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Original Research Article

Molecular xenomonitoring for the assessment of lymphatic filariasis transmission in Jaffna and Trincomalee districts in Sri Lanka

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ABSTRACT

Background: Lymphatic filariasis also known as elephantiasis is a neglected tropical disease causes by filarial parasites are transmitted to humans through infected female mosquito bite. The disease was formerly endemic in Sri Lanka and has been declared eliminated as a public health problem in 2016. However, LF transmission was not studied in detail in Jaffna and Trincomalee districts due to the long political unrest. The present study, LF transmission in mosquito was assessed by Molecular Xenomonitoring.
Methods: A prior investigation was carried out to detect anti-filarial IgG4 in urine with ELISA and circulating filarial antigen in capillary blood with Filariasis strip tests. Subjects become positive in both testes were included their house location for mosquito collection to conduct MX. Surrounding residents were selected as target for mosquito collection with CDC gravid trap. Collected mosquitoes were dried and sorted in 827 pools (≤20 mosquitoes/pool). DNA was extracted and purified from all the pools and examined by Polymerase Chain Reaction to detect filarial DNA.
Results: Total, 16,631 female *C. quinquefasciatus* mosquitoes were collected by CDC gravid traps and tested with PCR. All pools from Jaffna and Trincomalee districts were negative for *W. bancrofti* infection. But, four pools (0.67%) from Trincomalee district were positive for *B. malayi* infection which indicated possible resurgence of brugian filariasis.
Conclusions: The result suggested no evidence of infection of bancroftian filarisis. However, identification of *B. malayi* filarial DNA in post elimination phase is concerning and warrant further investigation.

Keywords: Lymphatic filariasis, Molecular Xenomonitoring, CDC gravid trap, Sri Lanka

INTRODUCTION

Many countries have adopted global programme to eliminate lymphatic filariasis (GPELF) strategies and carried out rounds of repeated annual Mass Drugs Administration (MDA). Due to multiple rounds of MDAs, LF infection rate gradually curbed down.[1,2] Some of the countries have reached elimination criteria set by World Health Organization (WHO), and others are on the track.[1] Sri Lanka has validated LF elimination as a public health problem in 2016[3]. However, Northern and Eastern provinces were not investigated for LF endemicity due to political unrest before 2009. Besides, National programme to eliminate lymphatic filariasis (PELF) in Sri Lanka needed evidence of LF transmission from unsurvey areas. For LF elimination, GPELF recommend rounds of annual MDA to interrupt transmission. After repeated rounds of annual MDAs, transmission assessment survey (TAS) used to verify interruption of LF transmission which gauze the effectiveness of MDA. Such TAS investigates filarial antigenemia among selected grade 1

and 2 primary school children which is an important surveillance tool using immunochromatographic Test (ICT) to detect CFA. However, TAS has about 95% certainty that infection rate in children is less than 2%[4]. Investigation showed, antibody testing of sentinel population and detection of filarial DNA in mosquito vector by Polymerase Chain Reaction (PCR) (Molecular Xenomonitoring/MX) were more sensitive than TAS for detecting persistence of *W. bancrofti* in Sri Lanka.[3-5] Beside this, several GPELF countries also had reported loss of sensitivity of TAS which becomes a major challenge in LF elimination effort.[6,7] In this scenario, MX can play significant role in detecting persistence of LF transmission and supplement TAS. The principle of MX included detection of filarial DNA in vector mosquito by PCR based method.[8] When mosquito effectively takes up filarial parasite from an infected individual through blood meal, the parasite can easily detect in mosquito by PCR based assay. The present study was focused on the screening of LF infection in vector *C. quinquefasciatus* mosquitos by PCR method to assess LF transmission in endemicity uncertain Jaffna and Trincomalee districts to supplement national PELF.

METHODS

A cross sectional study with purposive selection of houses for setting up of CDC gravid trap for mosquito collection was performed. The study was conducted from March 2018 to December 2019 in selected areas in Jaffna and Trincomalee districts.

Study location

The present study was conducted in Jaffna and Trincomalee districts.

