Evaluation of Direct Immunofluorescence Test for Diagnosis of Upper Respiratory Tract Infection by *Chlamydia pneumoniae*

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Background: *Chlamydia pneumoniae causes a variety of respiratory infections and is involved in cardiovascular diseases. Diagnosis of C. pneumoniae infection currently relies on antibody detection by microimmunofluorescence (MIF), which has limited use, and is the retrospective diagnosis for acute infection.*
Objective: *Find an effective early diagnosis of acute upper respiratory infection, or use in combination with MIF to accurately diagnose the infection by C. pneumoniae.*
Material and Method: *Direct immunofluorescence (DIF) was developed to detect C. pneumoniae in nasopharyngeal specimens obtained from patients with upper respiratory tract infection, and normal individuals. IgM and IgG antibodies against C. pneumoniae by MIF were determined for evaluation of the detected C. pneumoniae and seroconversion.*
Results: *DIF gave positive results in 29 of 37 (78.4%) samples from 31 patients. Fifteen samples positive by DIF illustrated antibody titers interpreted as acute C. pneumoniae infection, and eight DIF positive samples showed antibody titers of chronic infection. Negative results by both DIF and MIF were found in two patients and 23 of 25 by DIF but 20 of 25 by MIF in normal subjects. Five paired sera subsequently collected from three of the 31 patients illustrated seroconversion 2-4 months after the primary specimen collection, which gave positive results by DIF but negative for antibodies. Significant association was found between C. pneumoniae detection by DIF and antibodies by MIF when analysis was done in the group of patients and normal subjects ($p < 0.001$; Pearson chi-square test).*
Conclusion: *DIF could be an alternative assay for early diagnosis of C. pneumoniae infection, and may be used in combination with MIF for accurate diagnosis of acute C. pneumoniae infection.*

Keywords: *Direct immunofluorescence, Chlamydia pneumoniae, upper respiratory infection, microimmunofluorescence, Chlamydia pneumoniae antibody*

J Med Assoc Thai 2008; 91 (9): 1416-24
Full text. e-Journal: *http://www.medassocthai.org/journal*

Chlamydia pneumoniae is a common causative pathogen of acute and chronic respiratory infection in both immunocompetent and immunocompromised hosts. It involves a wide spectrum of respiratory diseases including the upper respiratory tract infection (pharyngitis, sinusitis, otitis, etc), and development of lower respiratory diseases, such as acute or chronic bronchitis, chronic obstructive airways disease and pneumonia. *C. pneumoniae* infection accounts for 10% to 20% of community-acquired pneumonia and is approximately 5% of bronchitis and sinusitis cases[1,2]. Based on seroepidemic studies, more than 50% of

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adults have antibodies against *C. pneumoniae*[3,4]. In addition to the respiratory diseases, this pathogen has been reported to induce extrarespiratory infections and immune disorder, as well as the pathogenesis of cardiovascular diseases, and atherosclerosis[5-9].

C. pneumoniae is an obligate intracellular bacterium that infects a wide variety of host cells including epithelial cells, endothelial cells, peripheral blood mononuclear cells, as well as those recently reported of polymorphonuclear and eosinophile/basophile[8-12]. Reinfection from primary infection by *C. pneumoniae* is common evidence throughout life, and associated with vasculitis in small and large vessels[13]. The organism remains viable in the host long after remission of an acute infection, and the clinical symptoms frequently recur after a short course therapy or administration of antibiotics not susceptible to the pathogen[3].

The diagnosis of *C. pneumoniae* infection, particularly with pneumonia, currently relies on serological testing. Microimmunofluorescence (MIF) assay is represented as the gold standard method in detection of IgG, IgM, and IgA antibodies raised against *C. pneumoniae* infection[14,15]. The titers of IgM and/or IgG antibodies by MIF can be used in distinguishing acute from chronic or past infections. However, in cases of reinfections, IgM antibody may be absent or present with low titers, and a high IgG antibody titer may persist for a long duration[15]. The evidence can make a diagnosis difficult to distinguish between acute infection and the persistent antibody from past infection. Furthermore, the appearance of MIF antibodies is slow. The interval of acute phase and convalescent serum samples is recommended for at least of a 3 week duration, and even a third serum sample obtained 2 months after onset of disease may be required for rising in antibody titer. The slow antibody response can cause misdiagnosis for acute *C. pneumoniae* infection, which generally uses a convalescent serum collected 2 weeks after the first serum sample. The delay of an appropriate antibiotic treatment for an acute primary upper respiratory infection is considerable for the disease progress to a severity of pulmonary disease, or chronic persistent infection, as well as complications involved in cardiovascular diseases.

