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RT-PCR assay for the detection of infective (L_3) larvae of lymphatic filarial parasite, *Wuchereria bancrofti*, in vector mosquito *Culex quinquefasciatus*

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Abstract

Background & objectives: Periodic monitoring of vector population for infection and infectivity rates is central to the evaluation of the filariasis elimination strategies in endemic areas to monitor the success of MDA and also to establish endpoints for intervention. The main objective of this study was to develop a RT-PCR assay, based on L_3 stage-specific primers to detect the presence of infective stage larvae of filarial parasite, *Wuchereria bancrofti* in the vector *Culex quinquefasciatus*.

Material & Methods: Subtracted probe development technique was employed for the identification of infective stage (L_3) specific genes. The subtracted cDNA was labeled by non-radioisotopic method and used for screening cDNA library of L_3 stage larvae of *W. bancrofti* constructed in UniZap XR. Recombinants were probed and identified from the library. The inserts of the recombinant clones were purified and sequenced. Primers were designed based on the sequence information of three recombinant clones for detecting L_3 larvae of *W. bancrofti* in the vector by RT-PCR assay. Preliminary laboratory evaluation was carried out to assess the sensitivity and specificity of WbL31 RT-PCR assay.

Results: cDNA library of L_3 stage of *W. bancrofti* constructed in UniZap XR vector, constituted 5×10^5 phages with 80–90% recombinant phages and the size of inserts varied from 0.1 to 1.0 kb. When subtracted cDNA was random prime labeled and used for screening cDNA library of L_3 stage of *W. bancrofti* constructed in UniZap XR, 18 clones were identified from the library. Three genes were found up-regulated in the L_3 stage, out of which WbL31 (cuticular collagen) was found to be useful in detecting L_3 larvae of *W. bancrofti* in the vector by RT-PCR assay with high specificity and sensitivity (98–100%).

Conclusion: Present paper marks first report on the development of an infective stage-specific RT-PCR assay (WbL31 RT-PCR assay) to detect L_3 stage *W. bancrofti* in the vector. This assay will have potential application in assessing the transmission of infection and hence in decision-making related to elimination programme.

Key words *Culex quinquefasciatus* – infective larva (L_3) – RT-PCR assay – *Wuchereria bancrofti*

Introduction

Lymphatic filariasis (LF) continues to be a major public health problem in tropical countries, causing severe morbidity and impediments to socioeconomic development, in spite of recent advancements in the vector control and chemotherapy. Globally, 1307 million people are at risk in 83 LF–endemic countries

and territories[1]. India alone contributes 40% of the global burden of this disease[2] and there are approximately 21 million people with symptomatic filariasis and 27 million who have asymptomatic microfilaraemia[3, 4]. Further, the disease prevalence is alarmingly the highest among the most productive age class of 15–44 years, which will affect the productivity of the affected communities[5]. WHO has identified filariasis as one of the eradicable diseases[6] and programme for elimination of this disease has been launched in different endemic countries as in India. In this context, the need to employ efficient tools to monitor the programme as well as subsequent certification is strongly felt[7,8].

Xenomonitoring, viz. the tools that assess the infection in vectors and human are useful for monitoring transmission of filarial infection. Vector infection and infectivity rates are used in the assessment of transmission and are conventionally determined by dissection and microscopic examination of vector mosquitoes for filarial parasites and their stages. This technique is cumbersome and hence not useful in large-scale surveillance and monitoring. Molecular tools (PCR assays) have been developed earlier for the detection of *Wuchereria bancrofti*[9–11], *Brugia malayi*[12–14] and *Onchocerca volvulus*[15, 16]. However, these assays are species-specific and can only differentiate the filarial species but not various stages of the parasite, namely mf, L_1, L_2 and L_3 (infective stage larvae) in vectors. Xenomonitoring, particularly using PCR to detect parasite DNA, is useful for assessing the rate at which mosquitoes get infected with mf through blood meal, while infective stage larvae (L_3) in the mosquito helps in defining the level of transmission from mosquito to humans. All the infected mosquitoes may not produce L_3 and hence may not be potentially infective. Knowing the level of infectivity in mosquitoes is very important as it gives the real time estimate of transmission. To withdraw or continue the intervention measure such as mass drug administration (MDA), an indicator of transmission such as infective stage-specific assay is highly essential to verify the absence of transmission of infection and to detect it if it reappears. Therefore, the present study was carried out to develop a RT-PCR assay for the stage-specific detection of infective stage larvae (L_3) of filarial parasite, *W. bancrofti* in vectors, which will help to assess risk and intensity of transmission.

Material & Methods

Parasite material: Microfilariae (mf) of *W. bancrofti* were purified from blood of mf positive patients residing in Puducherry town, which is a known endemic area for bancroftian filariasis[17]. Blood smears from 514 individuals were collected during 2100–2300 hrs by finger prick method and examined for mf after Giemsa's staining. About 5 ml blood was drawn from high-count (>100 mf/20 ml) mf carriers after obtaining written consent as approved by the ethical committee of the Centre. The mf were separated through Percoll-gradient centrifugation technique[18]. Around 6×10^5 mf of *W. bancrofti* were purified from the blood samples of 24 microfilaraemic human volunteers following this method.

