DEVELOPMENT OF EIA FOR DETECTION OF *CHLAMYDIA TRACHOMATIS* IN GENITAL SPECIMENS

Chariya Chomvarin[1], Yingrit Chantarasuk[1], Pramote Thongkrajai[1], Wiboonchai Yutanawiboonchai[2] and Nareas Waropastrakul[1]

[1]Department of Microbiology, Faculty of Medicine, Khon Kaen University, Thailand;
[2]Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand

Abstract. A double antibody sandwich enzyme immunoassay (EIA) for chlamydial antigen detection was developed using a monoclonal antibody against lipopolysaccharide (LPS) of *Chlamydia trachomatis* as a coating antibody. Polyclonal rabbit antiserum against partially purified antigen from elementary body (EB) antibody and horse-radish peroxidase conjugated goat anti-rabbit antibody were used as the primary and secondary antibody respectively. The developed EIA could detect protein of partially purified EB at the lowest concentration of 250 ng/ml. The assay was evaluated against the cell culture (CC), DNA hybridization assay (PACE2 system: Gen-Probe,San Diego, CA, USA) and a commercial enzyme immunoassay (kEIA)(Bioquest, NSW,Australia). The sensitivity, specificity, positive and negative predictive values of the developed EIA (dEIA) were 87, 96.2, 80, 97.7 for the specimens from females and 90.9, 90.7, 71.4, 97.5 for the specimens from males repectively. Cross reaction was not found with *Escherichia coli*, *Acinetobacter anitratus*, *β-Streptococcus* group A, *Enterobacter* spp, *Enterococcus*, *Lactobacillus* spp, *Neisseria* spp, but it was found with *Candida albicans* and herpes simplex virus type 1. The developed EIA can be applied successfully for both genders, particularly males. The cost per test is less than those for CC, kEIA and PACE2.

INTRODUCTION

A common bacterial cause of sexually transmitted disease in human is *Chlamydia trachomatis*. Genital infection with this organism may lead to costly acute illness and long term complication of urogenital organ (Iwen *et al*, 1991; Peeling, 1995). Infection with *C. trachomatis* is often asymptomatic or nonspecific in the clinical signs and symptoms, such as cervicitis in females and urethritis, epididymitis and prostatitis in males (Bowie *et al*, 1977; Barnes, 1989). Therefore, early screening for chlamydial infection is essential for treatment and prevention of the serious complications and further spread of the disease.

In Thailand, chlamydial investigation and treatment are applied only for patients who are considered at high risk of infection. Cell culture has been used as the gold standard for a long time. However, there are still several problems with the cell culture method. It is technically difficult, time consuming, costly and requires special handling of specimens (Thejls *et al*, 1994). Detection of chlamydial antigen in clinical specimens is another widely used method for laboratory diagnosis, such as EIA for antigen detection and DNA probe assay for nucleic acid detection (Chernesky *et al*, 1986; Clarke *et al*, 1993).

A widely used method to facilitate laboratory screening and diagnosis of chlamydial infection is EIA. The advantages of EIA are rapid and simple. A large number of specimens can be processed in relatively short time and there is no need for viable organisms and special instruments. Thus, in this study, we developed the polyclonal antibody against partially-purified elementary body (EB) of chlamydia and used in EIA development. The test was applied to evaluate 155 endocervical and 64 urethral male specimens in comparison with the cell culture, the DNA hybridization assay and the HRP Chlamydia EIA (kEIA) for diagnosis of *Chlamydia trachromatis* infection.

MATERIALS AND METHODS

Specimen collection and handling

350 females and 140 males who visited Khon Kaen STD clinic and AIDS center, Zone 6 and the outpatient clinic of the Division of Obstetrics and Gynecology, Khon Kaen Hospital were asked to donate clinical samples for this study. For each female patient, three endocervical swabs were collected, one was placed into sucrose phosphate transport medium for chlamydial culture, and another two were placed into the lysis buffer provided in

the collection kits for PACE2 and kEIA. Three urethral swabs from male patients were taken and manipulated in the same way.

