ORIGINAL ARTICLE

Detection of *Wuchereria bancrofti* DNA in wild caught vector and non-vector mosquitoes: implications for elimination of lymphatic filariasis

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Abstract

Background Transmission Assessment Survey (TAS) is the WHO recommended method used for decision-making to stop or continue the MDA in lymphatic filariasis (LF) elimination programme. The WHO has also recommended Molecular Xenomonitoring (MX) of LF infection in vectors as an adjunct tool in settings under post-MDA or validation period. Screening of non-vectors by MX in post-MDA / validation settings could be useful to prevent a resurgence of LF infection, as there might be low abundance of vectors, especially in some seasons. In this study, we investigated the presence of LF infection in non-vectors in an area endemic for LF and has undergone many rounds of annual MDA with two drugs (Diethylcarbamazine and Albendazole, DA) and two rounds of triple drug regimens (Ivermectin + DA).

Methods and results Mosquitoes were collected from selected villages of Yadgir district in Karnataka state, India, during 2019. A total of 680 female mosquitoes were collected, identified morphologically by species and separated as pools. The female mosquitoes belonging to 3 species viz., *Anopheles subpictus*, *Culex gelidus* and *Culex quinquefaciatus* were separated, pooled, and the DNA extracted using less expensive method and followed by LDR based real-time PCR assay for detecting *Wuchereria bancrofti* infection in vector as well as non-vector mosquitoes. One pool out of 6 pools of *An. subpictus*, 2 pools out of 6 pools of *Cx. gelidus*, and 4 pools out of 8 pools of *Cx. quinquefaciatus* were found to be positive for *W. bancrofti* infection by RT-PCR. The infection rate in vectors and non-vectors was found to be 1.8% (95% CI: 0.5–4.2%) and 0.9% (95% CI: 0.2–2.3%), respectively.

Conclusions Our study showed that non-vectors also harbour *W. bancrofti*, thus opening an opportunity of using these mosquitoes as surrogate vectors for assessing risk of transmission to humans in LF endemic and post MDA areas.

Abbreviations

TAS	Transmission Assessment Survey
WHO	World Health Organization
Mf	Microfilariae
LDR	Long dispersed repeat
CFA	Circulating filarial antigen
RT-PCR	Real time Polymerase Chain Reaction
MX	Molecular Xenomonitoring
NTD	Neglected Tropical Diseases
DA	Diethyl carbamazine and Albendazole
MDA	Mass Drug Administration
ICT	Immuno Chromatographic Test
OCP	Onchocerciasis Control Programme
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase Chain Reaction
IDA	Ivermectin, Diethylcabamazine and Albendazole
GPELF	Global Programme for Elimination of Lymphatic Filariasis
MMDP	Morbidity Management and Disability Prevention
CDC	Center for Disease Control and Prevention

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Introduction

To combat one of the most debilitating diseases, lymphatic filariasis (LF), the Global Programme for Elimination of Lymphatic Filariasis (GPELF) was launched in 2000, which involved annual Mass Drug Administration (MDA) with anti-filarial drugs for parasite control and Morbidity Management and Disability Prevention (MMDP) for disease control and management [1]. In 2019, 538.1 million people were treated for lymphatic filariasis (LF) in 38 countries that implemented MDA with 2 drugs Diethylcarbamazine and Albendazole (DA) to populations at risk of the disease [1]. Recently, a combination of 3 drugs viz., Ivermectin, Diethylcarbamazine and Albendazole (IDA) was administered to 45.2 million people in 11 countries [1]. In India, IDA was extended from 4 to 16 districts to treat more than 41 million people as recommended by the World Health Organization [1] The target set by GPELF in 2000 to eliminate LF as a public health problem globally was 2020, but this target was reset to 2030 in alignment with the new Neglected Tropical Diseases (NTD) Road Map [1]. New global estimates suggest a 74% reduction in the number of infected people since the start of GPELF [1].

The GPELF realized that, even when it is succeeded in reducing infection and disease, it would need robust method(s) to know if the transmission was truly interrupted and there was no recrudescence. The immunochromatographic test (ICT) to detect the circulating parasite antigen (CFA) was extremely important in the TAS, because of its ease of use, rapidity and ability to test at any time of day, thereby averting issues with nocturnal periodicity of the principal LF parasite. Still, the GPELF realized that situation may arise towards the 'endgame' when supplementary tool(s) might be required to ensure that LF was truly eliminated. This ignited an interest to use infection in vectors, apart from that in humans, as endpoints for monitoring LF elimination [2]. WHO has recommended Molecular Xenomonitoring (MX) as an adjunct tool to the TAS, to make decisions on stopping MDA [3, 4].