Direct detection of *C. pneumoniae* by culture and polymerase chain reaction (PCR) could provide accurate and timely diagnosis at the acute stage of infection[2,16-18]. However, *C. pneumoniae* is difficult to grow in cell culture, and this leads to its low sensitivity for the diagnosis. PCR is sensitive and specific but it is laborious, time consuming, not commercially available and has not been standardized for routine diagnosis. Direct immunofluorescence (DIF) has been demonstrated to be feasible for detection of virus antigens, as well as *C. pneumoniae* and used for diagnosis in patients with acute respiratory tract infection and community-acquired pneumonia[19]. Furthermore, DIF has been used in *C. pneumoniae* antigen detection in throat swabs and sputum in cases with acute and persistent lower respiratory tract infection[20]. In the present study DIF was developed to investigate *C. pneumoniae* in clinical specimens from patients with upper respiratory tract infection. MIF was also determined for *C. pneumoniae* antibodies. DIF was evaluated for an association of the presence of the pathogen and seroconversion. The assay was aimed for early diagnosis of acute upper respiratory infection, or use in combination with MIF for accuracy of diagnosis of the infection by *C. pneumoniae*.

Material and Method

Study population

Patients with clinical signs and symptoms of acute upper respiratory tract infection, who visited the outpatient clinic at Department of Otorhinolaryngology, Faculty of Medicine Ramathibodi Hospital between October 2005 and August 2007, were enrolled in the present study. The criteria for selection of the patients with clinical manifestations of acute upper respiratory infection compatible to *C. pneumoniae* infection were those who presented with at least two of the following symptoms: pharyngitis, coughing with or without nonpurulent sputum, rhinitis, fever, not more than 2 weeks before visiting the outpatient clinic. Patients with sinusitis, bronchitis, or asthma were included. The patients were excluded if they had had antibiotics after the onset of the symptoms. Thirty-one patients, aged 12 to 60 years (9 males and 22 females), were enrolled and informed consent was obtained in the present study. Two nasopharyngeal swab specimens were collected from each patient at the first visit by standard method using sterile cotton-tipped aluminum-shafted swabs (DELTALAB, Spain), and blood samples for acute phase sera were obtained from the patients at the time of collecting nasopharyngeal specimens. Convalescent sera were collected from the patients 2 weeks after the first serum specimens. Three of the 31 patients were randomly selected for prospective investigation of *C. pneumoniae* detection and seroconversion. Twenty-five healthy volunteers

without any signs and symptoms of upper respiratory tract infection for at least 2 months prior to collecting nasophryngeal specimens were included during the study period, and the paired sera were collected as described. The healthy subjects consisted of 7 males and 18 females with an age range from 22 to 64 years.

Specimen preparation

Nasopharyngeal swab specimens collected from the patients, and those from normal subjects were immediately placed immersing in transport medium (sucrose phosphate buffer) tubes. The specimens were kept at -70°C if they could not be processed at the day of collection. The swab specimens were vortex mixed in the transport medium for 1 min to dislodge pathogens from the swabs. Samples of two swabs collected from each patient, as well as those from normal subjects, were pooled as an individual tube, and then centrifuged at 1,800xg for 5 min. The samples were resuspended and transferred to 2 ml microcentrifuge tubes, and then centrifuged at 13,800 xg for 10 min. The supernatant was discarded to leave approximately 50 microliters of supernatant and pellets. The pellets were resuspended, and then 30 microliters were smeared on to a single well of Teflon-coated slides (Biomeriux, France). The smeared samples were dried in silica gel blue, and then fixed in cold acetone for 15 min, air dried before staining.

