Jpn. J. Infect. Dis., 56, 146-150, 2003

Original Article

Chlamydophila pneumoniae Infection among Young Children with Respiratory Diseases in Thailand

Piyada Wangroongsarb, Kanonkporn Geenkajorn, Wimol Pektkanchanapong, Apiram Vedjapoom[1], Mayura Kusum and Toshikatsu Hagiwara*

National Institute of Health and
[1]Queen Sirikit National Services of Child Health, Department of Medical Services,
Ministry of Public Health, Nonthaburi 11000, Thailand

(Received February 28, 2003. Accepted August 22, 2003)

SUMMARY: A total of 136 children aged 5 years and under with respiratory tract diseases were examined for *Chlamydophila pneumoniae* infection. By means of the micro-immunofluorescence test, an acute infection was suggested in 37 (27.2%) of them. Infection was found in 23 (43.4%) of 53 children with bronchitis, seven (70.0%) of 10 with pharyngitis, and two (22.2%) of nine with pneumonia. *C. pneumoniae* DNA was detected in seven of 55 children by means of nasopharyngeal swabs, and serological evidence was present in all of seven. Five of them were suggested the acute infection and four of the five showed IgG titers increasing four times and over. By age distribution, five of the seven DNA-positive children were 1 year old, and the remaining two were 2 and 4 years old, respectively. The clinical findings of the seven DNA-positive children were characterized as indicative of bronchitis ($n = 4$), pharyngitis ($n = 2$), and pneumonia ($n = 1$). In Thailand, *C. pneumoniae* infection occurs frequently among children aged 5 years and under, and may cause pharyngitis, bronchitis, and sometimes pneumonia. However, it is suggested that *C. pneumoniae* infection is not a major cause of severe pneumonia among children in that age group.

INTRODUCTION

Chlamydophila (*Chlamydia*) *pneumoniae* has been established as a major pathogen of respiratory tract infections, and been determined to be responsible for 5 to 15% of community-acquired cases of pneumonia, pharyngitis, bronchitis, and sinusitis (1,2). Furthermore, retrospective serological studies have demonstrated that *C. pneumoniae* infection appears to be common among school-aged children, and that around 50 % of adults throughout the world show evidence of a previous infection (1-3). However, infection among preschool-aged children has been influenced by geographical location and socioeconomic status. Namely, infection during childhood (less than 5 years old) is rare in the industrialized countries in the North Temperate Zone (2-4), whereas in tropical developing countries, such as the Philippines, infection in this age group is relatively common and may be severe (5). Given that acute respiratory diseases are a major killer of children younger than 5 years old in developing countries, more information concerning the role of *C. pneumoniae* infection is desirable (3).

In Thailand, a large number of severe pneumonia cases of patients under 5 years of age is reported every year (6). Some serological reports of *C. pneumoniae* infection (7,8) have suggested that infection is common among school-aged children and adults, and it may cause community-acquired pneumonia in this country as in other countries (1,2). However, the rate of infection among children less than 5 years of age in this country is still obscure, though a report concerning neonatal *C. pneumoniae* infection is available (9).

*Corresponding author: Mailing address: National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand. Fax: +66-2-589-9865, E-mail: toshikatsu@dmsc.moph.go.th

In this paper, we identified *C. pneumoniae* infection in children aged 5 years and under who suffered from respiratory diseases, and discussed the role of this organism in respiratory diseases among children in this country.

MATERIALS AND METHODS

Subjects: The study was conducted on children with respiratory diseases visiting the Children's Hospital from April to October of 1997. A total of 136 children, whose ages ranged from 1 month to 5 years, were enrolled in this study; 79 of them were male and 57 were female. The clinical symptoms and signs in the children, evaluated by physicians, were as follows: bronchitis ($n = 53$), pneumonia ($n = 9$), unspecified upper respiratory infection (URI, $n = 48$), influenza-like disease ($n = 12$), pharyngitis ($n = 10$), sinusitis ($n = 2$), and tonsillitis ($n = 2$). Informed consent was obtained from all patients' guardians.

Specimen collection: Serum specimens were collected for the detection of antibodies to *C. pneumoniae* from all 136 children during the initial visit. Second specimens were available from 70 of them, at 10 to 14 days after the first specimen collection. At the initial visit, throat swab specimens were collected from the nasopharynx of all patients for cultivation of *C. pneumoniae* and detection of DNA of the organism. The collected swabs were preserved in 0.2 M sucrose-0.02 M phosphate (2SP) transport medium (10) and stored at −70°C until examination.

