รายงานวิจัยฉบับสมบูรณ์

การแสดงออกของยีน Antisense Den2-prm RNA-EGFP ในยุงลายข้ามพันธุ์

โดย

รศ. นพ. เผด็จ สิริยะเสถียร

ตุลาคม 2553

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ภาควิชาปรสิตวิทยา
คณะแพทยศาสตร์
จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณเจ้าหน้าที่ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
Chula Reserch Center
อาจารย์ นายแพทย์ ปรีดา มาลาสิทธิ์ คณะแพทยศาสตร์ ศิริราชพยาบาล ในการอนุเคราะห์ PrM gene ของไวรัสไข้เลือดออกชนิดที่ 2
ศาสตราจารย์ นายแพทย์ ยง ภู่วรวรรณ ศูนย์เชี่ยวชาญเฉพาะทางด้านไวรัสวิทยาคลินิค คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

งานวิจัยชิ้นนี้ได้รับการสนับสนุนการสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

The DEN-2 prM RNA-3XP3-EGFP gene cassette was constructed into the *piggy Bac* transposable element vector under the control of the vitellogenin gene promoter (Vg). The *piggy Bac* transposable element-helper based was introduced into *Aedes aegypti* embryos by microinjection. 400 embryos were injected, 42 were surviving to be adults, and the survival rate was 10.5%. 7 mosquitoes were exhibited for green fluorescence protein (GFP), two lines of transgenic *Ae. aegypti* were established and the transformation efficiency was 4.76%. The insertion of the EGFP gene and piggy Bac were detected in the genome of the transgenic lines (G_1-G_3) by PCR and inverse PCR respectively. The results demonstrated that the transgenes were transferred into the next generation of the transgenic *Ae. aegypti* mosquito through normal Medilian inheritance. We report the possibility to create transgenic *Ae. aegypti* mosquitoes that are unable to transmit dengue viruses.

บทคัดย่อ

ชุดยีน DEN-2 prM RNA-3XP3-EGFP ได้ถูกแทรกอยู่ในพาหะนำยีน *piggy Bac* ภายใต้การควบคุมของยีน vitellogenin พาหะนำยีนและพาหะซึ่งทำหน้าที่ช่วยในการส่งถ่ายยีนได้ฉีดเข้าไปในตัวอ่อนยุงลายบ้าน (*Aedes aegypti*) โดยวิธีการฉีดด้วยเครื่อง microinjection จากตัวอ่อนจำนวน 400 ตัวที่ถูกฉีด ปรากฏว่ามี 42 ตัวที่รอดเป็นยุงลายบ้าน คิดเป็นอัตราการรอดชีวิตเท่ากับ 10.5% ในจำนวนนี้มี 7 ตัวที่พบว่าเรืองแสงสีเขียวของแมงกระพรุน (GFP) ซึ่งพบว่าสามารถส่งถ่ายยีนนี้ต่อไปได้ใน 2 ตัว คิดเป็นอัตราการส่งถ่ายยีนได้สำเร็จเท่ากับ 4.76% ยีนเรืองแสงสีเขียวของแมงกระพรุนและพาหะนำยีนสามารถตรวจสอบได้ในยุงรุ่น 1 ถึงรุ่นที่ 3 ด้วยวิธี PCR และ inverse PCR ตามลำดับ ผลการทดลองแสดงให้เห็นว่ายีนแปลกปลอมถูกส่งถ่ายไปในยุงลายบ้านรุ่นต่อๆไปตามกฏการถ่ายทอดของเมนเดล คณะผู้วิจัยได้รายงานความเป็นไปได้ในการสร้างยุงลายบ้านดัดแปลงพันธุกรรมซึ่งจะไม่สามารถถ่ายทอดเชื้อไวรัสไข้เลือดออก

Introduction

Mosquito borne diseases are still a major cause of morbidity and mortality in many countries. Human diseases transmitted by mosquitoes include malaria, filariasis, yellow fever, Japanese encephalitis and dengue fever (DF). The presence of multi-drug resistant strains of pathogens and the lack of availability of effective drugs for viral diseases have led to difficulty in controlling these diseases. New vaccine development also faces the problem of antigenic variation of the pathogens. Chemical control used to be the primary strategy for controlling mosquito borne diseases, but concerns about the impact of such compounds on human health and the environment together with the development of insecticide resistance in mosquitoes has limited the usefulness of this approach (Beerntsen *et al.,* 2000). Through advances in the knowledge of mosquito-pathogen relationships and the molecular biology of the mosquito, it may be possible to produce mosquito strains that are unable to transmit various parasites or viruses (Collins and James, 1996). There are a number of molecules when expressed or introduced into mosquitoes that are able to block the transmission of pathogens. For example, monoclonal antibodies directed against a circumsporozoite protein of the avian parasite, *Plasmodium gallinaceum*, blocks sporozoites from entering the salivary glands of the mosquito, *Aedes aegypti,* when injected into the hemolymph (Warburg *et al.,* 1992). Anti-sense RNA fragments directed to the premembrane (prM) coding region of the Dengue type 2 virus inhibit the replication of the virus within *Ae. aegypti* and thus prevent its transmission (Olson *et al.,* 1996). In similar experiments, the transmission of a West African yellow fever virus strain was blocked with antisense RNA molecules derived from the prM or polymerase coding regions of the virus (Higgs *et al.,* 1998). Intrathoracic inoculation of a double subgenomic Sindbis (dsSIN) virus TE/3'2J/anti-luc (Anti-luc) into transformed *Aedes aegypti* line which expressed luciferase (LUC) from the mosquito *Apyrase* promoter reduced the expression of LUC in transformed mosquitoes by 90% (Johnson *et al.,* 1999). Expression of such similar molecules in genetically transformed mosquitoes could lead to the production of strains that can be used in the control of transmission of pathogens (Coates *et al.,* 1999).

Dengue is the most important aboviral infection of man, approximately 100 million cases annually and 2.5 billion people at risk. There are 4 dengue virus serotypes (DEN1-4) contain a positive sense RNA genome (11Kb) that encodes three structural (C-preM-E) and seven nonstructural proteins (NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5). The three structural genes are translated into proteins that form the shell of mature viral particles. Non-structural proteins are assumed to be important during different stages of virus replication and maturation in the host cell (Hayes and Halstead, 1996). The epidemiology of the 4 dengue virus serotypes is similar, all have worldwide distribution in the tropics and are maintained in most tropical urban centres in a mosquito-human-mosquito cycle. The principal mosquito vector is *Aedes aegypti* which was originally indigeous to North Africa, but has spread throughput other parts of the tropical world *via* ships or other commercial vessels. This species become highly adapted to living in intimate association with humans and is a highly efficient epidemic vector in urban setting. Dengue virus is transmitted from human to human by the bites of female mosquito, *Ae. aegypti* can transmit dengue either immediately by a change of host or after an incubation period of 8-10 days during which time the virus multiplies in the salivary gland of the mosquito. Dengue virus infection causes a spectrum of clinical illness, ranging from inapparent infection to mild non-specific viral syndrome to classical dengue fever to severe and fatal haemorrhagic disease (dengue haemorrhagic fever, DHF).

The lack of specific treatment for dengue virus and unavailability of effective vaccine against the virus, the interruption of pathogen transmission by mosquito control provides the only effective approach to the control of dengue infection. Chemical control used to be the primary strategy for controlling mosquito borne diseases, but concerns about the effect on the environment and human health together with the presence of insecticide in mosquitoes have limited the usefulness of this approach. A possible alternative approach to control dengue is to create mosquito strains that are unable to transmit the virus.

A recombinant double stranded Sinbis (dsSIN) virus containing 567bp antisense RNA fragments directed to the premembrane (prM) coding region of the Dengue type 2 virus (DEN-2 prM RNA) when introduced into mosquitoes could inhibit the replication of dengue type 2 virus within *Ae. aegypti* mosquitoes and thus prevent its transmission (Olson *et al.,* 1996). The C6/36 (*Ae. albopictus*) cells transfected with dsSIN virus containing antisense DEN-2 prM RNA were completely resistant to DEN-2 challenge (Gaines *et al.,* 1996).

With the microinjection technique now developed and the availability of a number of transposable element based vector-helper systems, it is now possible to routinely introduce exogenous DNA into *Aedes* mosquitoes as described previously. If the gene encoding for antisense DEN-2 prM RNA could be introduced and expressed in mosquitoes as a part of a transposable element based expression construct, it may be possible to block dengue replication in mosquitoes. Building on this technology, we would like to create transgenic *Aedes* mosquitoes that are unable to transmit dengue virus.

Objectives

-To construct a *Hermes* transposable element based vector containing the *Apy*-gene and a gene encoding for antisense DEN-2 prM RNA-EGFP cassette.

-To evaluate the stability of the *Apy*-antisense DEN-2 prM RNA-EGFP cassette when introduced into the mosquito by microinjection.

Materials and Methods

Mosquito rearing

Ae. aegypti mosquitoes were maintained in an insectary of the Department of Parasitology, Faculty of Medicine, Chulalongkorn University. Conditions were set at $28^{\circ}C \pm 1^{\circ}C$ at 80% ± 5% relative humidity under 12/12 hours light/dark photo-period. Adults were supplied with a damp cotton wool pad contained 10% sucrose solution as a carbohydrate source. For blood feeding, female mosquitoes were allowed to feed on anaesthetized mice for 30 minutes twice a week subsequently. Eggs were laid by inseminated females 3-4 days after the bloodmeal on moist filter papers. Four days after oviposition, the egg papers were allowed to dry in the insectary and sealed in plastic bag for storage. Egg were stimulated to hatch by immersion in a dilute solution of hay infusion, prepared by soaking dried grass in tap water for 24 hours at $28^{\circ}C$. Twenty four hours post-hatching, and daily thereafter, the hay infusion was replace with fresh tap water to which grind mouse food was added.

Plasmid Construction

Amplification of Antisense Den2-PrM RNA gene

The antisense Den2-PrM RNA gene was amplified from the clone of pDisplay expression vector (gift from Dr. Preeda Malasit). The clone PrM gene was originally derived from dengue 2 virus strain 16681 (Sriburi *et al.*). Forward and reverse primers were designed to amplify the antisense Den2-PrM RNA gene which starts from nt 439-936 of whole genome nucleotide sequences. Bam HI restriction site and ATG start codon were added to 5' end of the forward primer. The primer sequences were 5'actggatccatgttccatttaaccacacgtaac3'. Asc I restriction site and TAA stop codon were added to 5' end of the reverse primer, the primer sequences were 5'ttggcgcgcctaatgtcattgaaggagtgaca3'. The restriction sequences were underlined. The PCR reaction was composed of 30ng of Den2-PrM clone pDisplay DNA, 50pmol of each primer, 20µM of dNTP mix, 10X Mg^{++} free PCR buffer, 2mM $MgCl_2$, 0.5 unit of Taq polymerase and distilled water to a final volume of 25µl and was performed in the thermal cycler using the following programme: 1 cycle of 95°C for 5 minutes, 35 cycles of 95°C for 1 minute and 55°C for 30 seconds and 72°C for 1 minutes and 1 cycle of 72°C for 10 minutes. 20µl of each PCR reaction was electrophoresed through a 2%, which was then stained with Et-Br and visualized on a transilluminator. Figure 1 shows the 522bp PCR product, corresponding to the antisense Den2-PrM RNA encoding region.