Various developmental stages of *W. bancrofti* (L_1, L_2 and L_3 stage larvae) were raised in the vector mosquito, *Cx. quinquefasciatus,* by employing artificial membrane feeding technique[19]. *Cx. quinquefasciatus* female mosquitoes were fed on a high-count microfilaraemic blood using this technique and dissected at various intervals. The L_1 and L_2 stage larvae were picked up after dissecting infected mosquitoes respectively on four and seven days post-infection. The infective stage larvae (L_3) were harvested using Bearman's funnel technique on Day 14 post-infection[19]. About 150 mf, 100 L_1, 200 L_2 and 3000 L_3 larvae were obtained through this method from a total of 2000 fed female mosquitoes. The remaining mosquitoes with various stages of parasite and an equal number of uninfected ones were preserved in TRI^R reagent (MRC, U.S.A.) for extraction of RNAs. The parasite material was stored in TRI^R reagent solution at –150°C and used for cDNA library construction.

Extraction of RNA: The mRNAs from different stages of filarial parasites were extracted using QuickPrep mRNA purification kit (Pharmacia Biotech, U.S.A.) as per the procedure suggested by the manufacturer. The mRNA pellet was dissolved in DEPC treated water and used immediately for construction of cDNA library or stored at –150°C for other purposes. The total RNA from infected/ uninfected mosquitoes were purified by using TRI^R reagent (Invitrogen, U.S.A.) as follows: mosquitoes were homogenized in 1 ml of TRI^R reagent, the homogenate was added with 0.2 ml of chloroform, vortexed briefly, stored at room temperature for 15 min and then centrifuged at 12,000 g for 15 min. The aqueous layer was transferred to a new tube for RNA extraction and added with 0.5 ml of isopropanol, mixed briefly, spun at 12,000 g for 15 min at room temperature and the pellet was washed with 75% ethanol. The pellet was dissolved in DEPC treated water and stored at –150°C until further use.

Construction of L_3 cDNA library: The mRNA (3 µg) purified by the above method was subjected to first strand synthesis driven by the random hexamer and cloned Murine Reverse Transcriptase using TimeSaver cDNA synthesis kit (Pharmacia Biotech, USA). The second strand was synthesized using second strand mix, which contained both RNAase H and DNA polymerase. The cDNA produced was extracted with phenol/chloroform and purified on a spun-column. EcoR I/Not I adaptors were ligated to the end of the cDNA, phosphorylated by Polynucleotide kinase and the unligated adaptors were removed by passing through spun-column. The ligated cDNA were precipitated, cloned into UniZAP XR (Stratagene) vector at EcoR I site and plated on XL1 Blue cells in the presence of X-gal and IPTG.

Preparation of subtracted cDNA: To identify infective stage (L_3) specific genes, the use of subtracted probe development technique was followed. In this method, mRNA was isolated from about 1000 L_3 larvae of *W. bancrofti* and the first strand of cDNA was synthesized using Subtractor kit (Invitrogen, La Jolla, California). The mRNA from 5000 mf of the same parasite were isolated and allowed to hybridize to the first strand cDNA of L_3 stages. The cDNA-RNA hybrids of L_3 stage were alkali treated to remove the template mRNA and then hybridized to the excess of photobiotinylated mRNA of mf stages. The resulting photobiotinylated mRNA-cDNA hybrids were complexed with free streptavidin and extracted from the hybridization mixture by selective phenol-chloroform mixture, leaving behind the unhybridized cDNA, which was the 'subtracted cDNA'.

Probe preparation and labeling: The subtracted cDNA was labeled by non-radioisotopic method using ECL random-prime labeling-detection system version II (Amersham Life Science, U.S.A.). The non-radioactive probes were prepared by labeling with fluorescein-11-dUTP. The cDNA was denatured by heating for 5 min and then chilling, followed by labeling using a reaction mix containing Klenow fragment, dNTPs, random primers, at 37°C for 1 h. After terminating the reaction, the labeled probes were used for screening.

