CHAPTER V

RESULTS

1. Detection of protein profile in mosquito cell extracts by SDS-PAGE

For analysis of the protein component recognized by human IgG antibodies, whole body extracts of *Aedes aegypti*, *Aedes albopictus* and C6/36 cell were separated on SDS-PAGE and stained with Coomasie blue. Figure 1 show the SDS-PAGE pattern and Figure 2 show the electrophoretically transferred patterns of the extracts on nitrocellulose membrane. The whole body extract of the different mosquito species and mosquito cell line, showed a similar SDS-PAGE pattern (Figure.1, lanes A-C). There were multiple components in these extracts and components with molecular weight of about 12, 14, 17, 26, 30, 35, and 41 kDa were most intensely stained. The components of C6/36 cell with molecular weight of about 12, 14, and 17 kDa were more intensely stained than the mosquito extract protein. This may be related to the difference of protein expression in development stage of mosquito life cycle. In general, protein extracts from C6/36 cell, *Aedes aegypti* and *Aedes albopictus* are similar. Figure 2 shows completely transferred of protein component from SDS-PAGE to the nitrocellulose membrane by electro-blotting and Amido Black staining.

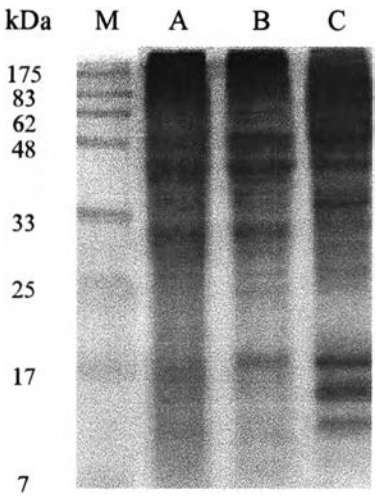

Figure 2 SDS-PAGE of the mosquito's cell extracts. The mosquito's cell extracts were separated by 15% SDS-PAGE and stained with Coomasie blue. Mosquito antigens used were whole body extracts of *Aedes aegypti* (lane A), *Aedes albopictus* (lane B), and C6/36 cell extract (lane C). The molecular weight standards (BioLab, New England) are MBP-β - galactosidase (175 kDa), MBP-paramypsin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β- lactoglobulin A (25 kDA), lysozyme (16.5 kDa), and aprotinin (6.5 kDa).

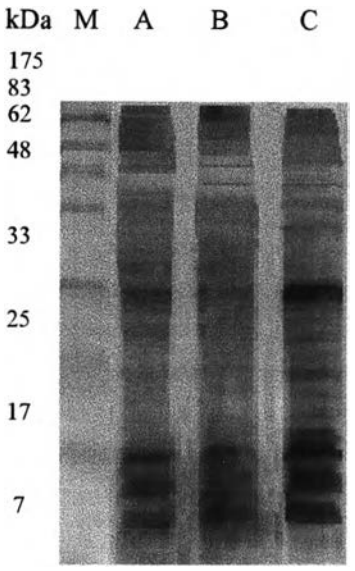

Figure 3 Electro-blotting patterns of different mosquito's cell extracts. The different mosquito's cell extracts separated by 15% SDS-PAGE and electrophoretaically transferred to nitrocellulose membrane. Mosquito antigens used were whole body extracts of *Aedes aegypti* (lane A), *Aedes albopictus* (lane B), and C6/36 cell extract (lane C). The molecular weight standards (BioLab, New England) are MBP-β- galactosidase (175 kDa), MBP-paramypsin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β- lactoglobulin A (25 kDA), lysozyme (16.5 kDa), and aprotinin (6.5 kDa).

2. Detection of anti-mosquito cell antibodies in rabbit's serum by Western blot analysis

After two rabbits had been immunization with C6/36 cell line, many anti-mosquito protein antibodies could be identified by Western blot analysis. From Figure2, lane A and lane B are protein extracted of whole body of *Aedes aegypti* and *Aedes albopictus*, respectively. Lane C is protein extracts of C6/36 cell. All of these proteins were separated on SDS-PAGE and followed by electro-blotting. Panel 1 of Figure 3 show non-reactivity of rabbit's preimmune serum. Panel 2, 3 and 4 of Figure 4 show reaction of rabbit anti-mosquito cell antibodies to mosquito protein from post-immune serum on day 52, 84, and 98, respectively. Multiple IgG-binding of C6/36 protein were identified in all immunoblots, from all post-immune sera. The patterns of them are similar in quantity but increasing in antibodies titer. They showed very strong IgG binding activity towards antigens of C6/36 cell with molecular weight of 34, 47, 54, 65, and 83 kDa as shown in Figure 3. While the reaction on protein extracted of *Aedes* mosquitoes show weakly reacted against the same molecular weight protein. However, almost of the immunoblot patterns from both C6/36 and mosquito's whole body extract protein are similar.

Mosquito antigens recognized by rabbit serum were compared between different cell sources. Molecular weight of mosquito antigens from the whole body extract of *Aedes aegypti* and *Aedes albopictus* and extract of C6/36 cell identified by rabbit antibodies are listed in the Table 3. All sources of mosquito cell shared almost antigens. However, some antigens were unique to a specific mosquito cell type.

