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Transmission of the nocturnal periodic strain of *Wuchereria bancrofti* by *Culex quinquefasciatus*: establishing the potential for urban filariasis in Thailand

S. TRITEERAPRAPAB[1]*, K. KANJANOPAS[3], S. SUWANNADABBA[3], S. SANGPRAKARN[1], Y. POOVORAWAN[2] AND A. L. SCOTT[4]

[1] *Department of Parasitology and* [2] *Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*
[3] *Filariasis Division, CDC, Ministry of Public Health, Bangkok, Thailand*
[4] *The Johns Hopkins University School of Public Health, Baltimore, MD 21205*

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SUMMARY

Control programmes have reduced the prevalence of Bancroftian filariasis in Thailand to low levels. Recently, there has been an influx of more than one million Myanmar immigrants into urban centres of Thailand. The prevalence of patent *Wuchereria bancrofti* infection in these immigrants (2–5%) has prompted concern in the public health community that the potential now exists for a re-emergence of Bancroftian filariasis in Thailand. It is possible that an urban cycle of transmission could become established. The Myanmar immigrants are infected with the nocturnal periodic (urban) type *W. bancrofti* for which *Culex quinquefasciatus* serves as the main vector. The Thai strains of *Cx. quinquefasciatus* have never been reported to transmit Bancroftian filariasis. Our results of feeding experiments demonstrated that the Thai *Cx. quinquefasciatus* are permissive for the development of Myanmar *W. bancrofti* to infective third-stage larvae thus establishing the potential for establishing an urban cycle of transmission in Thailand. We also adapted the *Ssp*I repeat PCR assay for the identification of infective mosquitoes that was capable of detecting a single infective stage larvae in a pool of 100 mosquitoes.

INTRODUCTION

Bancroftian filariasis, caused by the filarial parasitic nematode *Wuchereria bancrofti*, affects about 120 million people in the tropics and subtropics [1, 2]. In Thailand, control measures have reduced the prevalence of lymphatic filariasis to 3·7 per 100000 population [3] and have limited the endemic area to provinces on the Thailand–Myanmar border (Tak, Kanjanaburi, and Mae Hong-Sorn provinces). The nocturnal subperiodic strain of *W. bancrofti* (rural type) found in the infected Thai rural population employs *Aedes niveus* group as the main mosquito vector. Recently, it has been reported that Myanmar immigrants to Thailand carry *W. bancrofti* at a prevalence of 2–5% [3, 4]. It is estimated that more than one million Myanmar migrants have settled in the large urban centres of Thailand. These infected immigrants carry the nocturnal periodic form (urban type) of *W. bancrofti* which uses *Culex quinquefasciatus* as the main vector species [5]. *Cx. quinquefasciatus* is abundant in Thai cities.

Different strains of mosquitoes within the same species complex have different capabilities for supporting filarial parasite development [6, 7]. The strains of *Cx. quinquefasciatus* found in urban environments in Thailand have never been reported to transmit

* Author for correspondence.

Bancroftian filariasis. Thus the risk of introducing filariasis to the Thai urban populations remains unclear. As in other Southeast Asian countries, *Cx. quinquefasciatus* readily breeds in urban areas of Thailand. It is therefore important to determine whether the Thai strain of *Cx. quinquefasciatus* is permissive for the development of Myanmar *W. bancrofti*. The results of feeding experiments demonstrate that the Thai urban strain of *Cx. quinquefasciatus* is competent to transmit Myanmar Bancroftian filariasis thus establishing that the urban Thai population is now at risk. To facilitate the Filariasis Control Program in surveillance and monitoring the potential mosquito vectors, we also developed and validated the PCR-based assay to detect the *Ssp*I repeat of *W. bancrofti* [8, 9] in *Cx. quinquefasciatus*.

MATERIALS AND METHODS

Human participants

The study was approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. *W. bancrofti*-infected Myanmar volunteers were identified during a survey on Bancroftian filariasis [4, 10, 11]. As almost none of the Myanmar workers speak or read Thai or English, verbal informed consent in Myanmar was obtained from each volunteer in the presence of two witnesses. We had a translator for Thai and Myanmar languages for the communication. Each individual was informed as to the purpose and scope of the study. The volunteers were also educated about lymphatic filariasis and how to prevent the disease.

All participants were recent Myanmar immigrants over 16 years of age who were working at the Mae Sot District, Tak Province, Thailand. Physical examination and blood films for detection of microfilariae were performed. The volunteers who participated in this study were all asymptomatic and microfilaremic. After the study was completed, all of the volunteers received a standard course of treatment with diethylcarbamazine. The infected volunteers were assessed for microfilarial densities before and after mosquito feeding by the thick-blood smear technique.