Screening of the L_3 cDNA library of W. bancrofti: The cDNA libraries were plated using *Escherichia coli* (XL1 Blue) following the standard procedures[20]. Nitrocellulose membranes were placed on the top of the agar surface and the orientation was marked using a sterile needle and removed after 1 min and placed with the colony side up, on a pad of absorbent filter paper soaked in denaturing solution (1.5 M NaCl, 0.5M NaOH) for 7 min. Then the filter was placed in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCL pH 7.2, 0.001 M EDTA) for 3 min and repeated thrice with fresh neutralizing solution absorbed paper. Finally, the membranes were washed in 2x SSC, air-dried and UV cross-linked at 312 nm wave length for 2 min. Then the membranes were used for non-radioisotope hybridization using fluorescein-11-dUTP labeled probes, following the instruction of ECL kit (Amersham Life Science,

U.S.A.). Briefly, the membranes were pre-hybridized (5 x SSC, 0.1% SDS, 5% Dextran sulphate, 20 fold dilution of Liquid Block supplied, 100 µg/ml of denatured sperm DNA) and then hybridized with the probe for at 60°C over night. Hybridization and washing were carried out in bottles using a hybridization oven (Techne, U.K.). The membranes were washed as described earlier, blocked and then incubated with anti-fluorescein-HRP conjugate (1:1000 dilution) for one hour at room temperature. The membranes were washed and the detection of the signal was carried out using ECL detection reagents and subsequently exposing to blue light sensitive films. The films were developed using Kodak developer and fixer.

Insert size analysis of recombinants and sequencing: The phagemids of the 18 UniZap XR clones were excised *in vivo* and the insert size was checked by PCR amplification using T3 and T7 primers. The inserts of the clones were purified and sequenced by dideoxy chain terminator method in an automated sequencer (Applied Biosystems, U.S.A.). Sequences of the clones obtained were blasted and matched with those of known ESTs of *B. malayi* or *Caenorhabditis elegans*, available in the genome database (GenBank). Primers were designed based on the sequence information of the recombinant clones using Primer 3 computer software programme.

Stage-specificity of the primers: In order to ascertain their stage-specificity, the three primer pairs were tested for amplification of the cDNA generated from 50 mf, two each of L_1 and L_2, and one L_3 stage larvae. The PCR mix (50 µl) contained Buffer (Promega), 2.5–3.5 mM $MgCl_2$, 2 mM dNTP mix, 100 pM primers and two units of Taq DNA Polymerase. The PCR amplification cycles was: 95°C for 5 min, 94°C for 1 min, 54–62°C for 1 min, 72°C for 1 min, for 35 cycles with final extension at 72°C for 10 min. After PCR, 10 µl of products were run on 1.5% agarose gel and the bands were visualized after staining with Ethidium bromide followed by destaining.

Dot-blot hybridization to check the stage-specificity: The stage-specificity of WbL31 primer pair was tested further by dot-blot hybridization. These experiments were carried out on RNA of various mosquito stages of the parasite and their cDNAs, as well as mosquito and human RNA, using non-radioisotopic probes. The probe was prepared from the PCR products of the gene amplified using WbL31 primer and L_3 stage cDNA as template. The hybridization protocols followed were according to the ECL-Random prime labeling and detection system version II (Amersham Life Science, USA).

Preliminary evaluation of the infective (L_3) stage-specific RT-PCR assay: The preliminary evaluation of the specificity and sensitivity of the WbL31 primers in the specific detection of infective larvae in vectors was carried out by RT-PCR on the vector mosquito samples harbouring larvae of any of the developmental stages of *W. bancrofti*. Total RNA isolation carried out in mosquito samples using TRI^R reagent was utilized for reverse transcription using RT kit (Sensiscript, Qiagen, Germany), followed by PCR assay with specific primers as described earlier. Ten micro litre of the PCR products were run on 1.5% agarose gel and the bands were visualized after staining with Ethidium bromide and followed by destaining.

The evaluation of the RT-PCR assay was carried out in three phases: (i) for assessing the specificity of the assay, duplicate samples of mosquitoes with 50 mf or 2 each of L_1 or L_2 or 1 L_3 stage larvae were prepared and subjected to RT-PCR. The experiment was repeated five times; (ii) for determining the sensitivity of the assay, 10 samples containing mosquito spiked with 1 or 2 L_3 stage larvae and assayed along with 10 samples of uninfected mosquitoes; and (iii) to make the assay cost-effective RT-PCR assay was done with pools of mosquitoes. The size of the pools tested ranged from 10–100 each containing 1 or 2 L_3 stage larvae.

Standardization of a protocol for simultaneous extraction of RNA and DNA: After removing the aque-

ous layer for RNA extraction from the extraction mixture of the samples mentioned above, underlying interphase was separated for DNA extraction using TRI[R] reagent as per the instruction of the manufacturer. DNA pellet was air dried for 5–15 min, dissolved in 8 mM NaOH, stored overnight at 4°C and used as template DNA for Ssp I PCR assay for determining infection as reported by Chanteau *et al*[9].

Results

cDNA library of L_3 stage of *W. bancrofti* was constructed in UniZap XR vector. The library was amplified and the titre checked. The library constituted 5×10^5 phages with 80–90% recombinant phages.