Selection of houses for mosquito collection

For MX target sampling was used. Prior investigation had used a urine-based ELISA for the detection of anti filarial antibody (IgG4) among primary school children and circulating filarial antigen (CFA) was detected with immunochromatographic (ICT) strip test.[9] Houses of antibody and antigen positive subjects were targeted to place CDC gravid trap for mosquito collection. The filarial antibody and antigen positive subjects were selected as core households for mosquito collection by CDC gravid trap. Beside this, three more houses were selected from the surveyed houses closer to the positive households. Mosquito trap only set in houses having electricity supply. In case of refusal trap was placed to next home. Field survey was conducted by 3-4 groups comprising 4-5 individuals in each group. Mosquito trap was placed during dusk and collected at early morning.

Mosquito collection

Initially, two pilot entomological surveys were conducted in Jaffna and Trincomalee district to investigate abundance of LF vector *C. quinquefasciatus*. Afterwards, mosquito collection was conducted in full scale. The CDC gravid trap (Model 1712, John W. Hock. Company, Gainesville, Florida, USA) were used for mosquito collection (Figure 1). Hay infusion was used as liquid bait to attract *C. quinquefasciatus* which prefers to breed in polluted water specially blocked drainage system. The infusion was prepared from straw and water. The liquid bait for trapping was prepared as per manufacturer's instruction.

For the consent process, house head were contacted and explained the trapping procedure and objectives of mosquito collection. After getting consent from the house owner, traps were placed outdoor in shaded areas adjacent to the houses. CDC gravid traps were marked with household identification number using barcode stickers. Traps were placed to collect mosquitos from dusk (~6-7 pm) to dawn (~7-8 am) for one night only. House owner was provided instruction not to switch off the trap until field team member reach to their home and collect the trap. GPS coordinates including latitude and longitudes were taken for each house with personal digital assistants (PDA) (HP iPAQ 211, Hewlett Packard, Palo Alto, CA).

Mosquito identification and separation

Collection bags were carefully separated from the trap, and quickly transported from field to the laboratory in Faculty of Medicine, University of Jaffna. Mosquitos collected from Jaffna were knocked out by keeping at -40^{0}C freezer (Thermo Fisher, Scientific) for 10 minutes. However, due to lack of laboratory facilities, mosquitos collected from Trincomalee district were knocked out in the field with 10% ether for 10 minutes in large polythene bag. Afterwards, knocked out mosquitos were placed on white paper and sorted out. Identification of *C. quinquefasciatus* mosquito species were carried out morphologically using standard keys.[10] Key morphological features were used to identify *C. quinquefasciatus* were; Entirely dark proboscis without distinct median pale band, Tarsomeres entirely dark, Mesokatepisternum and mesepimeron without pattern of dark and pale areas, Postspiracular area without pale scale patch, Abdominal targa with basal pale bands and Scutal integument yellowish or pale brown. *C. quinquefasciatus* were observed under dissection microscope (ken-a-vision, Fisher Scientific) with 2X and 4X magnifications to confirm the morphology and separate other *Culex* mosquito species. Only gravid, semi gravid and blood feed female *C. quinquefasciatus* were sorted out and placed on clean petridish marked with trap identification number.

Drying and pooling of mosquitos

After sorting out, only female mosquitos were placed on clean petridish for drying. Drying was performed at 65°C for 3 hours in a digital oven (Thermo Scientific). The internal temperature of the oven was measured hourly

with a LCD digital oven thermometer (Thomas Scientific) to check actual internal temperature. After drying, condition of the mosquito was checked carefully, and if found completely dried up, mosquitoes were pooled in 1.5 ml microcnetrifuge tube (Eppendorf) with maximum 20 mosquitos per pool. Micro tubes were marked with respective identification number. Finally, micro tubes were sealed with paraffin film to keep them moisture free and dry, and placed them in a sealed plastic bag, and kept at -25°C until DNA were extracted and purified.