DIF assay for C. pneumoniae

Thirty-seven nasopharyngeal swab specimens were collected from 31 patients with upper respiratory tract infection, and twenty-five swab specimens were obtained from 25 normal subjects. The specimens were processed for direct detection of *C. pneumoniae* by DIF. The concentration of monoclonal anti-*C. pneumoniae* (DAKO, Denmark), and that of rabbit anti-mouse Ig-FITC (DAKO, Denmark) used in DIF were optimized for optimum dilutions prior to application in the assay. The monoclonal anti- *C. pneumoniae* used has been shown, by the manufacturer, not to cross-react with strains of Adenovirus, Respiratory Syncytial Virus, Influenza virus A and B, Parainfluenza virus 1,2, and 3, Herpes Simplex virus 1 and 2, *C. trachomatis* strain SA_2f, *C. trachomatis* strain A-K, LGV 1-3, some serotypes of *C. psittaci*, negative McCoy cells and negative yolk sac. The fixed and dried smears of nasopharyngeal specimens were overlayed with 20 microliters of the optimum dilution in PBS buffer, pH 7.2, of monoclonal anti-*C. pneumoniae*. The slides were incubated in a humid chamber for 20 min at room temperature. After washing twice in PBS, each 15 min, and subsequent rinsing with distilled water for 3-5 sec, 20 microliters optimum dilution of rablit anti-mouse Ig-FITC was applied. The FITC-conjugated anti-mouse Ig was diluted in 0.15 M PBS, pH 7.2, containing 1% Tween 20 and 0.02% Evan blue. After incubation and washing as above, the slides were covered with converslides using mounting medium (80% glycerol, pH 8.4). The immunofluorescent reactions were examined under an epifluorescence microscope (Nikon, Japan) with 400 x magnification. The bright apple-green dots of elementary bodies (EBs) and reticulate bodies (RBs) of *C. pneumoniae* were microscopic examined by the entire area of the smears, and scored according to the average range of the reactive fluorescing EBs and RBs/field. The scores were graded into five groups: negative, 0-< 4-6 fluorescing chlamydial EBs and RBs/field; positve 1+, ≥ 6-10 EBs and RBs / field; 2+, ≥ 10-20 EBs and RBs/field 3+, ≥ 20-30 EBs and RBs/field, and positive 4+, > 30 EBs and RBs/field. Positive control, prepared by a smear onto the Teflon-coated slide of *C. pneumoniae* strain YK41 prepared onto Hep-2 cell monolayer as previously described[21], was simultaneously performed for each set of the assay. Negative control, using a suspension of Hep-2 cells smeared onto the slides, and a well of the slide without smear of cell suspension used as reagent control were also included for each test run. The assay for DIF and interpretation of results of immunofluorescence reactions were carried out by the same person, and the staining reactions were blindly examined and interpreted by the other expert with experience in DIF for *C. trachomatis*. Interpretation was repeated if the results from both examiners were not in agreement, and occasionally justified by another expert.

MIF for C. pneumoniae antibodies

Acute and convalescent phase sera were collected from the patients, as well as the normal subjects for determination of antibodies raised against *C. pneumoniae*. IgG and IgM antibodies were carried out by MIF at National Institute of Health, Thailand, as previously described[14,21]. Briefly, *C. pneumoniae* YK41, *C. trachomatis* L2 and *C. psittaci* (supported by Dr. Toshino Kishimoto and Dr. Shuji Audo, National Institute of Infectious Disease, Japan), each of which was mixed by equal volumes with yolk sac, and then, each of the mixture of *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* was spotted onto each well of high Teflon-coated, 3 x 7-well slides (Matsunami, Japan). The spots were air-dried for 1 h, fixed for 10 min in