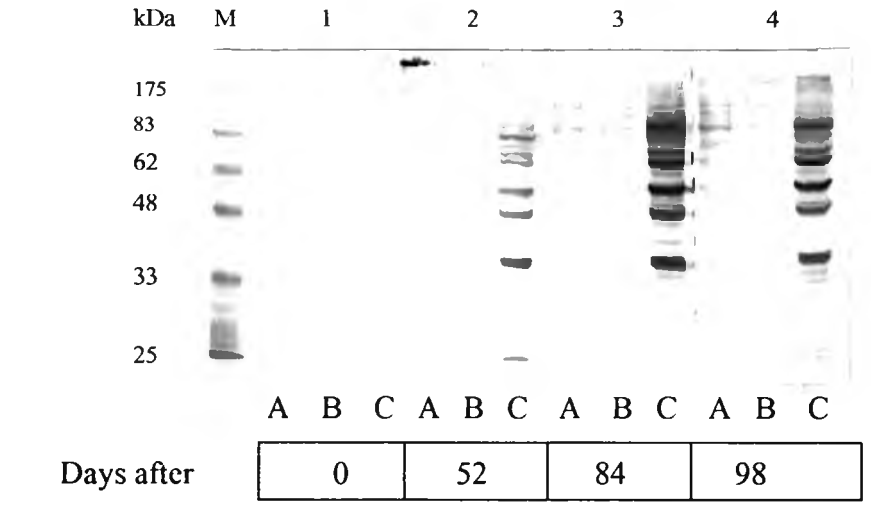

Figure 4 Detection of anti-mosquito cell antibodies in serum of experimental rabbit immunized with C6/36 cell line. Mosquito antigens used were whole body extracts of *Aedes aegypti* (lane A), *Aedes albopictus* (lane B), and C6/36 cell extract (lane C). They were separated by 12% SDS-PAGE and electroblotting onto nitrocellulose membrane . Rabbit immunized with C6/36 cell were bleed on day 0, 52, 84, and 98 days after first immunization.

Table 3. Western blot analysis of mosquito cell's protein antigens identified by rabbit's immuned serum.

MW (kDa)	Ae. aegypti Whole body	Ae. albopictus Whole body	C6/36
30			✓
32			✓
33			✓
34	✓	✓	✓*
40			✓
47			✓*
48			✓
54	✓	✓	✓*
55	✓		
63	✓		
65	✓	✓	✓*
69			✓
80	✓	✓	
81			✓
82			✓
83			✓*
120	✓	✓	
130	✓	✓	✓
150	✓	✓	
155			✓
170	✓	✓	✓

* positive result with strong IgG binding activity towards antigens

3. Detection of anti-mosquito cell antibodies in human serum by Western blot analysis.

To detect anti-mosquito cell antibodies, whole body extract of *Aedes aegypti* (A), *Aedes albopictus* (B) and C6/36 cell extract (C) was separated on SDS-PAGE and followed by electro-blotting for Western blot analysis with human serum from different source. The result shows in Figure 4 to 9.

A total of 30 guard's serum were used in immunoblot analysis. Most of the guard's serum (83%) can react with proteins from all three sources of antigens. The patterns and intensity of reaction is different qualitatively and quantitatively. The guard's serum reacts with low to high molecular weight protein (10-170 kDa). For dengue patient's serum, only 53% of them showed reactivity to mosquito protein with low intensity and few proteins antigens at molecular weight (28-34 kDa). The immunoblot pattern of both guard's serum (Figure 6) and dengue patient's serum (Figure 9) show weakly reactivity with C6/36 cell extracts. A total of ten cord blood from the Obstetric-Gynecology Department, King Chulalongkorn Memorial Hospital, were also used to react with mosquito's antigen. No reactivity can be detected from all cord bloods (data not shown). The antibodies can not be detected in 17% of guard, 47% of dengue patients and 100% of cord blood.

Mosquito antigens recognized by guard's serum in this study were compared with the mosquito antigen in saliva and salivary gland which reported in previous studies (105,106,115). Antigen identified varied depending on the each guard tested. Molecular weight of mosquito antigens from the saliva, salivary gland and whole body extract identified by human antibodies are listed in the Table 4. Molecular weight range from a low of 10 kDa to sized greater than 175 kDa. All sources of mosquito antigens shared and unique antigens were observed. Some antigens were unique to a mosquito cell or some antigen present in the saliva. *Aedes* mosquito's protein with molecular weight of 32.5 kDa and 83 kDa are identified in almost guard's serum

(10:30 and 12:30, respectively). Fifteen proteins of *Aedes* mosquito's whole body extract are identified in guard's serum while the previous studies showed eight proteins were identified from mosquito saliva and nine proteins were identified from mosquito's salivary gland. Immunoblot patterns were similary complex for all individual studied.