Sequencing

PCR products were purified through a spin column using the protocol described by the manufacturer. Purified products were quantified by electrophoresis on a 1.2% agarose gel sequenced using automated sequencing ABI Model 310 (Applied Biosystems). The sequence data obtained by automated sequencing was subjected to a DNA databank search using BLAST (Basic Local Alognment Search Tool, Altschul et al, 1990).The sequence of the PCR product corresponded to the PrM of Den2 virus.

Insertion of PCR product into plasmid vector using TA cloning® Kit (Invitrogen)

PCR products generated by *Taq* polymerase have 3’ A-overhang. The linearized vector supplied in the kit () has a single 3’ T residue, which allows PCR inserts to ligate to the vector. The method was followed the manufacturers specifications. The cloning was carried out using 1µl (10ng) of fresh PCR product without any further purification. The ligated vectors were transformed into TOPO 10’F competent cells and plate out on to LB agar plates containing 50µg /ml ampicillin, X-Gal 24µg/ml and IPTG 20µg/ml. White colonies were selected and confirmed as recombinant clones using plasmid minipreps, restriction digestions (Fig) and PCR.

Construction of Expression Vectors (piggyBac-Vg-Antisense Den2-PrM RNA-3XP3-EGFP)

Microinjection of mosquito embryos

The microinjection technique has been developed to introduce exogenous DNA into insect embryos. The technique for microinjection of mosquito embryos was based on that used to introduce DNA into the developing embryos of *D. melanogaster* (Rubin and Sprading, 1982). The germline transformation experiments involving microinjection have been carried out with aedine mosquitoes (McGrane *et al*, 1988; Morris *et al*, 1989) and with *An. gambiae* (Miller *et al*, 1987). Microinjection has proved to be an effective means of delivering recombinant DNA into *Ae. aegypti* embryos for analysis of embryo-specific putative promoter sequences (Morris *et al*,1995).

To facilitate germline transformation, the exogenous DNA must be introduced into developing embryo prior to pole cell formation. The introduced plasmid will be incorporated into the developing pole cells and integrate into the chromosomal DNA. The integrated DNA sequences may then be expressed throughout the somatic tissue of subsequent generations.

The microinjection technique performed in this experiment followed the method described by Morris *et al* (1997). Briefly, eggs were collected from female mosquitoes 3-4 days after a blood meal using moist filter papers as an oviposition site. 20-30 mid-dark grey embryos, which were approximately 90-120 minutes old were oriented with their posterior aligned. The oviposition paper was dried by blotting with dry filter paper and the embryos were transferred onto a coverslip with double-sided sticky tape attached their posterior poles close to the edge of the coverslip. The embryos were then left to desiccate for 15-60 seconds, with the state of desiccation observed under a dissecting microscope. To prevent further desiccation, embryos were covered with water-saturated halocarbon oil (Sigma). Embryos were injected with 0.2-0.5 nl of DNA solution into their posterior poles using microinjection apparatus (Femto Jet, Eppendrof). Microinjections were performed within 2 hours of oviposition. The injected embryos were incubated at 28°C and 80% relative humidity for 30 minutes and then transferred onto moist filter paper and returned to the insectary at 28°C and 80% humidity for 3-4 days before hatching.

Result

Amplification of the prM gene

The antisense Den 2 prM RNA gene was amplified using forward primer contains Bam HI restriction site and ATG start codon to the 5' end. The primer sequences were 5'actggatccatgttccatttaaccacacgtaac3'. Asc I restriction site and TAA stop codon were added to 5' end of the reverse primer, the primer sequences were 5'ttggcgcgcctaatgtcattgaaggagtgaca3'. The 522 bps of antisense Den 2 prM RNA gene was amplified (Fig1). The PCR products were sequenced and compared with data in Gen bank and confirmed to be the antisense Den 2 prM RNA gene.

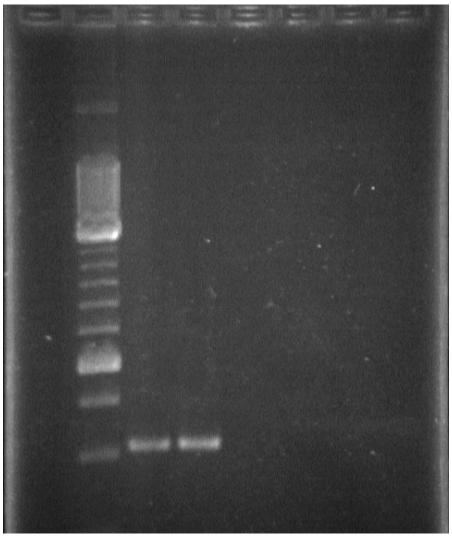

Figure 1. 2% agarose gel demonstrates the amplified antisense Den 2 prM RNA gene.

Construction of Expression Vectors (piggyBac-Vg-Antisense Den2-PrM RNA-3XP3-EGFP)

The antisense Den 2 prM RNA gene was insert into the vector piggyBac-Vg-3XP3-EGFP to construct Expression Vectors (piggyBac-Vg-Antisense Den2-PrM RNA-3XP3-EGFP). The inserted antisense Den 2 prM RNA gene in the expression vector was presented when digested the expression vector with *Bam* HI and *Asc* I (Fig 2).

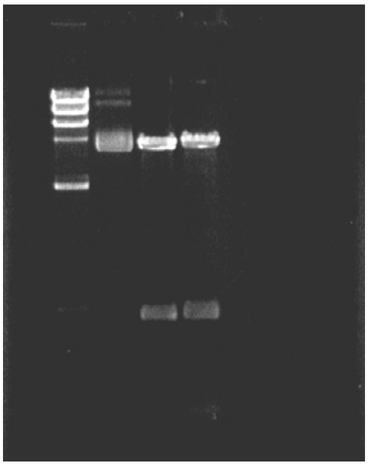

Figure 2. 2% agarose gel electrophoresis demonstrates the construced expression vectors (piggyBac-Vg-Antisense Den2-PrM RNA-3XP3-EGFP) digested with *Bam*HI and *Asc* I restriction enzymes.

Microinjection of *Ae. aegypti* embryos

The microinjection technique performed in this experiment followed the method described by Morris *et al* (1997) and Catteruccia *et al* (2000). In essence, eggs were collected from female mosquitoes 3-4 days after a blood meal using moist filter papers as an oviposition site. 20-30 mid-dark grey embryos (Fig. 3), which were approximately 90-120 minutes old were oriented with their posterior poles aligned (Fig. 4). The oviposition paper was dried by blotting with dry filter paper and the embryos were transferred onto a coverslip with double-sides sticky tape attached, with their posterior poles close to the edge of the coverslip. The embryos were then left to desiccate for 15-60 seconds, with the state of desiccation observed under a dissecting microscope. To prevent further desiccation, embryos were covered with water-saturated halocarbon oil (Sigma). Embryos were injected with 0.2-0.5 nl of DNA solution into their posterior poles (Fig. 4) using microinjection apparatus (FemtoJet, Eppendrof). Microinjection were performed within 2 hours of oviposition. We injected 2 mosquito species *Aedes aegypti* and *Ae. aegypti.* The embryos were injected with a mixture of the pBac [3xP3- EGFP afm, Vg-Def A] construct and the phsp-pBac helper plasmid at a final concentration of 0.3 and 0.25 μg/ml respectively in 5mM KCl and 0.1 mM NaH_2PO_4(pH 6.8). Injected embryos were incubated at 28°C and 80% relative humidity for 30 minutes and then transferred onto moist filter paper and returned to the insectary. 24 hours after injection, injected embryos were incubated at 37 °C for 60 minutes to facilitate the heat shock promoter. The injected embryos were then transferred into the insectary for 72 hours and stimulated to hatch in fresh water.

Percentage survival of injected embryos

The experiment was performed with two mosquito species *Aedes aegypti* and *Ae. aegypti.*The survival rate was calculated by

$$\frac{\text{Total number of adults surviving microinjection}}{\text{Total number of injected embryos}} \times 100$$

From 400 injected *Ae aegypti* embryos, 42 were hatch. The survival rate was 10.5 %. These 42 larvae were maintained in the insectary (Table 1).

Next step of experiment, we will detect the EGFP in these injected larvae (G_0) and mate them with wile type mosquito when they develop into adults.

Table 2 Survival rate from both experiments

Mosquito	No. of embryo injected	No. of embryo hatch	Servival Rate
Ae aegypti	400	42	10.5%

Detection of transformants and establishment of transgenic lines

Select transform mosquitoes (F_0) using fluorescence microscope

The injected larvae (G_0) were examined for transformation using fluorescence microscope. We observed green color in some injected larvae (Fig. 5). Then G_0 adults were set up to mate with wild type virgin females or males. The subsequent generation (G_1) were examined for the expression of green fluorescence protein under a fluorescence microscope. Three females of G_1 progeny showed transient GFP expression in their eyes during adult stages (Fig. 6, 7).

Figure 3. *Aedes aegypti* eggs

Figure 4. Microinjection, embryos were injected with 0.2-0.5 nl of DNA solution into their posterior poles

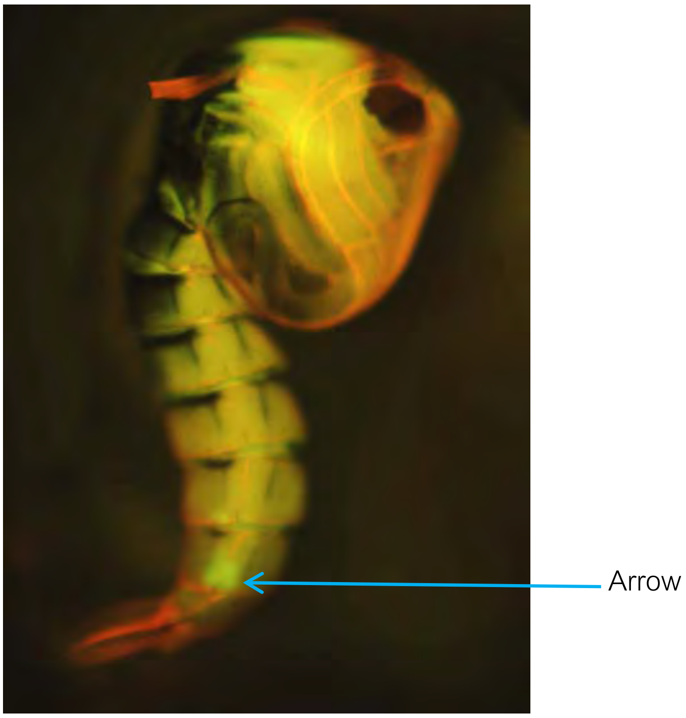

Figure 5. Transformed *Aedes aegypti* larva (G_o), suspected green fluorescence protein (GFP) was found at the abdominal end

