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

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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 -20ºC. 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).

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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-

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 28ºC ± 1ºC 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 -20ºC 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 4ºC and -20ºC. 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 -20ºC 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 -20ºC.

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 65ºC 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 -20ºC 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 95ºC for 5 minutes; followed by 35 cycles of 95ºC for 1 minute, 58ºC for 30 seconds and 72ºC for 1 minute; a last cycle of 72ºC 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).

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 4ºC and at -20ºC. Human host DNA was detected in mosquito specimens stored at 4ºC without preservative for 2 weeks, at room temperature in 70% ethanol for 3 weeks and at -20ºC without preservative for 4 weeks (Fig 2).

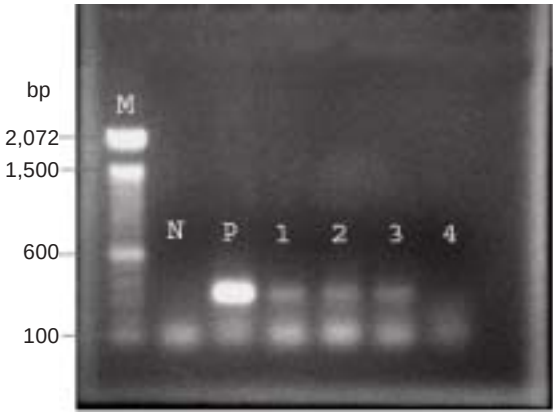

Fig 1–Time course analysis of human DNA from blood fed mosquito. Human DNA was PCR amplified as described in Materials and Methods. M, 100 bp marker; N, negative control; P, positive control; 1-4, female mosquitoes collected on day 1 to day 4 after blood feeding.

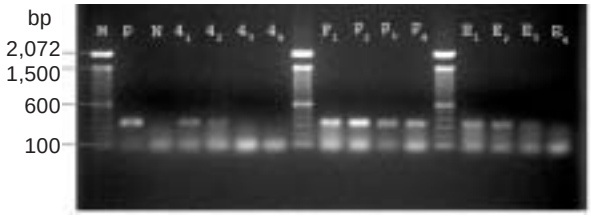

Fig 2–Preservation of mosquito specimen for detecting human host blood DNA. Human DNA was PCR amplified as described in Materials and Methods. M, 100 bp marker; N, negative control; P, positive control; 4_1-4_4, samples stored at 4ºC without preservative for 1-4 weeks; F_1-F_4, samples stored at -20ºC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

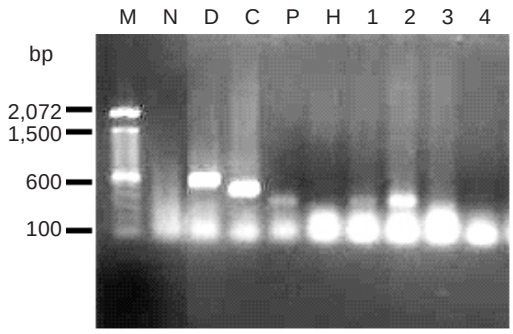

Fig 3–Agarose gel-electrophoresis of multiplex PCR of host DNA from field caught *Ae. aegypti*. Multiplex PCR was performed as described in Materials and Methods. M, 100 bp marker; N, negative control; D, C, P, H, positive control of dog, cow, pig and human DNA; 1-4, field caught mosquito specimens,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