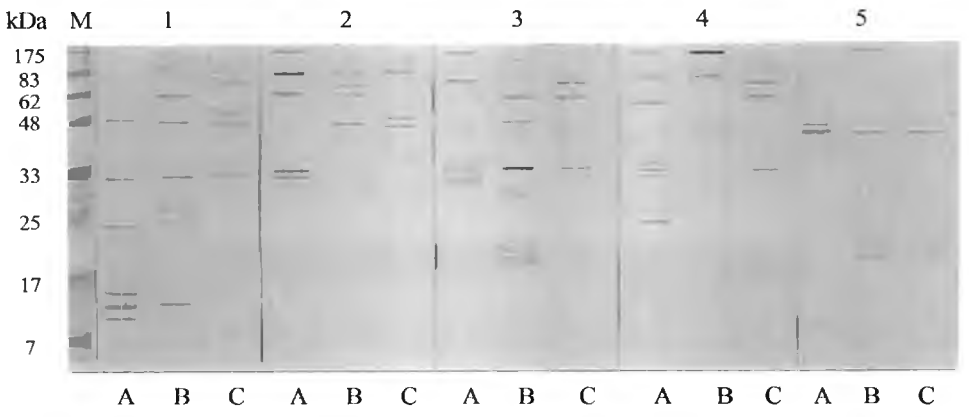

Figure 5 Immuno-blotting patterns of anti-mosquito cell antibodies from guard' s serum. Mosquito antigens used were whole body extracts of *Aedes aegypti* (lane A), *Aedes albopictus* (lane B), and C6/36 cell extract (lane C). They were separated by 15% SDS-PAGE and electroblotting into nitrocellulose membrane. The presence of IgG antibodies against the mosquito cell protein was detected immunochemically. Blots of panels 1 through 5 were incubated separately with sera from five guards.

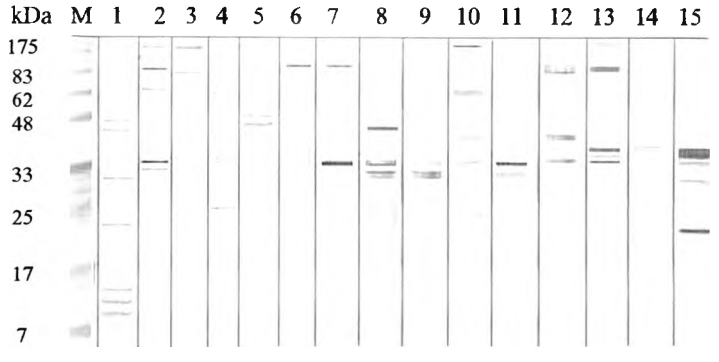

Figure 6 Immuno-blotting pattern of anti-*Aedes aegypti* whole body extract antibodies from guard' s serum. Mosquito antigens used were whole body extract of *Aedes aegypti*. They were separated by 15% SDS-PAGE and electroblotting into nitrocellulose membrane. The presence of IgG antibodies against the mosquito cell protein was detected immunochemically. Blots of panels 1 through 15 were incubated separately with sera from fifteen guards.

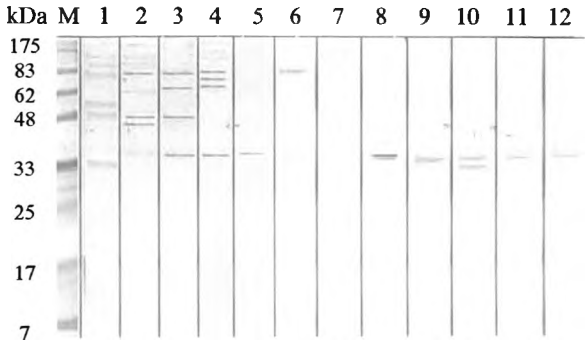

Figure 7 Immuno-blotting pattern of anti-C6/36 cell extract from guard' s serum

Mosquito antigens used were C6/36 cell's extracts. They were separated by 15% SDS-PAGE and electroblotting into nitrocellulose membrane. The presence of IgG antibodies against the mosquito cell protein was detected immunochemically. Blots of panels 1 through 12 were incubated separately with sera from twelve guards.

Table 4. Comparison of the mosquito protein's molecular weights, from different sources, identified in human's serum by Western blot analysis.

MW.	Ae.aegypti's saliva♣	Ae. aegypti's salivary gland ♠		whole body of Ae. aegypti ♥
10	✓			✓ (1:30)*
12				✓ (1:30)
14.9		✓		✓ (1:30)
18.5	✓			✓ (2:30)
21			✓	
23			✓	
24				✓ (2:30)
27				✓ (4:30)
28			✓	
29			✓	
30				✓ (2:30)
30.5	✓			✓ (1:30)
31			✓	
32				✓ (5:30)
32.5				✓ (10:30)
33	✓			✓ (8:30)
33.2		✓		
34			✓	✓ (3:30)
37	✓			✓ (1:30)
40.3		✓		
42			✓	
49.8		✓		
50	✓			
55.5	✓			
61.5	✓			
61.7		✓		
62			✓	✓ (3:30)
63.6		✓		
67.6		✓		
68	✓			
78			✓	
83				✓ (12:30)
106.2		✓		
114.9		✓		
175				✓ (5:30)

♣ The presence of human IgE antibodies against the Ae. aegypti's saliva proteins (105).

♠ The presence of human IgG antibodies against the Ae. aegypti's salivary gland proteins (106,115)

♥ The presence of human IgG antibodies against the Ae. aegypti's whole body proteins, present in this study.

* Number of guard's sera with IgG react with mosquito cell's proteins : number of total guard's sera.