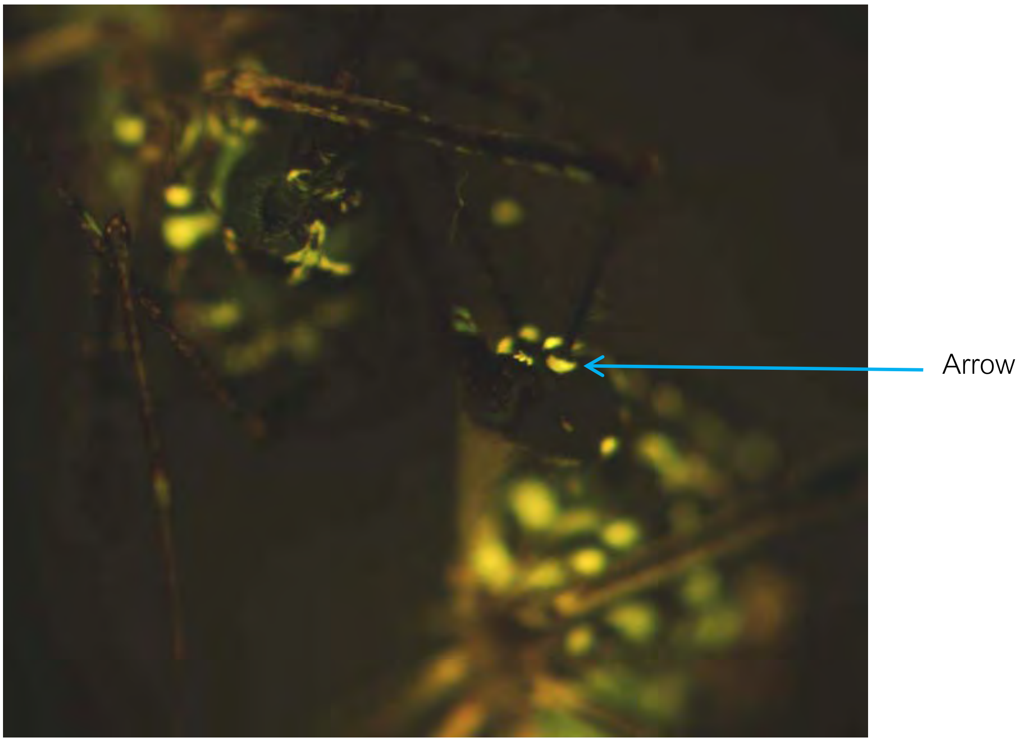

Figure 6. Transformed *Aedes aegypti* mosquito (G_1, right), suspected GFP was found at the eyes (arrow) compare to wild type mosquito (left)

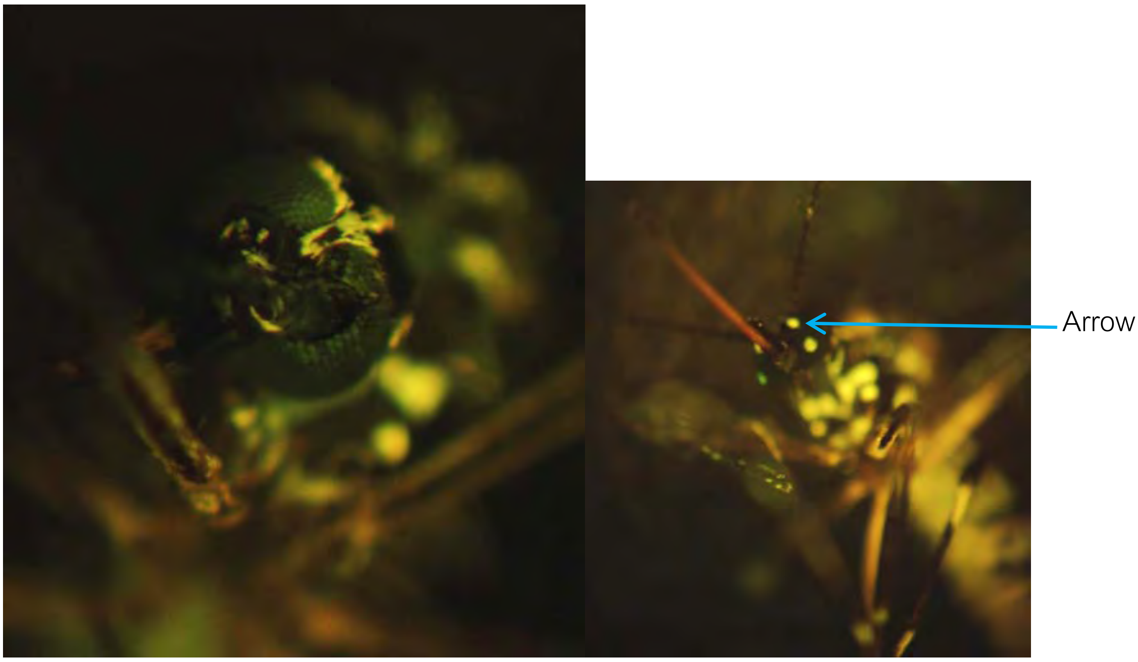

Figure 7. Transformed *Aedes aegypti* mosquito (G_1), suspected GFP was found at the eyes (arrow)

Detection of the EGFP gene in transformed mosquitoes using PCR and DNA sequencing

PCR were performed in order to detect the transgenes in the transformed mosquitoes. Genomic DNA of the transformed mosquitoes were prepared using Blood and Tissue Kit (QIAGEN).

PCR was used to amplify a region of the EGFP gene (Fig. 8). Primer sequences were designed to anneal specifically to the EGFP gene. Primers were purchased from Invitrogen, the primer sequenced are listed in table 2.

Each PCR reaction mixture containing 2.5µl of 10X buffer, 2mM each dNTP, 10pmol each primer (2F and 4R), 2.5mM $MgCl_2$, 3µl DNA extract, 1U of *Taq* polymerase (Invitrogen) and ddH_2O to a final volume of 25µl. The reaction was performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystem, Foster city, CA) by two-step PCR using the following conditions; initial denaturation step of 95°C for 3 min followed by 5 cycles of 95°C for 0.45min, 56°C for 0.45 min and 72°C for 1.5 min, followed by 25 cycles of 95°C for 0.45 min, 60°C for 0.45 min and 72°C for 1.5 min and followed by 72°C extension for 10 min. PCR products were detected on a 1% agarose gel (Fig.8-9).

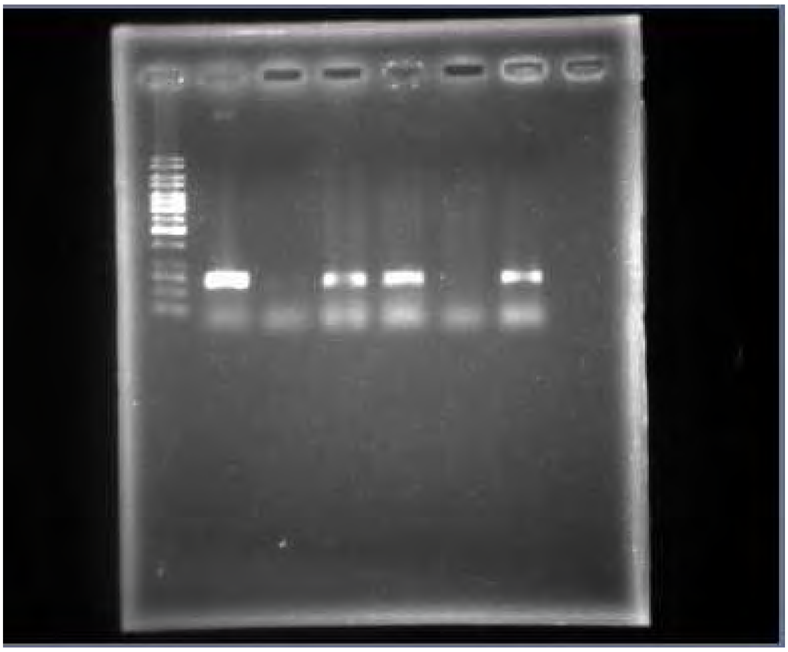

Figure 8. PCR for EGFP gene with genomic DNA extracted from G_1 transgenic mosquitoes; M=100bp marker; lane 1= positive control; lane 2= negative control; lane 3-6= genomic of G_1 mosquitoes

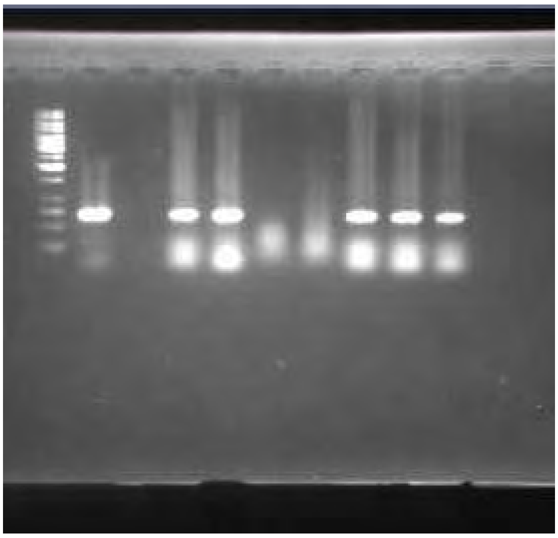

Figure 9. PCR for EGFP gene with genomic DNA extracted from G_1 transgenic mosquitoes; M=100bp marker; lane 1= positive control; lane 2= negative control; lane 3-9= genomic of G_1 mosquitoes

DNA Cloning and Sequencing.

The PCR products were purified from agarose gel using a Perfect® Gel Cleanup (Eppendorf; Germany) following the manufacturer's instructions. 50µl of PCR reaction was electrophoresed through a 1% agarose gel and stained with ethidium bromide. The desired PCR product was excised from the gel and placed in a 1.5ml microcentrifuge tube. The gel slice was weight, 3 volumes of Binding Buffer was added into the gel slice, the mixture was incubated at 50°C and vortexing every 3 min until the gel slice was completely dissolved. After the gel slice has completely dissolved, 1 volume of isopropanol was added into the solution mixture and mixed by inversion. The solution mixture was loaded into a Spin Column and centrifuged at 10,000g for 1 min then discarded the flow through. 700µl of Wash Buffer was add into the Spin Column and at 10,000g for 1 min then discarded the flow through. The column was spin again at 10,000g for 1 min to remove any residual Wash Buffer. The column was placed into a new collection tube, 25µl of Elution Buffer was added, the column was spin again at 10,000g for 1 min. Discard the column, DNA solution was subjected to a Perkin Elmer 310 Sequencer.

In order to obtain complete sequence, the PCR product was inserted into pGEM-T easy cloning vector (Promega) which contains T7 RNA polymerase promoter sequences. A sample of each fly species was randomly selected for sequencing.

The sequence data was 98% identical to EGFP sequences of the GenBank.

