Original Article

Identification of blood meal from field collected filarial vector mosquitoes, *Armigeres subalbatus* by multiplex PCR

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Abstract

Mosquitoes act as vectors of many diseases affecting humans and animal health, including Zika, malaria, dengue, chikungunya, viral encephalitis, and filariasis. One of the best strategies to control these vector-borne diseases is to control the mosquito vectors. Female mosquitoes require a blood source for egg development. As female mosquitoes take blood meals pathogens are released into the vertebrate host. Identification of the types of vertebrate blood sucked by the mosquito is essential information required to develop an effective strategy to control the mosquito populations and the related mosquito borne diseases. Objective of this study was to identify types of mammal blood in *Armigeres subalbatus* mosquito, the principal vector of filarial parasites especially *Dirofilaria* spp.. A total of 210 female *Ar. subalbatus* mosquitoes were collected from different place of Sai Kaew beaches, Samed Island, Rayong province, eastern Thailand. The blood meals of *Ar. subalbatus* were identified using multiplex PCR specific primers on mitochondrial cytochrome b gene. The result showed that 74 samples of *Ar. subalbatus* blood meals was positive for human (17.14%), pig (5.24%), cow (2.38%), dog (0.48%), and other mammals blood (10%). The 136 samples of negative detection by multiplex PCR were also positive 2 (0.95%) samples of avian blood, but 134 (63.81%) samples were not detected for vertebrate blood meal DNA. The benefits of this study are to understand the natural feeding behavior of *Ar. subalbatus* mosquitoes. Information obtained from the study would be applied to develop the effective control strategies for *Ar. subalbatus* and may provide indirect data suggesting what reservoirs are significant in the mosquito-borne diseases.

Keywords: *Armigeres subalbatus*, Mosquito, blood meal, Multiplex PCR

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Introduction

Mosquitoes are vectors for many pathogens of medical and veterinary importance (Sanchez-Vargas *et al*., 2004). Female mosquitoes need a blood meal for egg reproduction. The female mosquito can also transmit pathogens into the human or animal host whilst taking a blood meal (Foster, 1995). Identification of the blood meal from mosquitoes provides information on host feeding patterns and preference of mosquitoes in nature. In addition, it may assist in understanding indirect data to determine the reservoir hosts for any vector-borne diseases (Edman and Taylor, 1968; Edman, 1971; Edman *et al*., 1972; Lee *et al*., 2002). Several studies demonstrated mosquito blood meals using different methods including serologic techniques such as the latex agglutination (Boorman *et al*., 1977), enzyme-linked immunosorbent assay (ELISA) (Beier *et al*., 1988) and precipitin test (Gomes *et al*., 2001). However, these techniques have low sensitivity, are time-consuming and cross-reactions also occur. Nowadays, molecular techniques have been developed for detection of blood meal in haematophagous insects such as multiplex polymerase chain reaction (PCR) in mosquitoes (Kent and Norris, 2005) and real-time PCR in the phlebotomine sand fly (Sales *et al*., 2015). In this study, we were interested in identifying blood meals of field caught *Ar. subalbatus* collected from a tourist attractant island of eastern Thailand. *Ar. subalbatus* is known to be a vector of filarial heart worm, *Dirofilaria immitis* in dog (Cheong *et al*., 1981), *Wuchereria bancrofti* (Das *et al*., 1983), Japanese encephalitis (Liu *et al*., 2013), *Brugia pahangi* (Muslim *et al*., 2013) and most recently Zika virus was detected in this mosquito species (Tawatsin *et al*., 2018). *Ar. subalbatus* is commonly found in early morning and night biting mosquito and feeds on both human and animals (Srinivas *et al*, 1994). However, little is known about the prevalence and type of vertebrate blood feeding of *Ar. subalbatus*. Therefore, this study presented blood meal type from field collected filarial vector mosquitoes, *Ar. subalbatus* by multiplex-PCR based on mitochondrial cytochrome b (CytB) gene.

Materials and Methods

Mosquito collection: The study was approved by Institutional Animal Care and Use Committees of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA no. 006/2015). The mosquito samples were collected from different location on the Sai Kaew beaches, Samed Island, Rayong province using CDC light traps (25-W bulb) without CO_2 during November 20-22, 2015 for 3 nights. The 10 traps were placed around human settlements, open space, rock holes, bamboo trees, and small forests overnight from 6.00 pm to 6.00 am. *Ar. subalbatus* were collected from the light traps the following day and anesthetized using chloroform-soaked cotton balls. The mosquitoes were morphologically identified using taxonomic key (Barraund. 1934; Christophers. 1993; Darsie and Pradhan 1990). All *Ar. subalbatus* mosquitoes were differentiated according to their gender. After identification the mosquitoes were stored in microcentrifuge tubes, in a box containing dry ice and sent to the Vector Biology and Vector Borne Diseases Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University.