acetone, dried, and frozen at -20°C until use. Sera were two-fold serially diluted at 1:8 to 1:512 dilution in PBS for detection of IgG antibody, and in PBS containing anti-IgG for IgM antibody detection, and then applied onto the chlamydial antigen dots. The slides were incubated for 45 min at 37°C in a humid chamber, and then washed in PBS. FITC-conjugated rabbit anti-human IgG (DAKO, Copenhagen, Denmark), and FITC-conjugated rabbit anti-human IgM (DAKO, Copenhagen, Denmark), at 1:40 and 1:20 dilution respectively, were applied onto the immunoreaction dots, incubated and washed as described. The immunofluorescence reactions were examined, at x400 magnification, under an epifluorescence microscope (Nikon, Japan). Positive and negative control sera were included in each test run. Interpretation of an acute infection of *C. pneumoniae* was based on the criteria of the presence of IgM antibody $\geq$ 1:16, or a four-fold or greater of titer change between paired sera for IgG antibody, or the IgG $\geq$ 1:512 in either the first serum or the second serum. The stable IgG antibody titers of 1:16 to 1:256 were considered as the evidence of chronic or past infection[14,21].

Statistical analysis

The difference between *C. pneumoniae* detection by DIF in patients and in normal control subjects, the association between DIF results with antibody detection by MIF in the patients and normal controls, as well as the variables between DIF results and sex of the patients and normal subjects were analyzed using Pearson chi-square's test. Association between DIF results and age of the patients including normal controls was analyzed using independent t-test. Analysis was performed for 2-sided, and a p-value of < 0.05 was considered as statistical significance.

Results

C. pneumoniae in upper respiratory infection and normal individuals

Thirty-seven nasopharyngeal specimens were collected from 31 patients with upper respiratory tract infection, and twenty-five nasopharyngeal swabs were obtained from 25 normal subjects. The swab samples were processed for *C. pneumoniae* detection by DIF. A sample was interpreted as positive for DIF test when at least an average 6-10/field or more of bright apple-green dots of EBs and RBs of *C. pneumoniae* was seen. As shown in Table 1, positive results by DIF were found in 29 of the 37 (78.4%) samples from the patients with upper respiratory infection. Of the 29 nasopharyngeal samples giving positive results by DIF, *C. pneumoniae* detection was interpreted as positive 1+, 2+, 3+ and 4+ in 11 (37.9%), nine (30.0%), seven (24.1%), and two (6.9%) of the samples from the patients respectively. *C. pneumoniae* was detected and interpreted as positive 1+ or 2+ in 2 of 25 (8.0%) samples from normal individuals, as shown in Table 2. Twenty-three samples from 25 normal subjects were interpreted as negative results by DIF test. *C. pneumoniae* detected by DIF in patients with upper respiratory tract infection was significantly different from that detected in normal subjects ($p < 0.001$; Pearson chi-square test). There was no association between the positive results by DIF and sex ($p = 0.729$; Pearson Chi-square test), and age ($p = 0.630$; independent t-test) among the samples from the patients and normal individuals.

C. pneumoniae and seroconversion in patients and normal subjects

Thirty-seven nasopharyngeal swab specimens were collected from 31 patients for *C. pneumoniae* detection by DIF, and 37 acute phase serum samples were obtained for antibody levels from the patients at the time the nasopharyngeal specimens were collected. Convalescent sera were collected from the patients two weeks after the first sera. Six of the 37 nasopharyngeal specimens, and six of the paired-sera were the samples collected in addition to the primary specimen collection from three of the 31 patients, used for prospective investigation of the evidence of the pathogen and seroconversion. Twenty-five nasopharyngeal specimens and 25 paired-sera were also collected from 25 normal subjects. As shown in Table 1, DIF gave positive results in 29 of 37 (78.4%) samples, and *C. pneumoniae* antibodies were detected and interpreted as acute *C. pneumoniae* infection in 18 of 37 (48.6%) samples, and as chronic infection in 11 of 37 (29.7%) samples. Eight samples, positive by DIF, were interpreted as acute infection by MIF with IgG antibody titers $\geq$ 1:512. Six samples, positive by DIF, showed IgM antibody titers 1:32-1:64, and 1 sample was found for IgG titer change from $<$ 1:8 to 1:512. Three samples gave negative results by DIF, whereas IgM antibody was detectable at 1:16-1:32 dilution, and one sample with IgG titers $\geq$ 1:512. DIF gave positive results in 8 of the 11 samples interpreted as chronic/past infection by MIF with IgG antibody titers 1:16 to 1:256. Negative results by both DIF and MIF were found in the specimens from two patients.