Inverse PCR

In order to determine site of integration of the piggyBac in the genome of the transgenic *Aedes* mosquitoes, inverse PCR were performed. The genomic DNA was extracted and digested with *Dra* I restriction enzyme. The digested DNA was circularized by ligation with T_4 ligase overnight at 4°C, ligated DNA was used as template for inverse PCR. Each PCR reaction mixture containing 2.5µl of 10X buffer, 2mM each dNTP, 10pmol each primer (LHF and LHR for left hand insertion or RHF and RHR for right hand insertion), 2.5mM $MgCl_2$, 2µl of ligated DNA , 1U of *Taq* polymerase (Invitrogen) and ddH_2O to a final volume of 25µl. The reaction was performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystem, Foster city, CA) by PCR using the following conditions (for left hand insertion); initial denaturation step of 96°C for 2 min followed by

35 cycles of 96°C for 15 sec, 56°C for 30 sec and 72°C for 2 min, and followed by 72°C extension for 4 min. PCR products were detected on a 2% agarose gel (Fig.10). For right hand insertion the PCR were carry out with the same conditions except that annealing was performed at 65°C (Fig. 11).The PCR products were cloned in to pGEM-T and sequenced.

Primer	Sequence
LHF	5'-ATCAGTGACACTTACCGCATTGACA-3'
LHR	5'-TGACGAGCTTGTTGGTGAGGATTCT-3'
RHF	5'-TACGCATGATTATCTTTAACGTA-3'
RHR	5'-GGGGTCCGTCAAAACAAAACATC-3'

Table primer sequences for inverse PCR

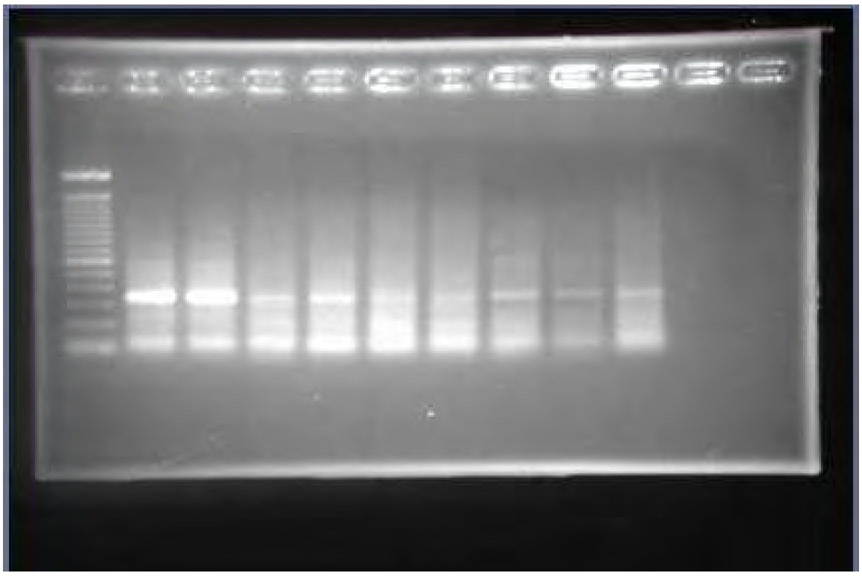

Figure 10. Inverse PCR for left hand insertion: lane M: 100 bp marker; lane 1 and 2: genomic DNA of G_1 transgenic mosquitoes; lane 3-6 genomic DNA of G_2 transgenic mosquitoes; lane 7: genomic DNA of G_3 transgenic mosquitoes; lane 8-9: genomic DNA of G_4 transgenic mosquitoes.

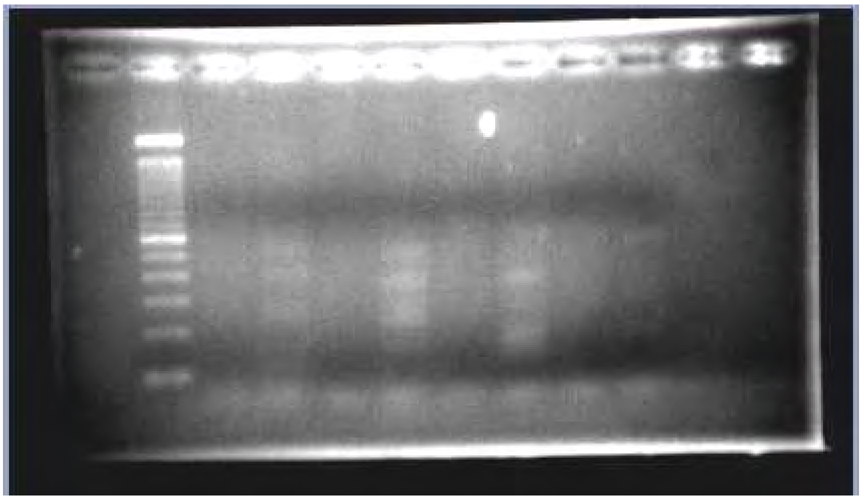

Figure 11. Inverse PCR for right hand insertion: lane M: 100 bp marker; lane 1 and 2: genomic DNA of G_1 transgenic mosquitoes; lane 3-6 genomic DNA of G_2 transgenic mosquitoes; lane 7: genomic DNA of G_3 transgenic mosquitoes; lane 8: genomic DNA of G_4 transgenic mosquitoes.

Discussion

400 *Ae. aegypti* embryos were injected, 42 were survive to be adults. Surviving rate of injected embryos was 10.5%. Among the 42 mosquitoes 7 were positive for GFP under the fluorescence microscope. All survived mosquitoes were set up to mate with wild type mosquitoes and the G_1 progeny were examined for GFP. Only two line from 7 was showed to be positive for GFP, transformation efficiency of this experiment was 4.7%. We used this line for detection of the EGFP gene and insertion of piggyBac in the genome. The EGFP gene was detected in G_1-G_4, this confirmed that the transgene was transferred to the next generation through normal Medilian inheritance.

The presence of the inverted terminal repeats of piggyBac was demonstrated by inverse PCR and the characteristic TTAA sequence at the borders of all the analyzed inserts from sequences of the inverse PCR products, confirmed that transformation resulted from precise transposition events. These results are similar to the previous reports in mosquito species such as *Ae. aegypti* and *Culex quinquefasciatus*.

Conclusions and future prospects

The aim of the work described in this experiment was to investigate the concept that exogenous gene could be inserted into the malaria vector *An. dirus*. If the exogenous gene could be expressed in the transgenic mosquitoes, it could inhibit development of malaria parasites in the mosquitoes. The findings of this work may be summarized as follows:

-The survival rate of microinjection of the *Ae. aegypti* was 10.5% and the transformation efficiency of *Ae. aegypti* with piggyBac transposable element based system was 4.76%.

- The experiments showed that piggyBac transposable element –helper based system was capable of mediating stable germline transformation in *Ae. aegypti*.
- GFP was efficient to detect transformants.
- piggyBac was inserted into the *Ae. aegypti* genome specifically at the TTAA site.

Future prospects

Once transgenic mosquitoes have been create, it is necessary to consider the problems likely to be faced in applying the technology in experimental and natural populations. There are several issues that must be addressed.

Fitness cost

Fitness cost is defined as the relative success with which a genotype transmits its genes to the next generation (Marrelli et al., 2006). There are two major components of the fitness cost, survival and reproduction which can be evaluated by analyzing several parameters, such as fecundity, fertility, larval biomass productivity, developmental rate, adult emergence, male ratio and mating competitiveness. Fitness of genetically modified mosquitoes can be reduced from the negative effect of transgene products such as a fluorescent marker and an anti-pathogen protein or from the insertional mutagenesis after a transposition event (Catteruccia et al., 2003; Marrelli et al., 2006). Catteruccia et al., (2003) demonstrated that the reductions of fitness of the transgenic *An. stephensi* mosquitoes are caused by the expression of an exogenous gene and the mutations from insertion of the transgene. The fitness cost of transgenic *Ae. aegypti* mosquitoes were examined by Irvin et al., 2004, the results showed that fitness of transgenic mosquitoes were reduced significantly compared to not-transgenic mosquitoes. The fitness of the genetically modified mosquitoes should therefore be evaluated under laboratory conditions for planning release strategies.

Stability

Stability of the integrated gene is crucial issue for application, in either mass-rearing factory of field. The problems posed by these two environments differ greatly, since in the former there will be control over the genotype while in the field it will not be the case except for the particular case of field release of sterile transformants. In the field, the release of fertile transformants may lead to potential interactions between the transgene and other genes or transposable elements that will affect stability (Asburner et al., 1998). The stability of transformants should therefore be tested under experimental conditions before any release can be completed.

Safety

There are other concerns that will dictate the use of transgenes in mass-rearing factory conditions. Transgenes that might be considered safe under laboratory conditions may face severe regulatory tests before being cleared for use in the factory or field. The problem of containment needs to be addressed. Transgenes requiring selection with a toxic agent or conferring pesticide resistance should not be used.

Field Objective

The objectives of field release will differ among target organisms but include eradication, population suppression and population replacement. However, there are some general principles. Firstly, the need for markers for field-release organisms, enabling the unambiguous distinction between transformants and the endemic population. Secondly, the consideration of the consequences of a successful field release of another transgenic strain of the same species. Some designs may be essentially “one-shot”, for example, any release of a mobile transgene that leads to the evolution of a transposition suppression may prevent any subsequent release of a transgene carried by the same vector (Ashburner et al., 1998).

For target species where population replacement is an objective there is an urgent need for research on how this may be achieved. One possible mechanism is meiotic drive. This describes any event occurring during meiosis, by which one particular chromosome is recovered preferentially over its homologue. Such a mechanism has been demonstrated in *Ae. aegypti* when linkage to the M^D locus was used to drive the marker gene, *re* (red eye) into cage population (Wood et al., 1977). Transposable elements, the possible basis of a gene transfer system, also have potential as drive mechanism. The efficiency of transposon-mediated drive mechanism is such that it is theoretically possible to spread even and unfavorable trait through a population, before natural selection can act on it, in spite of the sometime deleterious consequences of transposition itself (Ribiero and Kidwell, 1994). The rapid spread of a transposable elements through a natural population has already been demonstrated by the P element, which has invaded populations of *D. melanogaster* worldwide within the past fifty years (Anxolabehere et al., 1988), having invaded the species from the distantly related *D. willistoni*. Another advantage of transposable elements is that some have shown themselves capable of transferring across species boundaries (Houck et al., 1991). However, the impact of releasing fertile transgenic mosquitoes in the gene pool of the natural population needs to be fully assessed.

Risk evaluation and public acceptance

The release of any transgenic organism to the field will be governed by local and national regulatory agencies. Any proposal to release will generate concern among both the general public and pressure groups. Both the must be addressed earlier, rather than later, if field release is the intended end-point of technology development (Hoy, 2003).

Although stability of these transgenic mosquitoes in caged population could be made, but the fitness of the transgenic mosquitoes is diminished compare to wild type mosquitoes. Once the problem of the fitness of transgenic mosquitoes has been overcome, the safety and effect of releasing these transgenic mosquitoes into environment need to be fully assessed. Before contemplating release of transgenic mosquitoes containing active transposable elements, one must be aware of the possibility of horizontal transmission of the transgene to non-target species. Public acceptance of the release of transgenic mosquitoes must also be considered.

