CHAPTER III

RESULTS

1. Development of the rapid immunoperoxidase technique.

1.1 Preparation of antigen coated slide.

The suitable amount of antien for slide coating was McCoy cells suspension of 30-80% infection. Thus C. trachomatis serotype L_2 from 100% infected stock solution was diluted to various dilutions and inoculated onto the McCoy cells culture. As shown in table 1A (Appendix V), 30-80% infected cells was obtained using 1:1000-1:10,000 dilution of stock C. trachomatis serotype L_2. Consequently, dilutions at this range were used in subsequent infected cell preparations.

The optimal concentration of infected cells showing good distribution on the slide was $2x10^5$ cells/ml (Table 2A, Appendix V). Higher concentration ($5x10^5$ cells/ml) resulted in a confluent monolayer with excessive amount of cells whereas lower concentration ($1x10^5$, $1x10^4$ cells/ml) resulted in too few cells to be tested.

1.2 Determination of factors affecting the rapid immunoperoxidase test.

Various concentrations of rabbit anti-human Ig/peroxidase conjugate (1:20, 1:40, 1:60, 1:80) were titrated against known positive sera, control negative sera (normal human serum, 1:8 dilution) and PBS pH 7.4 (reagent control). As shown in table 3A, 4A and 5A (Appendix V), for IgG, IgM and IgA specific antibody detection, dilution of 1:20 for rabbit anti-human Ig/peroxdase conjugate produced best result and was

chosen for subsequent tests.

Optimal temperature and reaction time in each step of the rapid immunoperoxidase test were determined as shown in table 6A, 7A, 8A and 9A (Appendix V). A 30 minutes incubation at 37° C of chlamydial antigen-antibody reaction followed by a reaction tiime of 90 minutes for enzyme labelled antihuman Ig (IgG, IgA or IgM) at 37° C and 30 min at room temperature for substrate/chromogen reacton gave the best distinction between negative and positive control. This was summarized in diagram 2

Final suitable dilution of rabbit-antihuman Ig/peroxidase conjugate was again determined using the optimal condition as stated. As shown in table 10A, 11A and 12A (Appendix V), dilution of 1:20 for rabbit anti-human Ig/peroxidase conjugate produced best results in all 3 classes of specific antibody detection, thus was selected for subsequent tests. The optimum condition of avidin-biotin immunoperoxdase was summarized in diagram 3.

