Indian J Med Res 154, July 2021, pp 132-140
DOI: 10.4103/ijmr.IJMR_713_19

Quick Response Code:

Multi-centric evaluation of a stage-specific reverse transcriptase-polymerase chain reaction assay as a xenomonitoring tool for the detection of infective (L_3) stage *Wuchereria bancrofti* in vectors

Venkatesan Vasuki[1], Sugeerappa Laxmanappa Hoti[3], Swaminathan Subramanian[1], Abdul Mabood Khan[4], Velayudham Thenmozhi[2], Nagarajan Shriram Ananganallur[5], Namita Mahapatra[6] & Ramalingam Balasubramaniyan[1]

[1]ICMR-Vector Control Research Centre, Puducherry, [2]ICMR-Centre for Research in Medical Entomology, Madurai, Tamil Nadu, [3]ICMR-National Institute of Traditional Medicine, Belagavi, Karnataka, [4]ICMR-Regional Medical Research Centre, Dibrugarh, Assam, [5]ICMR-Regional Medical Research Centre, Port Blair, Andaman & Nicobar Islands & [6]ICMR-Regional Medical Research Centre, Bhubaneswar, Odisha, India

Received April 16, 2019

***Background & objectives*: An infective stage specific reverse transcriptase-polymerase chain reaction (RT-PCR) assay utilizing the abundant larval transcript-3 (Alt-3) gene of *Wuchereria bancrofti* was developed at ICMR-VCRC, Puducherry and found to be stage specific, and sensitive upon validation in the laboratory. This study was aimed at independently evaluating this assay for its utility as a monitoring/surveillance tool in the operational programme for elimination of lymphatic filariasis (LF) by four national research laboratories.**

***Methods*: Evaluation of the assay was carried out in a multi-centric mode in three phases. In phase I, a workshop was conducted to impart hands-on training to the scientists from the collaborating centres on the RT-PCR assay and in Phase II the assay was evaluated for specificity and sensitivity in detecting the infective (L_3) stage larvae of *W. bancrofti* in its vector, *Culex quinquefasciatus,* using 50 coded pooled samples. Phase III evaluation was done on wild-caught mosquito vectors from selected endemic areas of Assam and Bhubaneswar States and Andaman Nicobar islands.**

***Results*: Phase I data indicated that the assay was able to detect all the pools of mosquito samples contaning L_3 stage larvae of *W. bancrofti* as positive, even in the presence of other vector stages of the parasite indicating its stage specificity (100%). The assay was found highly sensitive (100%), detecting all the infected pools as positive and specific detecting all uninfected pools as negative. The results of phase II showed inter-laboratory variation. Phase III evaluation from all the centres suggested that the infectivity rate determined for pooled mosquitoes by the RT-PCR assay (0.5%) was comparable to that by dissection method (1.2%) (95% confidence interval overlaps).**

***Interpretation & conclusions*: Overall, the results from three of the four participating centres indicated that the assay is at least as sensitive and stage specific as the conventional mosquito dissection technique,**

© 2021 Indian Journal of Medical Research, published by Wolters Kluwer - Medknow for Director-General, Indian Council of Medical Research

and hence, may be useful as a xenomonitoring tool for Transmission Assessment Survey in Mass Drug Administration programmes for LF.

Key words Infective (L3) stage - L3 specific reverse transcriptase-polymerase chain reaction assay - multi-centric evaluation - *Wuchereria bancrofti*

Human lymphatic filariasis (LF) is endemic in 73 countries and territories of the tropical and subtropical regions of the globe, putting 1.39 billion people at risk of developing this debilitating disease[1]. LF continues to be an important public health problem in India, contributing about 44.3 per cent to the global burden. In an effort to eliminate LF, combined drug regimens of antifilarial drugs are being mass administered in >48 endemic countries[2]. The Global Programme to Eliminate Lymphatic Filariasis (GPELF) has two principal aims: (*i*) to interrupt LF transmission, and (*ii*) to manage morbidity and prevent disability[1]. Transmission of LF has two components: transmission from man to mosquito vector and transmission from the mosquito vector to the human host[3]. The success of the GPELF has led to a significant decrease in microfilaria and antigenemia levels in several countries that have accomplished several rounds of Mass Drug Administration (MDA)[4]. However, the current challenge is to decide on an appropriate time for cessation of MDA. Success of the global programme to eliminate LF through MDA depends on the availability of tools to monitor the outcome of the elimination efforts. The decision for cessation of MDA is complex and multiple decision support tools have been reported so far for arriving at the decision[5]. Transmission is a key parameter in this regard and can therefore be monitored and evaluated by measuring changes in infection status of either vectors or humans. Direct detection of microfilariae (Mf) of the parasite in the vector is indicative of both the presence of patent circulating Mf infections in humans and transmission of the infection from humans to the vector. Transmission of LF is a function of both the prevalence of mosquitoes with infective-stage larvae and the man-biting rate[6]. Detection as well as differentiation of the parasites and their stages in vectors is therefore essential to determine the infection (proportion of vectors harbouring any stage of the filarial parasite) and infectivity (proportion of vectors harbouring L_3 stage of the filarial parasite) rates for assessment of the transmission of filarial infection in endemic areas.