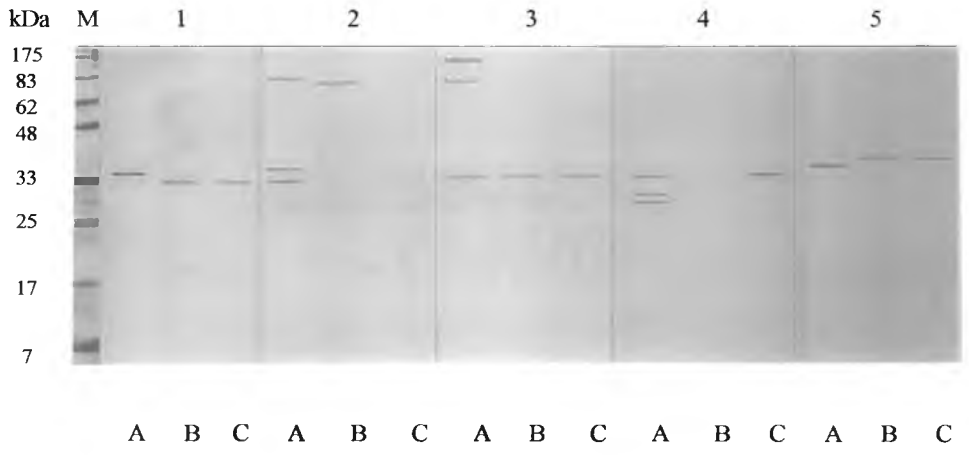

Figure 8 Immuno-blotting pattern of anti-mosquito cell antibodies from dengue patient's serum. Mosquito antigens used were whole body extracts of *Aedes aegypti* (lane A), *Aedes albopictus* (lane B), and C6/36 cell extract (lane C). They were separated by 15% SDS-PAGE and electroblotting into nitrocellulose membrane. The presence of IgG antibodies against the mosquito cell protein was detected immunochemically. Blots of panels 1 through 5 were incubated separately with sera from five dengue patients.

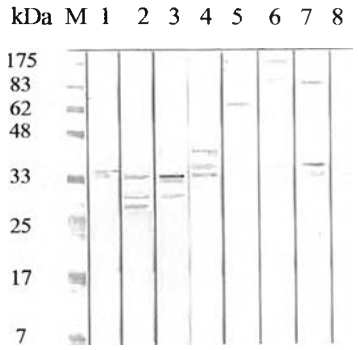

Figure 9 Immuno-blotting pattern of anti-*Aedes aegypti* whole body extract antibodies from dengue patient's serum. Mosquito antigens used were whole body extracts of *Aedes aegypti.* They were separated by 15% SDS-PAGE and electroblotting into nitrocellulose membrane. The presence of IgG antibodies against the mosquito cell protein was detected immunochemically. Blots of panels 1 through 8 were incubated separately with sera from eight patients.

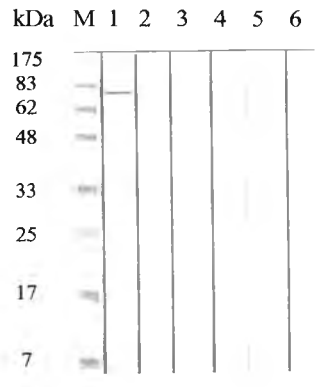

Figure 10 Immuno-blotting pattern of anti-C6/36 cell extract antibodies from dengue patient's serum. Mosquito antigens used were C6/36 cell extracts. They were separated by 15% SDS-PAGE and electroblotting into nitrocellulose membrane. The presence of IgG antibodies against the mosquito cell protein was detected immunochemically. Blots of panels 1 through 6 were incubated separately with sera from six patients.

4. Reactivity of the anti-mosquito cell antibodies by indirect immunofluorescence assay.

Detection of anti-mosquito cell antibodies was performed on C6/36 cell immobilized on glass slide. The reactivity of the preimmune sera and the immuned antisera from rabbits immunized with C6/36 cell were stained by IFA. The reactivity of the preimmune sera or PBS with C6/36 cell were negative (Figure 10). While the reactivity of the immune rabbit sera with C6/36 cell were strongly positive (Figure11). The anti-mosquito cell antibody from cord blood serum, guard's serum and serum of patient with dengue infection were shown in Figure 12 to Figure 16. Cord blood serum were used as negative control (Figure 13). The mosquito cell antibody from guard's serum show varies degree of intensity of fluorescence while dengue patient's serum show weakly intensity of fluorescence.

Figure 11 Immunofluorescence assay of preimmune rabbit's serum reacted with C6/36 cell line. The PBS is used as negative control.

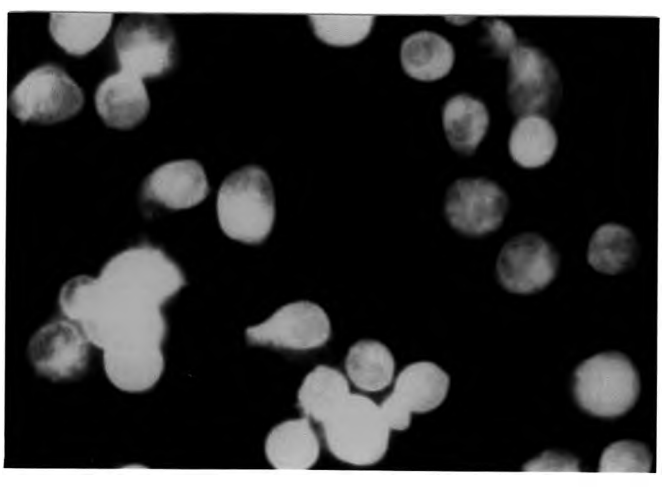

Figure 12 Immunofluorescence assay, the C6/36 cell were reacted with immunized rabbit's serum and counter stained with FITC conjugated to anti-rabbit IgG, show strong fluorescence staining.

Figure 13 Immunofluorescence assay of human's cord blood serum reacted with C6/36 cell line and counter stained with FITC conjugated to anti-human IgG.