Diagram 2 A summary of final optimum conditions for rapid immunoperoxidase

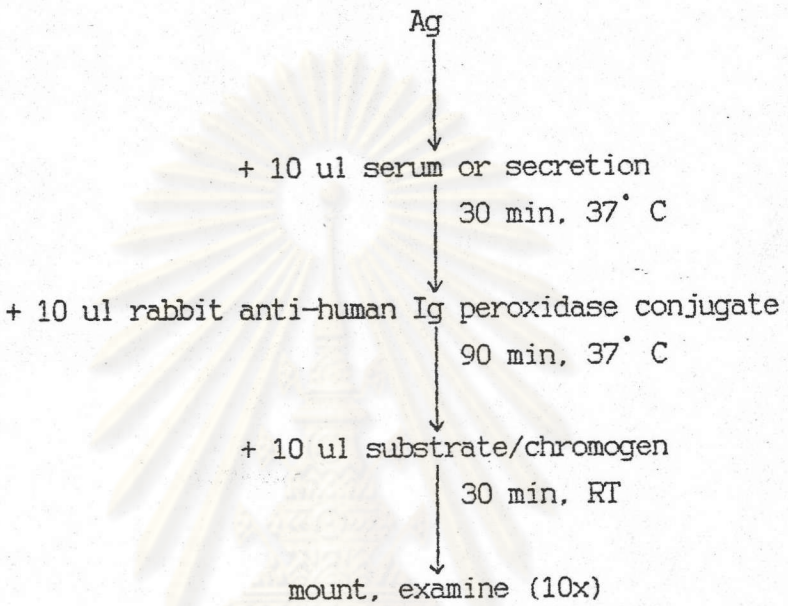
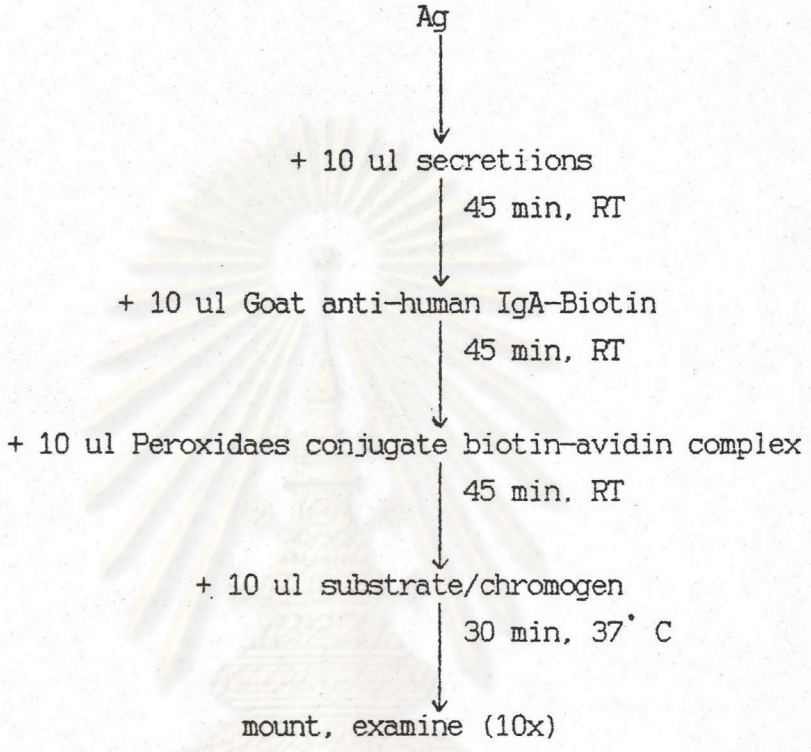


Diagram 3 A summary of final optimum conditions for avidin-biotin immunoperoxidase



1.3 Shelflife and applicable storing temperature for antigen slides

It was shown in table 6 that the titers of anti-chlamydial antibody were similar with antigen slides stored at various temperature indicated in the materials and methods.

Table 6 Effect of storing temperature on inclusion antigen slides for the titration of anti-chlamydial antibody

Number	Fresh antigen	storing condition			
		3 months		6 months	
		-20° C	-70° C	-20° C	-70° C
1	* 64	32	64	64	64
	** 64	64	64	64	64
2	* 8	<8	8	8	8
	** 8	8	8	8	8
3	* 16	8	16	16	8
	** 16	8	16	16	16
4	* 32	16	32	32	16
	** 32	16	32	32	32
5	* 8	<8	8	8	<8
	** 8	8	16	8	8
6	* 32	16	32	32	16
	** 32	16	16	16	32
7	* 32	16	64	32	16
	** 32	16	32	16	32
8	* 8	<8	16	16	8
	** 8	8	8	8	8
9	* 32	16	64	16	32
	** 32	16	64	16	32
10	* 8	<8	32	16	8
	** 16	16	8	8	16

* Reciprocal titer as determined by micro-immunofluoescent test

** Reciprocal titer as determined by rapid-immunoperoxidase test

2. Isolation of C. trachomatis and specific antibody detection from clinical specimens

2.1 Isolation of C. trachomatis

Urethral specimens were inoculated onto cycloherximide treated McCoy cells. C. trachomatis infected cells would present glycogen-containing inclusion body within cytoplasm detectable by iodine staining after 48-72 hrs of cultivation. This could be visualized as red-brown oval or round bodies, the color intensity of which varied depending on the length of incubation (Figure 10, 11). Of the 200 patients studied, 69 patients (34.5%) demonstrated chlamydia upon isolation. The serological manifestation of these patients were summarized in Table 15 appendix VI.

Of the 69 culture positive patients, serum IgG, IgM and IgA antibodies were found in 65, 5 and 3 sera respectively by m-IF and 67, 1 and 5 sera respectively by IP. As for chlamydial antibodies in secretions, 54 were IgG 2 IgM and 41 IgA by m-IF; and 36 IgG, 39 IgA by IP. Secretory IgA antibody was also detectable by avidin-biotin immunoperoxidase in 55 secretions.