Interruption of transmission of infection is largely being monitored using human infection measures such as antigenaemia[7], microfilarial loads[6] and antifilarial antibodies[8,9]. Detection of L_3 stage larvae of filarial parasites by dissection and microscopic examination is the gold standard method to estimate the transmission levels in endemic settings[10]. However, this method is cumbersome, subjective, has low through-put and not applicable in areas with ultra-low parasite prevalence in vectors. These attributes make this method unsuitable for use in assessing large scale programmes such as GPELF.

PCR-based methods for the detection of infective (L_3) stage larvae of *Wuchereria bancrofti* and *Brugia malayi* have previously shown to be useful detection tools[10,11] Molecular xenomonitoring (MX) using PCR based detection of filarial DNA in mosquito vectors, is a sensitive and less invasive tool which can help in indirect detection of filarial infection in communities[12-14]. Infective stage-specific reverse transcriptase-PCR (RT-PCR) assays, targeting two individual L_3 specific genes [Cuticular collagen 2 (CoL2, U370160) and Abundant larval transcript-3 (Alt-3, U370163) respectively] of *W. bancrofti*, were developed at ICMR-Vector Control Research Centre (VCRC), Puducherry and validated in the laboratory for stage specificity and sensitivity[11]. These assays could detect only a minimum number (1-2) of L_3 among all other stages in a pool of 25 mosquitoes. The intensity of the positive signal obtained with pools of 25 mosquitoes with single L_3 parasite was stronger in Alt-3-based than in Col 2-based assay. Therefore, RT-PCR assay targeting Alt-3 gene was taken forward for further validation.

The sensitivity and specificity of the Alt-3 assay was found to be 98 and 100 per cent [95% confidence interval (CI): 88-100% and 91-100%] respectively. Consequently, the process of detection of the infective stage parasite of *W. bancrofti* in vector mosquitoes by Alt-3-based RT-PCR assay was patented[15] (Indian patent no.257150). However, this assay needed an independent evaluation for its utility

as a monitoring/surveillance tool in the operational programme. Therefore, the objective of this study was to evaluate the Alt-3 gene-based RT-PCR assay as a xenomonitoring tool from the perspective of end points for the programme.

Material & Methods

Evaluation of the infective stage-specific RT-PCR assay was carried out in a multi-centric mode involving four national research laboratories located in four different regions of India namely ICMR-Regional Medical Research Centre (RMRC), Dibrugarh, Assam; ICMR-RMRC, Bhubaneswar, Odisha; ICMR-RMRC, Port Blair, Andaman & Nicobar Islands; Centre for Research in Medical Entomology (CRME), Madurai, Tamil Nadu from January 2012 to August 2013.

Uninfected *Culex quinquefasciatus* mosquitoes were obtained from the laboratory colony maintained at the rearing and colonization facility at ICMR-Vector Control Research Centre (VCRC), Puducherry. Mf of *W. bancrofti* (nocturnally periodic strain) were purified from blood collected from Mf positive patients residing in and around Puducherry town, which is a known endemic area for bancroftian filariasis[16] after procuring clearance from the Institutional Ethics Committee at CRME, Puducherry. Blood smears from microfilaraemic individuals were collected from >100 individuals during 2100-2300 hr by finger prick method and examined for Mf after Giemsa's staining. About 5 ml of blood was drawn from five high-count (>20-100 Mf/20 µl) Mf carriers after obtaining a written informed consent from the study participants. Mf separation from blood was done by Percoll-gradient technique[17].

Female *Cx. quinquefasciatus* mosquitoes were fed on high-count microfilaraemic blood (about 50 Mf/cc blood) by employing the artificial membrane feeding technique[18]. After dissecting infected mosquitoes on day four, L1 (short and sausage-shaped, length: 125-150 µm diameter 10-17 µm) stage and day seven, L2 (length: 200-300 µm; diameter 15-30 µm) stage post-infection respectively, were picked up using micro-needle and stored individually in sterile microfuge tubes. Infective stage larvae, L_3, (active filiform, length: 1400 µm; diameter 20 µm) were harvested using Bearman's funnel technique on day 14 post-infection[18]. Each parasite (L_1 or L_2 or L_3 larva) was stored individually in 20 µl Trizol® (Invitrogen, USA) in a microfuge tube at −80°C.

The evaluation of the assay was carried out in three phases (Fig. 1) and the experimental design of the study was as follows:

- In phase I, a workshop was conducted to impart hands-on training to the scientists from the collaborating laboratories and their technical staff on the RT-PCR assay for detecting the infective (L_3) stage larvae of *W. bancrofti* in its vector, *Cx. quinquefasciatus*. For assessing the stage specificity of the assay, pools of mosquitoes (25 numbers per pool) each containing a mix of different stages of the parasite were prepared. The positive pools contained (in the following order): (*a*) L_3 + Mf, (*b*) $L_3 + L_1$, (*c*) $L_3 + L_2$, (*d*) L_3 alone (+ve control) and the negative pools contained (*x*) Mf + L_1, (*y*) $L_1 + L_2$, and (z) only mosquitoes (−ve control, in duplicate, coded by an external expert and subjected to L_3 specific RT-PCR assay by each participant. Thus, 8 positive pools and 6 negative pools were distributed to each Centre. Further, the participants also assessed the sensitivity of the assay on coded samples of five infected (spiked with L_3 stage larva) and five uninfected mosquitoes (laboratory reared) pools, four pools each with Mf, L_1, L_2 and L_3, along with a positive control (L_3 only) and negative control (mosquitoes only), forming a total of 7 positive and 9 negative pools and these pools were not in duplicate (unlike the previous ones).
  Coding of the samples was done by an external expert to avoid bias or subjectivity by the participant in assessing the outcome of the experiment. Decoding means the results given by the participant for the coded (unknown) samples matched with the known original sample numbers given by the external expert.
  Before analyzing the coded samples in phase II, the participating centres pre-tested the known (12) samples; five each of positive and negative pools, a positive and negative control, were provided by the VCRC to each centre.
- In phase II, 50 mosquito pools comprising 25 infected (spiked with single *W. bancrofti* L3) and 25 uninfected (without L3) pools of 25 mosquitoes each were coded by the external expert and sent to each of the participating laboratories for processing further by RT-PCR assay.
- In phase III, field surveys were conducted in the LF endemic areas (Mf rate ≥1.0) of Assam and Odisha States and Andaman & Nicobar Islands. *Cx. quinquefasciatus* mosquitoes, the vector of *W. bancrofti* were collected from different locations

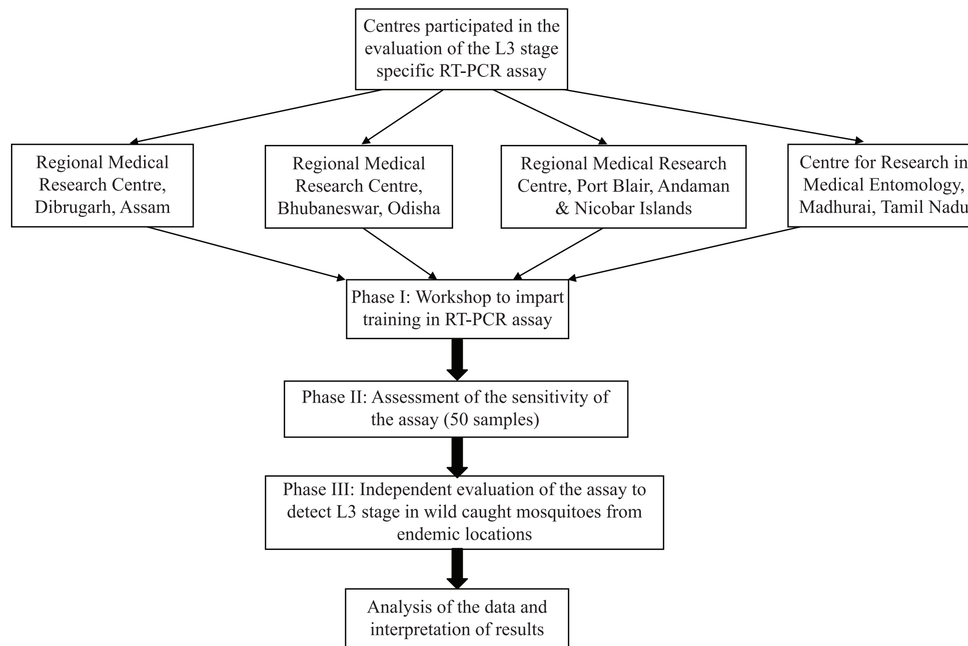

Fig. 1. Design of the multi-centric evaluation of a stage-specific RT-PCR assay for the detection of infective (L_3) stage *Wuchereria bancrofti* in vectors.

which included LF endemic tea gardens in Dibrugarh district of Assam State, endemic areas of Cuttack, Khurdha and Nayagarh districts in Odisha State, endemic areas of Thirukoyilur district in Tamil Nadu and two endemic locations *viz*. Haddo and Dairy Farm in South Andaman, Andaman & Nicobar Islands. From the selected endemic areas, indoor resting collections in households were made in the morning hours, following standard entomological procedures[19] for a period of one hour using mechanical aspirator. Collected mosquitoes were knocked down using diethyl ether, identified to species level and 25 gravid *Cx. quinquefasciatus* were pooled into a microfuge tube (1.5 ml) to which 350 µl Trizol® (Invitrogen, USA) was added. The tube was labelled, sealed with parafilm and stored at −20°C until further use. Pools of 25 mosquitoes each were processed by Alt-3-based RT-PCR assay and an equal numbers of mosquitoes were subjected to dissection and microscopy for detection of infective (L_3) stage *W. bancrofti* for assessing the transmission.

RNA extraction: Mosquito samples in 1.5 ml microfuge tubes were thoroughly homogenized with the pestle fitted to a hand-driven motor. After completing homogenization, the pestle was washed with 250 µl of Trizol® (Invitrogen, USA) and the homogenate was processed for extraction and purification of total RNA using AxyPrep^R Multi-source total RNA miniprep Kit (Axygen, USA) at room temperature following the protocol prescribed by the manufacturer with minor modifications. The extracted RNA was subsequently utilized as the template for reverse transcription for cDNA synthesis or stored at −80°C for future use.