Xenomonitoring (MX) is broadly defined as the detection of the pathogen in vectors, which is done conventionally by observing the presence of parasites stages in dissected vectors by microscopy. It is useful for monitoring infection in the community as it is non-invasive and does not require to contact humans. Initially xenomonitoring started in later 1970 in West Africa for assessing onchocerciasis infective larvae in blackflies for calculating annual transmission potential under the Onchocerciasis Control Programme (OCP) [2]. This technique was later adopted to LF vector surveillance and also for malaria surveillance in the communities [2]. Microscopic dissection is labour intensive and also requires well experienced technicians to dissect vectors for identification of larvae in the vectors. Later, PCR tools to detect DNA or RNA of the parasite in vectors, called Molecular xenomonitoring (MX) tools, were developed and found to be highly sensitive methods. A PCR assay developed to amplify *Ssp* I repeat sequence of 188 bp was found to be highly specific and sensitive to detect *W. bancrofti* in human blood [5] as well as in pools of vector mosquitoes, *Culex pipiens* [6]. MX is considered as a proxy for human infection in the community. The development of qPCR assays for detection of *W. bancrofti* and *B. malayi* DNA in different vectors has improved significantly since the development of a simple DNA extraction method for high-throughput detection of *W. bancrofti in* mosquitoes [7].

In India, all the 339 filariasis endemic districts are covered under LF elimination programme and MDA was stopped in 133 districts based on transmission assessment survey (TAS). With elimination goal around the corner, one can expect more number of endemic districts to move towards post-MDA/validation phases, which requires constant surveillance to ensure that the transmission interruption is sustained. It is to be noted that WHO has recommended MX to be used as a supplementary tool in assessing interruption of transmission [8–12] as it is more sensitive than TAS, particularly for post-MDA/validation surveillance. Generally, for MX of LF vectors known to transmit the filarial parasites are used as they are expected to harbour the parasites. But, our earlier studies have shown that even the mosquitoes in the LF endemic areas, that are not known to transmit the filarial infection (non-vectors) also harbour filarial parasites if they happen to feed on microfilaraemic blood, although they may not or poorly transmit the infection [13]. This opens up a possibility of using such non-vectors as surrogate vectors. Incidentally, the gravid traps used for collecting vector mosquitoes, also collect other mosquitoes which are non-vectors and hence provides an opportunity. Recently, it was reported that *Culex tritaeniorhynchus*, a vector for Japanese Encephalitis (JE) and a non-vector for LF, was found to harbour filarial parasite DNA [14]. In this study we attempted to assess the infection of filarial parasite by Real-time PCR assay in pools of wild-caught non-vector mosquitoes namely *Culex gelidus* and *Anopheles subpictus* collected from Yadgir, a LF endemic district in Karnataka state, India. The findings are discussed on the possibility of non-vectors transmitting LF infection and its implications on LF elimination.