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ปัญหาและอุปสรรคในการวิจัย

ถึงแม้ว่ายุงดัดแปลงพันธุกรรมไปสู่ยุงรุ่นต่อไปได้แต่อัตราการรอดของยุงรุ่นต่อไปนั้นต่ำมาก และการคงอยู่ของยีนแปลกปลอมนั้นสั้นมาก ซึ่งสามารถคงอยู่ไม่เกิน 3 รุ่น ทำให้เกิดปัญหาไม่สามารถทดสอบได้ว่ายุงดัดแปลงพันธุกรรมนั้นจะต้านทานไวรัสไข้เลือดออกได้หรือไม่ ปัญหาดังกล่าวอาจเกิดจากยีนแปลกปลอมมีขนาดรวมใหญ่มากเกินไป ถึงแม้จะพยายามฉีดในยุงลายหลายครั้งผลก็ยังไม่ดีพอที่จะเขียนรายงานในวารสาร อย่างไรก็ตามผู้วิจัยได้ทำวิจัยในเรื่องที่เกี่ยวข้องกับยุงลายบ้านและยุงแม่ไก่และได้ acknowledge สกว. ไว้ด้วยดังเอกสารในภาคผนวก

Output จากงานวิจัย

ผลงานตีพิมพ์

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การนำผลงานวิจัยไปใช้ประโยชน์

งานวิจัยนี้ได้ไปนำเสนอในการประชุมวิชาการ ใช้ในการเรียนการสอนนิสิตทั้งแพทยศาสตรบัณฑิตและระดับบัณฑิตศึกษา และสร้างเป็นส่วนของวิทยานิพนธ์นิสิต ได้ผลิตบัณฑิตระดับปริญญาโท 2 คน และงานวิจัย Prevalence of dengue virus in *Aedes* mosquitoes during dry season by semi-nested reverse transcriptase-polymerase chain reaction (semi-nested RT-PCR) ของนิสิตแพทย์ปี 3 ได้ไปประกวดในงาน AMSA ได้รับรางวัลที่ 1 และเทคนิคการตรวจหาเชื้อไข้เลือดออกในยุงลายจากงานวิจัยนี้ได้นำไปใช้ในงานวิจัยอื่นๆต่อมา และนำไปใช้ตรวจเพื่อการเฝ้าระวังการระบาดของไข้เลือดออกของกระทรวงสาธารณสุข

ภาคผนวก

IDENTIFICATION OF BLOOD MEAL OF FIELD CAUGHT *AEDES AEGYPTI (L.)* BY MULTIPLEX PCR

Padet Siriyasatien[1], Theerakamol Pengsakul[2], Veerayuth Kittichai[3], Atchara Phumee[3], Sakchai Kaewsaitiam[1], Usavadee Thavara[2], Apiwat Tawatsin[2], Preecha Asavadachanukorn[4] and Mir S Mulla[5]

[1]Department of Parasitology, [3]Medical Science Program, Faculty of Medicine, Chulalongkorn University, Bangkok; [2]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi; [4]Department of Statistic, Faculty of Commerce and Accountancy, Chulalongkorn University, Bangkok, Thailand; [5]Department of Entomology, University of California, Riverside, California, USA

Abstract. Laboratory bred female *Aedes aegypti* (L.) was used to determine sensitivity of multiplex PCR for detecting human blood meal. Human blood DNA was detected in live fully fed mosquitoes until 3 days after blood feeding, and for 4 weeks when stored at -20°C. Among 890 field caught female mosquito samples examined for vertebrate DNA by multiplex PCR, results were positive for human, pig, dog, cow and mixture of 2 host DNA at 86.1, 3.4, 2.1, 1.0 and 3.6%, respectively, while 3.9% of the samples were negative. Blood feeding pattern must be considered when mosquito control strategies become employed.

Key words: *Aedes aegypti*, blood meal, multiplex PCR

INTRODUCTION

Aedes aegypti is a major dengue vector in Thailand. Since the first report of dengue outbreak in Thailand in 1958, prevalence of the disease has increased dramatically and has spread throughout the country (Minister of Public Health, 1998). As a specific drug to treat the virus is unavailable and vaccine against dengue is during the development phase, mosquito control strategy is the only effective method to control the disease (Taksinvaracharn *et al*, 2004; Thavara *et al*, 2006).

Blood feeding patterns of the mosquito provide valuable data for disease transmission. The distribution of bites of the mosquito can be used to develop effective mosquito control strategies (Scott *et al*, 1993). Mosquito blood meals have been identified by various immunological techniques, such as capillary precipitin test (Tempelis, 1975), agar gel diffusion (Crans, 1969) and enzyme-linked immunosorbent assay (ELISA) (Burkot *et al*, 1981). Although such immunological methods have been widely used to identify mosquito blood meal (Burkot *et al*, 1981; Beier *et al*, 1988; Hunter and Bayly, 1991; Chow *et al*, 1993; Ponlawat and Harrington, 2005), these protocols still have some intrinsic problems leading to misidentification of the types of blood due to cross-reactivity of serum proteins from related species (Ngo and Kramer, 2003). Recently, molecu-

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Correspondence: Dr Padet Siriyasatien, APR Building, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Bangkok 10330, Thailand.
Tel: 66 (0) 2256 4387; Fax: 66 (0) 2252 5944
E-mail: padet.s@chula.ac.th

lar techniques have been developed to identify vertebrate DNA in mosquito blood meals (Ngo and Kramer, 2003; Kent and Norris, 2005). The advantages of these procedures are that they are highly species specific and sensitive. Multiplex PCR has been developed to identify vertebrate host DNA from mosquito using a primer set specific for human, dog, cow, pig and goat (Kent and Norris, 2005). The primers were designed to anneal specifically to cytochrome oxidase b gene of vertebrate hosts (Kent and Norris, 2005). The objectives of this study were to determine the time course of the multiplex PCR detection of host blood DNA in *Ae. aegypti* mosquito, to compare different methods for collecting mosquito specimens and to study the prevalence and type of vertebrate blood from field caught *Ae. aegypti*.

MATERIALS AND METHODS

Time course analysis

Five day-old female *Ae. aegypti* mosquitoes were allowed to feed on human blood through a membrane feeding apparatus. Fully fed mosquitoes were then reared in an insectary at 28°C ± 1°C and 80% ± 5% humidity, and supplied with a damp cotton wool pad containing 10% sucrose solution as a carbohydrate source. DNA extraction was performed at 24, 48, 72 and 96 hours after feeding, and extracted DNA samples were kept at -20°C until used.

To determine the best method for preserving host DNA in collected mosquito specimens, blood fed mosquitoes were collected in a microcentrifuge tube containing 70% ethanol and kept at room temperature, or in a microcentrifuge tube without ethanol, and kept at 4°C and -20°C. Host DNA was extracted from the collected mosquitoes every week for 4 weeks.

Mosquito collection

Mosquitoes were collected from various areas of Ratchaburi Province (approximately 100 km from Bangkok, Thailand) using an electronic aspirator during March-July 2008. The total number of female mosquitoes was 890, and after identification mosquitoes were stored in microcentrifuge tubes at -20°C until used.

DNA extraction

DNA from whole blood sample was extracted using an AquaPure Genomic DNA Isolation Kit (Bio-Rad, CA) followed the manufacturer's instruction. Human (*Homo sapiens*), dog (*Canis familiaris*), cow (*Bos tarsus*) and pig (*Sus scrofa*) blood samples preserved in EDTA were used, and the extracted DNA samples were kept at -20°C.

DNA was extracted from individual mosquito using the method described by Kent and Norris (2005). Mosquito abdomen was ground in 100 μl of extraction buffer [0.1M NaCl, 0.2M sucrose, 0.1M Tris-HCl, 0.05M EDTA, pH 9.1 and 0.5% sodium dodecyl sulfate (SDS)] and incubated at 65°C for one hour. A 15 μl aliquot of cold 8 M potassium acetate was added and the solution was incubated on ice for 45 minutes. The sample then was centrifuged at 15,000*g* for 10 minutes. To precipitate DNA, 250 μl of 100% ethanol were added to the supernatant, which was incubated at room temperature for 5 minutes and centrifuged at 15,000*g* for 15 minutes. DNA pellet was dried at room temperature, resuspended in 10 μl 0.1x SSC (15 mM NaCl, 1.5 mM sodium citrate) and 40 μl of double-distilled water and kept at -20°C until used.

Multiplex PCR

The procedure was modified from that of Kent and Norris (2005). PCR reaction (25 μl) contained 2.5 μl of 10x buffer, 2.5 μl of 50 mM $MgCl_2$, 1.5 μl of 100 μM of each primer (universal reverse primer and forward primers of human, pig, dog and cow cytochrome oxidase b gene), 0.5 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA), 2 μl of 10 mM dNTP mixture, 2 μl of extracted DNA and double-distilled water. Thermal cycling was performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystem, Foster city, CA) as follows: one cycle of 95°C for 5 minutes; followed by 35 cycles of 95°C for 1 minute, 58°C for 30 seconds and 72°C for 1 minute; a last cycle of 72°C for 7 minutes. A 10 μl aliquot of the PCR products was electrophoresed in 1.5% agarose gel at 100 volts, stained with ethidium bromide (0.5 μg/ml) and visualized using a Gel Doc EQ system (Bio-Rad, CA). Amplification of human, pig, cow and dog DNA produced the expected PCR amplicon of 334, 453, 561 and 680 bp, respectively.

RESULTS

Time course analysis

Human blood fed mosquitoes were used for time course analysis. DNA from fully blood fed mosquitoes were collected every 24 hours until 96 hours. Extracted DNA was PCR amplified and amplicon (334 bp) was visualized following electrophoresis and staining. Whole blood was used as positive control and sucrose fed mosquito as negative control. Human blood DNA was detected in mosquito until 72 hours after feeding (Fig 1).

Fig 1–Time course analysis of human DNA from blood fed mosquito. Human DNA was PCR amplified as described in Materials and Methods. M, 100 bp marker; N, negative control; P, positive control; 1-4, female mosquitoes collected on day 1 to day 4 after blood feeding.

Fig 2–Preservation of mosquito specimen for detecting human host blood DNA. Human DNA was PCR amplified as described in Materials and Methods. M, 100 bp marker; N, negative control; P, positive control; 4_1-4_4, samples stored at 4°C without preservative for 1-4 weeks; F_1-F_4, samples stored at -20°C without preservative for 1-4 weeks; E_1-E_4, samples stored in 70% ethanol at room temperature for 1-4 weeks.

Preservation of mosquito specimens for detecting host blood DNA

Blood fed mosquito specimens were kept for 1-4 weeks in microcentrifuge tube containing 70% ethanol at room temperature, and in microcentrifuge tube without ethanol at 4°C and at -20°C. Human host DNA was detected in mosquito specimens stored at 4°C without preservative for 2 weeks, at room temperature in 70% ethanol for 3 weeks and at -20°C without preservative for 4 weeks (Fig 2).