Evaluation of specimens for chlamydial infection by cell culture, PACE2 assay and EIA

Three methods were used for evaluation of chlamydial infection. For cell culture, cycloheximide-treated McCoy cell was used for *Chlamydia trachomatis* culture as described previously (Chomvarin *et al*, 1997). PACE2 assay using the chemiluminescent probe assay utilized an acridinium ester labeled single stranded DNA probe(s) complementary to rRNA of *Chlamydia trachomatis*. It was performed according to the manufacturer's instruction. One positive and three negative references were included in each running (Chomvarin *et al*, 1997). The kEIA was performed using the HRP chlamydia EIA kit, which used a monoclonal antibody directed against a genus specific lipopolysaccharide (LPS). The method was performed as described previously (Chomvarin *et al*, 1997). A sample was considered true positive for chlamydial infection when it had at least one of the following criteria : 1) a positive standard culture with primary inoculation, 2) positive in both non culture tests (PACE2 and EIA). The sensitivity, specificity, positive and negative predictive values of each assay would be calculated based on the above criteria.

Propagation of *C. trachomatis*

C. trachomatis positive specimens were used for the propagation of *C. trachomatis*, using the same procedure for isolation (Chomvarin *et al*, 1997). After 48 hours culture, cells were removed, disrupted with glass beads and centrifuged at 500*g* for 5 minutes. The supernatant was collected and inoculated into two vials of confluent McCoy cells. One vial was used for staining with immunofluorescent reagent to evaluate the infection while another would be used for stock culture if at least $5.5x10^6$ IFU/ml or 5-6 inclusions/ 400x power field was shown. Equal volume of stock media (4SP:0.4M sucrose and 0.02M phosphate) was added and the vial was stored at -70ºC.

Preparation and purification of chlamydial antigen

The procedure was modified from Mahony *et al* (1983). Briefly, three ml of chlamydial stock solution was plated to cycloheximide-treated McCoy monolayers in 25 cm^2 flasks and incubated at 37ºC for 2 hours. The inoculate was then removed and replaced with maintenance medium [(85ml RPMI1640, 5 ml fetal calf serum, 2 ml vancomycin (5,000 μg/ml), 1 ml gentamicin (500 μg/ml), 40 μl fungizone (2,500 μg/ml), 5 ml glucose (0.11 μg/ml), 0.2 ml cycloheximide (100 μg/ml)]. The culture was incubated further at 37ºC in 5% CO_2 for 72 hours. The infected cells were harvested by scraping and disrupted with 5 mm glass beads. The cell debris was removed by centrifugation at 500*g* for 10 minutes. The supernatant was centrifuged at 30,000*g* for 20 minutes to collect chlamydial antigen, which was then pooled and resuspended in 3 ml PBS. The antigen was then partially purified by centrifugation at 20,000*g* through a column of 2 ml of 30% (v/v) urograffin-76 for 40 minutes. The pellet was collected, resuspended and assayed for protein concentration using Lowry's method (Lowry *et al*, 1951).

Preparation of immune serum to EB antigen

Twenty, forty, sixty and eighty micrograms of partially purified EB antigen in incomplete Freund's adjuvant was injected intravenously into a New Zealand white rabbit at three day interval respectively. A final dose of 200 μg of antigen in incomplete Freund's adjuvant was injected intramuscularly on day 7 after the fourth injection. Two weeks later, the rabbit was bled and the serum was stored at -20ºC until used.