In order to get enriched copies of stage-specific genes (particularly, infective stage-specific genes) the subtracted probe development technique was employed. Approximately, 2×10^5 recombinants were probed and 18 clones were identified from the library. The insert size of the phagemids of the 18 UniZap XR clones ranged from 100–1000 bp. The inserts were sequenced and matched with those of known ESTs of *B. malayi* or *Caenorhabditis elegans* available in the genome database. Out of 18 clones, inserts of seven clones matched with cuticular collagen gene (U16030), five of them showed homology to enolase gene (EF190446) and six clones contained inserts showing homology with alt 3 gene (U80974) of *B. malayi*. Primer pairs were designed based on the sequence information of these three genes and designated as WbL31, WbL32 and WbL33 respectively.

When the stage-specificity of three primers was assessed, primer pair WbL31 could amplify the cDNA generated from L_3 stage larvae only resulting in a product size of 203 bp. Similarly, the primer pair WbL33 also amplified the cDNA generated from the L_3 stage larva alone yielding an amplicon of 111 bp. Both the primer pairs failed to amplify the cDNA of as many as 50 mf or two larvae each of L_1 and L_2 stages. The remaining primer (WbL32) did not amplify cDNA of any of the stages (Fig. 1). These results indicated that the primer pairs WbL31 and WbL33 specifically detected L_3 stage larva of *W. bancrofti* and their respective mRNA might occur as abundant messages while other primer pair although specific to L_3 stage, their mRNA might not occur as abundant species and hence did not amplify the respective cDNA from the minimum number of larvae tested. The primer pair WbL31 as shown below was designed based on the cuticular collagen gene and was taken up for further studies, as cuticular collagen gene has been studied extensively and reported to be highly expressed in mosquito derived L_3[21]. Amplified product of 203 bp was sequenced (Applied Biosystems, USA) and the sequence information was deposited in the GenBank (Acc. No. EU370160) (Fig. 2).

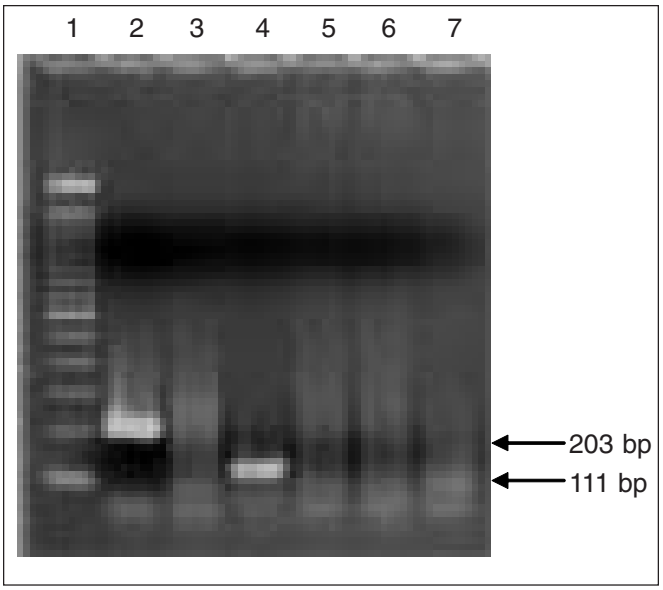

Fig. 1: Specific amplification of cDNA of infective stage larvae (L_3) of *W. bancrofti* by the L_3 stage-specific primers. Lane 1: 100 bp molecular weight marker; Lane 2: Primer WbL31; Lane 3: Primer WbL32; Lane 4: Primer WbL33; Lane 5 & 6: RT negative; and Lane 7: PCR control

WbL31F: 5'- TGG TGT TTC GAT TGT CCA GA - 3'
WbL31R: 5'- ATC ACA GTT CCA GGC ACT CC - 3'

The stage specificity of WbL31 primer pair was

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                ....|....|....|....|....|....|....|....|....|....|.
                          10         20         30         40         50
Bm-Col-II       TGGTGTTTCGATTGTCCAGATGCACCAGCCGGTCCAGCAGGAAATCCAGGA
WbL31-Cut.      TGGTGTTTCGATTGTCCAGATGCACCAGCTGGTCCACCAGGAAATCCAGGA
                 W  C  F  D  C  P  D  A  P  A  G  P  P  G  N  P  G

                ...|....|....|....|....|....|....|....|....|....|..
                         60         70         80         90        100
Bm-Col-II       CCGAAAGGTCCAAAGGGTAGACCTGGACCACCAGGAAAGGATGCAGATGGT
WbL31-Cut.      CCGAAAGGTCCAACGGGTAGATCTGGACCTCCAGGAAAGGATGCAGATGGT
                 P  K  G  P  T  G  R  S  G  P  P  G  K  D  A  D  G

                ..|....|....|....|....|....|....|....|....|....|...
                     110        120        130        140        150
Bm-Col-II       GGTAATAGAGGACCACCAGGACTGCCAGGTGAACCAGGACCAAATGGCGCA
WbL31-Cut.      GGTAATAGAGGACCACCAGGACTACCAGGTAATCCAGGACCAAGTGGCGTA
                 G  N  R  G  P  P  G  L  P  G  N  P  G  P  S  G  V