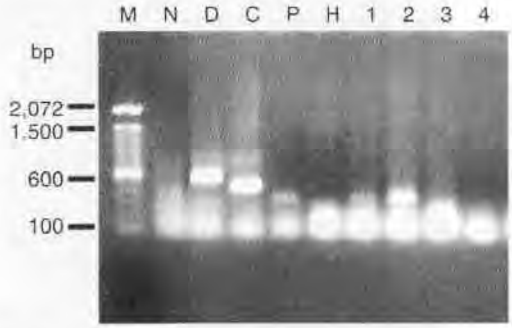

Fig 3–Agarose gel-electrophoresis of multiplex PCR of host DNA from field caught *Ae. aegypti*. Multiplex PCR was performed as described in Materials and Methods. M, 100 bp marker; N, negative control; D, C, P, H, positive control of dog, cow, pig and human DNA; 1-4, field caught mosquito specimens, which were positive for pig, pig, human and negative, respectively.

Identification of blood meal from field caught mosquito

Among the 890 mosquito samples, 766 (86.1%), 30 (3.4%), 18 (2.0%) and 9 (1.0%) were positive for human, pig, dog and cow DNA respectively, and 32 (3.6%) samples were positive for two types of host DNA and 35 (3.9%) were negative. Fig 3 shows a typical result.

DISCUSSION

Time course analysis study in *Ae. aegypti* mosquitoes fed on human blood demonstrated that host DNA was detected 72 hours after feeding. Kent and Norris (2005) showed that this method is able to detect vertebrate host DNA in *Anopheles stephensi* mosquito only up to 48 hours after feeding. Boake *et al* (1999) studies in black flies (*Simulium damnosum* s.l.) fed on human blood demonstrated that PCR is able to detect human DNA up to 72 hours post-feeding. Ngo and Kramer (2003) also demonstrated that avian blood DNA is detected in *Culex p. pipiens* L. up to 72 hours after feeding. Lee *et al* (2002) was able to detect Japanese quail DNA from *Cx. tarsalis* Coquillett 7 days post-feeding. Differences in duration of host DNA detection in blood feeding insects depend on several factors, including DNA extraction procedure, different digestive processes in black flies compared with mosquitoes or even differences in mosquito species (*An. stephensi* and *Ae. aegypti*).

Preservation method studies showed host DNA in blood fed *Ae. aegypti* mosquitoes stored at -20ºC without any preservative for more than 4 weeks. Less periods were obtained for mosquitoes kept in 70% ethanol at room temperature or at 4ºC.

High percentage (86.1%) of human DNA detected in *Ae. aegypti* mosquito is expected as it is considered as highly anthropophilic (Harrington *et al*, 2001). The negative result from this study was higher than the previous studies (Ngo and Kramer, 2003) and may have been caused by the primer set inability to anneal with other vertebrates, such as cat and other avian blood DNA.

In summary, blood feeding pattern of *Ae. aegypti* provides valuable data for dengue vector control. As the mosquito can maintain its life cycle by feeding on other vertebrate hosts, blood feeding pattern must be considered when mosquito control strategies are deployed.

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ANALYSIS OF SALIVARY GLAND PROTEINS OF THE MOSQUITO *ARMIGERES SUBALBATUS*

Padet Siriyasatien[1], Kuntida Tangthongchaiwiriya[1], Narisara Jariyapan[2], Sakchai Kaewsaitiam[1],
Yong Poovorawan[3] and Usavadee Thavara[4]

[1]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok; [2]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai; [3]Department of Pediatric, Faculty of Medicine, Chulalongkorn University, Bangkok; [4]Department of Medical Sciences, National Institute of Health, Nonthaburi, Thailand

Abstract. Quantitative studies of total salivary gland protein of *Armigeres subalbatus* mosquito revealed that the total salivary gland protein increased dramatically during the five days after emergence as adults. The amount of salivary gland protein of female and male mosquitos at day five after adult emergence were on the average 11.55 and 1.32 µg/pair gland respectively. SDS-PAGE studies showed that salivary gland protein profiles of *Armigeres subalbatus* demonstrated 9 major polypeptide bands of 68, 65, 60, 55, 40, 30, 28, 21, and 15 kDa. The 21 and 65 kDa bands were found only in the distal lateral region of the mosquito salivary gland and were depleted after the female mosquito took a blood meal.

INTRODUCTION

Mosquito-borne diseases still remain a major health problem in both human and veterinary sectors. Diseases transmitted by mosquitos include malaria, dengue hemorrhagic fever, Japanese encephalitis, yellow fever, and filariasis. The pathogens are transmitted to a vertebrate host when the female mosquito takes a blood meal. Many pathogens take up residence in the mosquito salivary glands before being transmitted to a new vertebrate host. In addition, the mosquito saliva may enhance or facilitate infectivity (Ribeiro, 1995; Osorio *et al*, 1996; Edwards *et al*, 1998). Mosquito salivary gland extracts contain α-glucosidases and α-amylases that initiate the digestion of carbohydrates present in dietary carbohydrate sources and other enzymes and peptides involved in blood feeding and ingestion such as anticoagulants, vasodilators, and platelet aggregation inhibitors (Ribeiro and Francischetti, 2003). The saliva also contains molecules that provoke a humoral and cellular immune response in the vertebrate host (Peng *et al*, 1995; Peng and Simons, 1997; Malafront *et al*, 2003).

The proteins present in the salivary glands of several mosquito species have been investigated (Mellink and van Zenben, 1976; Poehling, 1979; Al-Ahdal *et al*, 1990; Marinotti *et al*, 1990; Andrews *et al*, 1997; Saliman *et al*, 1999; Nascimento *et al*, 2000; Moreira *et al*, 2001; Jariyapan and Harnnoi, 2002; Suwan *et al*, 2002), however, little is known of *Armigeres subalbatus*, a major vector of a heart dog filaria, *Dirofilaria immitis*. In Thailand, *Ar. subalbatus* is the most common early morning and early night biting mosquito and is found throughout the country especially in the rural areas. It feeds on both human and domestic animals (Srinivas *et al*,1994) . The study on salivary gland protein of *Ar. subalbatus* mosquito has never been reported. Therefore, in this study the salivary gland proteins of *Ar. subalbatus* were determined and analysed. Our initial finding on the salivary gland proteins showed significant reduction of some major proteins after blood feeding.

MATERIALS AND METHODS

Maintenance of *Ar. subalbatus* mosquitos

Ar. subalbatus mosquitos were maintained in an insectary of the Department of Medical Sciences, National Institute of Health, Bangkok, Thailand. Conditions were set at 28°C ± 1°C at 80% ± 5% relative humidity under 12/12 hours light/dark photo-period. Adults were supplied

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Correspondence: Dr Padet Siriyasatien, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.
Tel: 66 (0) 2256-4387
E-mail:fmedpsy@md.chula.ac.th

with a damp cotton wool pad contained 10% sucrose solution as a carbohydrate source. For blood feeding, female mosquitos were allowed to feed on anesthetized mice for 30 minutes. Groups of mosquitos were reared simultaneously from the same cohort of eggs. Adult mosquitos aged 1 to 5 days after emergence were used.

Salivary gland dissection

Mosquitos were anesthetized on ice and salivary gland dissection was performed as described by Suwan *et al* (2002). Mosquito salivary glands were transferred to a microcentrifuge tube containing a small volume of PBS (phosphate buffer saline solution) and kept at -70ºC until used.

Protein quantification

Amount of total mosquito salivary gland protein was determined using a Bio-Rad Protein Assay (Bio-Rad) following the manufacturer's instruction. Two pairs of female or 10 pairs of male *Ar. subalbatus* salivary glands at day 1, 3, and 5 after emergence were used in this study. Each determination was repeated 3 times.

SDS-PAGE analysis and protein staining

SDS-PAGE was performed according to Laemmli (1970) and the proteins were silver stained using a Silver Stain kit (Bio-Rad) according to the manufacturer's instruction.

RESULTS AND DISCUSSION

The salivary glands of adult *Ar. subalbatus* are paired organs, located in the thorax. The female salivary glands display difference in structure when compared to the male ones. The female gland is composed of two identical lateral lobes and a shorter and wider median lobe (Fig 1). The lateral lobes can be divided into two regions, proximal and distal. The male gland consists of three morphologically homogenous lobes and is approximately one-fifth size of the female (Fig 1). Morphological pattern of *Ar. subalbatus* adult salivary glands followed the same pattern as described for *Ae. aegypti* (Mellink *et al*, 1976; Poehling, 1979), *Ae. albopictus* (Marinotti *et al*, 1996), *Ae togoi* (Jariyapan and Harnnoi, 2002), *Cx. pipiens* and *Ae. caspius* (Saliman *et al*, 1999).

Total protein contents of male and female mosquito salivary glands were determined. Table 1 demonstrates the average amount of total salivary gland protein content in male and female mosquitos at various times after emergence. Total salivary gland protein content of male mosquito on day 1 after emergence was 0.44 ± 0.10 µg/gland pair and increased to 1.32 ± 0.14 µg/gland pair at day 5. In females, total salivary gland protein content of mosquito at day 1 after emergence was 1.85 ± 0.53 µg/gland pair and increased dramatically to 11.55 ± 1.71 µg/gland pair at day 5. Comparison of protein content between male and female mosquitos at day 5 showed that a pair of male glands contained approximately 10% of that found in female ones. Similar results were found in *Cx. pipiens* (Saliman *et al*, 1999) and *Ae. togoi* (Jariyapan and Harnnoi, 2002).

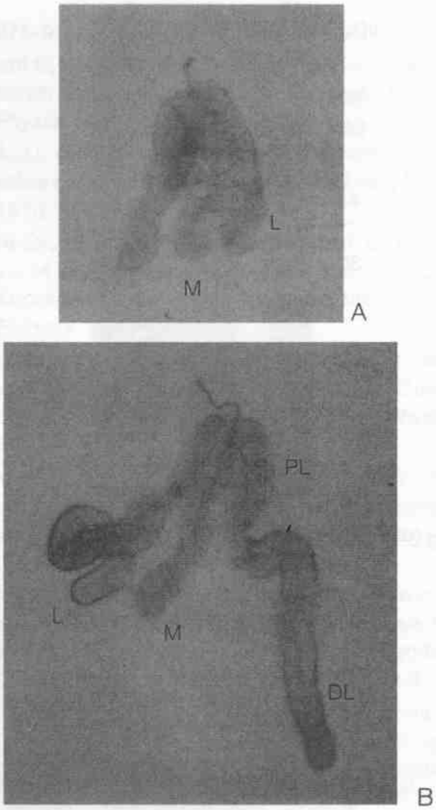

Fig 1–Salivary glands of *Armigeres subalbatus* mosquito. A: a male salivary gland; B: female salivary gland, M: median lobe; L: lateral lobe; PL: proximal region of lateral lobe; DL: distal region of lateral lobe. The photographs were taken using an Olympus (AH) microscope at 100x magnification.