2.2 Specific anti-chlamydial antibody detection

2.2.1 Anti-chlamydial antibody in serum.

All positive results at titers of $\geq$ 1:8 were considered as presence of antibody. Of the 200 sera tested, there were 173 and 186 chlamydial IgG antibody by m-IF (86.5%) and IP (93.0%) respectively. Of the 173 seropositive patients (as detected by m-IF), 65 have C. trachomatis upon culture (37.5%, Table 7) whereas 4 of the 27 sero-negative patients were culture positives (14.8%). Of the 186 sero-positive patients detected by IP, 67 have chlamydia upon isolation (36.0%, Table 8), whereas 2 out of 14 sero-negative samples show positive cultivation (14.28%). It is possible that serum antibody response may develop late in

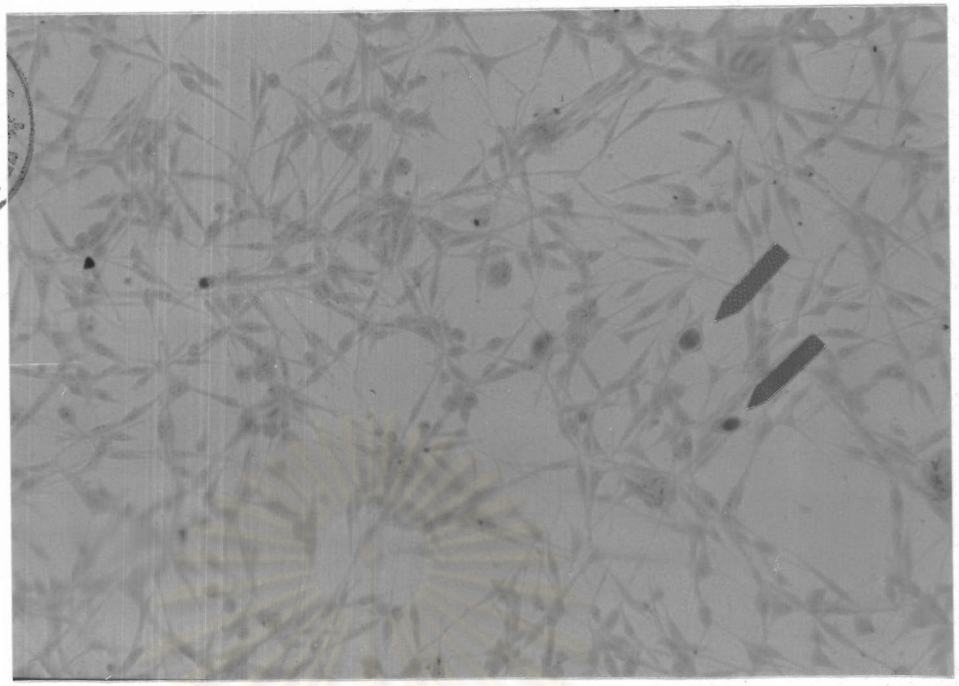


Figure 10 C. trachomatis inclusion bodies in McCoy cells with iodine staining (10x)