                .|....|....|....|....|....|....|....|....|....|...
                    160        170        180        190        200
Bm-Col-II       CCAGGTCAAGCAGGTCCAAAAGGACAACCAGGAGTGCCTGGAACTGTGAT
WbL31-Cut.      CCAGGTCAAGCAGGTCCAAAAGGACCACCAGGAGTGCCTGGAACTGTGAT
                 P  G  Q  A  G  P  K  G  P  P  G  V  P  G  T  V
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Fig. 2: Sequence comparison of cuticular collagen gene (WbL31) of *W. bancrofti* (Acc. No. EU370160) with its homologue of *B. malayi* (Acc. No.U16030) with 93% identity

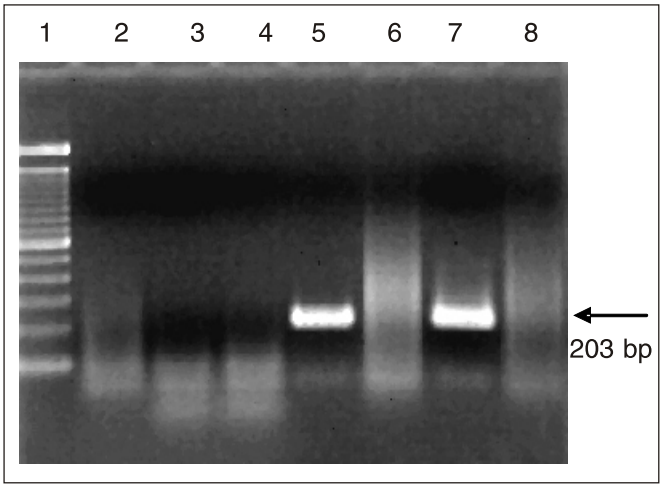

Fig. 3: Specificity of RT-PCR assay in the detection of infective stage larvae of *W. bancrofti* using primer WbL31 in mosquito vector. Lane 1: 100 bp molecular weight marker; Lane 2–5: *Cx. quinquefasciatus* with mf, L_1, L_2 and L_3 respectively; Lane 6: Negative control (mosquito); Lane 7: Positive control (L_3 larvae); and Lane 8: PCR control

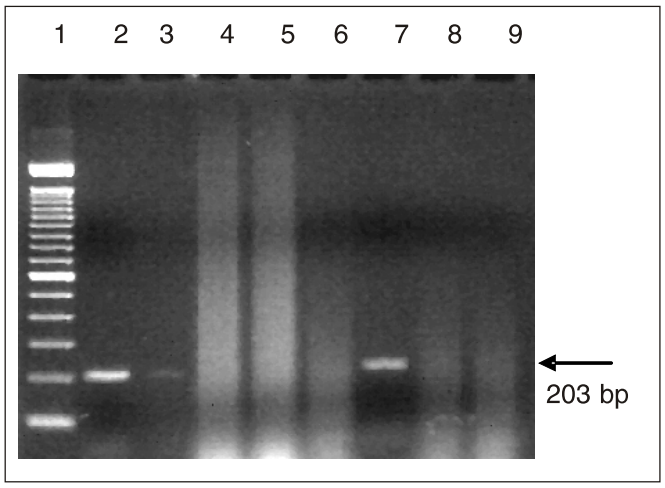

Fig. 4: Sensitivity of RT-PCR assay in the detection of infective stage larvae of *W. bancrofti* using primer WbL31 in pools of vector mosquito *Cx. quinquefasciatus*. Lane 1 : 100 bp molecular weight marker; Lane 2–5 & 6: *Cx. quinquefasciatus* in pools of 10, 25, 50, 75 and 100 respectively; Lane 7: Positive control (L_3 larvae); Lane 8: Negative control (mosquito); and Lane 9: PCR control

tested further by dot-blot hybridization. The results showed that the probe hybridized only to the mRNA and cDNA of L_3 stage larvae but not to mRNA and cDNA of other stages of the parasite, RNA of the vector *Cx. quinquefasciatus* and human.

When stage-specificity of the RT-PCR assay was determined by preliminary evaluation, positive signal with an amplicon of 203 bp was obtained in mosquito samples spiked with L_3 stage larvae alone and not in those containing either 50 mf or 2 L_1 or 2 L_2 (Fig. 3). All the 10 samples of mosquitoes spiked with either 1 or 2 L_3 stage larvae were found to be positive with 203 bp product. Results indicated that the assay was highly sensitive detecting even a single L_3 stage larva in the vector mosquito. While sensitivity of the RT-PCR assay on pools of mosquitoes ranging from 10–100 was assessed, pools of 10 with either 1 or 2 L_3 gave a strong positive signal, whereas pool size of 25 resulted in a faint signal. However, pool size of 50 and above did not give any meaningful result and hence needs to be optimized further for sensitivity (Table 1) (Fig. 4).