Reverse transcription-polymerase chain reaction (RT-PCR) for cDNA synthesis: First-strand cDNA was synthesized using Sensiscript Kit (Qiagen, Germany) following the manufacturer’s instructions. An aliquot of the finished reverse-transcription reaction was taken as template to the PCR mix for the amplification of the second strand.

The PCR reaction mixture (total volume, 25 µl) included 5.5 µl of sterile MilliQ water, 12.5 µl of Go Taq green mastermix (Promega, USA), and 2.0 µl of 25 pmol of each primer based on Alt-3 gene (forward: WbL33F and reverse: WbL33R):

WbL33F: 5’- GAGTCGTTTGGTTGGGGATA-3’ and WbL33R: 5’- TCTTCTTGCCCAGTACAGCA-3’. The amplification was carried out in a Thermal Cycler GeneAmp PCR system 2720 (Applied Biosystem, USA) using the following conditions: at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and 72°C for 10 min.

A sample of 10 µl of the PCR product was electrophoresed in 2 per cent (w/v) agarose gel along

with the molecular weight marker (100 bp), stained with ethidium bromide, to check for an amplicon size of 111 bp (GenBank: EU370163) on a UV transilluminator and the results documented in a gel documentation system (UVP gelDoc-It, UK).

Data analysis: The Kappa statistic was used to measure the degree of agreement between actual and test results based on pool screen method. The Kappa coefficients were interpreted as the agreement between actual and test is 0.01-0.20 slight; 0.21-0.40 fair; 00.41-0.60 moderate; 0.61-0.80 substantial; 0.81-1.0 almost perfect. Poolscreen V2.0 software developed by Katholi *et al*[20] was used to obtain the maximum likelihood estimate of the vector infectivity rate and its 95 per cent CI from the data generated from RT-PCR assay. For the mosquitoes dissected, the infectivity rate was calculated as the percentage of mosquitoes positive for L_3 larvae. The 95 per cent CI for the infectivity rate by dissection was calculated based on the normal approximates to binomial data. The difference in estimated infectivity rates between the two methods (Poolscreen and dissection) was evaluated by comparing 95 per cent CI's for the estimates.

Results

During the workshop (Phase I), stage specificity of the assay was tested by each participant in pools of mosquitoes containing mixed stages of *W. bancrofti*. This was done to simulate the infection of mosquitoes in field condition. The results were decoded by the same expert who coded them and it was observed that all the samples that contained L_3 stage larvae, irrespective of the presence of other stages, were found positive, while all those samples that did not contain L_3 larvae were negative (Fig. 2). Thus, the assay was able to detect L3 stage larva with specificity in pools of mosquitoes even in the presence of other vector stages of the parasite indicating its stage specificity (Table IA). Further, the participants also assessed the sensitivity of the assay on coded samples and the results indicated that the assay was sensitive in detecting all the L3 infected pools as positive and specific in detecting all the negative pools as negative (Fig. 3 and Table IB).

Results of Phase II evaluation by the participating Centres after decoding showed that the results of the two laboratories were 90-92 per cent concordant, but that of the other two laboratories were less concordant (42-60%), showing inter-laboratory variation. The Kappa statistic indicated that the agreement between actual and test detected were almost ideal for two laboratories (CRME and RMRC, Dibrugarh), and moderate for all the laboratories combined. However, for two laboratories, the results were not in agreement (Table II).

Phase III evaluation was done on wild-caught mosquito vectors from selected endemic areas. Forty to 100 pools of 25 mosquitoes each (coded by a third party) were subjected to *W. bancrofti* Alt-3 RT-PCR assay by each participating centre. Simultaneously, female mosquitoes were subjected to dissection and microscopy for the detection of L_3 stage larvae of *W. bancrofti* (Table III). The results from all the centres showed that the infectivity rate estimated by PCR assay (0.5%) was comparable to that by the dissection method (1.2%; 95% CI overlaps). Centre-wise analysis showed that the infectivity rates by PCR assay did not differ significantly from that by dissection method for two of the centres (RMRC, Dibrugarh and RMRC, Port Blair) but for RMRC, Bhubaneswar it differed significantly (no overlap of 95% CI).

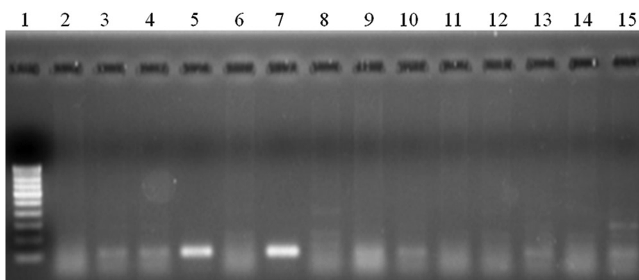

Fig. 2. Stage specificity of L_3-specific RT-PCR in detecting the infective stage *W. bancrofti* in pools of mosquitoes containing mixed stages of *W. bancrofti* in *Cx. quinquefasciatus* . Lane 1- Molecular weight marker (100 bp); lanes 2, 6, 8, 11, 12, 14 – mosquito pools with mixed stages without L_3; lanes 3, 4, 5, 7, 9, 10, 13, 15 – Mosquito pools with mixed stages including L_3.