Table 1. Results of DIF for *C. pneumoniae* and *C. pneumoniae* antibodies by MIF in patients with upper respiratrory tract infection

Patient no.	Sex	Age	DIF Result	MIF antibody titer				MIF interpretation
				Acute serum		Convalescent serum		
				IgM	IgG	IgM	IgG	
1	F	33	1+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
2	F	12	1+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
3	F	40	3+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
4	M	40	3+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
5	F	28	2+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
6	F	49	1+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
7	M	24	1+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
8	F	16	-	1:16	<1:8	1:16	<1:8	Acute infection
9	F	30	2+	<1:8	<1:8	1:32	1:8	Acute infection
10	F	58	3+*	1:16	1:64	1:32	1:8	Acute infection*
11	M	25	2+**	1:32	1:256	1:16	1:256	Acute infection**
12	M	45	2+	1:32	1:512	1:64	1:512	Acute infection
13	F	33	2+	1:64	1:512	1:32	1:512	Acute infection
14	F	58	3+*	1:32	1:128	1:64	1:256	Acute infection*
15	M	35	-	<1:8	≥1:512	<1:8	≥1:512	Acute infection
16	F	42	2+	<1:8	≥1:512	<1:8	≥1:512	Acute infection
17	F	56	3+	<1:8	<1:8	1:16	1:512	Acute infection
18	F	40	-	1:32	1:8	NA	NA	Acute infection
19	M	60	-***	<1:8	1:32	<1:8	1:16	Chronic/past infection***
20	F	14	3+	<1:8	1:64	<1:8	1:64	Chronic/past infection
21	M	45	1+	<1:8	1:256	<1:8	1:256	Chronic/past infection
22	F	54	4+	<1:8	1:64	<1:8	1:64	Chronic/past infection
23	F	51	-	<1:8	<1:8	<1:8	1:32	Chronic/past infection
24	F	50	1+	<1:8	1:32	<1:8	1:32	Chronic/past infection
25	M	31	-	<1:8	1:128	<1:8	1:128	Chronic/past infection
26	F	53	1+	<1:8	1:16	NA	NA	Chronic/past infection
27	F	60	1+	<1:8	1:32	NA	NA	Chronic/past infection
28	F	48	1+	<1:8	1:256	<1:8	1:128	Chronic/past infection
29	F	58	1+*	<1:8	1:16	<1:8	1:16	Chronic/past infection*
30	F	58	-	<1:8	<1:8	<1:8	<1:8	Negative
31	F	30	4+	<1:8	<1:8	<1:8	<1:8	Negative
32	M	60	2+***	<1:8	<1:8	<1:8	<1:8	Negative***
33	F	58	3+*	<1:8	<1:8	<1:8	<1:8	Negative*
34	M	25	2+**	<1:8	<1:8	<1:8	<1:8	Negative**
35	M	44	2+	<1:8	<1:8	<1:8	<1:8	Negative
36	F	26	-	<1:8	<1:8	<1:8	<1:8	Negative
37	M	60	1+***	<1:8	<1:8	<1:8	<1:8	Negative***

+, positive result by DIF; -, negative result by DIF; NA, not available
*,**,***, DIF and MIF results of samples from 3 patients obtained as primary collection, and subsequent collection for investigation of seroconversion

Six nasopharyngeal specimens obtained from five patients gave positive results by DIF, whereas antibody titers both IgG and IgM were interpreted as negative results of < 1:8 (as shown in Table 1). In this patients group, nasopharyngeal as well as paired serum samples were additionally collected from three patients for prospective investigation for seroconversion. The patients had mild to moderate symptoms of upper respiratory infection at each specimen collection. Three paired-sera consecutively collected from a patient produced IgM and IgG antibodies interpreted as acute infection 2 and 4 months after the primarily

Table 2. Results of DIF for *C. pneumoniae* and *C. pneumoniae* antibodies by MIF in normal subjects