Table 1. Sensitivity of the WbL31-RT-PCR in detecting L_3 stage *W. bancrofti* in mosquitoes

Sample	No. of samples tested	No. of positive samples
Individual mosquito with 2 L_3	10	10
Individual mosquito with 1 L_3	10	10
Mosquito pool of 10 with 2 L_3	10	10
Mosquito pool of 10 with 1 L_3	10	10
Mosquito pool of 25 with 2 L_3	10	10
Mosquito pool of 25 with 1 L_3	10	10
Mosquito pool of 50 with 2 L_3	10	0
Mosquito pool of 50 with 1 L_3	10	0
Mosquito pool of 75 with 2 L_3	10	0
Mosquito pool of 75 with 1 L_3	10	0
Mosquito pool of 100 with 2 L_3	10	0
Mosquito pool of 100 with 1 L_3	10	0

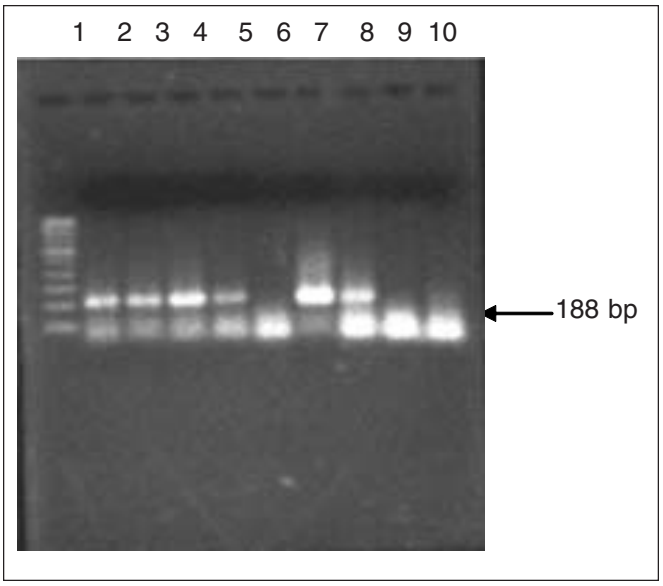

Fig. 5: DNA extracted simultaneously from the same mosquito samples containing any stage of the parasite, *W. bancrofti*, after isolating RNA, was amplified by Ssp I PCR. Lane 1: 100 bp molecular weight marker; Lane 2–5 & 7: *Cx. quinquefasciatus* containing any stage of the parasite; Lane 6: Uninfected mosquito; Lane 7: Positive control (L_3 larva); Lane 8: Negative (Mosquito) control; and Lane 9: PCR control

RNA and DNA were extracted from the same samples of mosquitoes with any stage of the parasite either mf, L_1, L_2 or L_3, simultaneously. When the DNA was subjected to Ssp1 PCR assay, mosquito samples with any stage parasite gave positive signal with an amplicon of 188 bp (Fig. 5). The RNA extracted simultaneously, was tested for WbL31 RT-PCR assay as stated above and found to yield positive signal only to L_3. Thus, RNA and DNA extracted simultaneously from the same sample were suitable for the stage-specific RT-PCR and species-specific Ssp I PCR assays, respectively.

Discussion

This is the first report on the development of a stage-specific RT-PCR assay to detect the presence of infective (L_3) stage larvae of filarial parasite, *W. bancrofti* in the vector *Cx. quinquefasciatus*. It may

be noted that though PCR-based methods have been developed for the diagnosis of several species of filarial parasites, namely *Onchocerca volvulus*[22], *B. malayi*[14], *B. pahangi*[23], *W. bancrofti*[24] and *Dirofilaria immitis*[25], no report is available on the existence of specific assay for the detection of infective stage larvae of these filarial parasites.

In recent years considerable efforts have been made to develop specific and sensitive PCR methods for the diagnosis of *W. bancrofti*[26, 27]. Earlier studies detected PCR-amplified DNA with subsequent DNA probe hybridization condition with increased sensitivity and species specificity[28]. Further, simpler PCR methods, developed based on the repeat sequence of 188 bp and designated as Ssp I, were sensitive and specific[9]. This assay was adapted by groups working in different geographical areas[11, 25, 29,30] as a potentially useful diagnostic tool for estimation of filarial endemicity in villages with high and low prevalence of filariasis[31]. Additionally, rapid and simple DNA extraction protocol was developed to make Ssp I PCR assay cost effective[32]. Such findings provide indication for the possible use of molecular approach in monitoring transmission as a means for evaluation of large-scale control programmes. However, to withdraw or continue the intervention measure (MDA), an indicator of transmission such as infective stage-specific assay is highly essential to verify the absence of transmission of infection and to detect it if it reappears.

