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Original Article

Molecular DNA identification of blood sources fed on, for Culicine mosquitoes (Diptera: Culicidae) collected in the Songkhla province, southern Thailand

Theerakamol Pengsakul[1*], Napadol Sudsom[2], Gregory Foakes[3], Kartik Bhatt[3], Marisa Eisenberg[3], and Padet Siriyasatien[4, 5]

[1] *Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

[2] *Nan Provincial Public Health Office, Mueang, Nan, 55000 Thailand*

[3] *Departments of Epidemiology and Mathematics, School of Public Health, University of Michigan, Ann Arbor, Michigan, 48109 United States of America*

[4] *Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand*

[5] *Excellence Center for Emerging Infectious Diseases, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Pathum Wan, Bangkok, 10330 Thailand*

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Abstract

Culicine mosquitoes are medically important vectors. Therefore, mosquito control measures are a crucial strategy to interrupt disease transmission. Collection of data on mosquito feeding patterns is crucial for developing an effective vector control strategy. The objective of this study was to use molecular biology methods to identify the sources of DNA in mosquito blood meals. The DNA from blood meals in the mosquito stomachs was extracted and amplified with multiplex PCR, using specific primer sets based on the mitochondrial cytochrome *b* gene, to identify the DNA sources among human, pig, goat, dog, cow, and chicken. Among the 297 mosquito samples collected in the Songkhla province of Thailand, in *Aedes* spp. mosquitoes the percentages positive for human, dog, pig, chicken, cow, a mixture of 2 vertebrate DNAs, or of 3, and negative (no identified DNA) were 61.90, 2.38, 2.38, 0.60, 0.60, 4.18, 1.20 and 26.79% respectively. In *Culex* spp. blood meals the rank order was different: fractions positive for chicken, human, dog, cow, goat, pig, a mixture of 2 or 3 vertebrate DNAs, and negative were 40.83, 10.00, 5.00, 4.17, 1.67, 0.83, 8.32, 3.32 and 25.83% respectively. This study shows that feeding behaviors of the two species differ, with most *Aedes* spp. blood meals containing human blood, while *Culex* spp. had primarily consumed chicken blood. An improved understanding of the feeding behaviors of mosquitoes could contribute to new, more effective strategies for the control of mosquito populations.

Keywords: cytochrome b gene, multiplex PCR, *Aedes* spp., *Culex* spp., blood meal

* Corresponding author.
Email address: theerakamol.p@psu.ac.th

1. Introduction

Over 3,450 species of mosquitoes have been identified globally, and 412 species among these have been described in Thailand (Chansang, Chansang, Benjaphong, & Tipyasook, 1997). The majority of mosquitoes are found in tropical areas. Only female mosquitoes feed on blood, necessary for the development of their eggs. The mosquito species may differ in their blood feeding patterns and host preferences (Busula *et al.*, 2015). On the other hand, the feeding patterns affect the transmission of vector borne diseases between humans and animals, and in turn affect the prevalence and spreading of diseases in humans.

The dynamics of a mosquito population is dependent on a variety of factors such as rainfall, geography, and human behavior. In the rainy season, the population of mosquitoes is high (Barrera, Amador, & MacKay, 2011). In addition, multiple animal hosts may contribute to the maintenance and growth of mosquito populations in certain areas. The feeding behaviors of mosquitoes that feed on blood have thus received increasing attention in epidemiological research. In 2009, Watts *et al.* determined various types of mosquito blood meals in Florida, USA, by using DNA sequencing (Watts, Fitzpatrick, & Maruniak, 2009). They found that the mosquitoes had fed on many types of animals, such as horses, cows, armadillos, deer, raccoons, rabbits and owls; but mostly preferred mammalian blood.