Rearing of mosquitoes

Cx. quinquefasciatus larvae were collected from the urban area in Mae Sot, Tak province and maintained in an insectory until they had developed into mature adult mosquitoes. The temperature in the insectory was maintained at 25 ± 2 °C with a relative humidity of 80 ± 10%. All mosquitoes had access to glucose solution.

Feeding the mosquitoes

Each feeding experiment employed 200–400 *Cx. quinquefasciatus*. After starving the mosquitoes for 24 h, female mosquitoes were allowed to feed for 30 min on the forearms of the *W. bancrofti* infected volunteers between 20.00 and 24.00 hours. Microfilarial density in each volunteer was determined in triplicate before and after mosquito feeding and were between 36 and 1503 microfilariae per ml of blood. Fed mosquitoes were collected with aspirators, placed in cages and maintained as outlined above.

Detection of *W. bancrofti* larvae in *Cx. quinquefasciatus*

At 24-h intervals after blood-feeding, the mosquitoes were dissected to determine the numbers of developing larvae and stages of development. The head, thorax and abdomen were separated, dissected and placed in normal saline solution at 37 °C. The number and stage of the *W. bancrofti* larvae that emerged from the tissues were recorded.

Extraction of DNA from mosquitoes

Extraction of DNA from heads and whole mosquitoes was performed as described [9] with modifications [8]. Briefly, mosquitoes were pooled, dried and crushed with a sterile pestle in a 1·5-ml Eppendorf tube. The crushed material was washed in buffer (0·1 M NaCl, 30 mM Tris–HCl [pH 7·8], 30 mM ethylenediaminetetraacetic acid [EDTA] 10 mM 2β-mercaptoethanol, and 0·5% Nonidet P40), centrifuged for 2 min at 12000 ***g*** at room temperature and the supernatant was discarded. To release DNA, pelleted material was treated with 0·1 M NaOH, 0·2% sodium dodecyl sulphate for 1 h at 37 °C. The solution was neutralized by the addition of 2 N HCl. After centrifugation, the DNA-containing supernatant was mixed thoroughly with 1 ml of 4·5 M guanidine isothiocyanate, 50 mM Tris–HCl (pH 6·4), 1·2% Triton X100, and 20 mM

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EDTA. The DNA was precipitated by addition of 40 μl of a silica particle suspension (Sigma) and incubation at room temperature for 10 min. Following centrifugation at 12000 ***g*** for 10 sec, the silica particles were washed twice with 4·5 M guanidine isothiocyanate in 50 mM Tris–HCl (pH 6·4). The silica pellet was dried at 56 °C for 10 min with the tube caps removed, suspended in 100 μl TE buffer (10 mM Tris–HCl [pH 8], 1 mM EDTA) and incubated at 56 °C for 10 min to elute the DNA. The supernatant was used in a polymerase chain reaction (PCR) assay to detect *Ssp*I repeats of *W. bancrofti*.

Polymerase chain reaction assay

The PCR reaction was performed with 1 or 2 μl of the mosquito/parasite DNA extract as template in a final volume of 50 μl. The reaction included 2 units of *Taq* polymerase, 400 pM each of NV1 and NV2 primers, and 200 μM of each deoxynucleotide in 50 mM KCl, 10 mM Tris–HCl (pH 9), 0·1% Triton X100, and 1·5 mM $MgCl_2$ [9]. The sequence of NV1 and NV2 were 5′-CGTGATGGCATCAAAGTAGCG-3′and 5′-CCCTCACTTACCATAAGACAAC-3′, respectively. DNA was denatured at 94 °C for 5 min, followed by 30 cycles of 15 s at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final 10 min extension at 72 °C. The 188 bp *Ssp*I PCR product was run on a 2·5% agarose gel and stained with ethidium bromide. The positive control template was *W. bancrofti* genomic DNA (kindly provided by Dr N Raghavan, the Johns Hopkins University School of Public Health, Baltimore, MD).

RESULTS

Independent of the blood levels of microfilariae, 4 days following feeding, 90–94% of the Thai strain of Myanmar *Cx. quinquefasciatus* contained first-stage larvae (L1) of the nocturnal periodic strain of *W. bancrofti*. At 9 days post-feeding 84–86% of the mosquitoes harboured second-stage larvae (L2) (data not shown). There was no correlation between the survival of mosquitoes and the microfilarial density in the blood meal (data not shown). After day 9 post-feeding, parasite development in the vector became highly asynchronous as has been reported previously [12, 13]. By 14 days post-feeding, there was a wide variation in the proportion of mosquitoes that harboured L3s and in the number of L3s per mosquito. This aspect of vector-stage development was studied in more detail.