In the present study, cuticular collagen gene was found to be up-regulated in L_3 stage larva of *W. bancrofti* through subtracted hybridization protocol. This gene was reported to be highly expressed in the mosquito derived L_3 of *B. pahangi* by differential screening approach. The mRNA for the collagen genes was found to be abundant in the mosquito derived L_3 and up-regulated[21]. A similar collagen gene (Bm-col-2) was also shown to be transcribed at higher levels in the vector derived L_3 stage in *B. malayi*[33]. It has been demonstrated that many L_3 specific proteins in filarial nematodes are synthesized during parasite development in the mosquito, in readiness for the transfer to the mammalian host[34]. Recently, in the initial analysis of the genome of the human filarial parasite, *B. malayi,* collagens and collagen processing, have been identified as one of the several systems likely to be fruitful targets for the discovery of additional drugs. It has also been reported that *B. malayi* has ~82 genes that encode for a collagen repeat including cuticular collagens and basement membrane collagens[35].

The present investigation has led to the development of an assay using reverse transcriptase PCR (RT-PCR) to amplify RNA for the cuticular collagen gene preferentially expressed by infective (L_3) stage *W. bancrofti*. The results of the laboratory evaluation of the RT-PCR assay demonstrated high specificity and sensitivity in detecting even a single infective stage larva in vector mosquitoes. The assay could detect single infective stage larvae in a pool of 10–25 mosquitoes, which would be a cost-effective approach to assess the ongoing transmission in an endemic area. There is need, however, to carry out further optimization of the assay for mosquito pool size of 25 and above.

In this study, a protocol was also developed to assess infection and infectivity simultaneously. The protocol involved extraction of DNA and RNA of the parasite simultaneously from the same vector sample, followed by Ssp I PCR and then WbL31 RT-PCR, respectively. The protocol could identify the mosquitoes, which were infected as well as infective in stepwise manner. The advantages of the protocol are twosome. Firstly, by carrying out Ssp IPCR, one can map and monitor the LF control programme such as MDA, and then by WbL31 RT-PCR, one can decide whether to withdraw the MDA, based on the absence and presence of infective stage larvae (indicator of active transmission) in vectors. Secondly, since RT-PCR is expensive, infection can be assessed initially in mosquitoes and then infectivity in those mosquitoes found to be infected, will economize the diagno-

sis. In conclusion, the success of this assay in specifically identifying the infective (L_3) stage larvae of *W. bancrofti* will be of great value in assessing the transmission of infection and hence in decision-making related to elimination programme.

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References

1. Global programme to eliminate lymphatic filariasis. *WHO Weekly Epidemiologic Rec* 2006; *81*(22): 221–32.
2. Ramaiah KD, Das PK, Michael E, Guyatt H. The economic burden of lymphatic filariasis in India. *Parasitol Today* 2000; *16:* 251–3.
3. Sabesan S, Palaniyandi M, Das PK, Michael E. Mapping of lymphatic filariasis in India. *Ann Trop Med Parasitol* 2000; *94:* 591–606.
4. Das PK, Ramaiah KD, Augustin DJ, Kumar A. Towards elimination of lymphatic filariasis in India. *Trends Parasitol* 2001; *17:* 457–60.
5. Ramu K, Ramaiah KD, Guyatt HL, Evans DB. Impact of lymphatic filariasis on the production of male weavers in a south India village. *Trans R Soc Trop Med Hyg* 1996; *90:* 669–70.
6. Ottesen, EA. The global programme to eliminate lymphatic filariasis. *Trop Med Int Health* 2000; *5:* 591–4.
7. Dean M. Lymphatic filariasis : the quest to eliminate a 4000 year old disease. Hollis Publishing Company, USA, Health & Development International and Glaxo Smithkline 2001.
8. Weil GJ. Diagnostic tools for filariasis elimination programmes. *Scientific working group on lymphatic filariasis,* Geneva, Switzerland, 10–12 May 2005. Working paper No.4, *UNICEF/*UNDP/*WorldBank/WHO Special Programme for Research and Training in Tropical Diseases (TDR)* 2005.
9. Chanteau S, Luquiaud P, Failloux AB, Williams SA. Detection of *Wuchereria bancrofti* larvae in pools of mosquitoes by the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 1994; *88:* 665–6.
10. Nicolas L, Luquiaud P, Lardeux F, Mercer DR. A polymerase chain reaction assay to determine infection of *Aedes polynesiensis* by *Wuchereria bancrofti. Trans R Soc Trop Med Hyg* 1996; *90:* 136–9.
11. Ramzy VK, Farid HA, Kamal IH, Ibrahim GH, Molrsi ZS, Faris R, Weil GJ, Williams SA. A polymerase chain reaction based-assay for detection of *Wuchereria bancrofti* in human blood and *Culex pipiens. Trans R Soc Trop Med Hyg* 1997; *91:* 156–60.
12. Williams SA, Desimone SM, McRenolds LA. Species-specific oligonucleotide probes for the identification of human filarial parasites. *Mol Biochem Parasitol* 1988; *28:* 163–70.
13. Poole CB, Williams SA. A rapid DNA assay for the species-specific detection and quantification of Brugia in blood samples. *Mol Biochem Parasitol* 1990; *40:* 129–36.
14. 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.
15. Oskam L, Schonne GJ, Kroon CCM, Lujan R, Davies JB. Polymerase chain reaction for detecting *Onchocerca volvulu*s in pools of blackflies. *Trop Med Int Health* 1996; *1:* 522–7.
16. Toe L, Boakye A, Adjami A, Back C, Merriweather A, Unnasch TR. Detection of *Onchocerca volvulus* infection by O-150 polymerase chain reaction analysis of skin scratches. *J Infect Dis* 1998; *178:* 282–5.
17. Das M. Vectors of filaria with special reference to India. *J Commun Dis* 1976; *8:* 101–9.
18. Chandrasekar R, Rao UR, Rajasekaraiah GR, Subramanyam N. Isolation of microfilariae from blood on iso-osmotic percoll gradient. *Indian J Med Res* 1984; *79:* 497–501.
19. 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.
20. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: laborarory manual*. IInd edn. Cold Spring, U.S.A : Harbor Laboratory Press 1985; p. 8.3–15.109.