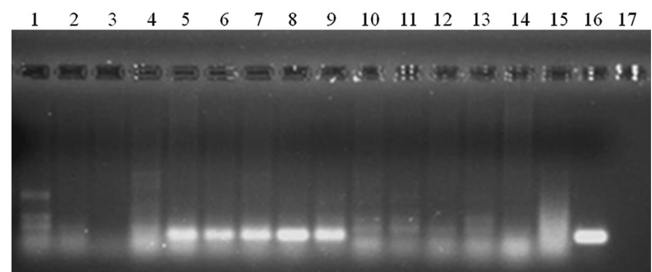

Fig. 3. Specificity and sensitivity of L_3-specific RT-PCR in detecting the infective stage *W. bancrofti* in *Cx. quinquefasciatus* by the participant. Lane 1: Molecular weight marker (100 bp); lanes 2, 3, 4; pools containing Mf, L_1, L_2; lanes 5-10: pools containing L_3 larvae; lanes 11-15: pools without L_3; lane 16: positive control (L_3 larvae; lanes 17: negative control (mosquito).

Table IA. Stage specificity of the L_3 specific reverse transcription PCR assay in detecting the infective (L_3) stage *W. bancrofti* in pools of *Cx. quinquefasciatus* mosquitoes containing mixed stages of *W. bancrofti* parasites

Name of the centre	Number of positive pools	Number detected as positive (sensitivity %)	Number of negative pools	Number detected as negative (specificity %)
CRME, Madurai	8	8 (100)	6	6 (100)
ICMR-RMRC, Dibrugarh	8	8 (100)	6	6 (100)
ICMR-RMRC, Port Blair	8	8 (100)	6	6 (100)
ICMR-RMRC, Bhubaneswar	8	8 (100)	6	6 (100)
Overall	32	32 (100)	24	24 (100)

CRME, Centre for Research in Medical Entomology; RMRC, Regional Medical Research Centre

Table IB. Sensitivity of the L_3 specific RT-PCR assay in detecting the infective (L_3) stage *W. bancrofti* in pools of *Cx. quinquefasciatus* mosquitoes

Name of the centre	Number of positive pools	Number detected as positive (sensitivity %)	Number of negative pools	Number detected as negative (specificity %)
CRME, Madurai	7	7 (100)	9	9 (100)
ICMR-RMRC, Dibrugarh	7	7 (100)	9	9 (100)
ICMR-RMRC, Port Blair	7	7 (100)	9	9 (100)
ICMR-RMRC, Bhubaneswar	7	7 (100)	9	9 (100)
Overall	28	28 (100)	36	36 (100)

Table II. Results of phase 2 evaluation of 50 samples by RT-PCR assay for detection of infective stage (L_3) larvae of *W. bancrofti* in *Cx. quinquefasciatus* vectors

Name of the centre	Number of positive pools	Number detected as positive (sensitivity %)	Number of negative pools	Number detected as negative (specificity %)	Concordant results (%)	κ statistic, K (95% CI)
CRME, Madurai	25	21 (84)	25	24 (96)	45 (90)	0.80 (0.63-0.97)
ICMR-RMRC, Dibrugarh	25	23 (92)	25	23 (92)	46 (92)	0.84 (0.69-0.99)
ICMR-RMRC, Port Blair	25	10 (40)	25	11 (44)	21 (42)	0.00 (0.00-0.11)
ICMR-RMRC, Bhubaneswar	25	15 (60)	25	15 (60)	30 (60)	0.00 (0.00-0.47)
Overall	100	69 (69)	100	73 (73)	142 (71)	0.42 (0.29-0.55)

CI, confidence interval

Discussion

MX tools are being increasingly used in the recent years *e.g.*, for filarioid helminths[21,22], multidrug malaria parasites[23] and *Trypanosoma brucei*[24]. Species specific conventional PCR assays for *W. bancrofti* and *B. malayi* were developed earlier[25,26] and subsequently, a real-time PCR assay for *W. bancrofti* was developed[27]. The latter assay has been used for assessing the post-MDA scenario in American Samao[28], Sri Lanka[29] and Togo[30]. These studies have amply elucidated the use of MX tools in the end game of GPELF. However, the species-specific PCR assay used in these studies are useful only for determining the infection rates in vectors thus leaving an iota of doubt regarding the assessment of ongoing transmission of infection in an endemic area. A tool that can detect the infective stage of the parasite and hence can measure the infectivity rate in vectors would be much rather useful. In view of this, a conventional RT-PCR based assay utilizing the infective stage-specific gene, Alt-3, for detecting infective (L_3) stage larvae of the lymphatic filarial parasite, *W. bancrofti*, in vector mosquito *Cx. quinquefasciatus* was developed[11]. This assay is based on the stage-specific primers designed based on the gene, Alt 3, upregulated in infective stage larvae of the filarial parasite, *W. bancrofti*. Molecular detection of

Table III. Evaluation of the assay on wild caught *Culex quinquefasciatus* in comparison with dissection and microscopy