Materials and methods

Study area

The study areas were villages in two taluks in Yadgir of Yadgir district, Karnataka state, India, having a population of 1,174,271 (as per census of 2011) and known to be endemic for bancroftian filariasis with persistent infection levels according to multicentric trial that was conducted to assess the safety, efficacy and tolerability of IDA against DA. The Mf prevalence ranged between 1.7 and 7.1% in the villages of Yadgir district [15–19]. The mosquito survey was conducted during the month of July 2019, at the time of intermittent rainy season and mosquitoes were collected from 4 wards of Yadgir urban area and four villages in and around Yadgir town (Fig. 1). The main vector for *W. bancrofti* transmission in this area is *Culex quinquefasciatus*.
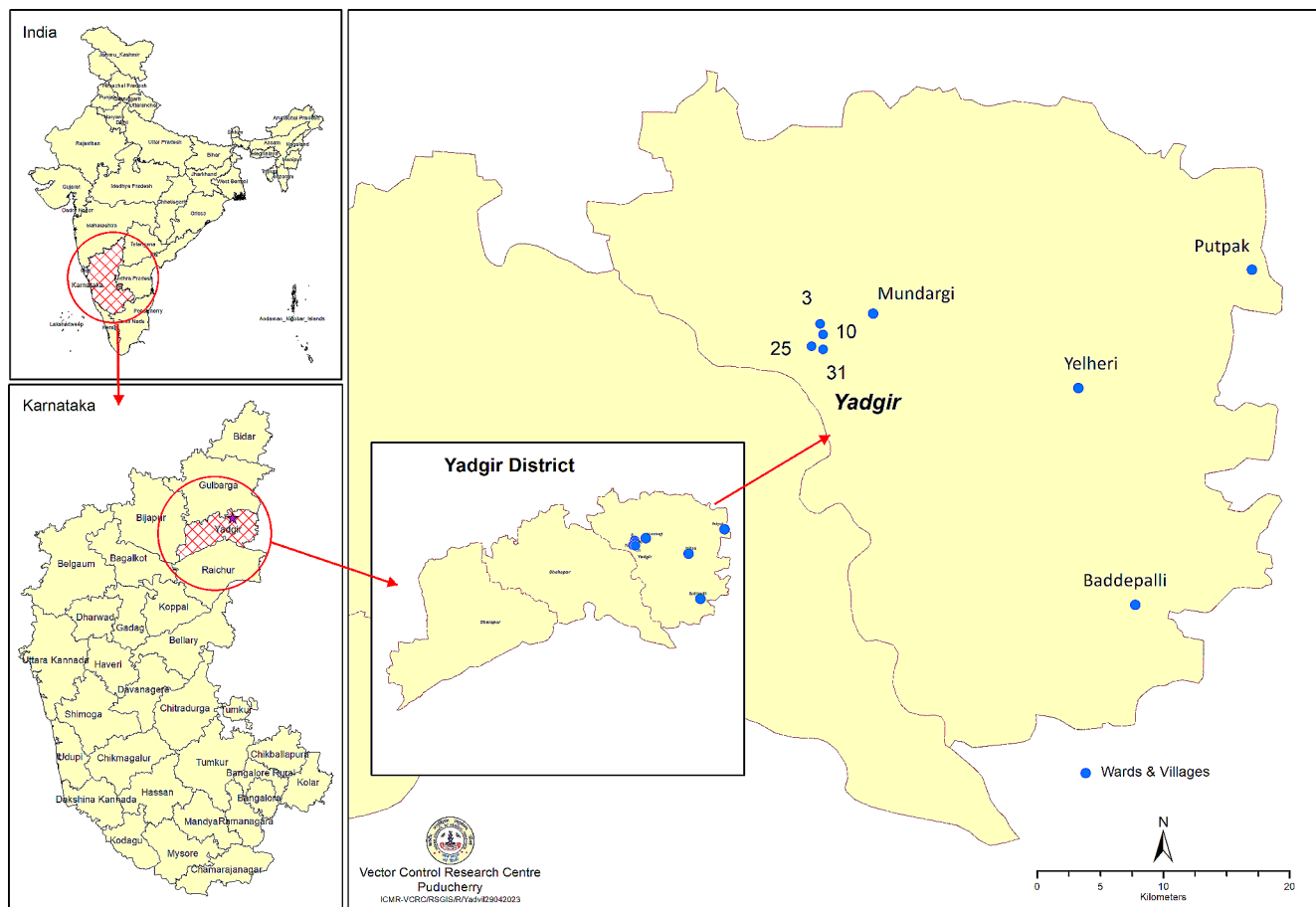
Mosquito collection

Mosquitoes were collected by using modified CDC Gravid traps with hay infusion as oviposition attractant (Subramanian et al. 2017). The traps were set up at 6.00 pm, close to houses, in the selected villages. The traps were setup in dark places near septic tanks, open bathrooms, shrubs without direct sunlight and air movement, avoiding traditional kitchens. The cloth cage was removed 6.00 am next day morning and were transported to the field laboratory for identification and segregation of mosquitoes. The mosquitoes trapped in the cage of the trap were collected using battery operated suction pump.