21. Hunter SJ, Martin SAM, Thompson SJ, Tetley L, Devaney E. The isolation of differentially expressed cDNA clones from the filarial nematode *Brugia pahangi*. *Parasitol* 1999; *119:* 189–98.

22. Meredith SEO, Lando G, Gbakima AA, Zimmerman PA, Unnasch TR. *Onchocerca volvulus:* application of the polymerase chain reaction to identification and strain differentiation of the parasite. *Exp Parasitol* 1991; *73:* 335–44.

23. Williams SA, Poole CB, Landry D, Glover J, McReynolds LA. *Brugia malayi* and *Brugia pahangi*; synthetic biotin labeling of oligonucleotide probes for use in species specific detection assay. *Exp Parasitol* 1993; *77:* 235–45

24. Zhong M, McCarthy J, Bierwert L, Lizotte-Waniiewski M, Williams SA. A polymerase chain reaction assay for detection of the parasite *Wuchereria bancrofti* in human blood samples. *Am J Trop Med Hyg* 1994; *54:* 357–63.

25. Nicolas L, Scoles GA. Multiple polymerase chain reaction for detection of *Dirofilaria immitis* and *Wuchereria bancrofti* in their common vector *Aedes polynesiensis. J Med Entomol* 1997; *34:* 741–4.

26. Dissanayake S, Piessens WF. Cloning and characterization of a *Wuchereria bancrofti* DNA in mosquitoes with a non-radioactive probe. *Mol Biochem Parasitol* 1990; *39:* 147–50.

27. Siridewa K, Karunanayake EH, Chandrasekharan NV, Abeyewickreme W, Franzen L, Aslung L, Petterson U. Cloning and characterization of repetitive DNA sequence specific for *Wuchereria bancrofti. Am J Trop Med Hyg* 1994; *51:* 495–500.

28. Dissanayake S, Xu M, Piessens WF. Detection of amplified *Wuchereria bancrofti* DNA in mosquitoes with a non-radioactive probe. *Mol Biochem Parasitol* 1991; *45:* 49–56.

29. Dissanayake S, Rocha A, Noroes J, Medeiros Z, Dreyer G, Piessens, WF. Evaluation of PCR-based methods for the diagnosis of infection in bancroftian filariasis. *Trans R Soc Trop Med Hyg* 2000; *94:* 526–30.

30. Kamal IH, Fischer P, Adly M, El Syed AS, Morsy ZS. Ramzy RMR. Evaluation of a PCR-ELISA to detect *Wuchereria bancrofti* in *Culex pipiens* from an Egyptian village with a low prevalence of filariasis. *Ann Trop Med Parasitol* 2001; *9:* 833–41.

31. Farid HA, Hammad RE, Hassan MM, Morsy ZS, Kamal IH, Weil GJ, Ramzy RM. Detection of *Wuchereria bancrofti* in mosquitoes by the polymerase chain reaction: a potentially useful tool for large-scale control programmes. *Trans R Soc Trop Med Hyg* 2001; *95:* 29–32.

32. Vasuki V, Hoti SL, Sadanandane C, Jambulingam P. A simple and rapid extraction method for the detection of *Wuchereria bancrofti* infection in vector mosquito, *Culex quinquefasciatus* by Ssp 1PCR assay. *Acta Tropica* 2003; *86:* 109–14.

33. Scott AL, Yenbutr P, Ward Eisinger S, Raghavan N. Molecular cloning of the cuticular collagen gene *Bmcol-2* from *Brugia malayi. Mol Biochem Parasitol* 1995; *70:* 221–5.

34. Ibrahim MS, Richie TL, Scott AL. Surface associated antigens of *Brugia malayi* L2 and L3 parasites during vector stage development. *Mol Biochem Parasitol* 1992; *52:* 97–100.

35. Ghedin E, Wang S, Spiro D, Caler E, Crabtree J, Allen JE, *et al*. Draft genome of the filarial nematode parasite, *Brugia malayi*. *Science* 2007; *317:* 1756–60.

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