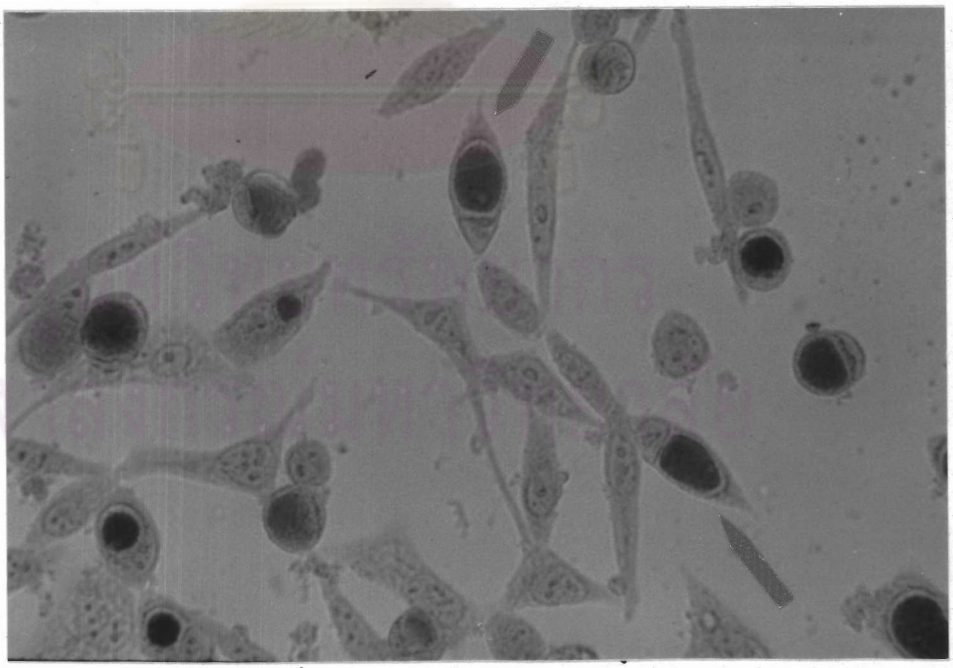


Figure 11 C. trachomatis inclusion bodies in McCoy cells with iodine staining (40x)

some patients.

Incidence of serum IgM and IgA chlamydial antibodies were demonstrated in table 10. Of the 200 sera tested, only 17 and 10 were positive for IgM and IgA antibodies by m-IF in comparison to 2 positive IgM and 10 positive IgA antibodies by IP.

With regards to C. trachomatis cultivation, 5 of the 17 IgM seropositive group and 3 of the 10 IgA seropositive group by m-IF show positive result on chlamydial isolation (29.4 and 30.0% respectively, Table 7). One of the 2 IgM and 5 of the 10 IgA seropositive by IP, have chlamydia on isolation (50% each, Table 8).

All patients with specific IgM antibodies by IP also demonstrate IgM positivity by m-IF and 6 of 10 IgA positive sera by IP show positive IgA antibody by m-IF.

Comparison of antibody titers for serum IgG antibody detectable by IP and m-IF was demonstrated in Figure 12. The IgG antibody titer obtained by the two methods correlated well with each other, with a correlation coeffient (r) of 0.97. The result of serum IgG chlamydial antibody detected by IP was compared with that by m-IF in Table 9. The sensitivity, specificity, positive predictive value and negative predictive value were 98.84%, 44.44%, 91.94% and 85.71% respectively.

2.2.2 Anti-chlamydial antibody in secretion or urethral discharge.

Of the 200 urethral discharge tested, there were 54 (27.0%) IgG, 3 (1.5%) IgM and 58 (29%) IgA antibodies by m-IF; 47 (23.5%) IgG and 53 (26.5%) IgA antibodies