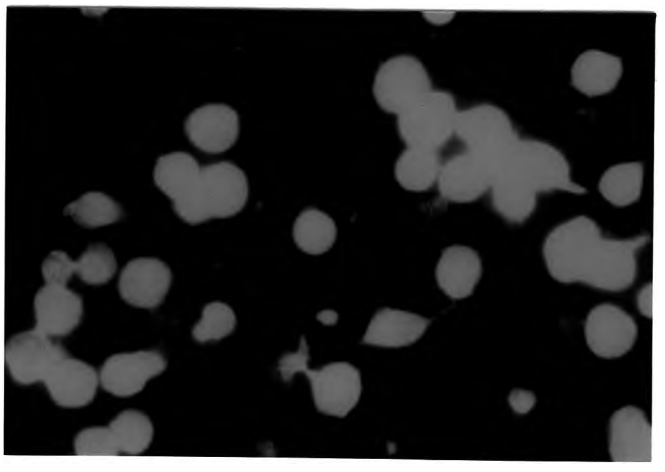

Figure14 Immunofluorescence assay of guard 's serum with strong reactivity. Immunofluorescence assay of the C6/36 cell reacted with guard 's serum, and counter stained with FITC conjugated to anti-human IgG.

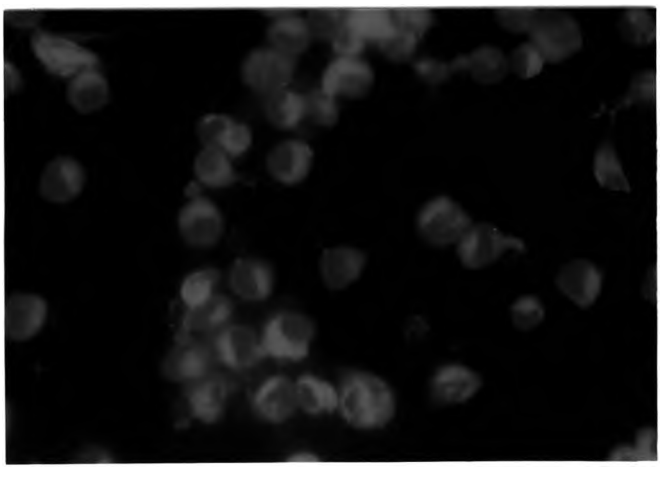

Figure 15 Immunofluorescence assay of guard 's serum with weak reactivity. The C6/36 cell reacted with guard 's serum, and counter stained with FITC conjugated to anti-human IgG.

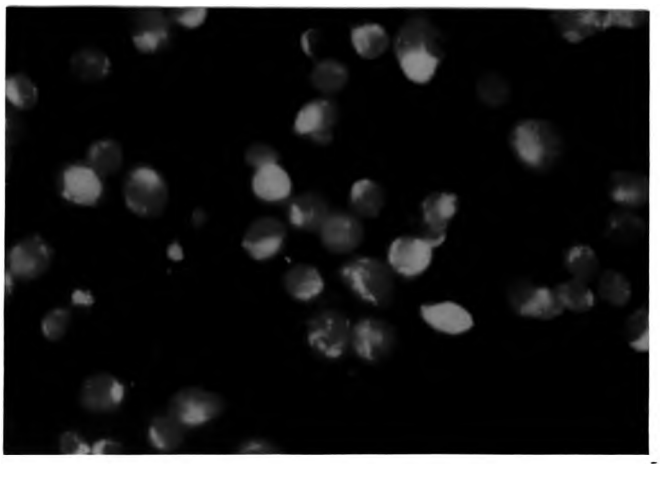

Figure 16 Immunofluorescence assay of dengue patient 's serum with strongly reactive. The C6/36 cell reacted with dengue 's serum, and counter stained with FITC conjugated to anti-human IgG.

Figure 17 Immunofluorescence assay of dengue patient 's serum with weak reactivity. The C6/36 cell reacted with dengue 's serum, and counter stained with FITC conjugated to anti-human IgG.

5. Detection of mosquito cell antibodies against mosquito's salivary gland by indirect immunofluorescence assay.

Salivary gland of *Aedes aegypti* mosquito (Figure 17) were dissected and fixed on glass slide. Anti-mosquito cell antibodies from immuned rabbit's serum reacted with mosquito cell that gave positive signal (Figure 18) when compared to control negative serum of preimmune rabbit. The result indicated that C6/36 cell antigens also presence in salivary gland.

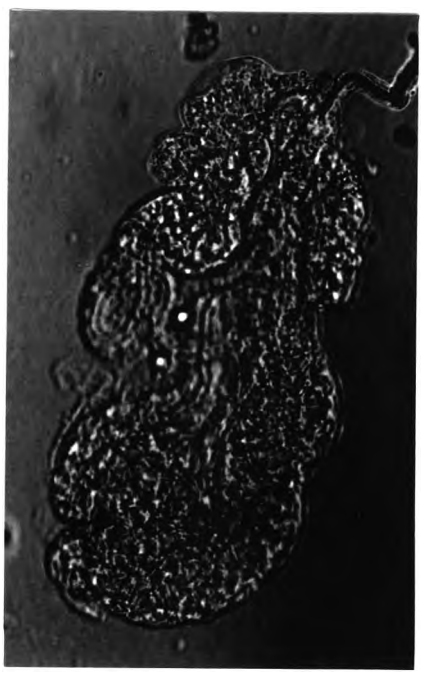

Figure 18 Microscopic photograph of salivary gland of female *Aedes aegypti* mosquitoes. Salivary gland of female *Aedes aegypti* mosquitoes were dissected for indirect immunofluorescence assay.