SDS-PAGE analysis of the proteins present in the salivary glands of male and female mos-

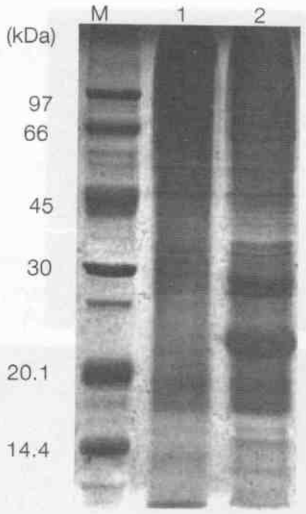

Fig 2–Protein electrophoresis pattern of salivary glands of adult male and female *Armigeres subalbatus* at day 5 after adult emergence. Ten pairs of sugar fed female salivary glands (lane 2) and 15 pairs of male salivary glands (lane 1) were submitted to SDS-PAGE in a 12% polyacrylamide gel followed by silver staining. Molecular mass markers (M) are in kilodalton (kDa).

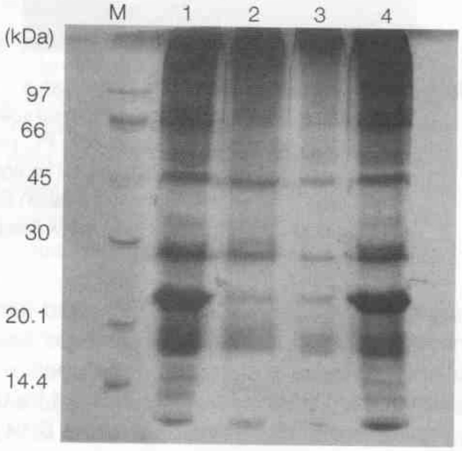

Fig 3–Electrophoretic profile of polypeptides from female *Armigeres subalbatus* mosquitos salivary gland lobes. Proteins were separated on a 12% SDS-PAGE gel and silver stained. Lane 1,ten whole female salivary glands; lane 2, twenty proximal lateral lobes; lane 3, ten median lobes; lane 4, twenty distal lateral lobes. M: molecular weights markers of sizes (kDa) indicated on the left side of the picture.

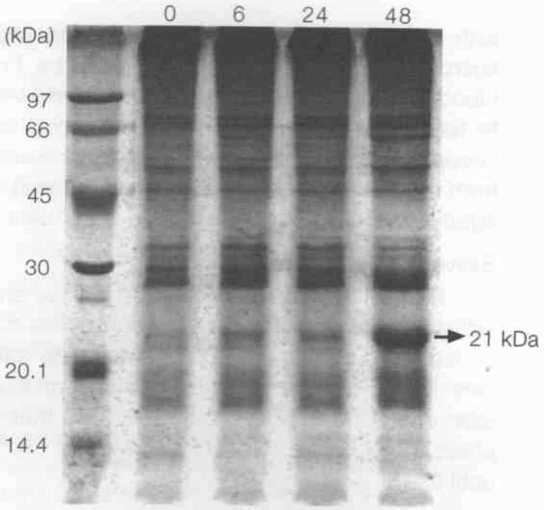

Fig 4–Protein electrophoretic profile of salivary glands of blood fed *Armigeres subalbatus* mosquitos. Ten pairs of salivary glands were dissected from blood fed mosquitos at 0, 6, 24 and 48 hours after a blood meal and submitted on 12% SDS-PAGE and silver stained. M: molecular weight markers of sizes (kDa) indicated on the left side of the picture. Number at the top indicate hours after a blood meal.

Table1

Total salivary gland protein contents of male and female *Armigeres subalbatus* mosquitos after the adult emergence.

	Post emergence (day)					
	Male			Female		
	1	3	5	1	3	5
Protein content (µg/gland pair)^A	0.44 ± 0.10	0.49 ± 0.14	1.32 ± 0.14	1.85 ± 0.53	6.53 ± 0.78	11.55 ± 1.71

A = mean ± SD; n = 10

quitos at day 5 of emergence was performed. The analysis of both female and male salivary gland proteins revealed the presence of at least 9 major and several minor protein bands (Fig 2). Protein bands with estimated molecular masses of 68, 60, 55, 40, 30, 28 and 15 kDa were found in salivary glands of both sexes whereas the 65 and 21 kDa protein bands were observed only in females.

The different morphological regions of the female salivary glands displayed distinct protein profiles (Fig 3). The 65 and 21 kDa proteins appeared predominantly in the distal-lateral lobe. The protein profile of the female proximal-lateral lobe was similar to that of the male salivary gland. The protein profiles at 0, 6, 24, and 48 hours after a blood meal was also analyzed (Fig 4). Immediately after blood feeding, the 65 and 21 kDa protein bands were barely detected, but both proteins started to appear gradually 6 hours later and returned to the unfed level in 48 hours. Further investigations in molecular, biochemical, and immunological aspects of *Ar. subalbatus* salivary glands will provide information for better understanding of the role of mosquito salivary gland proteins in blood feeding and disease transmission.

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Prevalence of Dengue Virus in *Aedes* Mosquitoes During Dry Season by Semi-Nested Reverse Transcriptase-Polymerase Chain Reaction (Semi-Nested RT-PCR)

Rupporn Tuksinvaracharn*, Pongsakorn Tanayapong*,
Saravech Pongrattanaman*, Pokrath Hansasuta MD, DPhil (Oxon)**,
Parvapan Bhattarakosol PhD**, Padet Siriyasatien MD, DTM&H, PhD***

** Undergraduate student, M.D. programme, Faculty of Medicine, Chulalongkorn University*
***Department of Microbiology, Faculty of Medicine, Chulalongkorn University*
****Department of Parasitology, Faculty of Medicine, Chulalongkorn University*

Dengue hemorrhagic fever remains a major health concern in Thailand. Much effort has focused on the prevention and control of the disease. Detection of dengue virus infection rate in mosquitoes would evaluate dengue control programs and predict the epidemics of dengue hemorrhagic fever. To determine dengue virus infection rate in mosquitoes by Semi-nested RT-PCR. A total of 400 mosquitoes were collected from Rom Kao Community representing a crowded community and another 9 non-crowded communities in Bangkok. Mosquitoes were then divided into 40 pools, each contained 10 mosquitoes. A total of 391 Aedes aegypti and 9 Aedes albopictus were screened for dengue virus. The mosquito infection rate in the Rom Klao community was 5% of the mosquito pool equal to that found in non-crowded communities. Both groups were found to have dengue virus serotype 3. The present study suggests a circulation of dengue virus serotype 3 in both crowded and non-crowed communities, the infection rates of which are indifferent during the dry season.

Keywords : *Dengue virus, Aedes aegypti, Aedes albopictus, Mosquito infection rate*

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Dengue hemorrhagic fever (DHF) continues to present a major health problem in many areas of the world especially in Southeast Asia. The infection is caused by the dengue virus which has four serotypes (Den1, Den2, Den 3 and Den 4). Dengue virus is transmitted to humans via mosquito bites. In Thailand, there are two major dengue vectors described, *Aedes aegypti* and *Ae. albopictus* (1,2).

The first outbreak in Thailand was documented in 1958, and a total of 2158 cases were reported. The prevalence of the disease has increased dramatically in recent decades. Morbidity and mortality rate of DHF is highest in children especially at the age of 5-9 and 10-14. More recently, DHF in adults has been documented in Thai patients (3). At present, the transmission of the disease has spread throughout the country (4). Due to the lack of specific treatment of the dengue virus and unavailability of effective vaccine against the virus, the interruption of pathogen transmission by mosquito control provides the only effective approach to the control of dengue infection(5,6). The most effective strategy to control a dengue outbreak is to eliminate mosquitoes and larval breeding habitats. The strategy is commonly operated before the rainy season when the population densities of mosquitoes are lower, and hence easier to control (7-9). The surveillance of dengue infection in mosquitoes at such times provides useful data for prevention and control of the disease before the beginning of an epidemic in the rainy season. In Thailand, the dengue control relies solely on surveillance for *Aedes* larval habitat and mosquito population density. Although the surveillance has been regularly operated for many years, it has never prevented an outbreak of dengue infection in Thailand. This phenomenon coincided with the reemergence of dengue outbreaks in Singapore despite the reduction of *Aedes* mosquito population to a relatively low level(10). As described previously, entomologic surveillance *per se* may not serve as an effective tool to forecast a dengue outbreak. Monitoring of the dengue virus in field mosquitoes is more relevant and can complement the current dengue surveillance. It directly detects the prevalence of dengue virus serotypes (11) and pockets of

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Correspondence to : Siriyasatien P. Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

infected mosquitoes, thus serving as a sensitive indicator for mosquito control. The present study demonstrates data of a dengue vector survey and prevalence of dengue infection from field-caught mosquitoes from crowded and non-crowded communities in Bangkok during the dry season.

Material and Method

Mosquito collection

This project was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Mosquitoes were collected from crowded and non-crowded communities in Bangkok using human bait collection according to WHO methods (6). Rom Klao community in Lad Krabang district was chosen and defined as a crowded community (crowded community is a highly urbanized area with a house density of at least 15 houses per 1600 square meters: defined by Bangkok Metropolitan Authority). Ten female mosquitoes were collected daily from each house from 10.00 am to 5.00 pm from the 25th of March to the 10th of April 2003. The total number of female mosquitoes was 400, which were equally collected from crowded and non-crowded communities. All mosquitoes were aliquoted, ten female mosquitoes per microcentrifuge tube and stored at -70°C until used.

Detection of dengue virus

Viral RNA extraction

Viral RNA was extracted from 10 pooled mosquitoes. The mosquito's wings and legs were removed then the mosquitoes were quickly frozen by dry ice and ground in 150 µl of RNase-free water and centrifuged at 12,000 rpm for 5 minutes. 100 µl of supernatant was preceded to RNA extraction using RNeasy mini kit (QIAGEN, Germany). The procedure followed the manufacturer's recommendation. Total RNA was eluted in 30 µl of RNase-free water.

Oligonucleotide primers

The primers' sequence designed by Lanciotti *et al*(12) was used in the present study. Six oligonucleotide primers within the core and pre-membrane protein gene (C-prM) of dengue viruses were used. Two consensus primers (D1 and D2) were designed to be homologous to the genomic RNA of all four dengue serotypes, whereas the type-specific nucleotide primers (TS1, TS2, TS3 and TS4) were designed to anneal specifically to each of their respective genomes. These primers were positioned such that a differently sized product was generated from each type.

Semi-nested RT-PCR

The procedure performed in the present study was modified from the study by Lanciotti *et al.* The first step RT-PCR was performed using Superscript one step RT-PCR with Platinum® *Tag* (Invitrogen, USA). The protocol followed the manufacturer's instruction. In 50 µl of reaction was performed by mixing 25 µl of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 2.4 mM $MgSO_4$), 1.8 µl of 50 mM $MgSO_4$, 0.5 µl of 25 pmol primers (D1 and D2) of each primer, 1 µl of RT/Platinum® *Taq* Mix (Invitrogen), 2 µl of RNA template and RNase-free water to the total of 50 µl. The thermal cycler was programmed to begin with one cycle of 55°C for 30 min followed by a 2-min incubation at 94°C and 40 cycles of 94°C for 15 sec, 55°C for 20 sec and 68°C for 3 min.