Mosquito identification, segregation and pooling

The collected mosquitoes were anaesthetized using ether, morphologically identified the species viz., *Culex gelidus*, *Anopheles subpictus* and *Culex quinquefasciatus* using the key [20]. Species wise, female mosquitoes were segregated as fully fed, semi gravid and gravid. These female mosquitoes were pooled (1–25 per pool) species-wise in 2.0 ml microfuge tubes and dried overnight on a dry bath at $95°C$ before DNA extraction.

Fig. 1 Location of mosquito collection sites (villages / wards) in Yadgir taluks of Yadgir district, Karnataka state, India

Table 1 Number of mosquitoes collected, pools tested and positive for *W. bancrofti* parasite DNA, and rate by mosquito species

Mosquito species	Total no of mosquitoes	Number of pools tested	Number of pools positive for *W. bancrofti* DNA	Pools positive for DNA (%)	*W. bancrofti* parasite DNA prevalance, (95% CI)
Culex quinquefasciatus	314	8	4	50.0	1.77 (0.54–4.18)
Culex gelidus	121	6	2	33.3	1.87 (0.34–5.29)
Anopheles subpictus	245	6	1	16.7	0.44 (0.02-2.00)
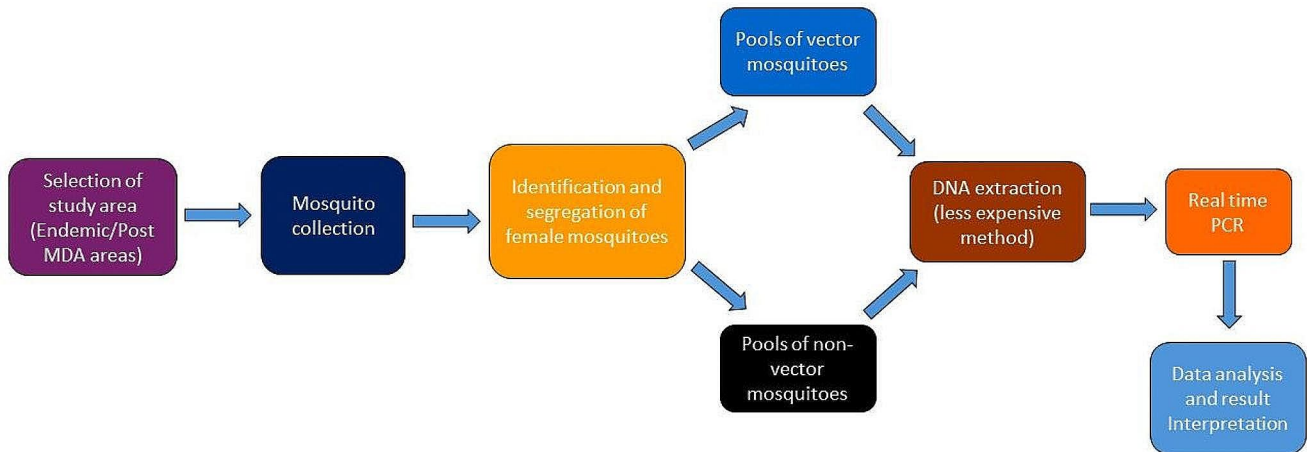
Fig. 2 Schematic representation of work flow for the screening of *W. bancrofti* infection in vector and non-vector mosquitoes

DNA extraction

DNA was extracted from pools of dried mosquitoes by TE buffer (pH 8.0) based method [21]. 100 µl of TE buffer with minor modification in which cost-effective steel ball bead (3 mm) was added instead of Zinc Ball bearing beads (4.5 mm) for homogenization using a tissue lyzer (Qiagen II) for 15 min with 25 frequencies followed by keeping the samples in boiling water bath up to 10–11 min. The homogenate was centrifuged at 14,000 rpm for 10 min and the supernatant separated and used for LDR based real time PCR assay.