Detection of antibodies to *C. trachomatis*

Antigens including partially purified EB, commercial EB (Syva company, San Jose, USA), chlamydial LPS (Bioquest, NSW, Australia), whole cell lysate of *E. coli* ATCC 25992, and *A. anitratus* at a concentration of 280 μg/ml were used to test the rabbit immune serum by dot immunoassay technique. Briefly, 20 μl of the antigen solution was blotted onto a 0.45 μm nylon blotting membrane (Costar, USA) under an adjustable vacuum. The membrane was air dried, washed for 3 times with 1x Tris-NaCl Tween (TNT) for ten minutes each, followed by blocking with 5% skim milk in 20mM Tris buffer saline (TBS), pH 7.5 at 4ºC for overnight. The membrane was washed again for 3 times and incubated with 1:100 immune rabbit serum in 5% skim milk in TBS at 37ºC for 2 hours. The membrane was then washed and incubated in HRP goat anti-rabbit IgG (Promega, USA) diluted to 1:1,000 for 2 hours. After 3 washes, the membrane was then incubated in 0.06 g% DAB substrate (3, 3´- diaminobenzidine tetrahydrochloride, Sigma) at room temperature in the dark for 15 minutes.

The reaction was terminated by washing with tap water. The positive result was indicated by brown color. Dot immunoassay was also performed using mouse mAb against a commercial chlamydial LPS (Bioquest, NSW, Australia) diluted to 1:150, followed by HRP antimouse IgG (Dako, Denmark) as the comparison to rabbit immune serum.

Development of an EIA assay

Standardization of the assay: The optimal condition for the EIA was established by checkerboard titration. A range of 2-16 μg/ml of 100 μl mAb to chlamydial LPS antigen in coating buffer (carbonate-bicarbonate buffer) was used to coat EIA plates (Nunc, Denmark) at room temperature for overnight. The plate was further washed 3 times with PBS-Tween and blocked with EIA blocking solution (5% skim milk and 4% BSA in coating buffer) for 1 hour. The 100 μl of pooled positive and negative control sera were added to each well and incubated for 2 hours. After 3 washes, 100 μl of rabbit immune serum of various dilution (1:100 -1:800) in EIA diluting solution (1% skim milk and 4%BSA in PBS-Tween) was added and incubated at 37ºC for 2 hours. The plate was washed and 100 μl of 1:1,000 to 1:6,000 HRP conjugated goat anti rabbit IgG was added, incubated for another 1 hour, followed by 6 washings. The 100 μl of OPD substrate solution was added to the wells and incubated for 30 minutes. The reaction was terminated by adding 50 μl of 2M H_2SO_4. The optical density was read by a MicroEIA Autoreader(Dynatech) at 490 nm.

Analytical sensitivity and specificity of the developed EIA: Using the decided optimal conditions, the dEIA was used to detect chlamydial partially purified EB at the concentration of 0.0025 to 1 μg/ml. Various antigens were also tested, including *E. coli, A. anitratus, Enterobacter* spp, *Enterococcus, β-Streptococcus* group A, *Lactobacillus* spp, *Candida albicans, Neisseria* spp. These antigens were tested at the concentration equivalent to $1.5x10^8$ organisms/ml.

Herpes simplex virus (HSV) type 1 was cultured by using vero cells for 48 hours and the cell debris was removed by centrifugation. The supernatant was used as the antigen for testing as above.

Evaluation of dEIA for clinical application

Clinical application of the dEIA was carried out by testing against clinical specimens which were already evaluated by kEIA, cell culture and PACE2. Sensitivity, specificity and predictive values were calculated using a standard formula (Ilstrup, 1996). True positive result, which used in the formula, was obtained according to the criteria mentioned previously. Agreement between the HRP chlamydial EIA and the dEIA was assessed by using Kappa statistics (Landis and Koch, 1977).

Within-run precision was determined by testing the pooled positive samples twenty times on the same plate. To determine the between-run precision, the same samples were tested two times on three plates running on the other day.