Serology: The micro-immunofluorescence (micro-IF) test to assay serum IgG and IgM antibodies to *C. pneumoniae* was used as described previously (11,12). In brief, immunoglobulin-specific *Chlamydia* antibodies were measured with FITC-labeled anti-human IgG and IgM (Dako Co., Copenhagen, Denmark). Acute infection was based on the following criteria (9). A fourfold or greater titer change

between paired sera, or the presence of IgM ≥ 16 or IgG ≥ 512 in either the first serum or the second serum. The stable IgG titers (16 to 256) were considered evidence of a previous infection.

Culture: Culture of *C. pneumoniae* was performed on cyclohexamide-treated HEp-2 cells grown on coverslips in culture plates, as described elsewhere (13) but with slight modification. In brief, 200-250 μl of each specimen was inoculated into four wells of a 24-well culture plate, then the plate was centrifuged at 1,500 rpm at room temperature for 1 h. The supernatant was replaced by *Chlamydia* isolation medium containing 1 μg/ml cycloheximide. After incubation at 35°C, 5% CO_2 for 3 days, a coverslip in a well was fixed with ethanol and stained with fluorescent-conjugated *Chlamydia* genus-specific monoclonal antibody (DENKA SEIKEN Co. Ltd., Tokyo) or *Chlamydia pneumoniae* specific monoclonal antibody (DENKA SEIKEN). Inclusion bodies of the organism were identified as *C. pneumoniae* if they were stained positively with both antibodies. Subculture was discontinued after three negative passages.

Polymerase chain reaction (PCR) amplification: *Chlamydia* DNA was extracted from the nasopharynx swabs by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A pair of primers derived from highly conserved genus-specific regions of the major outer membrane protein (MOMP) genes of *Chlamydia* (13) was used in this study. Using the Ready-to-Go PCR Beads (0.2 μl tubes, Amersham Pharmacia Biotech Ltd., Piscataway, N.J., USA), PCR amplification was carried out. PCR conditions were as follows. After denaturation at 94°C for 3 min, samples were amplified for 40 cycles. An amplification cycle consisted of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. PCR products were analyzed by electrophoresis with 3% agarose gel.

Restriction fragment length polymorphism (RFLP) analysis: An amplified DNA was digested with restriction enzymes *Alu*I and *Pvu*II, according to the manufacturer's instructions. DNA fragments were analyzed on 3% agarose gel as described previously (14). In brief, both *C. pneumoniae* and *C. trachomatis* are digested with *Alu*I and are not with *Pvu*II. However, their respective restriction profiles are different.

Statistical analysis: Analysis was carried out with the chi-square test.

RESULTS

Serology: A total of 73 out of 136 children examined by the micro-IF test had antibodies to *C. pneumoniae* in the IgG and/or the IgM serum fractions. As shown in Table 1, in 37 (27.2%) out of 136, an acute infection was suggested on the basis of the criteria of the micro-IF. Eight of 37 showed increasing IgG antibody titers (fourfold and greater) between the paired sera, and the remaining 29 showed titers of 16 and higher in the IgM serum fraction (geometric mean antibody titer: 24.3) in the first and/or the second serum. The prevalence rate of acute infection among children aged under 6 months was significantly high, though there was no significant difference by gender (data not shown). Among 36 cases categorized as IgG positive in Table 1, 10 showed stable titers (geometric mean antibody titer: 16.0) between the paired sera suggesting a previous infection in nine; one showed an IgG titer of 1:16, which was suspected to be of maternal origin. Eighteen of 36 showed a twofold titer change between the paired sera, and for the remaining eight only the first serum (geometric mean antibody titer: 18.0) was examined because no second serum sample was available. Clinical findings and serological results among those children are shown in Table 2. Bronchitis and URI were found to be major clinical findings. However, *Chlamydia* acute infection, suggested by the micro-IF, was frequently found among children with pharyngitis (70.0%) and bronchitis (43.4 %) and, subsequently, pneumonia (22.2%). Among children aged less than 6 months, the incidence rate of bronchitis was high and in two-thirds of these cases acute infection was suggested. No pneumonia or pharyngitis cases of *C. pneumoniae* infection were suggested by the micro-IF test among children of that age.