Microfilarial density and infectivity rate

In order to study the effect that microfilarial density in the blood meal had on infective rates and on the number of L3s found per mosquito, we performed a study involving 11 infected volunteers who had microfilarial levels that ranged from 36–1503 parasites/ml of blood. The infectivity rates of L3s in *Cx. quinquefasciatus* varied between 26% and 94·4% (Fig. 1A). On average, one L3 is recovered from mosquito fed on blood meal with the lowest microfilarial density, but the average number increased to 3·6 L3/mosquito on the highest microfilarial density meal.

In general, there was a positive correlation between the percentage of *Cx. quinquefasciatus* harbouring L3s and the density of *W. bancrofti* microfilariae in the blood meal (Fig. 1A). When the microfilarial density of the blood meal increased from 36–958 Mf/ml blood, the proportion of mosquitoes harboring L3s increased 26–94·4%. There was an apparent slight decrease to 82·5% when the mosquitoes were fed on blood with a microfilarial density of 1503 per ml of blood.

Microfilarial density and the number of L3 recovered from mosquitoes

The number of L3s recovered per mosquito also increased with higher microfilarial densities (Fig. 1B). There was a significant correlation between microfilarial density and number of L3s per mosquito ($R^2 = 0{\cdot}8605$; $P < 0{\cdot}005$).

Polymerase chain reaction for detection of *W. bancrofti* from *Cx. quinquefasciatus*

To develop tools for screening field samples of mosquitoes for *W. bancrofti*, we tested whether the 188-bp *Ssp*I repeat would be amplified in the Myanmar strain of *W. bancrofti* genomic DNA. The *Ssp*I PCR assay was sensitive enough to amplify *Ssp*I repeats from a single L3 spiked into pools of 5, 10, 20 or 50 mosquitoes (data not shown). When a single infected mosquito was mixed into pools of 10, 20, 50

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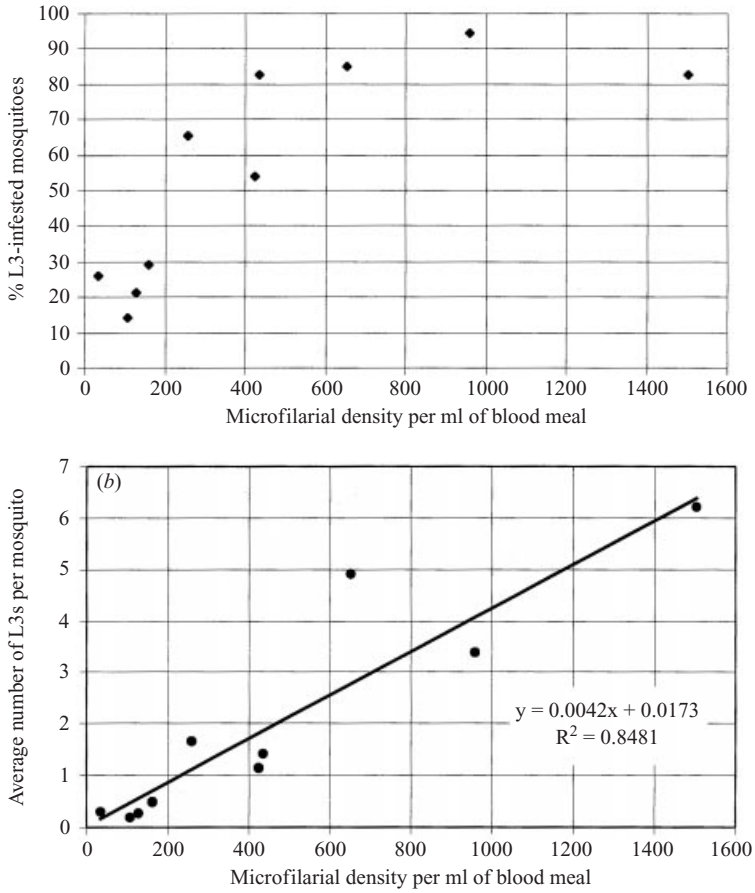

Fig. 1. A: Relationship between microfilarial densities in the blood of each volunteer and percentage of L3-infection in mosquitoes. B: Relationship between microfilarial density in blood and number of L3s per mosquito. Total of 100–400 mosquitoes were fed on each volunteer once. Microfilarial density was the average values in each volunteer before and after mosquito feeding. At 14 days after blood meal, 19–336 mosquitoes were dissected from each feed. Each data point represents one feed from each volunteer.