Real time PCR assay

In our earlier study, DNA obtained through TE-based extraction method was suitable for successful amplification of 188-bp amplicon of *W. bancrofti* from pools of ten mosquitoes by conventional *Ssp* I PCR assay [21]. One micro litre of the extracted DNA was used as template in LDR real-time PCR (CFX96 Real time PCR system, Bio-Rad, Germany) as described earlier [22] along with 1 ng (strong positive), 100 pg (moderate positive) and 10 pg (low positive) of genomic DNA samples as positive controls and NTC (water) controls. Real-time PCR reactions were performed with 12.5 µl of Fast start essential

Probe Master Mix (Roche) along with 10 µm of "long dispersed repeat" forward primer (LDR1-5′ATTTTGAT CATCTGGGAACGTTAATA-3′; reverse primer LDR2-′ CGACTGTCTAATCCATTCAGAGTGA) and 10 µm Taq man probe (6FAMATCTGCCCATAGAAATAAC-TACGGTGGATCTGTAMRA) in a final volume of 25 µl. Cycling parameters used were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The Cycle threshold (Ct) values of samples (Table 1) ranging from 1.0 to 39.0 were considered positive, and samples that failed to reach the fluorescence threshold beyond 39 were considered indeterminate and repeated to confirm the negativity or positivity of those samples [22] (Fig. 2). The positive real-time PCR product of *W. bancrofti* were analyzed by 2% of agarose gel electrophoresis for the detection and confirmation of LDR specific band of the real-time PCR assay.

Data analysis

The percentage of pools positive (pool positivity rate) for *W. bancrofti* DNA was calculated as the ratio of number of pools positive for *W. bancrofti* DNA to total number of pools screened. The prevalence of *W. bancrofti* parasite DNA (maximum likelihood estimates with 95% confidence interval) was estimated with Pool Screen software 2.0.3 [23, 24]. (Table 1).

Results

A total of 680 mosquitoes were collected and out of this 314 were vectors of filariasis (*Cx. quinquefasciatus*), and 366 were non-vectors (245 *An. subpictus* and 121 *Cx. gelidus*) (Tables 1 and 2). The vector mosquitoes (*Cx. quinquefasciatus*) formed 46%, while non-vectors (*Cx. gelidus* and *An. subpictus*) constituted 52% of the total collections. Majority of the collections were from areas of Yadgir municipality and Baddepalli under Ajlapur Primary Health Centre (PHC), followed by Putpak village under Kandkur Community Health Centre (CHC) (Table 2). *Cx. gelidus* were collected in more numbers from 4 wards of urban areas of Yadgir municipality, and *Cx. quinquefasciatus* collected from only 2 wards. On the other hand, majority of collections from Baddepalli were *An. subpictus* with a small number of *Cx. quinquefasciatus* collections. High density of *Cx. gelidus* was observed in urban areas of Yadgir municipality whereas in the semi-urban or surrounding areas, high density of *An. subpictus* was observed. All collections from Putpak village under Kandkur CHC, Mundargi under Honagera PHC, and Yelheri under Yelheri CHC were *Cx. quinquefasciatus*.

The mosquitoes collected were grouped into 20 pools. Upon subjecting to RT-PCR assay for *W. bancrofti* infection, 4 pools out of 8 pools of *Cx. quinquefaciatus* (pool positivity rate: 50%), 2 pools out of 6 pools of *Culex gelidus (33.3%)*, and 1 pool out of 6 pools of *An. subpictus* (16.7%) were found to be positive for *W. bancrofti* infection (Table 2). However, the pool positivity rates were not significantly different (*P* value = 0.53) and corroborated with the overalapping 95% CI for parasite DNA prevalence 1.77%, (95%CI: 0.54–4.18) in *Cx. quinquefasciatus* and 1.87%, (95% CI: 0.34–5.29) in *Cx. gelidus*. The prevalence of *W. bancrofti* DNA in vectors and non-vectors was found to be 1.8% (95% CI: 0.5–4.2%) and 0.9% (95% CI: 0.2–2.3%) respectively, higher than the provisional threshold of 0.25% with upper limit of 2.3% (Table 1) indicating the risk of on-going transmission.

Discussion

India has targeted LF elimination by 2027, ahead of the global target of 2030 [25]. In view of this, surveillance and monitoring for residual infection in endemic and Post-MDA areas to assess on-going transmission is necessary, either through routine blood surveys in humans and entomological surveys for vectors.

In one of the study sites, Ward 31 of Yadgir, though pools of both the vector mosquito, *Cx. quinquefasciatus*, and non-vector mosquito, *Cx. gelidus* were positive for *W. bancrofti* infection by Real-time PCR there was no significant difference in both pool positivity rates and *W. bancrofti* parasite DNA rates. This observation indicates the possibility of transmission or circulation of *W. bancrofti* and/or the susceptibility of non-vectors in these areas. However, their role in transmission needs to be verified by the presence of infective stage (L3) of the parasites, either by dissection or by L3 stage-specific PCR assay. This finding imposes surveillance and monitoring of the local non-vectors also for LF infection.