Patient no.	Sex	Age	DIF Result	MIF antibody titer				MIF interpretation
				Acute serum		Convalescent serum		
				IgM	IgG	IgM	IgG	
1	M	28	-	<1:8	<1:8	<1:8	<1:8	Negative
2	M	22	-	<1:8	<1:8	<1:8	<1:8	Negative
3	F	32	-	<1:8	<1:8	<1:8	<1:8	Negative
4	F	26	-	<1:8	<1:8	<1:8	<1:8	Negative
5	F	33	-	<1:8	<1:8	<1:8	<1:8	Negative
6	M	64	-	<1:8	<1:8	<1:8	<1:8	Negative
7	F	60	-	<1:8	<1:8	<1:8	<1:8	Negative
8	F	48	-	<1:8	<1:8	<1:8	<1:8	Negative
9	F	45	-	<1:8	<1:8	<1:8	<1:8	Negative
10	F	33	-	<1:8	<1:8	<1:8	<1:8	Negative
11	F	30	-	<1:8	<1:8	<1:8	<1:8	Negative
12	F	55	-	<1:8	<1:8	<1:8	<1:8	Negative
13	M	28	-	<1:8	<1:8	<1:8	<1:8	Negative
14	F	48	-	<1:8	<1:8	<1:8	<1:8	Negative
15	F	28	-	<1:8	<1:8	<1:8	<1:8	Negative
16	M	49	-	<1:8	<1:8	<1:8	<1:8	Negative
17	F	27	-	<1:8	<1:8	<1:8	<1:8	Negative
18	F	47	-	<1:8	<1:8	<1:8	<1:8	Negative
19	F	33	-	<1:8	<1:8	<1:8	<1:8	Negative
20	F	26	-	<1:8	<1:8	<1:8	<1:8	Negative
21	M	28	1+	1:8	1:128	1:8	1:128	Chronic/past infection
22	F	46	2+	1:8	1:64	<1:8	1:64	Chronic/past infection
23	M	28	-	1:8	1:128	1:8	1:128	Chronic/past infection
24	F	44	-	<1:8	1:128	<1:8	1:128	Chronic/past infection
25	F	44	-	<1:8	1:16	<1:8	1:16	Chronic/past infection

+, positive result by DIF; -, negative result by DIF

collected specimen of which MIF illustrated negative results. In this patient, MIF gave a result interpreted as chronic/past infection eight months later. IgM antibody titers at 1:32 and 1:16, and IgG titer 1:256 were detected and interpreted as acute infection for the second samples subsequently collected three months from the primary sample in the other patients. Seroconversion was found and interpreted as chronic/past infection with IgG titer 1:32 and 1:16 for the samples collected eight months after the first and second specimen collection in the third patient. *C. pneumoniae* was detectable by DIF in nasopharyngeal specimens from these three patients unless the swab collected eight months after the first onset in the third patient. Appropriate antibiotics had not been administrated to the three patients until the detectable antibodies by MIF were interpreted as acute or chronic *C. pneumoniae* infection. No statistically significant association was found between *C. pneumoniae* detected by DIF and acute or chronic infection interpreted by MIF in the 37 patient samples analyzed ($p = 0.770$; Pearson Chi-square test). This is also the case in the analysis of 33 samples of the patients, excluding the four samples of the three patients that primarily gave negative MIF but subsequently developed antibody titers ($p = 0.357$; Pearson chi-square test). However, statistically significant association was found between *C. pneumoniae* detected by DIF and acute or chronic infection by MIF when analysis was done with 62 samples from the patients and normal subjects ($p < 0.001$; Pearson Chi-square test).

Discussion

C. pneumoniae causes a wide spectrum of disease with a variety of human cells being infected. There is no ideal method used presently for routine laboratory diagnosis of *C. pneumoniae* infection. An accurate and timely diagnosis of *C. pneumoniae* acute

primary infection or reinfection is an important requirement for management of the patients. It is also important to prevent development of life-threatening pneumoniae, persistent infection, and complications with cardiovascular diseases[6,15,22,23].