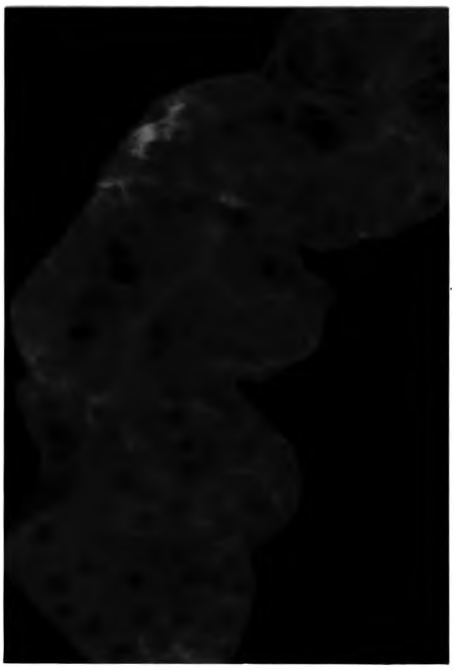

Figure 19 Immunofluorescence staining of salivary gland of female *Aedes aegypti* mosquito with rabbit anti-mosquito cell antibodies. Salivary gland of female *Aedes aegypti* mosquito were dissected in phosphate buffer saline and fixed with cold acetone on glass slide. The salivary gland were incubated with immuned rabbit serum (1:20). After washing with PBS, they were incubated with goat anti-rabbit IgG conjugated with FITC (1:200) and washing with PBS before examined by fluorescence microscopy.

6. Detection of mosquito cell antibodies against mosquito's protein by enzyme linked immunosorbent assay (ELISA)

The anti-mosquito cell antibodies can be semi-quantitated by ELISA method. The mosquito cells extracted antigens were immobilized on microtiter plate. The optimal concentrations of the antigen, and antibody were assessed by checker board titrations. The optical density are directly correlated with antibodies level. The results of ELISA are showed in Figure 20 and 21.

6.1 Anti-mosquito cell antibodies from C6/36 cell-immunized experimental rabbits.

Before start the immunization program, rabbit were collected for preimmune blood as negative control. After finished the immunization program, immune sera were tested for anti-mosquito cell antibodies by ELISA. The result show the increasing of antibody level in immune sera when compared with antibodies level from primary or secondary immunization.

6.2 Anti-mosquito cell antibodies from human serum.

Guard' serum and dengue patient' serum were determined for anti-mosquito cell antibodies by ELISA. Figure 21 show significantly higher anti-mosquito cell antibodies in guard's serum compared to dengue patient's serum, $p<0.05$; student's t test. Most of dengue patient's sera showed lower OD. value in ELISA, having a mean$\pm$SD antibodies of 0.132 ± 0.032 by using C6/36 cell extract as antigen and 0.269 ± 0.089 by using *Aedes aegypti* whole body extract as immobilized antigen. The antibodies in guard's sera show significantly higher antibodies titer, with a mean$\pm$SD antibodies of 0.209 ± 0.089 by using C6/36 cell extract as antigen and 0.338 ± 0.1 by using *Aedes aegypti* whole body extract as immobilized antigen. Moreover, there are correlation between the three assay, the higher OD. value in anti-mosquito cell

antibodies by ELISA, the stronger fluorescence staining, and the intense staining results of Western blot analysis. While, almost dengue patient's serum show lower OD. value in ELISA, weak fluorescence staining and lower number of antibodies detection in Western blot analysis.