In India, primary vector of LF is *Cx. quinquefaciatus*. The development of filarial larvae in vectors having vector competency is related to limitation and facilitation proportionally which implies microfilariae (Mf) developing into L_3. This in turn is dependent on the number of Mf actually ingested by the vectors and whether increase or decrease of L_3 larvae [26]. Vector parasite relationship limits the transmission capacity.

Most of the vector competency studies are laboratory studies based on the development of *B. malayi* larvae in mosquitoes [27, 28]. Some studies reported that non -vector transmitting the parasite in the community possibly due to sudden climatic changes which may be adopted to develop parasite larvae in the non-vector mosquitoes acting as vector in some countries [29–37].

Limited laboratory and field studies were carried out to find *B. malayi* infection in non-vector mosquitoes by dissection but not much was reported on the development

Table 2 Details of mosquito collection and Real-time PCR results

Mosquito collection place	Mosquito species	Number of mosquitoes collected	Number of pools tested	Number of pool positive
Ward No 3, Yadgir, CHC	*Culex gelidus*	22	1	0
Ward No 10, Yadgir,CHC	*Culex gelidus*	30	2	1
	Culex quinquefasciatus	44	1	0
Wod No 25, Yadgir, CHC	*Culex gelidus*	61	2	0
Ward No 31, Yadgir, CHC	*Culex gelidus*	8	1	1
	Culex quinquefasciatus	38	1	1
Baddepalli, Ajlapur,PHC	*Culex quinquefasciatus*	42	1	1
	Anopheles subpictus	245	6	1
Putpak, Kandkur CHC	*Culex quinquefasciatus*	112	3	1
Mundargi, Honagera PHC	*Culex quinquefasciatus*	41	1	0
Yelheri, Yelheri CHC	*Culex quinquefasciatus*	37	1	1

of L_3 larvae in the experiment. These reports have shown that *B. malayi* DNA was detected in *Cx. pipiens* and other *Culex* species [27, 28]. Various field studies have proved non-vectors are as competent as the vectors in transmitting *B. malayi* and *W. bancrofti* [14]. Studies elsewhere also reported that the field collected mosquitoes of various species *viz., Cx. tritaeniorhynchus* and *Cx.gelidus* as a JE vector in India [38]. In Indonesia, *B. malayi* DNA was detected in *Cx. tritaeniorhynchus* by PCR assay [14]. This was not previously considered primary vector of *B. malayi*, but this result suggested that these mosquitoes might play a role in transmitting *B. malayi* in the Balangan district, Indonesia [14]. *Anopheles gambiae* and *An. funestus* are main vectors in Ghana and a recent report showed that *An. melas* could also transmit *W. bancrofti* microfilaraemia at low level [29]. *Mansonia* species *viz., Ma. africana, Ma. uniformis*, which are non-vectors were found to harbour larvae of *W. bancrofti* larvae upon dissection and confirmed by PCR assay [30] and there was a first report in Ghana and in West Africa since in Guinea [30]. The study reported that *Anopheles* species had genetic differentiation and insecticide resistance influencing the vector density in West Africa [31]. It was found that *Culex quinquefaciatus* a non-vector harboured *W. bancrofti* larvae in Nigeria [32, 33].

Earlier report indicated that second and third stage (infective) larvae, one each were found in wild caught *An. subpictus* mosquitoes collected from Indonesia which were identified by dissection [34] *An. subpictus* was reported to be the most important vector of *W. bancrofti* on Flores, an island near Alor [35, 36] as has been seen in Yadgir. This mosquito which was earlier considered as a local non-vector has been found to naturally pick up LF infection, which also could lead to active transmission in the study area. In a similar study reported the presence of *W. bancrofti* DNA in non-vectors such as *Cx. quinquefasciatus, Ae. aegypti, Ae.* (Finalaya) *spp* and *Ae.upolensis* in American Samoa [39]. Spacio-temporal effect influences the adaptability behaviour in LF vectors *Anopheles* species of *An. arabiensis, An. gambiae, An. merus, An. melas,* and *An. funestus.* in Saharan Africa [40]. MX has been observed as a potential predictor of human filarial infection transmitted by different filarial vectors in wide-range of environmental situations [41, 42].