Immunological assays such as agar gel diffusion (Sahu, 1998), capillary precipitin test (Tempelis, 1975), and Enzyme-Linked Immunosorbent Assay (ELISA) (Beier *et al.*, 1988, Chow, Wirtz, & Scott, 1993; Hunter & Bayly, 1991), have been used to identify mosquito blood meals. While immunological techniques have been widely used, they cannot distinguish all types of mosquito blood meals because of cross-reactivity confusing serum proteins from closely related species (Siriyasatien *et al.*, 2010). Thus, immunological techniques are often used to identify sources of mosquito blood meals only up to the family or the order (Ngo & Kramer, 2003; Santiago-Alarcon, Palinauskas, & Schaefer, 2012). By contrast, polymerase chain reaction (PCR) can be used to classify the DNA found in mosquito blood meals with high accuracy of determining the species (Kent & Norris, 2005). At present, there are many types of PCR-based analyses, such as conventional PCR, allele-specific PCR (ASPCR), nested PCR, and multiplex PCR (Kent & Norris, 2005; Siriyasatien *et al.*, 2010). Multiplex PCR is convenient as it directly identifies the DNA, providing speed and cost-effectiveness to blood meal identification (Lee *et al.*, 2002). The cytochrome *b* gene acts in the electron transport chain processes of mitochondria, has an overall DNA length around 1140 bp, and can be found in all animals. This gene has distinct characteristics for each species, making it an ideal choice for identifying the blood meal sources (Jain, Brahmbhatt, Rank, Joshi, & Solanki, 2007). In this study we used multiplex PCR to identify mosquito blood meal sources from the cytochrome *b* genes.

Thailand is located in Southeast Asia, and southern Thailand has a tropical monsoon climate. This area has rainfall throughout the year and contains the highest annual rainfall regions of the country (Chufamanee & Lønholdt, 2001; Trisurat, Eawpanich, & Kalliola, 2016), making it an area favorable to the mosquitos. Thus the current study focused on Songkhla province in southern Thailand as the study area.

In this study, we collected Culicine mosquitoes in Songkla province, to study the blood feeding behavior of mosquitoes in this target area. We selected only female mosquitoes with blood in the stomach, and then extracted DNA from that blood. We then identified the blood meal sources using PCR with primers specific to human, pig, goat, dog, cow, and chicken. The results may facilitate designing mosquito control strategies locally, and inform about the blood feeding behaviors of the mosquitoes. Moreover, knowledge of the mosquito feeding targets may lead to better interventions of vector borne diseases, particularly their transmission between animals and humans. While the study contributes to the local control of mosquito borne diseases, its results and methods may have wider generality and applicability.

2. Materials and Methods

2.1 Mosquito collection

Female Culicinea mosquitoes (*Ae. aegypti, Ae. albopictus, Culex* spp. and *Mansonia* spp.) were collected from 16 districts (Mueang Songkhla, Sathing Phra, Chana, Na Thawi, Thepha, Saba Yoi, Ranot, Krasae Sin, Sadao, Na Mom, Khuan Niang, Bang Klam, Singhanakhon, Khlong Hoi Khong, Rattaphum, and Hat Yai) of the Songkhla province using a hand-held net based on the World Health Organization (WHO, 2003) guidelines, in the living areas from house to house and not collected in one place for more than 15 to 20 minutes. The time of collection was 06:00-18:00 during March to December 2014. After identification, the mosquitoes were stored in Cryo-tubes in liquid nitrogen until use.

2.2 DNA extraction

DNA from each blood sample was extracted using an E.Z.N.A. Tissue DNA Kit (OMEGA Biotek, USA) following the manufacturer's instructions, and was kept at -20°C until use. The DNA was amplified using multiplex PCR, and then the PCR product was used to identify specific vertebrate DNAs in the mosquito blood meal by using a primer set specific for human (Human741F: 5' ggcttacttctcttcattctctcct 3'), pig (Pig573F: 5' cctcgcagccgtagatctt 3'), goat (Goat894F: 5' cctaatcttagtacttgtaccattcctc 3'), dog (Dog368F: 5' ggaattgta ctattattcgcaaccat 3'), cow (Cow121F: 5' catcggcacaaatttagtcg 3'), and chicken (Chick1123R: 5' gaagaggataagtaggatggtgaag 3'), with agarose gel electrophoresis.