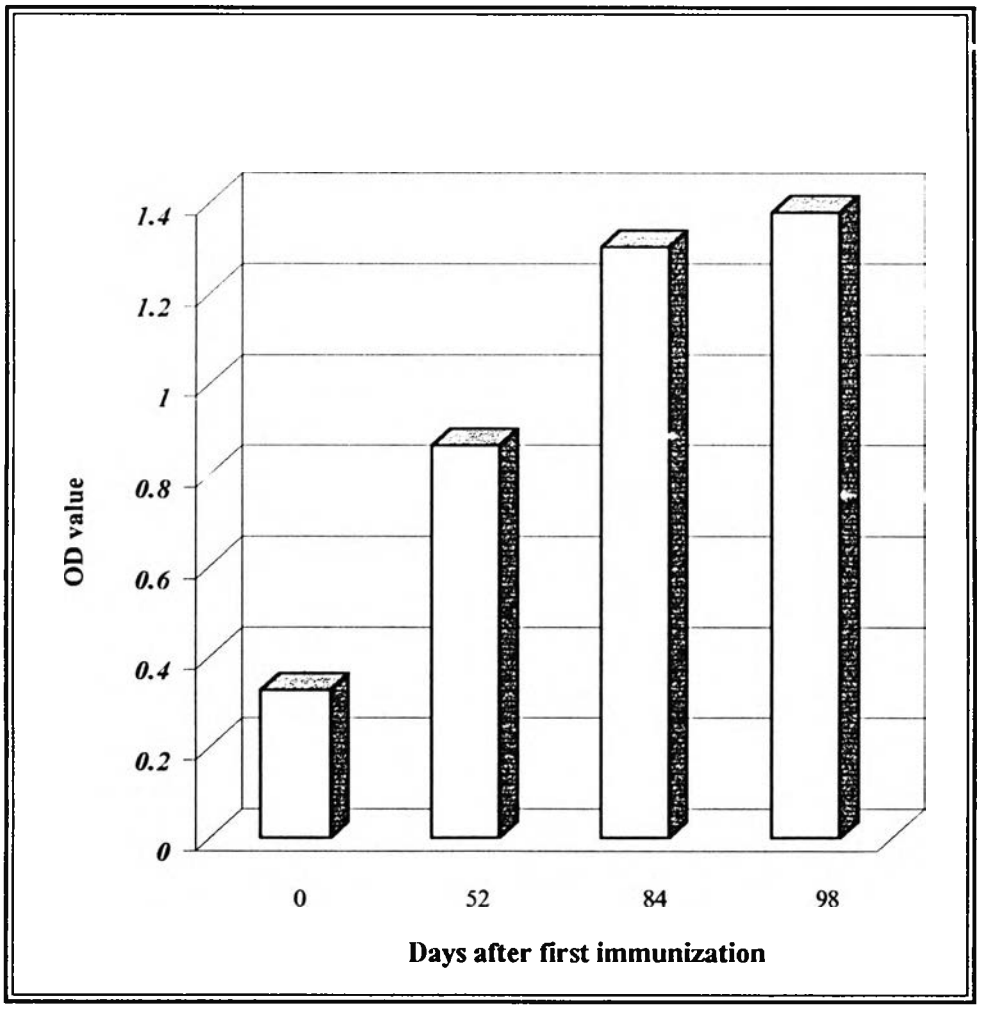

Figure 20 Reactivities of rabbit anti-mosquito cell antibodies to mosquito cell in ELISA. The OD. value of ELISA in anti-mosquito cell antibodies detection from rabbit's serum immunized with C6/36 cell, 0, 52, 84, and 98 days after first immunization.

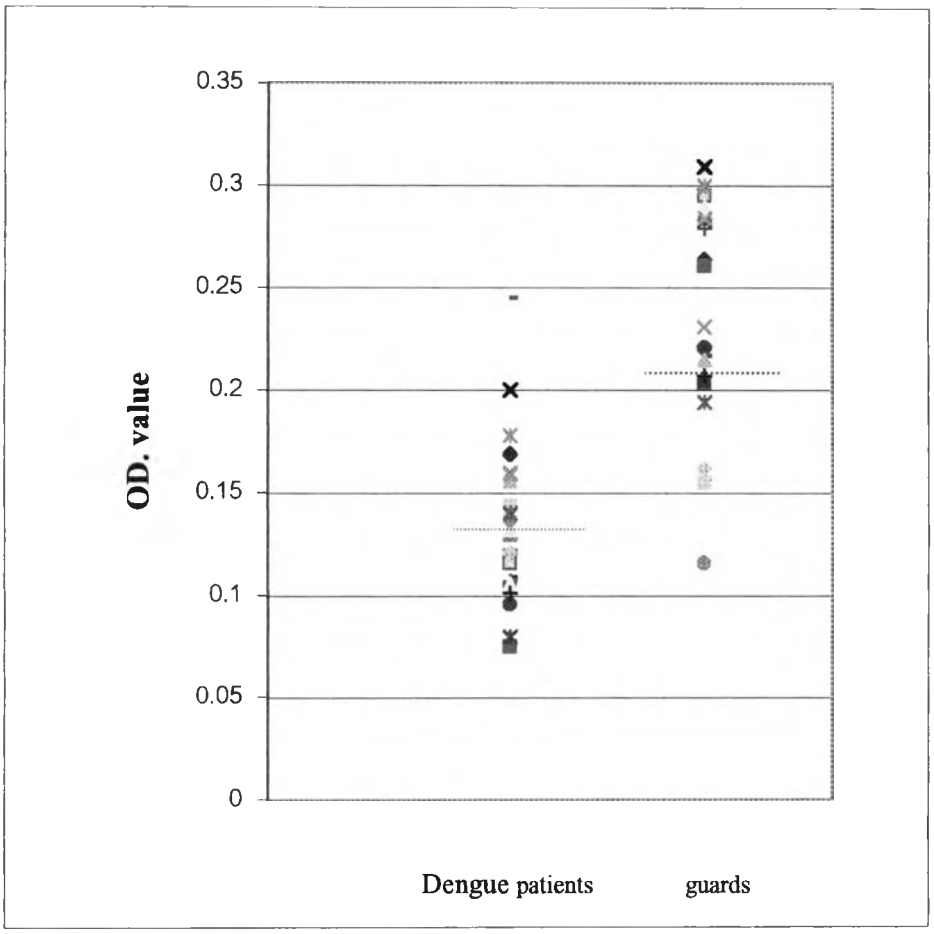

Figure 21 Reactivities of human anti-mosquito cell antibodies to mosquito cell in ELISA. The OD value of ELISA in detection of anti-mosquito cell antibodies against C6/36 cell extracts from guard' s serum and serum of patients with dengue infection.

7. Detection and typing of dengue viruses by RT-PCR

RT-PCR assay was developed to reversed transcribed dengue viral RNA and amplify four differently sized type-specific products. This protocol is an adaptation of RT-PCR assay described by Lanciotti et al. (115). Seven oligonucleotide primers are included in this assay: in the RT-step and first PCR amplification, one primer that targets a region of the capsid gene conserved in all four dengue virus serotypes and 3' another one of conserved primer target on region of the membrane glycoprotein precursor. In the second PCR amplification, five primers are included in the PCR reaction: one 5' primer that targets a region of the capsid gene conserved in all four dengue virus serotype and four 3' primers, each of which is complementary to sequence unique to each serotype. These primers are positioned such that a differently sized product is generated from each type, as show in Figure 23, lane 1 to 4 (dengue-1, 467 bp; dengue-2, 204 bp; dengue-3, 275 bp; dengue-4, 377 bp). Several modifications were made to the original protocol. The dengue-2 specific primer was redesigned for easy classification of different serotype, in addition, DEN-G conserved primer was designed for more complete nested-PCR than the original protocol that was semi-nested PCR assay. The concentration of the different primers were adjusted to optimized the amplification of all four products. Patient's serum that positive by PCR were used for detection of anti-mosquito cell antibodies in subsequent experiment.