Isolation: *C. pneumoniae* was not isolated from nasopharyngeal swabs taken from 136 children and examined by cell culture.

DNA detection: A total of 55 nasopharyngeal swabs (male = 33, female = 22) could be tested by PCR-RFLP. The others provided an amount of specimen to scan to use; they had been used for previous isolation attempts. The clinical findings and serological results among the children tested are shown in Table 3. The numbers of children with acute, IgG-positive and -negative infection were 16, 21, and 18, respectively. Age distribution was as follows; under 6 months, 6 months to 11 months, 1 year, 2 years, 3 years, and 4 years and over were 6, 8, 20, 9, 7, and 5, respectively. *Chlamydia* DNA was detected in seven of these children. Clinical findings and serological results of the seven DNA-positive cases are shown in Table 4. After digestion of those PCR products with enzymes *Alu*I and *Pvu*II, all of the amplified DNA products were identified as *C. pneumoniae* DNA (data not shown). Of the seven, five were suspected to have acute infection based on the micro-IF results, and the remaining two showed low titer of IgG antibody to the organism. Therefore, the DNA could be detected in 31.5% (5/16) of children, suggesting acute infection. Further *C. pneumoniae* DNA was found to be detected in all cases that showed increasing antibody titer in the IgG serum fraction. By clinical findings, *Chlamydia* DNA was detected in all of the following: two children with pharyngitis, four of 25 with bronchitis, and one of seven with pneumonia. By age distribution, the DNA was detected in five of 20 children aged 1 year, one of nine aged 2 years and one of five aged 4 years.

Table 1. Age-related incidence of *C. pneumoniae* infection in children with respiratory diseases

Age	No. tested	Serological test results		
		Acute	IgG positive[1)]	% Acute
<6 m	8	5	2[2)]	62.5
>6 m to <1 y	15	1	2	6.7
1 y	38	10 (2)[3)]	11	26.3
2 y	22	7 (3)	4	31.8
3 y	34	9 (1)	11	26.5
4 to 5 y	19	5 (2)	6	26.3
Total	136	37 (8)	36	27.2

[1)]: Cases that showed IgG antibody titers (16-32), excepting cases of acute infection.
[2)]: One case aged 2-months had stable IgG antibody titers suspected to be of maternal origin.
[3)]: Figure in the parentheses indicates the number of cases showing a fourfold titer increase.

Table 2. Clinical findings and serological results in children with respiratory diseases by age

Age	No. of case	Clinical findings					
		Pneumonia	Bronchitis	Pharyngitis	URI	Like influenza	Tonsillitis/Sinusitis
<6 m	Specimen	0	5	0	1	2	0
	Acute	0	3	0	1	1	0
	IgG positive[1)]	0	1	0	0	1	0
≥6 m to <1 y	Specimen	3	6	1	4	1	0
	Acute	0	1	0	0	0	0
	IgG positive	0	1	0	1	0	0
1 y	Specimen	4	16	2	12	3	1
	Acute	1	7	2	0	0	0
	IgG positive	1	7	0	3	0	0
2 y	Specimen	0	4	5	10	2	1
	Acute	0	1	4	2	0	0
	IgG positive	0	0	0	3	1	0
3 y	Specimen	2	13	0	14	4	1
	Acute	1	8	0	0	0	0
	IgG positive	1	2	0	7	0	1
4 to 5 y	Specimen	0	9	2	7	0	1
	Acute	0	3	1	1	0	0
	IgG positive	0	4	0	1	0	1
Total	Specimen	9	53	10	48	12	4
	Acute	2	23	7	4	1	0
	IgG positive	2	15	0	15	2	2
	% Acute	22.2 (2/9)	43.4 (23/53)	70.0 (7/10)	8.3 (4/48)	8.3 (1/12)	0 (0/4)

[1)]: See footnote 1) of Table 1.

Table 3. Clinical findings and serological results in children examined for *C. pneumoniae* DNA

Serological results	Clinical findings						
	Pneumonia	Bronchitis	Pharyngitis	URI	Like influenza	Tonsillitis/Sinusitis	Total
Acute	3	8	2	3	0	0	16
IgG positive[1)]	1	10	0	8	2	0	21
IgG negative	3	7	0	3	4	1	18
Total	7	25	2	14	6	1	55

[1)]: See footnote 1) of Table 1.