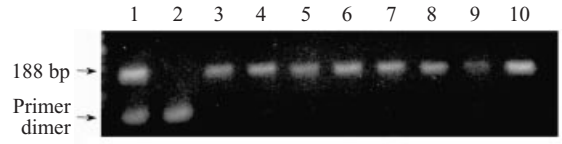

Fig. 2. Detection of *W. bancrofti* in *Cx. quinquefasciatus* by PCR asssays of pools of 50 and 100 whole mosquitoes. Lane 1, 0·1 pg *W. bancrofti* DNA as control; lane 2, DNA from 50 uninfected *Cx. quinquefasciatus*; Lanes 3 and 4, 48 uninfected mosquitoes +2 putatively infected; Lanes 5 and 6, 49 uninfected mosquitoes +1 putatively infected; Lanes 7 and 8, 98 uninfected mosquitoes + putatively infected; Lanes 9 and 10, 99 uninfected mosquitoes +1 putatively infected.

or 100 mosquitoes, the PCR assay still showed a positive result (Fig. 2). Therefore, the *Ssp*I-PCR assay could be used as a screening test for field collected mosquitoes.

DISCUSSION

This is the first report that the Thailand strain of *Cx. quinquefasciatus* is permissive for the development of the nocturnal periodic strain of Myanmar *W. bancrofti*. Each area that is endemic for Bancroftian filariasis has its particular mosquito vector that is primarily responsible for disease transmission. The results of a number of studies present contrasting views of the vectorial capacity of *Cx. quinquefasciatus*. Laboratory-reared *Cx. quinquefasciatus* in Florida, USA, is not susceptible to *W. bancrofti*, whereas *Aedes aegypti and A. taeniorhynchus* are [14]. A study in Liberia showed that *Cx. quinquefasciatus* had low susceptibility to local *W. bancrofti*, but were susceptible to East African strains of *W. bancrofti* [6]. In the Pacific islands, *Cx. quinquefasciatus* is considered a poor insect host for *W. bancrofti*, whereas the same species of mosquito seems to be a highly efficient

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vector in Africa [15]. In Papua New Guinea infective larvae are found only in *Anopheles koliensis*, but not in *An. punctulatus* or *Cx. quinquefasciatus* [16]. Interestingly, in western New Guinea, where *Cx. quinquefasciatus* is a good laboratory host, infection rates in natural populations are much higher in *An. farauti* than in *Cx. quinquefasciatus* [17]. However, regardless of geographic location, it has been suggested that *Cx. quinquefasciatus* should always be regarded as a potential vector, particularly in urban areas [18, 19].

Vector competence is determined by (1) the ability it takes up the parasite from the mammalian host; (2) the ability of the parasite to develop to the infective-stage; and (3) the ability of the vector to transmit the infective stage [20]. The data presented here demonstrated that the Thai strain of *Cx. quinquefasciatus* meets the first two determinants of vector competence. The presence of infective-stage larvae in the head of the mosquito suggests that this strain *Cx. quinquefasciatus* would be capable of transmission to a new host if given the opportunity. The infectivity indices of mosquitoes were proportional to the level of microfilariae in *W. bancrofti* (Fig. 1). The data agreed with those previously reported for *Culex* and other vector species [12, 21–24]. Therefore, it is likely that the higher the density of microfilariae in the peripheral blood of human hosts, the higher the proportion of blood feeding mosquitoes which become infected. Mosquitoes fed on very high microfilaremia have been reported to have a shorter life span than those fed on lower microfilaremia [25, 26]. However, as reported from Brazil, we did not find such adverse effects [12, 26].

In addition to early detection and prompt treatment of infected cases, verification of *W. bancrofti* in mosquito populations by a PCR assay can be used as a tool to monitor and evaluate the filariasis control programmes. The high senstitivity and specificity of PCR will help us identify *W. bancrofti* in mosquitoes more effectively and efficiently than the routine micro-dissection technique [27, 28]. The PCR assay makes it possible to test thousands of mosquitoes per day compared to 100–200 mosquitoes per day that can be surveyed by conventional micro-dissection techniques [9].

Environmental alteration associated with indiscriminate urbanization provides ample opportunities for the breeding of *Cx. quinquefasciatus* in urban areas. In India, the annual transmission potential of Bancroftian filariasis is found to be higher in urban than in rural areas [29]. With the influx of a large population of infected individuals into urban areas, *Cx. quinquefasciatus* are in place to establish an urban cycle of *W. bancrofti* transmission in Thailand.

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