DNA extraction and purification from mosquito pools

Mosquitoes were pooled as maximum 20 mosquitos per pool. For DNA extraction and purification from *C. quinquefasciatus*, ≤20 female whole mosquitos were mixed with 180µl of 1×PBS.Then homogenized using a sterile disposable plastic homogenizer (Nippi Co. Ltd. Japan). The sludge was added to 20µl of proteinase K and 200 µl of buffer AL (QIAGEN, Germany). The mix was incubated for 70^{0}C for 10 minutes. After incubation, the mix was added with additional 20 µl of proteinase K, and again incubated for 56^{0}C for 3 hours. To sediment mosquito debris, the sample was centrifuged at 14,000 rpm (17,000 g) for 5 minutes. The supernatant was transferred into a new tube for DNA purification using QIAamp DNA Mini Kit (QIAGEN, Germany) according to manufacturer's manual.

Polymerase chain reaction to detect W. bancrofti and B. malayi DNA in mosquito pools

The extracted and purified DNA from mosquito pools were tested for *W. bancrofti* and *B. malayi* DNA by PCR assay as previously described.[11,12] 1 µl of extracted and purified DNA sample was used for the PCR assay. Two oligonucleotide primers were used for the assay namely NV-1 and NV-2. These primers amplify a fragment of 188bps, a highly repeated DNA sequence in *W. bancrofti* genome called SspI repeat and *B. malayi* genome termed HhaI. The sequences of these primers are; NV-1: 5′CGTGATGGCATCAAAGTAGCG-3′ and NV-2: 5′ CCCTCACTTACCATAAGACAAC-3′. The reaction mixture was in a total volume of 25 µl. The master mix contained, 5 µl of 5X Phusion® HF reaction buffer (New England BioLabs Inc.), 0.5 µl of 10 nM dNTPs, 0.25 µl of each primer mix (50 pmole/µl each), 0.25 µl of Phusion®HF DNA polymerase (2,000 U/ml) (New England BioLabs Inc.,), and 18 µl of nuclease free water (Sigma Aldrich). A 10 pg and 100 pg DNA of *W. bancrofti* and *B. malayi* was used as positive controls. Positives and negative PCR controls were run for every sample batch. PCR Amplification was performed in a thermal cycler (Takara, Japan) which was programmed for 40 cycle of denaturation for 94^{0}C for 1min, annealing for 55^{0}C for 1 min and extension for 72^{0}C for 1 min, preceded by an initial denaturation of 5min at 95^{0}C. After finishing of all cycles, final extension reaction was continued for 10 min. at 72^{0}C. The PCR products were analyzed by electrophoresis on 2.5% agarose gel.

RESULTS

Mosquitos from *Culex*, *Armigeres*, *Aedes* and *Toxorhynchites* genera were trapped into the collection bag. However, relative abundance of *Culex* mosquito was the highest followed by *Armigeres*. Female *C. quinquefasciatus* is the only reported vector of LF in Sri Lanka.

Table 1: Results of screening of *C. quinquefasciatus* mosquitos collected from Jaffna district by PCR assay.

Locality	No. of mosquitoes tested	No. of pools tested	No. of pools positive for *W. bancrofti*	No. of pools positive for *B. malayi*
Karaveddy	1,447	71	0	0
Jaffna	918	44	0	0
Point Pedro	813	40	0	0
Chankanai	757	37	0	0
Chavakachcheri	680	34	0	0
Total	4,615	226	0	0

Table 2: Results of screening of *C. quinquefasciatus* mosquitos collected from Trincomalee district by PCR assay.

Locality	No. of mosquitoes tested	No. of pools tested	No. of pools positive for *W. bancrofti*	No. of pools positive for *B. malayi*
Muthur	4,740	237	0	1
Trincomalee	3,458	173	0	1
Kinniya	2,040	102	0	0
Sampur	1,780	89	0	2
Total	12,018	601	0	4

Hence, priority was given on *C. quinquefasciatus* to pool them for analysis by PCR. About 16,631 gravid, semi gravid and blood fed female *C. quinquefasciatus* were collected from more than 75 trap locations in Jaffna (35) and Trincomalee (40) districts. About 12,700 (72.26%) *C. quinquefasciatus* were collected from approximately 40 different locations in Trincomalee district, and about 4,613 (27.74%) *C. quinquefasciatus* were collected from approximately 35 locations in Jaffna. Collected mosquitos were sorted in 827 pools with ≤20 mosquitos per pools. About 98% (811) pools contained 20 mosquitos. Only 2% (16) pools had less than 20 mosquitos. The maximum number of mosquitoes were placed in a pool was 20. The lowest pool size was 4. Out of 827 pools, 226 (27.33%) pools were from Jaffna and 601 (72.67%) pools were from Trincomalee. Each of the pool was marked with identification number which represents location of mosquito samples. During mosquito collection, rainfall and routine fumigation by national dengue control programme have reduced mosquito collection in Jaffna and Trincomalee districts.