2.3 Multiplex PCR

Multiplex PCR was used to amplify the DNA with a mixture of group-specific primers based on the mitochondrial cytochrome *b* gene, to identify the DNA of six blood hosts: human (Human741F), pig (Pig573F), goat (Goat894F), dog (Dog368F), cow (Cow121F), and chicken (Chick1123R) as described previously (Siriyasatien *et al.*, 2010; Kent & Norris, 2005). This alternative multiplexed PCR generated a control product with UnRev1025A (5' ggttgtcctccaattcatgtta 3') and UnFor403 (5' tgaggacaaatatcattctgagg 3') for all six species, and chicken-specific product produced by UnFor1029 (5' taacctgaatcggaagccaacc 3'). The PCR products were electrophoresed in 2% agarose gel at 100 volts, stained with ethidium bromide (0.5 µg/ml) and visualized using a Uvitec UVIdoc HD2 (UVITEC Cambridge, UK).

Each PCR amplification reaction was done in a final volume of 25 µl, containing 9.5 µl double-distilled water, 12.5 µl My TaqHs Red Mix buffered (Bioline, USA), 1 µl primer (20µM of each primer: the mixed primer consisted of 0.125 µl each of Human741F, Pig573F, Goat894F, Dog368F, Cow121F, Chick1123R, UnFor1029 and UnRev1025A), and 2 µl template (DNA extract). The PCR amplification steps included initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 58°C and 1 min extension at 72°C, and a final elongation step at 72°C for 7 min. After amplification, 10 µl of the PCR product was analyzed by electrophoresis on a 2% agarose gel containing 0.5 µg/ml ethidium bromide.

2.4 Host feeding pattern analysis

The blood meal results from 2% agarose gel electrophoresis were labeled by the host sources of DNA in the blood meal, and also the mosquito species was recorded for each sample. These data were used to determine the blood feeding patterns of the collected Culicine mosquitoes.

3. Results

3.1 Mosquito collection

A total of 1,366 adult Culicine mosquitoes were collected, from which 297 samples of mosquito blood meals were obtained, with 4 genuses represented: 168 of 2 *Aedes spp*. (96 of *Ae. aegypti* and 72 of *Ae. albopictus*), 120 of 9 *Culex spp.* (60 of *Cx. quinquefasciatus,* 18 of *Cx. hutchinsoni,* 5 of *Cx. tritaeniorhynchus,* 6 of *Cx. papuensis,* 10 of *Cx. malayi,* 12 of *Cx. fuscocephala,* 3 of *Cx. gelidus,* 5 of *Cx. termi and* 1 of *Cx. foliates),* 7 of *Armigeres subalbatus* and 2 of *Mansonia* spp. (unidentified species).

3.2 DNA analysis

Among the 297 blood samples, 214 (72.1%) were positive for at least one of the host DNAs tested for, and 83 (27.9%) of the samples were negative (Figure 1). Among the 83 negative samples, 68 (22.9%) were positive for some other (not one of the tested for hosts) mammalian DNA and 15 (5.1%) of the samples were negative (Figure 2).

In a total of 168 *Aedes* spp. blood meals, 62 (36.9%) of *Ae. albopictus* and 61 (36.3%) of *Ae. aegypti*, were positive for at least one of the hosts tested, namely human, dog, pig, chicken, cow, mixture of 2 host DNAs, and mixture of 3 host DNAs, at 61.9, 2.4, 2.4, 0.6, 0.6, 4.2, and 1.2%, respectively, and 45 (26.8%) of the samples were negative (Figure 3).