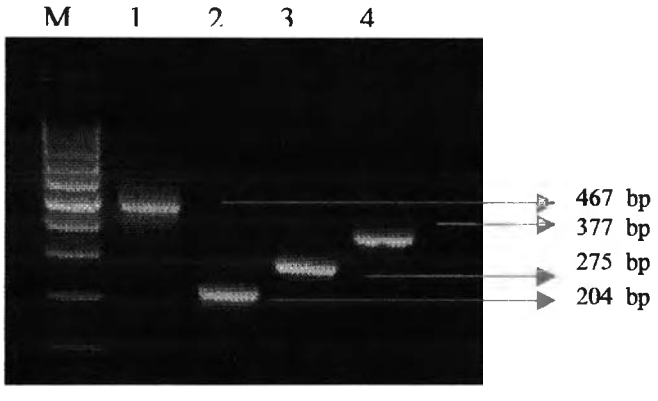

Figure 22 Agarose gel electrophoresis of dengue PCR products from different serotypes. Detection and typing of dengue virus by RT-PCR assay, the differently size of PCR product from each dengue serotype was analyzed by 2% agarose gel electrophoresis, lane M, 100-bp ladder; lanes 1 to 4 (dengue-1, 467 bp; dengue-2, 204 bp; dengue-3, 275 bp; dengue-4, 377 bp).

7.1 The sensitivity of dengue PCR

The sensitivity of this assay was determined by testing serial dilutions of stock recombinant plasmid contain dengue serotype 2 New Guinea C strain sequence from 5' UTR to NS1. The PCR assay was able to detect 150 molecules of recombinant plasmid. The PCR results of the sensitivity assay were shown in Figure 24. All assays had reproducibility scores of 10 for 10 for 150 molecules of recombinant plasmid but scores decreased for smaller numbers of molecule.

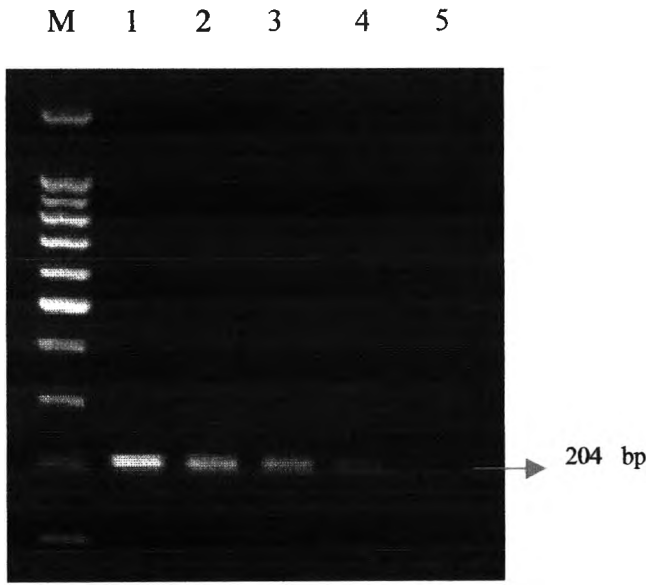

Figure 23 The sensitivity of dengue PCR for serial dilution of recombinant plasmid contain dengue serotype 2 New Guinea C strain. The recombinant plasmid were serially diluted and subjected to nested PCR. The PCR product were electrophoresed on 2% agarose gel and detected under UV light. M, DNA ladder; lanes1-5, 1200, 600, 300, 150, 75 molecules of recombinant plasmid.

7.2 The specificity of dengue PCR

The recombinant plasmid of dengue type 2 New Guinea C strain was used for evaluated specificity and sensitivity of RT-PCR assay. The correct size of PCR product was observed and analyzed by sequencing. For other dengue serotypes, the RNA, which was isolated from each patients diagnosed as dengue infection by clinical and laboratory data, was subjected to the RT-PCR assay. The correct size of first PCR product (511 bp) was obtained in some cases depended on the high titer of virus in the circulation. Each PCR product was correctly typed when assayed by second round of amplification with one concensus primer, DENG and type specific primers, DEN-1, DEN-2, DEN-3 and DEN-4. The dengue primers were followed Robert S. Lancoitti et al.(115). With some modification. DENG and DEN-2 primers were designed from available published sequences with the aid of a primer3 program (www.genome.wi.mit.edu/). The standard nucleotide Blast (blastn), were used to selected the most specific primer for each serotype and for ability to distinguish serotype by the size of DNA fragments. The primers have been blast with other related flavivirus and found that the primers are dengue specific. The specificity was also verified by performing the nested PCR from serum of patient without dengue infection; HIV and hepatitis infection and normal human serum. In addition, DNA sequencing was performed on product of each serotype and compare with reference sequences (dengue viruses serotype1, NC_001477; serotype2, NC_001474; serotype3, NC_001475; serotype 4, NC_ 002640) from published database by Blast program