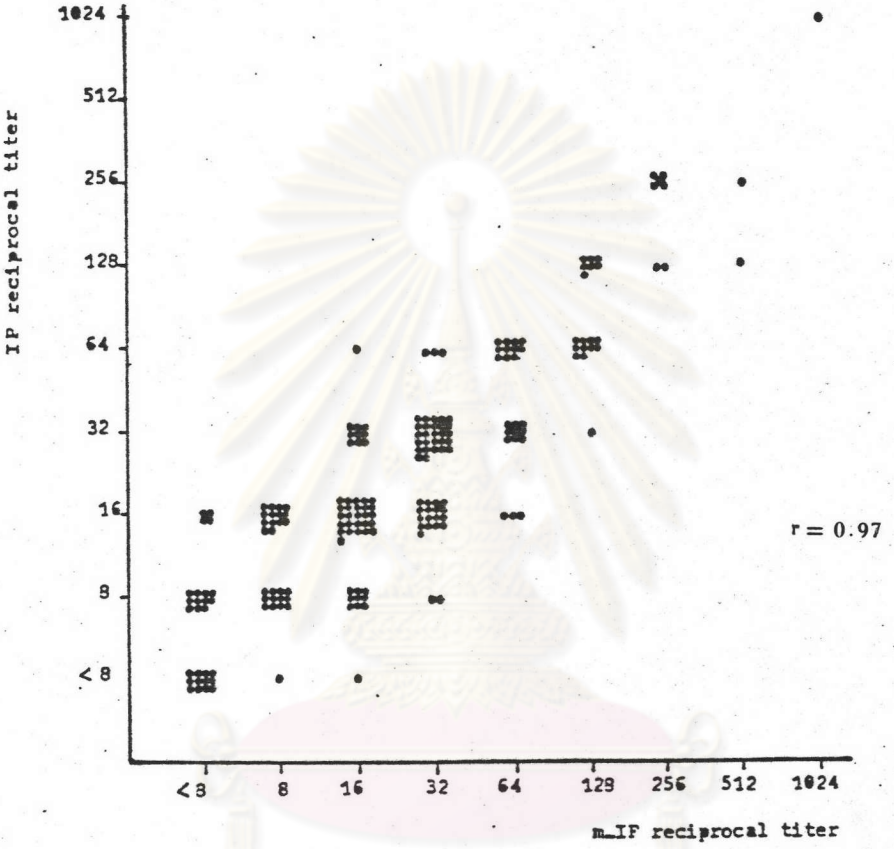


Figure 12 Scatter diagram of serum IgG antibody titer detected by rapid immunoperoxidase assay (IP) and micro-immunofluorescence (m-IF)

by IP. IgM antibodies were undetectable by IP technique. When avidin-biotin IP method was used, the detectability of serum IgA antibody was increased to 84 (42%) (Table 10).

All patients with IgG antibody in secretion were also positive for serum IgG. Interestingly, the presence of IgA antibody in secretion did not correlate with that of serum IgA but did very well with serum IgG antibodies. Of the 3 patients with IgM antibody in secretion, 2 also posses IgA antibody.

In comparison with m-IF test, the results of IgG, IgA antibody in secretion by IP and IgA antibody by avidin-biotin IP were shown in table 18, 19 and 20 appendix VI and summarized in Table 11. The sensitivity, specificity, positive predictive value and negative predictive value were 85.19%, 99.32%, 97.87% and 94.77% respectively for IgG antibody, 79.31%, 95.07%, 86.79% and 91.84% respectively for IgA antibody, and 94.83%, 79.58%, 65.48% and 97.41% espectively for avidin-biotin IgA.

Chlamydial isolation was found in 41 of 54 patients exhibiting IgG antibodies (75.9%), 2 of 3 exhibiting IgM and 40 of 58 exhibiting IgA antibodies (68.9%) by m-IF technique. Whereas in immunoperoxidase test, chlamydial culture was positive in 36 of 47 patients with IgG antibodies (76.5%) and in 39 of 53 with IgA antibodies (73.5%). Avidin-biotin IP was also used to detect IgA antibody in which 55 of 84 patients with specific IgA antibodies (65.4%) showed C. trachomatis upon culture (Table 12).

Correlation of IgG and IgA antibodies in secretion by IP technique with chlamydial infection was

determined by Chi-square (x2) method. As demonstated in Table 13 and appendix VI Table 21, 22 the incidence of IgG/IgA antibodies in the positive culture group were significantly higher than that in the negative culture group (55 versus 29, Table 13). When the result of chlamydial isolation was utilized as golden standard for definitive diagnosis, the avidin-biotin immunoperoxidase method could be evaluated with a sensitivity, specificity, positive predictive value and negative value of 79.71%, 77.86%, 65.48% and 87.93% respectively.

In addition to chlamydial cultivation, the presence of IgA antibodies in secretion was also indicative for possible chlamydial infection (47). Consequently, both techniques were combined for more accurate diagnosis of chlamydial infection and employed as golden standard for further evaluation of the avidin-biotin IP technique in Table 14. Hence the estimated cases of current chlamydial infection amounted to 86 and the sensitivity, specificity, positive predictive value of avidin-biotin secretory IgA for detecting chlamydial infection were increased to 81.40%, 87.72% and 83.33% respectively with a negative predictive value of 86.21%.