In a total of 120 *Culex* spp. blood meals, 42 (35%) of *Cx. quinquefasciatus,* 15 (12.5%) of *Cx. hutchinsoni,* 11 (9.17%) of *Cx. fuscocephala,* 5 (4.2%) of *Cx. malayi,* 5 (4.2%) of *Cx. termi,* 4 (3.3%) of *Cx. tritaeniorhynchus,* 4 (3.3%) of *Cx. papuensis* and 3 (2.5%) of *Cx. gelidus,* were positive for at least one of the hosts tested, namely chicken, human, dog, cow, goat, pig, mixture of 2 host DNAs, and mixture of 3 host DNAs, at 40.83, 10.00, 5.00, 4.17, 1.67, 0.83, 8.32 and 3.32%, respectively, and 31 (25.8%) of the samples were negative (Figure 4).

These results show that the *Aedes* spp. mosquitoes tend to prefer human blood, while the *Culex* spp. mosquitoes prefer chicken blood. This information provided an understanding of the mosquito feeding behavior, and can

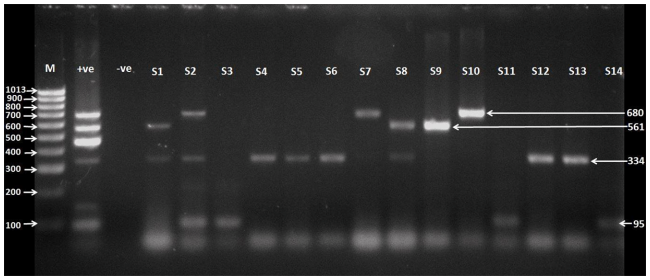

Figure 1. Ethidium bromide–stained agarose gel showing host specific cytochrome b polymerase chain reaction products, amplified from whole blood DNA extractions of blood recovered from Culicine mosquitoes that fed on it. Control products amplified from whole blood are shown in lanes S9 (cow, 561 bp) primer Cow121F, S10 (dog, 680 bp) primer Dog368F, S13 (human, 334 bp) primer Human 741F, and S14 (chicken, 95 bp) primer Chick1123.

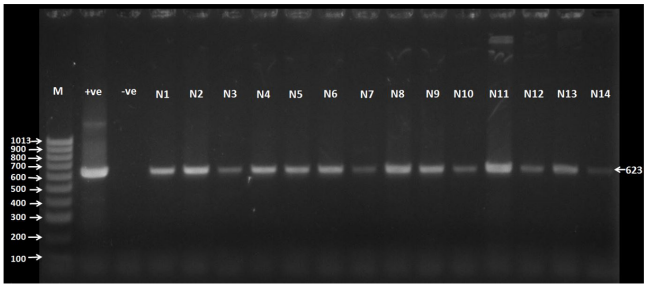

Figure 2. The alternative primers UnFor403 and UnRev1025A used in place of Human741F, Pig573F, Goat894F, Dog368F, Cow121F and Chick1123, show a 623-bp control band for all mammalian species. Lane M is for 100 bp Hyper leader, and lanes N1 to N14 are control bands.

facilitate the development of strategies to control the mosquito population.

4. Discussion

We collected two *Mansonia* spp. and seven *Armigeres* spp. mosquitoes; these are very small sample sizes and do not warrant analysis. So we focused on the blood meal DNA in *Aedes* spp. and *Culex* spp. that had much larger sample sizes, and the host animals of blood meals were different between these two genuses. The *Aedes* spp. sample consisted of two species, *Ae. aegypti* and *Ae. albopictus*, both of which primarily fed on humans, followed by dogs and pigs in the order of apparent preference. Moreover, we found blood from more than one type of host in one mosquito. These results can be assessed against a prior study at Bernalillo, New Mexico, USA (Greenberg, Lujan, Di Menna, Wearing, & Hofkin, 2013). That study reported that 96.7% of *Ae. vexans* mosquitoes preferred mammalian blood. In 1993, In the northern parts of America, 64% of the *Ae. albopictus* blood meals contained mammalian blood (Savage, Niebylski, Smith, Mitchell, & Craig, 1993). In Thailand, another study showed that most of the *Ae. aegypti* blood meals in 2001 at Chachoengsao province contained human blood (Harrington, Edman, & Scott, 2001). These studies along with the current results support that the *Aedes* spp. mosquito primarily feeds on mammalian, mainly human, blood.