While Mf prevalence of 1% was found with the presence of both the species of parasites *B. malayai* and *W. bancrofti,* xenomonitoring revealed infection of vectors with parasites indicating ongoing transmission 5 years Post-MDA in Thailand [43]. Elsewhere, MX showed diurnally sub-periodic *W. bancrofti* infection in *Aedes niveus* post nine rounds of MDA in Nancowry Islands and Andaman Nicobar Islands [44]. Another study that monitored resting and feeding behaviour of vectors showed co-infection of *W. bancrofti* and *Plasmodium falciparum* in the vectors of Burkin Faso suggesting the

possibility of an integrated surveillance [45]. MX detected *W. bancrofti* in vector mosquitoes in the city of São Luís and hence the risk of transmission in the community of Brazil [46].

As both vector and non-vectors are influenced by seasonal climatic changes, a sudden surge in vector density of these species could trigger adoptability of parasite development which could lead to higher chances of picking up LF infection from infected individuals. Hence, it is necessary to have non-vectors also under vector surveillance, especially in the endgame of elimination [29–38].

Recently MX tool expanded to other vector species similar to our study that showed vector species *Anopheles* to be positive for *W. bancrofti* infection in Country Kenya [47], *Cx. quinquefasciatus* in Tanzania [48], *Anopheles i*n Coastal Kenya [49]. The assessment of various collection methods and different region LF vectors were explained [50]. Recently as per guidelines of WHO Comparative assessment of national protocol and mini-TAS were assessed in Indian settings of two non-endemic districts of Odisha. In this study MX, a non invasive method to assess LF transmission in community was found to be cost effective compared to mini-TAS [51].

This is a preliminary study in Yadgir that has made an attempt to see the possible role of local vectors and non-vectors in the transmission of LF in an endemic area. In Yadgir, in spite of several rounds of MDA the expected decline in the infection levels in humans were not achieved and this could be due to many reasons like sub-optimal coverage, high densities of the vector and local non-vectors, persisting high Mf density in infected individuals, lack of awareness about MDA etc. Added to these, this finding of the risk of non-vectors circulating parasite of LF is really a cause of concern, particularly for the local health authorities and as such for the country, in achieving the LF elimination. Studies in many countries indicated that non-vectors are acting as vectors for transmitting filariasis infection [14, 30, 31]. This finding sounds an alert that particularly in high endemic areas, routine surveillance of vector mosquitoes alone may not be sufficient and regular/frequent monitoring of local non-vectors for LF infection, will help the programme to take appropriate vector control measures to bring down the man-vector contact, particularly when there is an on-going MDA programme in place. The findings in this study also emphasize the need for the combination of both vector control alongside of MDA which will reduce the mosquito (both vector and non-vector) density thereby reducing transmission to the human hosts.

Conclusion

The findings of this study indicated the presence of LF infection in vector and non-vector mosquitoes and hint at the risk of transmission in the community. Microscopy is

the gold standard but dissection and observation of parasite larvae in mosquitoes is difficult to differentiate from mosquito muscles, and hence trained and experienced personnel is needed to avoid false negative which may lead to wrong decision in the process of elimination. MX over comes these short comings and serves a complimentry tool for assessing LF transmission in human by processing large number of mosquito samples using less expensive method of DNA extraction. The cost of DNA extraction is cheaper and is about US dollar 0.21 /per pool to detect infection in pools of vector and local non-vector mosquitoes in the endemic / resurgence in post-MDA area. The present study has hinted at the probable changes in transmission dynamics of vector and therefore on the need for the future surveillance to be focused on both vector and non-vector and also competency study, particularly in areas where the infection is persistent after many rounds of MDA. The antigen based assays are less sensitive than molecular assay. The MX could be the potential surveillance tool for vector surveillance and may also offer early signal for the parasite circulation in the community. This study suggests that vector control for seasonal vector abundance and reinforcement of surveillance activities may be helpful for LF elimination programme.

Data availability Extracted DNA and supporting data for the real time PCR assay are stored properly. The raw data used for the analysis during this study are available upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable, oral permission obtained from head of the residence for keeping Gravid trap for mosquito collection outside of the residence.

Consent for publication Not applicable.

Consent for publish Not applicable.

Competing interests The authors declare no competing interests.

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