Table 7 Serum IgG, IgM and IgA chlamydial antibodies by micro-immunofluorescence versus C. trachoatis isolation

Chlamydial Antibody[a]			Isolation of C. trachomatis		% positive
	number positive	titer	positive	negative	
IgG	173	1:8-1:1024	65	108	37.5
IgM	17	1:8-1:16	5	12	29.4
IgA*	10	1:8-1:32	3	7	30.0
IgG+IgM	14		4	10	
IgG+IgM+IgA	2		1	1	
IgG+IgA	8		2	6	

[a] total number of sera = 200

* coexist as IgG+IgM+IgA or IgG+IgA

Table 8 Serum IgG, IgM and IgA chlamydial antibodies by rapid immunoperoxidase versus C. trachomatis isolation

Chlamydial Antibody[a]			Isolation of C. trachomatis		% positive
	number positive	titer	positive	negative	
IgG	186	1:8-1:1024	67	119	36.0
IgM	2	1:8	1	1	50.0
IgA	10	1:8-1:64	5	5	50.0
IgG+IgM	1		-	1	
IgG+IgM+IgA	1		1	-	
IgG+IgA	9		4	5	

[a] total number of sera = 200

Table 9 Comparison of serum IgG antibody detected by rapid immunoperoxidase (IP) and micro-immunofluorescence (m-IF)

Test		m-IF		Total
		Positive ($\geq$ 1:8)	Negative (<1:8)	
IP	positive ($\geq$ 1:8)	171	15	186
	negative (< 1:8)	2	12	14
Total		173	27	200

Sensitivity 98.84%
Specificity 44.44%
Positive predictive value 91.94%
Negative predictive value 85.71%

Table 10 Incidence of serum and secretory chlamydial antibodies detected by micro-immunofluorescence (m-IF) and rapid immunoperoxidase (IP)

Test (N =200)	No. of patients demonstrating antibody		
	IgG	IgM	IgA
Serum m-IF	173 (86.5%)	17 (8.5%)	10 (5.0%)
IP	186 (93.0%)	2 (1.0%)	10 (5.0%)
Secretion m-IF	54 (27.0%)	3 (1.5%)	58 (29.0%)
IP	47 (23.5%)	-	53 (26.5%)
avidin-biotin IP	ND	ND	84 (42.0%)

ND = Not done

Table 11 Evaluation of chlamydial antibodies in secretions as detected by rapid immunoperoxidase (IP) in comparison with micro-immunofluorescence (m-IF)

Antibody class, (Test)	Sensitivity (%)	specificity (%)	Positive predictive value (%)	Negative predictive value (%)
IgG (IP)	85.19	99.32	97.87	94.77
IgA (IP)	79.31	95.07	86.79	91.84
IgA (avidin-biotin IP)	94.83	79.58	65.48	97.41

Table 12 Secretory IgG, IgM and IgA chlamydial antibodies versus C. trachomatis cultivation

Secretory chlamydial Ab	Total number positive	Isolation of C. trachomatis		% positive
		positive	negative	
m-IF				
IgG	54	41	13	75.9
IgM	3	2	1	66.7
IgA	58	40	18	68.9
IP				
IgG	47	36	11	76.5
IgM	0	0	0	
IgA	53	39	14	73.5
avidin-biotin IgA	84	55	29	65.4

Table 13 Comparison of secretory IgA antibody as demonstrated by avidin-biotin immunoperoxidase with isolation of C. trachomatis

avidin-biotin IP	Isolation of C. trachomatis		Total
	Positive	Negative	
Secretory IgA positive	55	29	84
Secretory IgA negative	14	102	116
Total	69	131	200

Sensitivity 79.71% Specificity 77.86%
Positive predictive value 65.48% Negative predictive value 87.93%
x^2 = 61.50; Significant at $P < 0.01$

Table 14 Comparison of secretory IgA antibody as demonstrated by avidin-biotin immunoperoxidase and micro-immunofluorescence and/or isolation of C. trachomatis

avidin-biotin IP	Isolation of C. trachomatis / secretory IgA by m-IF		Total
	Positive	Negative	
Secretory IgA positive	70	14	84
Secretory IgA negative	16	100	116
Total	86	114	200

Sensitivity 81.40% Specificity 87.72%
Positive predictive value 83.33% Negative predictive value 86.21%