RESULTS

Specificity of rabbit immune serum

Two weeks after the complete course of immunization, the rabbit was bled and the serum was assayed for presence of antibodies against chlamydial antigen. Dot immunoassay showed that the serum reacted with control EB and partially perified EB. No cross reaction was seen when the serum was tested against two Gram negative bacteria, *E. coli* and *A. anitratus* (Fig 1). The immune serum also recognized chlamydial LPS (data not shown).

Optimal conditions and specification of the dEIA

Checkerboard titration revealed the optimal condition of the dEIA as the following: concentration of mAb for coating plate was 8 μg/ml; con-

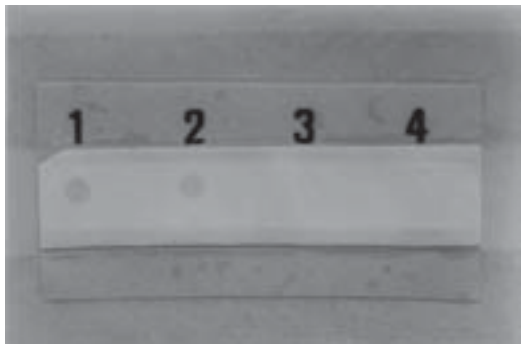

Fig 1– Dot immunoassay of rabbit antiserum to *C. trachomatis*. The antigens were blotting onto a nylon membrane, reacted with rabbit antiserum to chlamydial EB and probed with HRP conjugated goat anti-rabbit IgG. The DAB substrate solution was applied. The positive result was indicated by the deposition of a brown dot. (1) control EB (2)partially purified EB (3) *E.coli* ATCC 25922 and (4) *A.anitratus.*

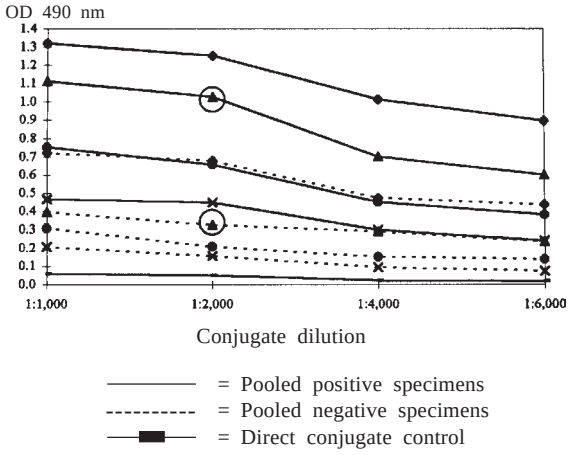

Fig 2–Checkerboard titration for optimal concentration of monoclonal antiboby, rabbit antiserum and HRP conjugated goat-antirabbit antibody. Plate was coated with monoclonal antibody (8 μg/ml). Rabbit antiserum dilution were 1:100 (◆), 1:200 (▲), 1:400 (●) and 1:800(X). The optimum dilutions of rabbit antiserum and conjugated antibody were 1:200 and 1:2,000 respectively.

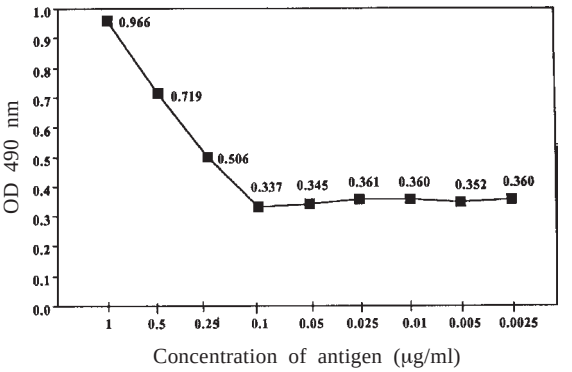

Fig 3–Tritration curve for minimum concentration of chlamydial antigen as detected by dEIA.

centration of rabbit immune serum and HRP conjugated goat anti rabbit IgG was 1:200 and 1:2,000 respectively (Fig 2). For the conditions above, the OD of positive control was 0.658 and that of the negative control was 0.367.