Evaluating centre	Number of locations	Number of *Cx. quinquefasciatus* collected	Number of pools	Number of pools detected as positive (%)	Number of *Cx. quinquefasciatus* dissected	Number positive for L_3	Infectivity rate (%, 95% CI) by	
							PCR assay	Dissection
ICMR-RMRC, Dibrugarh	10	2500	100	19 (19)	938	13	0.84 (0.48-1.39)	1.39 (0.64-2.13)
ICMR-RMRC, Bhubaneswar	3	1000	40	2 (5)	1550	29	0.20 (0.002-0.7)	1.87 (1.2-2.5)
ICMR-RMRC, Port Blair	2	1025	41	0	1000	0	0.0	0.0
ICMR-CRME, Madurai*	-	-	-	-	-	-	-	-
Overall	15	4525	181	21	3488	42	0.50 (0.32-0.82)	1.20 (0.80-1.57)

*Not completed

L_3 stage parasites primarily included preservation and efficient extraction of RNA from parasites in pools of mosquitoes. The assay developed by us was able to detect a single L_3 parasite spiked in a pool of 25 whole mosquitoes (Table IA). However, this assay required independent multi-centric validation using wild-caught mosquitoes before being employed in the LF elimination programme. The validation was thus conducted in LF endemic areas with the participation of four laboratories, in three phases.

In phase I, assay conducted on a limited number of pooled mosquito samples showed that it was sensitive and specific. Further, the assay conducted on coded pooled mosquitoes containing mixed stages of the parasites also showed it to be stage specific, detecting the L_3 stage even in the presence of other parasite stages. In the phase II evaluation, the results of RT-PCR assays performed in the collaborating laboratories on non-coded samples (pre-test) also showed that the assay is highly sensitive and specific, detecting all positive samples as positive and negative samples as negative (Tables IA and B). Followed by this, the sensitivity and specificity of the RT-PCR assay validated on coded samples in their respective laboratory settings indicated inter-laboratory variation, which could be due to issues pertaining to storage and transportation of samples and reagents and handling of RNA extraction protocol (Table II).

The purpose of phase III evaluation was mainly to see whether the assay can be employed for detecting infective stage parasite in wild-caught vectors, while also enabling the establishment of the assay in the collaborating centres. At RMRC, Dibrugarh, the assay was evaluated on 100 pools (each containing 25 mosquitoes) from 10 different LF endemic locations and comparison with staging by dissection and microscopy. The infectivity rates obtained by both methods were statistically comparable (Table III). The investigators also tested pools of mosquitoes from non-endemic areas and those reared in the laboratory and found that both the lots were negative by RT-PCR assay. Thus, the results of the evaluation at this centre suggested that the RT-PCR is specific, sensitive and can be employed for field studies.

Similarly, this assay was conducted by RMRC, Bhubaneswar on mosquitoes collected from three endemic locations (Cuttack, Khurdha and Nayagarh), and the location-wise analysis of the data indicated that in two of the locations the infectivity rates were

comparable (95% CI overlap) with that obtained by the dissection method (Supplementary Table). However, in the vectors collected from Nayagarh, infectivity rates obtained by the two methods were not comparable (Supplementary Table). This may be due to the large difference in the number of mosquitoes assessed by the two methods. While 1550 mosquitoes were subjected to dissection and microscopy which yielded infectivity rate of 2.1 per cent (95% CI: 1.0-3.2), only 5 pools (125 mosquitoes) were subjected to RT-PCR assay (infectivity rate: 0%). Thus, the difference in infectivity rate determined by combining data from all the three locations could be due to small numbers of pools (5 pools of 25 mosquitoes each *i.e.* 125 mosquitoes) from Nayagarh subjected to PCR assay compared to that subjected to dissection.

At RMRC, Port Blair, 1025 mosquitoes (41 pools) from two locations were collected and none were found positive by RT-PCR, while dissection of 1000 mosquitoes from the same locations also indicated the absence of L_3 stage parasite in any of the mosquitoes, thus indicating that the results of the two methods agree with each other. It may be noted that these areas have undergone several rounds of MDA and the Mf rate as reported by the National Vector Borne Disease Control Programme (NVBDCP) is extremely low[31]. At CRME, Madurai, collection of mosquitoes from the endemic locations and detection of L_3 stage by RT-PCR assay could not be completed. In all the above-mentioned laboratories, the personnel were trained and the central laboratory in each centre is well established and the assay conditions standardized.

The impact of MDA in reducing the filarial infection in humans is assessed by Transmission Assessment Survey (TAS) following the protocol recommended by WHO. However, TAS cannot detect low-level infection in certain endemic areas. To achieve elimination of LF, robust surveillance and monitoring tools are needed. MX, a process of screening wild mosquitoes for parasite infection/infectivity is a useful tool to assess whether the parasite transmission is present in an endemic community, during and/or following control/elimination programmes. The MX programme results reported from recent studies[19-22] indicate the potential of MX as an alternate or supplementary tool for the enhanced disease surveillance system.