DNA extraction: Two hundred microliter of human (*Homo sapiens*), dog (*Canis familiaris*), cow (*Bos tarsus*), pig (*Sus scrofa*), and chicken (*Gallus domesticus)* blood samples, and individual female *Ar. subalbatus* mosquito samples were transferred into 1.5 ml microcentrifuge tubes containing 400 µl of lysis buffer. *Ar. subalbatus* mosquito samples were ground with sterile plastic pestles prior to DNA extraction processes. Samples were then processed for total DNA extraction by a tissue DNA extraction kit (Invisorb® Spin Tissue Mini Kit, Invitek, Germany) according to the manufacturer's instructions. DNA was eluted in 80 µl of elution buffer; quantity and quality were determined by a Nanodrop 2000c apparatus (Thermo Scientific, USA). The genomic DNA was stored for an extended time at -80°C for long-term storage.

Multiplex-PCR: Multiplex-PCR amplification was used to detect mammal DNA, the reactions were performed in PCR Mastercycler® pro (Eppendorf, Germany) under the following conditions: denaturation at 95 °C for 5 min, followed by 40 cycles of 95°C for 1 min, annealing at 58°C for 1 min; and extension at 72°C for 1 min, with the final extension at 72°C for 7 min. The forward and reverse primers are described in Table 1.

PCR was used to detect avian blood, with the conditions as follow conditions: initial denaturation at 93°C for 5 min, followed by 35 cycles of denaturation 93°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, and a final extension after the last cycle at 72°C for 7 min the method was modified from Cicero and Johnson (2001). The products were analyzed by 1.5% agarose gel electrophoresis, stained with 0.5 µg/ml ethidium bromide and visualized with Quantity One quantification analysis software, version 4.5.2 Gel Doc EQ system (Bio-Rad, USA).

Results

A multiplex polymerase chain reaction (PCR) base on CytB gene was used to identify host blood meals from field-collected *Ar. subalbatus* mosquitoes. First of all, we screened the mammal's blood meal DNA in *Ar. subalbatus* using the primer UNFOR403 and UNREV1025. The result revealed that 74 of 210 mosquitoes showed apparent DNA in the blood meal (Figure 1B). Among the 74 samples, for 36 (17.14%), 11 (5.24%), 5 (2.38%), 1 (0.48%), and 21(10%) were positive with human, pig, cow, dog, and other mammals blood DNA, respectively (Figure 1A). For avian blood detection, we found 2 of 210 positive samples by using specific primers (L15557 and H16065) (Figure 1C, Table 2). *Ar. subalbatus* detected the types of vertebrate blood, which the most is human, other mammals, pig, cow, dog, and avian, respectively.

Table 1 Sequences of specific primers used for PCR amplification

Primer	5'- 3' sequence	Target host	Product size (bp)	References
Human741F	GGCTTACTTCTCTTCATTCTCTCCT	Human	334	
Pig573F	CCTCGCAGCCGTACATCTC	Pig	453	
Cow121F	CATCGGCACAAATTTAGTCG	Cow	561	
Dog368F	GGAATTGTACTATTATTCGCAACCAT	Dog	680	Kent and Norris, 2005
UNFOR403	TGAGGACAAATATCATTCTGAGG	Other mammals	623	
UNREV1025	GGTTGTCCTCCAATTCATGTTA		-	
L15557	GACTGTGACAAAATCCC[A/G/C/T]TTCCA	Avian	508	Cicero and Johnson, 2001.
H16065	GGTCTTCATCT[C/T][A/T/C]GG[C/T]TTACAAGAC		-	

Table 2 The number and percentages of blood meal identification in *Ar. subalbatus* mosquitoes by using multiplex PCR

Blood type	Mammals [n (%)]					Avian [n (%)]	Not detected
	Human	Pig	Cow	Dog	Other mammals		
	36 (17.14)	11 (5.24)	5 (2.38)	1 (0.48)	21 (10)	2 (0.95)	134 (63.81)
Total	**74 (35.24)**					**2 (0.95)**	**134 (63.81)**