Using the conditions derived from checkerboard titration, various concentration of partially purified EB were tested. As shown in Fig 3, the dEIA could not discriminate the antigen at the concentration of 100 ng/ml (0.1μg/ml) and below. Discrepency was seen when the antigen was 250 ng/ml and higher, for which the OD varied.

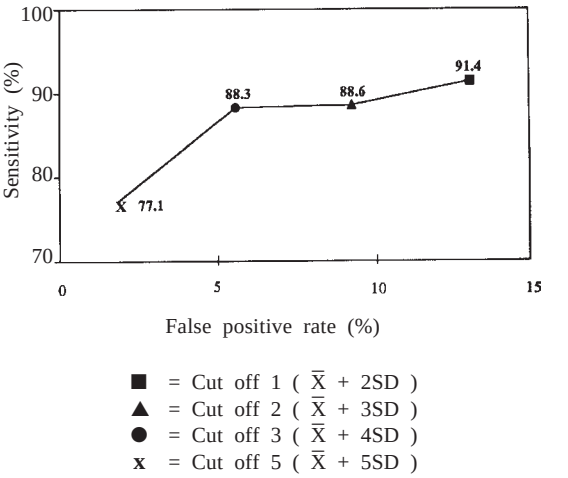

Fig 4–ROC curve of cut off points for dEIA test.

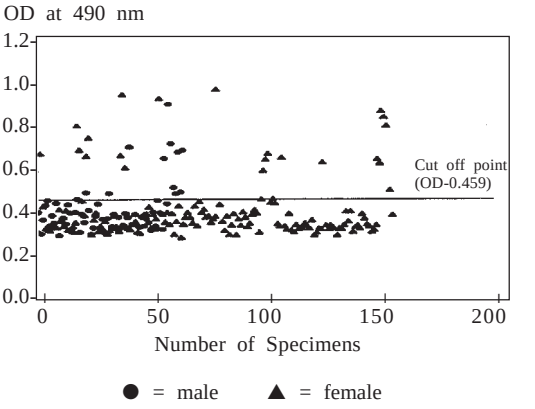

Fig 5–Scatter diagram of OD values from 219 samples of clinical specimens detected by dEIA.

To evaluate the precision and consistency of the dEIA, it was shown that the with in run precision and the between run precision had the coefficient variation of 3.3 and 2.5 % respectively (data not shown).

Cross reaction was found when using the dEIA to test *C. albicans* cells at the concentration of $1.5x10^8$ organism/ml and also from HSV type1 (data not shown). No cross reaction was found with *E. coli*, *A. anitratus*, *Enterobacter* spp, *Enterococcus*, *β-Streptococcus* group A, *Lactobacillus* spp, *Neisseria* spp at the same concentration.

Application of the dEIA to clinical specimens

The dEIA was used to detect chlamydial antigen

Table 1
Number and percentage of 155 endocervical swabs and 64 male urethral swabs possesing *C. trachomatis* positive test by CC, kEIA and PACE2.

Test methods and results			No. (%)		Total	Evaluation
CC	kEIA	PACE 2	Females	Males	No. (%)	
+	+	+	17 (11.0)	7 (11.0)	24 (11.0)	TP
+	+	-	3 (1.9)	-	3 (1.4)	TP
-	+	+	1 (0.7)	2 (3.1)	3 (1.4)	TP
-	+	-	3 (1.9)	1 (1.6)	4 (1.8)	TN, FP
+	-	+	-	2 (3.1)	2 (0.9)	TP, FN
+	-	-	2 (1.3)	1 (1.6)	3 (1.4)	TP, FN
-	-	+	1 (0.7)	-	1 (0.5)	TN
-	-	-	128 (82.6)	51 (79.7)	179 (81.7)	TN
	Total		155 (100)	64 (100)	219 (100)	

TP = True positive; TN = True negative; FP = False positive for kEIA
FN = False negative for kEIA

Table 2
Number and percentage of 155 endocervical swabs and 64 male urethral swab possesing *C. trachomatis* positive test by CC, developed EIA(dEIA) and PACE2.