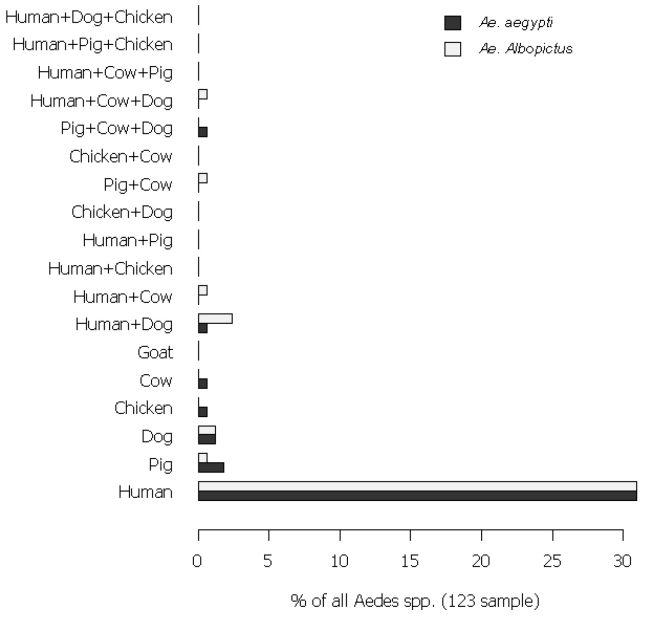

Figure 3. Comparison of blood feeding sources between *Ae. Albopictus* and *Ae. aegypti* mosquitoes.

Aedes spp. mosquitoes are the viral vector for many viruses, including Dengue virus (Failloux, Vazeille, & Rodhain, 2002), Yellow fever virus (Briscoe, 1962), Chikungunya virus (Zinser, Ramberg, & Willott, 2004), and Zika virus (Olson & Ksiazek, 1981). This study suggests that since *Aedes* spp. tends to feed on human blood, it is more likely to spread vector borne diseases in regions with high population density of humans.

For *Culex* spp. in this study, we found 8 species, namely *Cx. quinquefasciatus, Cx. hutchinsoni, Cx. tritaeniorhynchus, Cx. papuensis, Cx. malayi, Cx. fuscocephala, Cx. gelidus*, and *Cx. termi*. These eight species of *Culex* spp. had mainly fed on chicken blood, with human and dog following in rank order. These results differ from a prior study in Arizona, USA (Zinser, Ramberg, & Willott, 2004), which found that *Cx. quinquefasciatus* mostly prefers human blood (50%) followed by bird blood (32%). On the