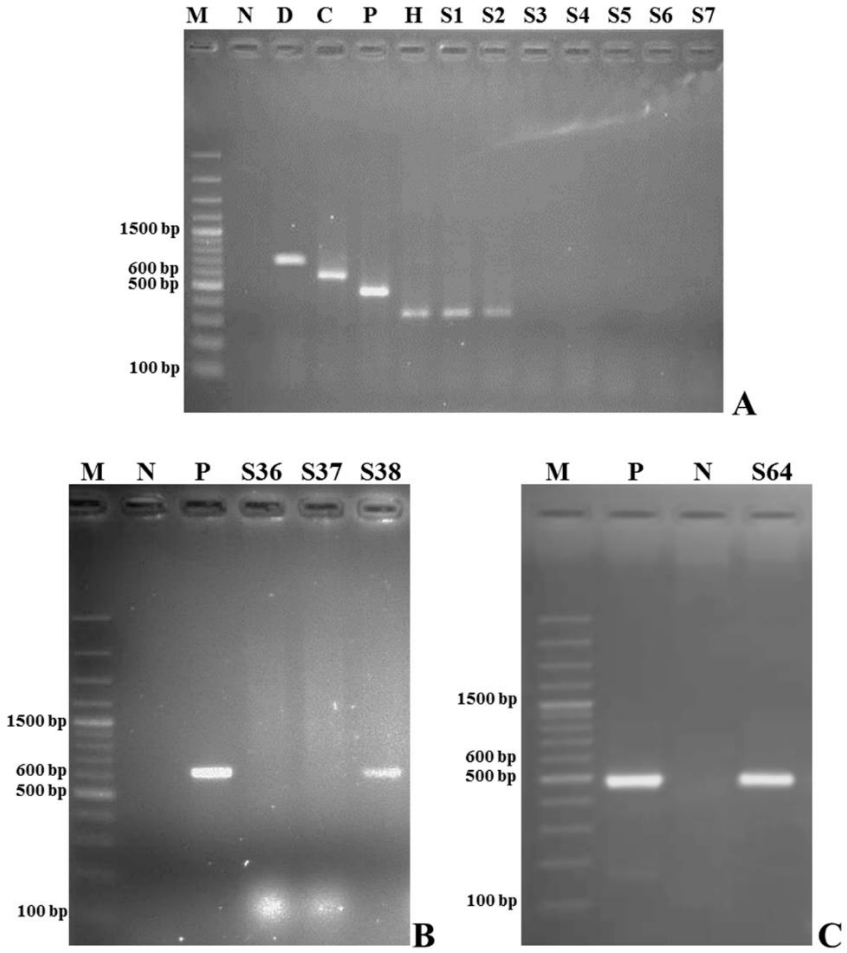

Figure 1 PCR amplification of CytB gene against Human's blood, Pig' blood, Cow' blood, Dog' blood DNA (A), Mammals' blood DNA (B), and Chicken' blood DNA (C) from *Ar. subalbatus*. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Lane D, C, P, and H: Dog' blood, Cow' blood, Pig' blood, and Human's blood, respectively, lane S1-S7, S36-S38 and S64: *Ar. subalbatus* sample, lane M: molecular mass marker (100 basepairs [bp]), lane P: positive control, lane N: negative control (no DNA template: double-distilled water)

Discussion

The blood meal source of hematophagous arthropods such as mosquitoes, ticks, stable flies, sand flies, and black flies have been detected by serological technique and molecular technique (Washino and Tempelis, 1983; Kirstein and Gray, 1996; Pitzer *et al.*, 2011; Sant' Anna *et al.*, 2008; Hunter and Bayly, 1991). Washino and Tempelis (1983) revealed that eight serological techniques: have been used to identify blood meal of mosquitoes, Ring test, Precipitin test, Agar gel diffusion, Microplate, Gel surface precipitin test, Fluorescent antibody technique FA) , Passive hemagglutination inhibition technique (PHI) , and Enzyme- linked immunosorbent assay (ELISA) . However, the predominant serological methods were precipitin test and ELISA techniques. Moreover, the molecular techniques have been used to detect a blood meal source of vector- borne disease mosquitoes, especially, *Anopheles* spp., *Culex* spp., and *Aedes* spp. (Kent, 2009). Recently, MALDI-TOF/MS technique has been used to detect blood source of blood- feeding arthropods because this technique can detect specific species (Niare *et al.*, 2016). In the advent of molecular techniques, several molecular techniques have been used to identify blood meal in some arthropods. These techniques are more specific species to identify the blood source in arthropods when compared to serological technique. Moreover, for the time course of detection, the serological technique can be detected of blood meal source of mosquitoes specimen up to 48 hours when stored at room temperature (Washino and Tempelis, 1983) while, MALDI-TOF/MS technique can be detected within 24 hours (Niare *et al*., 2016; Tandina *et al*., 2018) but molecular technique can be detected up to 96 hours at the same condition (Kent and Norris, 2005; Siriyasatien *et al*., 2010). Therefore, this study was focused on blood meal identification of *Ar. sulbabatus* by using multiplex-PCR, due to the sensitivity and specificity described previously.