Table 4. Clinical findings and serological results in children found to be *C. pneumoniae* DNA positive

Case No.	Gender	Age	Clinical finding	Ig fraction	1st	2nd	Serodiagnosis
1	Male	1 y	Pneumonia	IgG	16		IgG positive[1)]
				IgM	<16		
2	Male	1 y 1 m	Pharyngitis	IgG	<16	512	Acute
				IgM	32	16	
3	Female	1 y 1 m	Bronchitis	IgG	<16		Acute
				IgM	32		
4	Male	1 y 4 m	Bronchitis	IgG	<16	128	Acute
				IgM	<16	16	
5	Male	1 y 11 m	Bronchitis	IgG	16	32	IgG positive
				IgM	<16	<16	
6	Male	2 y	Acute bronchitis	IgG	<16	512	Acute
				IgM	32	16	
				IgM	32	16	
7	Male	4 y 8 m	Pharyngitis	IgG	<16	32	Acute
				IgM	16	<16	

[1)]: See footnote 1) of Table 1.

DISCUSSION

Antibodies to *C. pneumoniae* were demonstrated in 73 of 136 children aged 5 years and under and having respiratory diseases. Among antibody-positive cases, acute infection of *C. pneumoniae* was suggested in 37. Prevalence rate of the acute infection was significantly high (62.5%) among children aged under 6 months. In Thailand, Nubthapisud et al. (9) reported that acute infection was suggested by the micro-IF test in two infants, aged 24 days and 2 months, respectively, with respiratory diseases, and they suggested that the organism was possibly transmitted from mother to infant during or a short time after the birth. Our results may support this hypothesis, though epidemiological information on infection in member of a patient's family was obscure in our study.

In this study, *Chlamydia* acute infection suggested by the micro-IF test was frequently found among children with pharyngitis (70.0%), bronchitis (43.4%), and, subsequently, with pneumonia (22.2%). *C. pneumoniae* DNA was detected in four of 25 children with bronchitis, two children with pharyngitis, and one of seven with pneumonia. All of seven DNA-positive children showed serological evidence of infection, and five of them were suggested by the micro-IF test to have acute infection. This is the first available report in this country of detection of *C. pneumoniae* DNA in patients with respiratory disease. *C. pneumoniae* DNA was detected in five out of 20 aged 1 year old examined. However, there was no case of detection in a patient less than 6 months of age, though the prevalence rate of acute infection suggested by the micro-IF test was very high. Failure of detection of *C. pneumoniae* DNA may be due to the fact that the number of children tested was too small and due to the difficulties of throat swab collection from such young patients.

In tropical and developing countries, *C. pneumoniae* infection among children aged less than 5 years old seems to be severe, and such children show high antibody titers to the organism (3,5). However, our study in Thailand indicated that the prevalence rate of pneumonia was found to be lower than that of pharyngitis, and the antibody titer to *C. pneumoniae* in cases with pneumonia was not remarkably high. In Japan, Yamazaki et al. reported an outbreak of *C. pneumoniae* infection in a family (15). According to their report, one of two children suspected to have *C. pneumoniae* infection did possess the antibody to *C. pneumoniae*. Further, Iwata et al. (16) reported 11 cases of children less than 5 years of age in whom *C. pneumoniae* was found by isolation and/or PCR test. However, some of the pneumonia cases had low antibody titers to *C. pneumoniae*. Therefore, clinical and serological findings of *C. pneumoniae* infection among preschool-aged children in this country may be similar to such findings in Japan, though the prevalence rate of infection among children of similar ages in Japan was relatively low (17).

In this study, we failed to isolate *C. pneumoniae* from nasopharyngeal swabs of children in whom *C. pneumoniae* DNA had been detected. As described elsewhere (18), the organism is rather susceptible, compared with other *Chlamydia* spp., to inactivation by rapid freezing, and, further, it grows poorly in tissue cultures. We did not estimate the cause of failure of isolation, but we consider that organism storage conditions might have been unsuitable.

In conclusion, *C. pneumoniae* infection occurs frequently among children aged 5 years and under in Thailand, and the infection causes phraryngitis, bronchitis, and sometimes pneumonia. Although the infection generally seems not to be severe, further studies should be conducted to clarify the pathogenicity of the organism in respiratory diseases occurring in children.

ACKNOWLEDGMENTS

We are indebted to the Japan International Cooperation Agency (JICA), which made this research project possible financially.