Serological testing by MIF is currently the most commonly used method for diagnosis of *C. pneumoniae* infection. However, MIF is limited and offers only retrospective diagnosis for acute infection. The difficulty in obtaining appropriate paired serum samples for MIF and the prevalence of high background of IgG antibody in adult populations are the problems of interpretation and management for the patients. It has been documented that in primary infection, IgM antibody appears ~ 2-3 weeks, and IgG antibody may not reach a high titer until 6-8 weeks from the onset of illness[15]. In this statement, the paired-sera obtained at the interval of two weeks apart can provide misdiagnosis of acute primary infection to the patients. However, it must be delayed for an appropriate treatment with antibiotics susceptible to *C. pneumoniae* when convalescent sera, collected later than two weeks from the onset of the disease, is used for the diagnosis. In the present study, IgM as well as IgG antibody raised against *C. pneumoniae* were detected in most patients with positive DIF results. However, the MIF antibodies appeared, and interpreted as acute infection in the paired-sera subsequently collected from two patients at two months, and at two and four months respectively, after the primary collected specimens. A fourth paired-sera collected six months later from one of these two patients gave MIF antibodies interpreted as chronic/past *C. pneumoniae* infection. Delayed seroconversion and persistent infection is considerable for these two patients. The authors also had occasions in collecting nasopharyngeal specimens and paired sera at 4-months and 8-months after the primary specimen collection from the patient. In this patient IgM antibody was not demonstrated, but IgG antibody titers were 1:32 and 1:16, and interpreted as chronic/past infection by MIF in the sera collected eight months after the first round of specimen collection. It is acknowledged that *C. pneumoniae* infection does not induced good protective immunity, and reinfection can occur while IgM antibody may not appear. In the present study, the results of detection for *C. pneumoniae* by DIF and antibodies by MIF are in accordance with those reported by direct detection and PCR method. In those, *C. pneumoniae* was detectable, whereas seronegative results were found in some patients[3,18,22,24]. However, in the present study, antibodies were detectable two months or more after the first sample collection in three patients prospectively investigated for the seroconversion.

The results of the developed DIF are dependent on several factors, including the appropriate specimen collecting, the load of the organism in clinical specimens, and the sufficient quantity of the organism obtained after dislodging from collecting swabs. The skill in interpretation of DIF results also is an important requirement. In the present study, DIF gave negative results in three patients with IgM and IgG antibodies showing low titers of 1:16, and 1:32. In these patients, it is possible for *C. pneumoniae* infection, which is mild, to lead to low load of the pathogen obtained from the specimen collection. Two patients with IgG titers 1:128 and $\geq$ 1:512 gave negative results by DIF. The process of dislodging the pathogen could be insufficient eluting out of the pathogen from the swab samples, which resulted in a decreased yield of *C. pneumoniae*.

In normal individuals, *C. pneumoniae* detection by DIF gave negative results. Furthermore, IgG and IgM antibodies were not detected in most cases, although two of 25 (8.0%) cases were DIF positive. IgG antibody titers at 1:16 -1:128 were detected in five cases. Asymptomatic carriage or even the persistence or colonization of the pathogen is considerable for these subjects. In analysis of DIF for *C. pneumoniae* and MIF for antibodies among the patients and the normal subjects, significant association was found in both assays.

During the time of the present study, the authors obtained nasopharyngeal swab specimens and sera from three hospitalized newborn patients (age 1½ - 2½ months) to investigate the etiology of pneumonia. The patients had a cough for 2-3 days prior to the onset of dyspnea and hypersecretion with chest radiograph appeared interstitial infiltration. Atelectasis at right upper lung was present in one of the three patients. The patient samples were processed for DIF and MIF as described. *C. pneumoniae* was detected in the nasopharyngeal swabs, and interpreted as DIF positive 1^+ in all three patients, whereas IgM antibody titers were detected at < 1:8 dilution (data not shown). These three infants were successfully cured by treatment with erythromycin, an antibiotic susceptible to *C. pneumoniae*, at 30-40 mg/day for 14 days. Study of direct detection of *C. pneumoniae* by PCR in adult patients with community-acquired pneumoniae also illustrated PCR positive results but seronegative was found in single serum from the

patients[18]. Although real-time PCR has been described to be superior to conventional PCR, and is likely the most candidate for direct detection of the pathogen[15,17,25], the assay needs an expensive automation and is not available in general laboratories.