Test methods and results			No. (%)		Total	Evaluation
CC	dEIA	PACE 2	Females	Males	No. (%)	
+	+	+	16 (10.3)	8 (12.5)	24 (11.0)	TP
+	+	-	3 (1.9)	1 (1.6)	4 (1.8)	TP
-	+	+	1 (0.7)	1 (1.6)	2 (0.9)	TP
-	+	-	5 (3.2)	4 (6.3)	9 (4.1)	TN, FP
+	-	+	1 (0.7)	1 (1.6)	2 (0.9)	TP, FN
+	-	-	2 (1.3)	-	2 (0.9)	TP, FN
-	-	+	1 (0.7)	1 (1.6)	2 (0.9)	TN
-	-	-	126 (81.3)	48 (75.0)	174 (79.4)	TN
	Total		155 (100)	64 (100)	219 (100)	

TP = True positive; TN = True negative; FP = False positive for dEIA
FN = False negative for dEIA

in 219 clinical specimens. Thirty-four of these specimens were considered positive according to the criteria mentioned in material and methods, using cell culture, kEIA and PACE2 (Table 1). The appropriate cut off point for clinical application was derived from the reciever operating characteristic (ROC) curve. For each cut off point, the sensitivity was plotted against the false positive rate (Fig 4) The OD of $\bar{X}$+4SD which derived from mean of the OD of the pooled negative control + 4SD was 0.459, yielded the most acceptable indication for cut off point. It showed 88.3, 95.1, 4.9 and 94.1% sensitivity, specificity, false positive and accuracy respectively.

Based on the cut off point, 39 specimens were found positive by dEIA (Table 2). The scatter of OD values from 219 samples of clinical specimens detected by dEIA is shown in Fig 5.

When the results were combined with CC and PACE2, it was found that sensitivity, specificity, positive and negative predictive values of the test in clinical application were 88.1, 94.6, 77.5, 97.6% respectively (Table 3).

Table 3
Sensitivity, specificity and positive and negative predictive values of kEIA and dEIA compared with True Positive Test criteria for diagnosis of *C. trachomatis* infection.

Gender (No.)	Status (No.)	Methods	% (No. Of positive specimens/No. tested)			
			Sensitivity	Specificity	PPV[a]	NPV[b]
Female (155)	Symptomatic (81)	kEIA	100.0 (7/7)	100.0 (74/74)	100.0 (7/7)	100.0 (74/74)
		dEIA	85.7 (6/7)	96.0 (71/74)	66.7 (6/9)	98.6 (71/72)
	Asymptomatic (74)	kEIA	87.5 (14/16)	94.8 (55/58)	82.4 (14/17)	96.5 (55/57)
		dEIA	87.5 (14/16)	96.6 (56/58)	87.5 (14/16)	96.6 (56/58)
	Combined (155)	kEIA	91.3 (21/23)	97.7 (129/132)	87.5 (21/24)	98.5 (129/131)
		dEIA	87.0 (20/23)	96.2 (127/132)	80.0 (20/25)	97.7 (127/130)
Male* (64)	Symptomatic (64)	kEIA	75.0 (9/12)	97.6 (41/42)	90.0 (9/10)	93.2 (41/44)
		dEIA	90.9 (10/11)	90.7 (39/43)	71.4 (10/14)	97.5 (39/40)

PPV[a]: positive predictive value, NPV[b]: negative predictive value
* Chlamydia positive specimens were found only in symptomatic men.