REFERENCES

1. Kuo, C. C., Jackson, L. A., Campbell, L. A. and Grayston, J. T. (1995): *Chlamydia pneumoniae* (TWAR), Clin. Microbiol. Rev., 8, 451-461.
2. Kauoppinen, M. and Saikku, P. (1995): Pneumonia due to *Chlamydia pneumoniae*: prevalence, clinical features, diagnosis, and treatment. Clin. Infect. Dis., 21, S244-252.
3. Grayston, J. T. (1994): *Chlamydia pneumoniae* (TWAR) infection in children. Pediatr. Infect. Dis. J., 13, 675-685.
4. Miyashita, N., Fukano, H., Yoshida, K., Niki, Y. and Matsushima, T. (2002): Seroepidemiology of *Chlamydia pneumoniae* in Japan between 1991 and 2000. J. Clin. Pathol., 55, 115-117.
5. Saikku, P., Ruutu, P., Leinonen, M., Panelius, J., Tupasi, T. E. and Grayston, J. T. (1988): Acute lower-respiratory -tract infection associated with Chlamydial TWAR antibody in Filipino children. J. Infect. Dis., 158, 1095-1097.
6. Division of Epidemiology, Ministry of Public Health (1999): Annual Epidemiological Surveillance Report. p. 275-283 (text in Thai, figure and table in English).
7. Nubthapisud, P., Tatiyakavee, K., Prapphal, N., Likinukul, S. and Grayston, J. T. (1992): The prevalence of *Chlamydia pneumoniae*, strain TWAR, antibody in school children: a preliminary study. Chula. Med. J., 36, 273-277.
8. Leowattana, W., Mahanoda, N., Bhuripanyo, K. and Pokum, S. (2000): Seroprevalence of *Chlamydia pneumoniae* infection in Thailand. J. Med. Assoc. Thai., 83, S1-S5.
9. Nubthapisud, P., Prapphal, N., Likinukul, S. and Vejabhuti, A. (1995): *Chlamydia pneumoniae* infection in Thai children; Serological diagnosis. Southeast Asian J. Trop. Med. Public Health, 26, 339-341.
10. Grayston, J. T., Wang, S. P., Kuo, C. C. and Campbell, L. A. (1989): Current knowledge on *Chlamydia pneumoniae*, strain TWAR, an important cause of pneumonia and other acute respiratory diseases. Eur. J. Clin. Microbiol. Infect. Dis., 8, 191-202.
11. Wang, S. P. and Grayston, J. T. (1986): Microimmunofluorecsence serological studies with TWAR organism. *In* Oriel, D., Ridway, G., Schachter, J., Taylor-Robinson, D. and Ward, M. (ed), Chlamydial Infections. Proceedings of the 6th International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge.
12. Wangroongsarb, P., Phuekfen, P., Naigowat, P. and Hagiwara, T. (2002): *Chlamydophila pneumoniae* specific antibodies in Thai patients with myocardial infarction. Jpn. J. Infect. Dis., 55, 49-51.
13. Maass, M. and Harig, U. (1995): Evaluation of culture conditions used for isolation of *Chlamydia pneumoniae*. Am. J. Clin. Pathol.,103, 141-148.

14. Yoshida, H., Kishi, Y., Shiga, S. and Hagiwara, T. (1998) : Differentiation of *Chlamydia* species by combined use of polymerase chain reaction and restriction endnuclease analysis. Microbiol. Immunol., 42, 411-414.
15. Yamazaki, T., Nakata, H., Sakurai, N., Kuo, C. C., Wang, S. P. and Grayston, J. T. (1990): Transmission of *Chlamydia pneumoniae* in young children in a Japanese family. J. Infect. Dis., 162, 1390-1392.
16. Iwata, S., Akita, H., Sato, Y., Sunakawa, K., Komota, T., Bannai, H., Yoshida, H. and Hagiwara, T. (1999): Clinical feature of *Chlamydia pneumoniae* infection. Modern Media, 45, 33-40 (text in Japanese).
17. Miyashita, N., Fukano, H., Yoshida, K., Niki, Y. and Matsushima, T. (2002): Seroepidemiology of *Chlamydia pneumoniae* in Japan between 1991 and 2000. J. Clin. Pathol., 55, 115-117.
18. Maass, M. and Dalhoff, K. (1995): Transport and storage conditions for cultural recovery of *Chlamydia pneumoniae*. J. Clin. Microbiol., 1793-1796.