a genitourinary clinic and the impact of antibodies to *Chlamydia pneumoniae* and *Chlamydia psittaci* on the sensitivity and the specificity of *Chlamydia trachomatis* serology tests. Sex Trans Dis 1993; 20:61-65.
 26. Hyman CL, Roblin PM, Gaydos MS, Quinn TC, Schachter J et al. The prevalence of asymptomatic nasopharyngeal carriage of *Chlamydia pneumoniae* in subjectively healthy adults: assessment by polymerase chain reaction-enzyme immunoassay and culture. Clin Infect Dis 1995; 20:1174-1178.
 27. Hahn DL, Anttila T, and Saikku P. Association of *Chlamydia pneumoniae* IgA antibodies with recently symptomatic asthma. Epidemiol Infect 1996; 117:513-517.
 28. Cook PJ, Davies P, Tunnicliffe W, Ayres JG, Honeybourne D et al. *Chlamydia pneumoniae* and asthma. Thorax 1998; 53:254-259.
 29. Von Hertzen L, To "yryla" M, Gimishanov A, Bloigu A, Leinonen M et al. Asthma, atopy and *Chlamydia pneumoniae* antibodies in adults. Clin Exp Allergy 1999; 29:522-528.
 30. Larsen FO, Norn S, Mordhorst CH, Stahl Skov P, Milman N et al. *Chlamydia pneumoniae* and possible relationship to asthma. APMIS 1998; 106:928-934.