Direct detection of *C. pneumoniae* by DIF provides an alternative approach in diagnosis of acute *C. pneumoniae* infection in patients with upper respiratory tract infection. DIF should be used in combination with antibody detection by MIF for accurate diagnosis of *C. pneumoniae* infection. The assay is applicable for *C. pneumoniae* detection in pneumonia cases by using nasopharyngeal swab specimens, which would be a high valuable tool, particularly with patients of very low age. A large scale study of the cases in such group is needed for validation and prior to the use for diagnosis of the lower respiratory tract infection.

Acknowledgement

The present study was supported by Research Grant from the Faculty of Medicine Ramathibodi Hospital, Mahidol University No 48025/year 2005.

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การประเมินการทดสอบวิธี direct immunofluorescence สำหรับการวินิจฉัยการติดเชื้อ Chlamydia pneumoniae ที่ทางเดินหายใจส่วนต้น

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Chlamydia pneumoniae เป็นสาเหตุของโรคติดเชื้อทางเดินหายใจหลายแบบ และเกี่ยวข้องกับโรคหลอดเลือดหัวใจ การวินิจฉัยการติดเชื้อ Chlamydia pneumoniae ในปัจจุบันเป็นการตรวจหาแอนติบอดีโดยวิธี microimmunofluorescences (MIF) ซึ่งมีข้อบ่งใช้ที่จำกัด และเป็นการวินิจฉัยการติดเชื้อเฉียบพลัน แบบย้อนหลัง การตรวจหาเชื้อ Chlamydia pneumoniae โดยตรง จากสิ่งส่งตรวจที่เก็บจาก nasopharynx ของผู้ป่วยได้ถูกพัฒนาขึ้นโดยวิธี direct immunofluorescence (DIF) เพื่อการวินิจฉัยการติดเชื้อ ที่ทางเดินหายใจส่วนต้น การตรวจหาแอนติบอดีชนิด IgG และ IgM โดยวิธี MIF ในผู้ป่วยรวมถึงคนปกติได้กระทำเพื่อประเมินผลการตรวจหา Chlamydia pneumoniae โดย DIF และการสร้างแอนติบอดีจากการติดเชื้อ พบว่าสิ่งส่งตรวจ 37 ตัวอย่างที่ได้จากผู้ป่วย 31 ราย ให้ผลบวกโดย DIF 29 ใน 37 (78.4%) โดยที่ 15 ตัวอย่างของผู้ป่วยนี้ตรวจพบ แอนติบอดีโดย MIF ซึ่งแปลผลของการติดเชื้อเป็นแบบเฉียบพลัน และ 8 ตัวอย่าง แสดงผลของMIF เป็นการติดเชื้อเรื้อรังหรือในอดีต ผู้ป่วย 2 ราย ให้ผลลบทั้ง DIF และ MIF ในคนปกติ 25 ราย DIF ให้ผลลบ 23 ราย ขณะที่ MIF ให้ผลลบ 20 ราย ซีรัมคู่ (paired-sera) ซึ่งเก็บต่อมาจากผู้ป่วย 3 ราย จากกลุ่มผู้ป่วย 31 ราย นี้ ตรวจพบ แอนติบอดี 2-4 เดือน หลังจากสิ่งส่งตรวจครั้งแรกซึ่ง DIF ให้ผลบวกแต่ MIF ให้ผลลบ พบว่ามีความสัมพันธ์ทางสถิติระหว่างการตรวจหาเชื้อโดยตรงโดยวิธี DIF และการตรวจหาแอนติบอดีโดยวิธี MIF เมื่อทำการวิเคราะห์รวมในผู้ป่วยและคนปกติ ($p < 0.001$; Pearson Chi-square test) การตรวจหาเชื้อโดยตรงโดยวิธี DIF สามารถใช้ในการวินิจฉัยการติดเชื้อในระยะแรกเริ่ม และอาจใช้ร่วมกับวิธี MIF เพื่อการวินิจฉัยที่ถูกต้อง ของการติดเชื้อแบบเฉียบพลัน