The second step amplification reaction was attempted to identify type-specific DNA products. A typical 25 µl of PCR reaction was performed containing 2.5 µl of 10X buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 25 pmol of each primer (D_1, TS_1, TS_2, TS_3 and TS_4), 0.5 unit of *Taq* polymerase (GibcoBRL), 4 µl of 20 mM DNTP mixture, 10 µl of product from the first step and RNase-free water to 25 µl. The thermal cycler was programmed to begin with one cycle of 94°C for 5 min incubation followed by 30 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min, the last 1 cycle of 72°C for 5 min and final holding at 4°C. 10 µl of the product from the second step was electrophoresed through a 1.5% Agarose gel at 100 volts, stained with ethidium bromide and visualized on a UV transilluminator.

Partially extracted dengue virus antigens from mice microglia were used as positive controls. The antigens were purchased from the Ministry of Public Health, Thailand. They were standard Den-1 Hawaii, Den-2 strain TR 1751, Den-3 strain H87 and Den-4 strain H241. Uninfected laboratory-reared *Aedes aegypti* mosquitoes were used as negative controls.

Results

Mosquito collection

During the study period, there was no dengue case reported from the area of study. A total of 400 female mosquitoes were collected. The mosquitoes were identified by morphometric analysis, 391 were *Ae. aegypti* and 9 were *Ae. albopictus*. All *Ae. albopictus* mosquitoes were collected from the non-crowded community. The landing activities of *Aedes* mosquitoes collected from Rom Klao community seemed to have two highest peaks, one in the early morning and the other in the late afternoon (Fig. 1). *Ae. aegypti* was found only in the crowded community while both species (*Ae. aegypti* and *Ae. albopictus*) were captured in the non-crowded community. The number of *Ae. aegypti* was greater than that of *Ae. albopictus*.

Sensitivity of semi-nested RT-PCR

In order to determine the sensitivity of the test, dengue virus serotype 3 strain H87 was used. The amount of dengue virus was determined by hemagglutination test and converted into viral particles (10^7 particles/ml is equivalent to 1 HA unit). Serial 10-fold dilutions of extracted dengue virus serotype 3 were reverse transcribed and amplified. The limit of detection was approximately 25 viral particles/µl (Fig. 2).

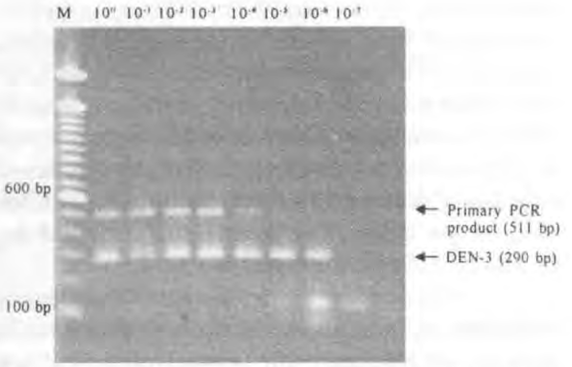

Fig. 2 Agarose gel electrophoresis of the second-round semi-nested PCR quantitated Dengue serotype 3 RNA. Serial 10-fold dilutions were reverse transcribed and amplified as indicated above each lane. Molecular weight (M) markers are shown of the left

Detection of Dengue viruses in mosquitoes

A total of 400 female mosquitoes were grouped into 40 pools (20 pools from each community). Dengue virus was detected in 2 pools, one from the crowded community and another from the non-crowded community. Only dengue virus serotype 3 was found. Both infected mosquito pools contained only *Ae. aegypti* mosquitoes (Fig. 3).

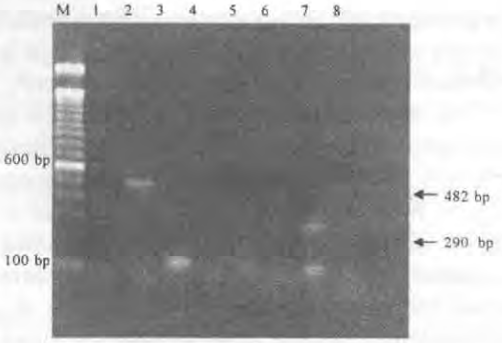

Fig 3. Agarose gel electrophoresis of dengue virus detection in field caught mosquito. Molecular weight (M) markers are shown of the left., lane 1: virus-free mosquito sample were as negative control, lane 2: dengue virus serotype 1 was used as a positive control, lane 3-8:Field caught mosquito samples, dengue virus serotype 3 was detected in lane 7

Discussion

The prevalence of *Ae. aegypti* and *Ae. albopictus* mosquitoes in the present study were 97.75 and 2.25% respectively. The magnitude of differences between the two species in the present result is different from that of the previous study by Thavara *et al* on Samui Island, Thailand during the rainy season. *Ae. aegypti* in their study contributed 75.4% of indoor biting activity, whereas *Ae. albopictus* contributed 24.6% of indoor biting activity. Relatively low frequencies of *Ae. albopictus* in the present study may be due to the lack of their natural habitats during the dry season. In addition, *Ae. albopictus* breeding potential is expected to decline in highly urbanized areas due to a reduction of outdoor natural site. Moreover, the biphasic landing activities demonstrated in the present study was similar to that reported by Thavara *et al*. However, the landing activities shown in the present study were peaked at early and late afternoon (Fig. 1), while Thavara *et al* reported the peaks of landing activities during the morning and afternoon hours.

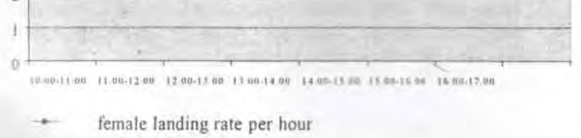

Fig. 1 Graph illustrating *Aedes* mosquito landing rate between 10.00 am to 5.00 pm

There have been a number of studies on detection of dengue virus in field-caught mosquitoes. The mosquito infection rates in Thailand ranged from 3.6% to 61.5% in previous studies (10,13-16). The rates of infection were different due to various factors including virus stain, susceptibility of mosquito species, sensitivity of virus detection techniques, study sites, and period of investigation. Indeed, the differences of these infection rates were primarily due to the sensitivity of detection employed in each study. The technique used in the present study, semi-nested RT-PCR, is believed to be one of the most sensitive methods to detect RNA virus in specimens. The present study showed the detection of 25 viral particles.

Although the authors proposed that dengue virus infected mosquitoes were more prevalent in crowded communities, the present result did not agree with the hypothesis. The number of infected mosquitoes in crowded and non-crowded communities was not different from the infection rate of 5% from both communities. The incidence of dengue infection in crowded communities is usually higher than that of non-crowded communities. The reason for this, despite equally prevalent infected mosquitoes, might be that more people would be accessible to the mosquitoes in a crowded area *vis-à-vis* in a non-crowded community.

Ae. aegypti mosquitoes were confirmed to be the most important vectors for dengue virus transmission in the present study. *Ae. albopictus* mosquitoes, however, should be underestimated in this context, since the present study was done during the dry season when their natural habitats were depressed. Moreover, preference was given to *Ae. aegypti* mosquitoes because collection of mosquitoes in the present study was done indoors.

Dengue virus serotype 3 was identified in mosquito pools both from crowded and non-crowded communities. Interestingly, dengue virus serotype 3 was recently not isolated from patients from Bangkok Metropolitan areas where the present study was carried out. Whether this study result would predict dengue serotype prevalence in the following dengue season remains to be seen and warrants further study.

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การสำรวจความชุกของเชื้อ *Dengue virus*ในยุงลายช่วงฤดูแล้งโดย วิธี *Semi-nested Reverse Transcriptase - Polymerase Chain Reaction (Semi- nested RT- PCR)*

รับพร ทักษิณวราจาร, พงศกร ตนายพงศ์, สราเวช พงศ์รัตนามาน, ปกรัฐ หังสสูต, ภาวพันธ์ ภัทรโกศล, เผด็จ สิริยะเสถียร

ไข้เลือดออกยังคงเป็นปัญหาสาธารณสุขที่สำคัญของประเทศไทยและหลายประเทศในเอเชียตะวันออกเฉียงใต้ สาเหตุของโรคเกิดจากเชื้อ Dengue virus ซึ่งมีอยู่ 4 serotypes คือ DEN-1, DEN-2, DEN-3 และ DEN-4 โดยมียุงลายบ้าน (Aedes aegypti) และยุงลายสวน (Aedes albopictus) เป็นพาหะนำโรคที่สำคัญ เนื่องจากยังไม่มียาและวัคซีนสำหรับการรักษาและป้องกันไข้เลือดออก การควบคุมยุงลายซึ่งเป็นพาหะนำโรคจึงเป็นวิธีที่มีประสิทธิภาพที่สุดในการป้องกันและควบคุมการระบาดของโรค ปัจจุบันการเฝ้าระวังโรคโดยอาศัยปริมาณลูกน้ำยุงลาย และจำนวนยุงลายเป็นหลักแต่ยังไม่สามารถควบคุมการระบาดของโรคได้อย่างมีประสิทธิภาพ ดังนั้นการสำรวจปริมาณยุงลายที่มีเชื้อไวรัสไข้เลือดออกจึงควรนำมาพิจารณาร่วมในการเฝ้าระวังโรค สำหรับประเทศไทยจำนวนผู้ป่วยโรค ไข้เลือดออกจะเปลี่ยนแปลงขึ้นลงตามฤดูกาล โดยมักจะมีการระบาดในฤดูฝน ดังนั้นการตรวจหาเชื้อไวรัสไข้เลือดออกในยุงลายช่วงฤดูแล้งอาจสามารถนำมาใช้ในการทำนายการระบาดของโรคที่จะเกิดขึ้นในฤดูฝน เนื่องจากปริมาณยุงลายในช่วงฤดูแล้งมีน้อยและจำนวนยุงลายที่มีเชื้อไข้เลือดออกอาจมีไม่มาก ดังนั้นการตรวจหาเชื้อไวรัสในยุงลายจึงต้องเป็นวิธีที่มีความไวและความจำเพาะสูง วิธี semi-nested RT-PCR เป็นเทคนิคที่มีความไว และความจำเพาะสูงพอที่จะตรวจหาเชื้อไวรัสแม้จะมีปริมาณน้อยในยุง ผู้วิจัยได้ทำการสำรวจโดยการจับยุงลายในกรุงเทพมหานครจำนวน 400 ตัว โดยจับจากชุมชนร่มเกล้าซึ่งเป็นชุมชนแออัด และจากบ้านที่ไม่อยู่ในชุมชนแออัดจาก 9 เขต พบว่ายุง 391 ตัวเป็นยุงลายบ้านและ 9 ตัวเป็นยุงลายสวน ยุงถูกแบ่งเป็น 40 กลุ่มเพื่อหาไวรัสไข้เลือดออกโดยวิธี RT-PCR พบว่าทั้ง 2 แหล่งมียุงติดเชื้อไข้เลือดออก 5% และเป็น serotype 3 จากการสำรวจหาเชื้อไข้เลือดออกในยุงลายจากชุมชนทั้ง 2 แห่งในช่วงฤดูแล้ง ไม่พบว่ามีความแตกต่างกัน