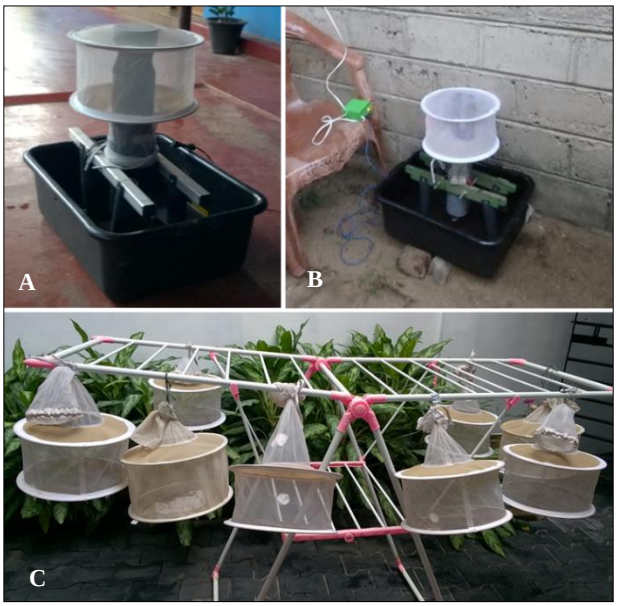

Figure 1 (A-C): Trapping and collection of mosquitoes by CDC gravid trap.

Analysis of mosquito pools by PCR

From Jaffna district, total 4,613 *C. quinquefasciatus* mosquitos were analysed in 226 pools. All the pools were tested for *W. bancrofti* and *B. malayi* infection. MX of mosquitos trapped from Jaffna did not find any evidence of LF infection. On the other hand, in Trincomalee district total 12,018 *C. quinquefasciatus* were sorted and tested in 601 pools. All 601 pools were negative for *W. bancrofti* infection. However, surprisingly, 4 pools were positive for *B. malayi* infection (Figure 2). The positive pools were from Trincomalee Town, Muthur and Sampur areas.

DISCUSSION

For assessing LF transmission and elimination progress, it is essential to monitor LF transmission in human host and vector population. LF infection in human host can be detected by assessing mircrofilaria (mf) and CFA in the blood. The detection of mf and CFA are invasive and may cause non-participation in repeated survey. For nocturnally periodic LF, blood must collect at midnight which is also logistically cumbersome and sometime expensive. Besides, it is challenging to detect mf and CFA when LF infection is very low. At a low LF transmission rate, the sensitivity of CFA detection by ICT reported to be low for LF diagnosis in human host.[4,13] In the vector population, LF detection was based on manual dissection of mosquitos which was considered as the gold standard for detecting LF infection in vector population. But, in low prevalence setting it required huge collection and dissection which is time consuming and laborious. LF prevalence cannot be accurately assessed by dissection at low transmission. The technique also depends on skilled entomologist, and it is more labor intensive and less sensitive than molecular technique.[14,15] Developed of molecular technique allowed to detect filarial parasite DNA in vector mosquitos which is termed as Molecular Xenomonitoring. MX is based on the principle of detecting filarial parasite DNA in mosquitoes by Polymerase Chain Reaction based method.