Table 4
Agreement between dEIA and kEIA in female and male specimens.

dEIA	kEIA	Females	Males	Total
+	+	23	9	32
+	-	2	5	7
-	+	1	1	2
-	-	129	49	178

Agreement between dEIA and kEIA are shown in Table 4. When compared with p-values and the strength of agreement (Kappa, K-values), it was found that the agreement between two EIA methods of both gender was statistically significant at $p<0.01$ (p-values for the agreement between two methods are statistically significant difference when they are less than 0.05) and when K-values was 0.85 (K-values between 0.81-1.00 means almost perfect). However, when compared in each gender, it was found that the strengths of agreement in both symptomatic females and males were 0.74 and 0.69 respectively (K-values between 0.61-0.80 means substantial) whereas that of symptomatic females was 0.89 respectively (data not shown).

DISCUSSION

Rabbit immune serum to partially purified EB, combined with a monoclonal antibody to LPS chlamydial antigen was used to develop an EIA. The serum had been shown to recognize both partially purified EB and purified EB with no cross reaction with *E. coli* and *A. anitratus*. The developed assay could detect partially purified EB as low as 250 ng/ml. It was not surprising that this dectection limit is higher than that was reported by Mohanty *et al* (1996), in which purified EB was used in preparing the antibody and testing the sensitivity of the assay. In addition, they used purified polyclonal IgG and mAb to MOMP antigen in their system. The system detecting LPS was shown to have the tendency to be less sensitive than the one that detecting MOMP (Mohanty *et al*, 1996). However, LPS antigen was more stable and more soluble than MOMP (Schachter, 1991). Thus, specimen procession is then easier and less stringent.

The dEIA in this study was shown to have a narrow range of variation with intra- and inter-run CV of less than 10%. This was acceptable for general EIA (Catty and Raykundalia 1989).

Among infections of male and female genital tract, the dEIA showed cross reaction with HSV and *C. albicans* infection. This could be its disadvantage. However, considering clinical characteristics of these two infections, ulcer in HSV infection and characteristic leukorrhea in candidiasis, through physical examination should help to differentiate these two infections. Moreover, microscopic examination of vaginal discharge and cervical scraping should reveal the characteristic morphology of HSV infected cells and the presence of yeast or mycelia in candidiasis.

In clinical evaluation, the dEIA showed the overall value of 88.1 and 94.6 % sensitivity and specificity respectively. For endocervial specimens, the dEIA was less sensitive than the commercial kit. On the contrary, it yielded higher sensitivity for urethral specimens. Despite subtle difference, the agreement between two tests was satisfied. Thus, the dEIA could be appropiately applied for female and male specimens for detection of chlamydial infection.

Many factors had been shown to effect the sensitivity of an EIA for chlamydial infection. Degradation of antigen may occur after storing at -70ºC for a few years (Olsen and Sambol, 1993). In contrast, freezing for a short period, 8 weeks as was reported, may modify the antigen and increase the sensitivity of the test (Kluytmans *et al*,1993). Those observations may explain the different sensitivity of the dEIA for female and male specimens, which were collected through a few years, stored for various periods and thawed to be assayed at the same time. Moreover, environmental factors, such as pH, composition of secretion, cyclical change, etc which were different in males and females may play a role in the preservation or degradation of chlamydial antigens (Mahony and Chernesky, 1985).

Results in the present study suggested that the prepared rabbit immune serum could be used to develop an assay for diagnosis of chlamydial infection. Purification and use of IgG fractions of the serum may yield a better sensitive of the assay for detecting chlamydial infection. The application is not limited to genital infection since chlamydial infection was found in other organs as well. In addition, EIA in this study applied the monoclonal antibody against LPS for coating plate. High consideration should be taken into account in applying the test to other chlamydial infection since the LPS is genus specific.

In conclusion, the dEIA can be applied satisfactorily for the detection of genital chlamydial infection, in both males and females. The cost per test is cheaper than CC, commercial EIA and PACE2. With some precautions, it could be used as a routine procedure for the diagnosis of genital tract infection.

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