Previous studies of blood meal identification by using ELISA technique of *Ar. subalbatus* have been reported in India and Thailand. The results revealed that preference host of *Ar. subalbatus* in India were human (93.33%) and cow (6.66%) respectively (Wilson and Sevarkodiyone, 2015). Similar to previous results in Thailand, showed that mixed human-monkey blood (50%) and dog blood (50%) respectively (Khaklang and Kittayapong, 2014).

This is the first study of using the molecular techniques to identify blood meal of *Ar. subalbatus* in Thailand. In the present study, we used multiplex-PCR assays for the identification of *Ar. subalbatus* blood meals. This assays can detect even a low level of host DNA in field-collected females stored at -20°C. There are reports that important factors such as the environment of mosquito collection, digestion of the blood meal, storage conditions of samples after field collection, and the methods used for detection and identification affect the detection of blood source in mosquitoes (Santos *et al.*, 2019). Molecular techniques can show false negatives when detecting the host of individual blood- fed mosquitoes because host DNA concentrations may be insufficient for detection. Kent and Norris (2005) suggested that blood meals failed during first round PCR amplification, but re-amplification PCR showed 16% of PCR products had visible band. Re- amplification was required because the host DNA was at low concentration in the source extraction. However, no positive band was found with re-amplification PCR. The *Ar. subalbatus* samples were collected outside near human residence. Therefore, our preliminary data revealed that most of the vertebrate blood type detected was human, other mammals, pig, cow, dog, and avian, respectively. No co- blood meal feeding was present in this study. The previous study in Thailand by Khaklang and Kittayapong (2014) investigated blood meals of *Armigeres* spp. collected from a tourist island, Koh Chang, Thailand. Using by ELISA technique it was determined vertebrate hosts were dog (50%) and mixed human & monkey (50%). *Ar. subalbatus* from rural areas of Southern Tamil Nadu, India showed a host preference for humans (93.33%) and cows (6.66%) using double-gel diffusion tests (DGD) (Wilson and Sevarkodiyone, 2015) . However, our study was found human, dog, and cow DNA from *Ar. subalbatus* was 17.14%, 2.38%, and 0.4%, respectively, detected by multiplex-PCR. The reasons were suspected that the detection of blood meal may be non- detection of blood meal in the mosquitoes. Moreover, the sample size of this study was quite low in number and taken from a small area**.** To increase the accuracy of blood patterns in *Ar. subalbatus*, a larger sample size and several regions might be needed**.**

Several reports showed that *Ar. subalbatus* has been mentioned as a vector of animal filariasis in Southeast Asia and it is suspected for a potential vector of *W. bancrofti* and Zika in Thailand (Cheong *et al*., 1981; Das *et al.* , 1983; Liu *et al.* , 2013; Muslim *et al.* , 2013; Thongsripong *et al*., 2013; Tawatsin *et al*., 2018). Recent report of ocular dirofilariasis in a Thai patient who reside in Bangkok, Thailand, the capital of the country has been documented (Sukudom *et al.*, 2018). In this study, we found human DNA is the most detected in this mosquito species. Therefore, this report is increasing the awareness of *Ar. subalbatus* as a potential of vector for disease transmission to human. Moreover, the human behavior has been changed such as traveling into forest areas for holidays, this would also increasing the potential risk of disease caused by a close contact between human and wild animals which would transmitted by *Ar. subalbatus* mosquitoes.

In conclusion, blood feeding pattern of *Ar. subalbatus* investigated by the molecular techniques showed that they prefer to feed on humans more than animal. The results indicated that they are more anthropophilic mosquito. Several pathogens have been found in *Ar. subalbatus* mosquitoes. The present study provides data for the future study of diseases transmitted by *Ar. subalbatus* mosquito and, therefore, the vector control strategies of some diseases especially those diseases transmitted by *Ar. subalbatus* mosquito should be re-evaluated.

Competing interests: The authors declare that they have no competing interests.

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