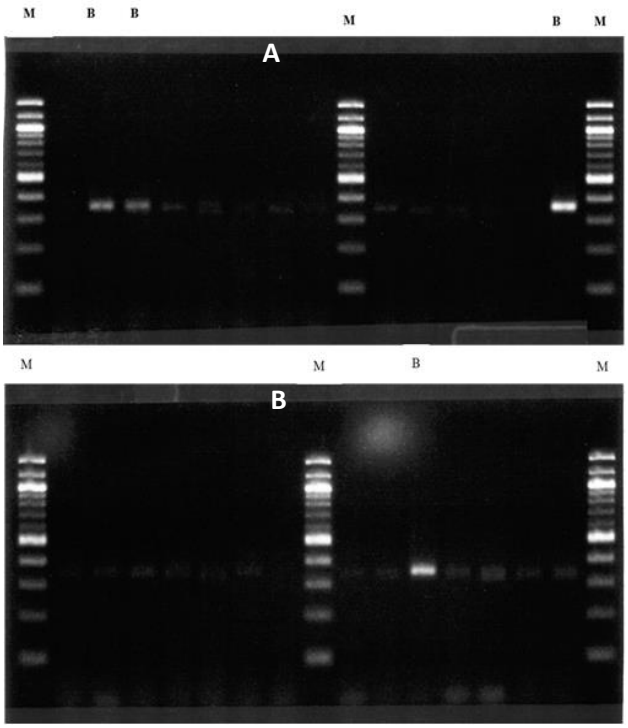

Figure 2 (A and B): *Brugia malayi* positive mosquito pools from Trincomalee district; M: 100 bp DNA ladder (New England BioLab Inc.) and B: *Brugia malayi* DNA.

MX for the detection of persistence of LF transmission

Mosquito collection is easy, non-invasive, and participant may allow repeated collection if necessary. In vector mosquitos, determination of infection rate can gauge the LF transmission status. MX is more sensitive method than mf detection for tracking LF infection.[4,16] MX provides an indirect indication of the potential for ongoing transmission of LF in low endemic areas.[17,18] Filarial DNA can be detected by MX in both vector and non-vector mosquitoes for two weeks or longer after they ingest mf positive blood.[19] MX is an indirect method of detection of transmission since ingested mf is detected not L3.[20] However, the advances of technology have allowed the specific amplification of ribonucleic acid applying reverse transcriptase PCR (RT-PCR) and made it possible to estimate infectivity ratio of mosquito.[21,22] MX can be used as an important tool for monitoring progress of LF elimination which is efficient in detecting LF transmission at low level which will help to identify hidden foci, residual transmission or hotspot for further control activities. Several studies have suggested that MX was sensitive technique to detect residual transmission and have potential to assess the success of LF elimination programme.[4,8,11,12,14,23-25] In Egypt, a series of studies revealed that, MX was used to estimate relative prevalence of *W. bancrofti* in villages with low prevalence rate.[16] Follow up study in American Samoa, highlighted the potential application of MX as a surveillance tool to track LF transmission.[24] Estimating parasite load in the vector mosquitoes has great potential for the monitoring and evaluation of LF elimination programme. WHO has also recommended the use of MX to supplement TAS.[4,26,27] In such case, MX can be used as an important tool to supplement TAS for stopping MDA and post MDA surveillance. However, critical reviews of MX have reported some drawbacks as well.[14,16] First, MX required better laboratory infrastructures, supply, and skilled personnel. Second, MX may not be a feasible method where LF is transmitted by *Anopheles, Aedes* and *Mansonia* mosquitos due to difficulty in trapping of sufficient number of mosquitoes for PCR analysis. As a result, improved mosquito technique will be necessary to collect enough mosquitoes to get more accurate estimate of infection.

Application of MX for the detection of persistence of LF transmission in Sri Lanka

WHO has validated that, Sri Lanka has eliminated LF as a public health problem. For the validation process, TAS was conducted in endemic areas using ICT test among systematically selected primary school children. Most of the areas passed TAS as per WHO criteria. However, post elimination survey of LF transmission by MX has revealed, the presence of *W. bancrofti* infection in *C. quinquefasciatus* mosquitos in some endemic areas. These areas passed TAS as per WHO criteria which means reduced sensitivity of TAS.[4] A detail study by Rao et al covering LF endemic Galle district of Southern province reported the widespread infection in vector *C. quinquefasciatus* mosquitos in coastal evaluation units (EU). MX detected higher infection rate in coastal EU than inland EU. Besides, MX of wild caught mosquitos from Ratnapura district detected *B. malayi* DNA in *C. quinquefasciatus* mosquitos (Unpublished data) which was different type of finding.