PCR based approach *i.e.*, MX to detect infection/infectivity in mosquitoes has a particular advantage of a real-time assessment of the active transmission which would be useful for evaluating the success of GPELF by monitoring the decline of transmission risk following MDA. The potential of this RT-PCR based assay to rapidly screen pools of 25 mosquitoes/tube and 40 tubes per day (about 1000 mosquitoes per day) will prove particularly valuable when the occurrence of infection in the mosquitoes falls below one per cent, whereas a technician can dissect and detect infection only about 40-50 mosquitoes per day. Thus, the ability to rapidly screen such large numbers of mosquitoes is advantageous in determining the presence or absence of infectivity (L_3) in mosquitoes in a defined location or region of the country following the completion of an LF-elimination programme. It would also provide important information regarding possible endpoints for MDA and for detecting resurgent infection following cessation of MDA as an effective surveillance tool for post MDA monitoring. The limitation of possible degradation/contamination of RNA or cDNA due to two steps involved in this assay may be overcome by converting it as a quantitative RT-PCR in real-time format with higher sensitivity. In the present study the multi-centric evaluation of stage-specific RT-PCR assay developed for detecting infective (L_3) stage larvae of the lymphatic filarial parasite, *W. bancrofti*, in vector mosquito *Cx. quinquefasciatus* showed that it is stage-specific and sensitive, and has potential for application in the assessment of transmission of LF in the national elimination programmes.

Financial support & sponsorship: This study received funding from department of Biotechnology (DBT), Government of India and Indian Council of Medical Research -Task Force, New Delhi.

Conflicts of Interest: None.

References

1. World Health Organization. Global Programme to Eliminate Lymphatic Filariasis: Progress report on mass drug administration in 2011. *WHO Wkly Epidemiol Rec* 2012; *87* : 345-56.
2. Ottesen E, Hooper PJ, Bradley M, Biswas G. The Global Programme to Eliminate Lymphatic Filariasis: Health impact after 8 years. *PLoS Negl Trop Dis* 2008; *2* : e317.
3. Paily KP, Hoti SL, Das PK. A review of the complexity of biology of lymphatic filarial parasites. *J Parasit Dis* 2009; *33* : 3-12.
4. World Health Organization. Global Programme to Eliminate Lymphatic Filariasis: Progress report on mass drug administration in 2007. *WHO Wkly Epidemiol Rec* 2008; *83* : 333-48.
5. Sunish IP, Rajendran R, Mani TR, Munirathinam A, Tewari SC, Hiriyan J, *et al*. Transmission intensity index to monitor

filariasis infectionpressure in vectors for the evaluation of filariasis elimination programmes. *Trop Med Int Health* 2003: *8* : 812-9.