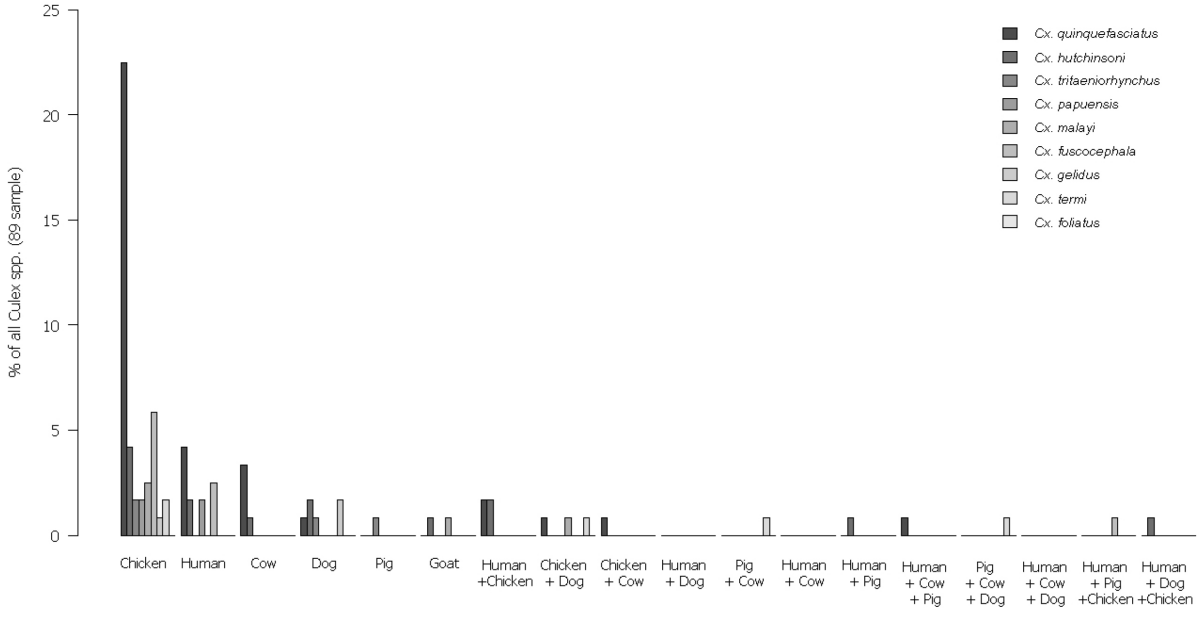

Figure 4. Comparison of identified animal source for blood found in *Culex* spp mosquitoes.

other hand, several studies also support the findings of our study, indicating that *Culex* spp. mostly feeds on avian blood. A 1990 study in California, USA, found that 99% of *Culex* spp. blood meals contained avian blood (Reisen, Meyer, Tempelis, & Spoehel, 1990), and a 1988 study in North Carolina, USA, reported the main target host of *Culex* spp. to be birds (Irby & Apperson, 1988).

The environment is an important factor affecting the blood feeding behavior of mosquitoes. In a 1992 study of *Culex* spp. blood meals, urban and forest areas were compared (Niebylski & Meek, 1992). The study found that the main feeding target in urban areas was dogs followed by birds and humans, while the main feeding target in forests was birds, followed by dogs and humans. In light of these supporting results, we suggest that *Culex* spp. prefers avian blood followed by mammalian blood, but the results will differ depending on which animals are available in the surrounding areas. *Culex* spp. in Songkhla province preferred chicken blood from the avian group. Further, it is unclear to what degree this preference simply reflects the density or availability of particular species of hosts, rather than the intrinsic preferences or attraction to one species over another.

Culex spp. is an important vector that carries diseases to human, from animals or other humans, the diseases including Japanese encephalitis virus (Takahashi, 1976) and Filariasis (*Wuchereria bancrofti*) (Omori, 1962). Since the feeding behavior of *Culex* spp. mosquitoes includes both humans and other hosts, not only are they a major culprit spreading vector borne diseases, but they may also play a role in conserving the pathogen in animal species as reservoirs, causing periodic re-emergence of infectious diseases in humans.

Mosquito blood meals were examined by multiplex PCR, using specific primers for cytochrome *b* gene, distinguishing between human, pig, goat, dog, cow, and chicken. In the cases where the host source of a blood meal was not identified, this failure can be attributed to the limited set of primers used.

5. Conclusions

Our study has provided blood feeding pattern of Culicine mosquitoes collected in Songkhla province, showing that the mosquitoes are able to feed on blood from multiple sources. This allows a disease to continue circulating in the mosquitoes and the environment, emerging repeatedly in humans. Thus, new vector control interventions must also focus on preventing other animal species from being bitten by mosquitoes. Additionally, the primers could be selected to cover a broader range of animal hosts, and this could be addressed in further studies.

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