Assessment of LF transmission in Jaffna and Trincomalee districts by MX

Usually, districts are considered as Evaluation Unit (EU). In the present study, MX was used to assess LF transmission in these endemicity uncertain districts. It was essential to examine about 150-200 pools of mosquito from each EU.[4] From Jaffna district, total 4,613 *C. quinquefasciatus* mosquitos were gathered in 226 pools. All the pools were tested for *W. bancrofti* and *B. malayi* infection. MX of mosquitos trapped from Jaffna did not find any evidence of LF transmission. On the other hand, in Trincomalee district, total 12,018 *C. quinquefasciatus* were sorted and tested in 601 pools. All 601 pools were negative for *W. bancrofti* infection. However, interestingly 4 pools were positive for *B. malayi* infection, and the positive pools were from Trincomalee town, Muthur and Sampur areas. *B. malayi* transmitted by *Mansonia* spp. of mosquito was formerly endemic in certain area of Trincomalee district. A detail survey was not possible in theses area due to political unrest. MX of wild caught mosquitos from Ratnapura district also detected *B. malayi* infection in *C. quinquefasciatus* mosquitos (Unpublished data). *C. quinquefasciatus* is the only reported vector of bancroftian filariasis in Sri Lanka. Though, very few number of mosquito pools found positive for *B. malayi* infection in Trincomalee district, it may be necessary to conduct further investigation to find out, if *C. quinquefasciatus* is transmitting *B. malayi*. Such finding possibly can be explained in two ways. Firstly, probable vector shift, where *C. quinquefasciatus* might serve as secondary vector for *Brugia*. Secondly, possible zoophilic nature of *C. quinquefasciatus* mosquitos which associated with zoonotic transmission which can be a matter of concern.

Collection, identification and pooling of mosquitos were the most labour intensive and time-consuming part of MX. Large number of mosquitoes are necessary for more accurate estimation of parasitic DNA rate in mosquito when infection in low. For the present study, total 16,631 *C. quinquefasciatus* mosquitos were collected using CDC gravid trap. This trap is useful and novel tool to collect gravid mosquitoes for surveillance purposes which helped us to collect large number of mosquitoes.[28] CDC Gravid trap was designed to catch female gravid mosquito in search for an aquatic source. Gravid trap used in the present study worked very well in field condition. Many *C. quinquefasciatus* mosquitoes were trapped with very few numbers of damaged mosquitoes. The present MX study used target sampling of households (HHs) for

trapping mosquito by CDC gravid trap. The houses positive for anti-filarial antibody (IgG4), CFA and neighbour residents of the positive HHs were selected as target. By this sampling technique, about 16,631 female *C. quinquefasciatus* mosquitos were collected from 75 HHs, which were sorted in 827 pools with 20 mosquito/pool. Study in India reported to use sample of 5,000 mosquitos in 200 pools (25 mosquito/pool) from 200 systematically selected houses.[25] In Sri Lanka, MX study used approximately 7,500 mosquitos in 300 pools (25 mosquito/pool) from 150 systematically selected houses. A different sampling technique (target sampling) was used in the present study with a big sample size (16,631 mosquitos). Larger sample may be necessary from many HHs to get more accurate result, when infection is low.[25] However, it was reported that, sampling from 75 or150 HHs was not statistically inferior to sampling from 300 houses.[4,25] Sampling of optimum number of HHs played important role in cost reduction by minimizing mosquito trapping sites. Drying of collected mosquitos is one of the important steps of MX. Different time and temperature scheme had been reported by the investigator for drying of mosquitos. Given that variable time and temperature combination, a new protocol was developed for mosquito drying, where mosquito was dried at 90°C for 1 hour in a digital electric oven. The new protocol was found effective in drying mosquitoes for MX and saved valuable time.

Limitations

The study had some limitations as well. The houses were selected conveniently for the mosquito sample collection. More houses could have been selected in the villages that were antigen positive. Different protocol was used for the mosquito drying prior to sampling.

CONCLUSION

The result of the present study suggested no evidence of infection of bancroftian filarisis in vector *C. quinquefasciatus* mosquitos collected from Jaffna and Trincomalee district. However, presence of *B. malayi* infection in mosquito collected from Trincomalee district. Identification of *B. malayi* filarial DNA in *C. quinquefasciatus* in post elimination phase is concerning and warrant further surveillance to check possible reoccurrence of brugian filariasis.

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