6. Weil GJ, Ramzy RM. Diagnostic tools for filariasis elimination programs. *Trends Parasitol* 2007; *23* : 78-82.
7. Weil GJ, Lammie PJ, Weiss N. The ICT filariasis test: A rapid-format antigen test for diagnosis of bancroftian filariasis. *Parasitol Today* 1997; *13* : 401-4.
8. Lammie PJ, Weil G, Noordin R, Kaliraj P, Steel C, Goodman D, *et al*. Recombinant antigen-based antibody assays for the diagnosis and surveillance of lymphatic filariasis – A multi-center trial. *Filaria J* 2004; *3 :* 9.
9. Rahman A, Hwen-Yee C, Noordin R. Pan LF-ELISA using BmR1 and BmSXP recombinant antigens for detection of lymphatic filariasis. *Filaria J* 2007; *6 :* 10.
10. Intapan PM, Chomnang T, Lulitanond V, Maleewong W. Rapid detection of *Wuchereria bancrofti* and *Brugia malayi* in mosquito vectors (Diptera: Culicidae) using a real-time fluorescence resonance energy transfer multiplex PCR and melting curve analysis. *J Med Entomol* 2009; *46 :* 158-64.
11. Vasuki V, Hoti SL, Patra KP. RT-PCR assay for the detection of infective (L3) larvae of lymphatic filarial parasite, *Wuchereria bancrofti*, in vector mosquito *Culex quinquefasciatus*. *J Vector Borne Dis* 2008; *45 :* 207-16.
12. Laney SJ, Buttaro CJ, Visconti S, Pilotte N, Ramzy RM, Weil GJ, *et al*. Reverse transcriptase-PCR assay for detecting filarial infective larvae in mosquitoes. *PLoS Negl Trop Dis* 2008; *2 :* e251.
13. Schmaedick MA, Koppel AL, Pilotte N, Torres M, Williams SA, Dobson SL, *et al*. Molecular xenomonitoring using mosquitoes to map lymphatic filariasis after mass drug administration in American Samoa. *PLoS Negl Trop Dis* 2014; *8 :* e3087.
14. Subramanian S, Jambulingam P, Chu BK, Sadanandane C, Vasuki V, Srividya A, *et al*. Application of a household-based molecular xenomonitoring strategy to evaluate the lymphatic filariasis elimination program in Tamil Nadu, India. *PLoS Negl Trop Dis* 2017; *11 :* e0005519.
15. Hoti SL, Vasuki V. A process for diagnosis of infective (L3) larvae of *Wuchereria bancrofti* in vector mosquito, *Culex quinquefasciatus*. Indian Patent Number (257150) Granted On September 6, 2013.
16. Sabesan S, Vanamail P, Raju KH, Jambulingam PJ. Lymphatic filariasis in India: Epidemiology and control measures. *Postgrad Med J* 2010; *56 :* 232-3.
17. Chandrasekar R, Rao UR, Rajasekaraiah GR, Subramaniyam N. Isolation of microfilariae from blood on iso-osmotic percoll gradient. *Indian J Med Res* 1984; *79* : 497-501.
18. Paily KP, Hoti SL, Balaraman K. Development of *Wuchereria bancrofti* (Nematoda: Onchocercidae) in *Culex quinquefasciatus* (Diptera: Culicidae) after repeated feeding on microfilaraemic blood. *Trop Biomed* 1995; *12* : 73-6.
19. World Health Organization. *Lymphatic Filariasis: The Disease and Its Control. Fifth Report of the WHO Expert Committee on Filariasis. WHO Tech Rep Ser, 702*. Geneva: World Health Organization; 1979. p. 1-71.
20. Katholi CR, Toe L, Merriweather A, Unnasch TR. Determining the prevalence of *Onchocerca volvulus* infection in vector populations by PCR screening of blackflies. *J Inf Dis* 1995; *172* : 1414-7.
21. Ubleis SS, Cuk C, Nawratil M, Butter J, Schoener E, Obwaller AG, *et al*. Xenomonitoring of mosquitoes (Diptera: Culicidae) for the presence of filarioid helminthes in eastern Austria. *Can J Infect Dis Med Microbiol* 2018. doi: 10.1155/2018/9754695.
22. Rydzanicz K, Golab E, Rozej-Bielicka W, Masny A. Screening of mosquitoes for filarioid helminthes in urban areas in south western Poland-common pattern in European setaria tundra xenomonitoring studies. *Parasitol Res* 2019; *118* : 127-38.
23. Sorosjinda-Nunthawarasilp P, Bhumiratana A. Ecotope-based entomological surveillance and molecular xenomonitoring of multidrug resistant malaria parasites in Anopheles vectors. *Interdiscip Perspect Infect Dis* 2014. doi: 10.1155/2014/969531.
24. Cunningham LJ, Lingley JK, Haines LR, Ndung'u JM, Torr SJ, Adams ER. Illuminating the prevalence of *Trypanosoma brucei* s.l. in Glossina using LAMP as a tool for xenomonitoring. *PLoS Negl Trop Dis* 2016; *10* : e0004441.
25. Chanteau S, Luquiaud P, Failloux AB, Williams S. Detection of *Wuchereria bancrofti* larvae in pooled mosquitoes by the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 1994; *89* : 665-6.
26. Lizotte MR, Supali T, Felix P, Williams SA. A polymerase chain reaction assay for the detection of *Brugia malayi* in blood. *Am J Trop Med Hyg* 1994; *51* : 314-21.
27. Rao RU, Atkinson LJ, Ramzy RM, Helmy H, Farid HA, Bockarie MJ, *et al*. A real time PCR-based assay for detection of *Wuchereria bancrofti* DNA in blood and mosquitoes. *Am J Trop Med Hyg* 2006; *74 :* 826-32.
28. Lau CL, Won KY, Lammie PJ, Graves PM. Lymphatic filariasis elimination in American Samoa: Evaluation of molecular xenomonitoring as a surveillance tool in the endgame. *PLoS Negl Trop Dis* 2016; *10 :* e0004722.
29. Rao RU, Samarasekera SD, Nagodavithana KC, Punchihewa MW, Dassanayaka TD, Gamini PK, *et al*. Programmatic use of molecular xenomonitoring at the level of evaluation units to assess persistence of lymphatic filariasis in Sri Lanka. *PLoS Negl Trop Dis* 2016; *10 :* e0004722.
30. Dorkenoo MA, De Souza DK, Ramesh A, Cameron M, Spence K, Hoek Spaans R, *et al*. Molecular xenomonitoring for post-validation surveillance of lymphatic filariasis in Togo: No evidence for active transmission. *PLoS Negl Trop Dis* 2018; *11* : 52.
31. Directorate of National Vector Borne Disease Control Programme. *Elimination of lymphatic filariasis India-2013-14.* Available from: *https://nvbdcp.gov.in/WriteReadData/l892s/TAS-National-Guidelines-2013-14.pdf*, accessed on March 3, 2020.

For correspondence: Dr Sugeerappa Laxmanappa Hoti, ICMR-National Institute of Traditional Medicine, Nehru Nagar, Belagavi 590 010, Karnataka, India
e-mail: hotigood@gmail.com

Supplementary Table. Comparison of RT PCR assay results with dissection method for L_3 detection in vectors of LF in three locations of Odisha

Study site	RT-PCR assay results						Dissection results					
	No. of pools	Total No. of mosquitoes	No. of pools positive for L_3	Infectivity rate (%)	LCL	UCL	No. of dissected	L1/L2 %	No. of positive for L_3	Infectivity rate %	LCL	UCL
Cuttack	20	500	1	0.20	0.006	1.05	578	ND	8	1.38	0.43	2.34
Khurdha	15	375	1	0.28	0.009	1.41	308	ND	7	2.27	0.61	3.94
Nayagarh	5	125	0	0.00	0.000	0.00	664	ND	14	2.11	1.02	3.20
Total	40	1000	2	0.20	0.002	0.71	1550	ND	29	1.87	1.20	2.55