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RESEARCH ARTICLE

MULTIPLEX REVERSE TRANSCRIPTASE PCR ASSAY FOR THE DETECTION OF INFECTIVE STAGE *WUCHERERIA BANCROFTI* AND ITS VECTOR *CULEX QUINQUEFASCIATUS*

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ABSTRACT

There is currently a global program attempting to eliminate lymphatic filariasis (LF) by administering drugs to affected communities with the goal of interrupting transmission of the parasite. At present the global programme for the elimination of lymphatic filariaisis (GPELF) uses indirect human measures to evaluate the success of its primary goal, the interruption of transmission. An infective stage (L3) detection assay provides a more direct measure of transmission risk and may be useful as a sensitive and non-invasive method for monitoring GPELF programs. In this study, we have developed a multiplex reverse transcription polymerase chain reaction (RT-PCR) assay for the simultaneous detection of infective stage (L3) *Wuchereria bancrofti* and the vector species *Culex quinquefasciatus*. The assay could detect a single L3 in an optimum pools size of 25 mosquitoes when each of the two sets of L3 specific primers (WbL31 and WbL32) were used, indicating the respective diagnostic bands of 203 bp and 111 bp, along with an amplicon of 500 bp for the vector. The detection potential of the assay in terms of sensitivity and specificity of the primer WbL3-1 was 85% and 100%, whereas the same for the primer WbL3-2 was 95% and 100% respectively based on the decoded results of the assessment of 20 coded samples and the variation in the sensitivity was insignificant. This assay may be useful as a non-invasive surveillance tool for early detection of LF resurgence following suspension of MDA, if the detection potential of the assay would be evaluated on larger number of field caught mosquitoes.

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INTRODUCTION

Globally, lymphatic filariasis (LF) is the most threatening of the mosquito-transmitted parasitic diseases next to malaria (Hotez *et al*., 2004). Lymphatic filariasis (LF) is prevalent in tropical and sub-tropical countries, and an estimated 120 million people are affected in 73 endemic countries (WHO, 2012) with an estimated 1.393 billion people residing in areas where filariasis is endemic, necessitating mass drug administration (MDA). In India, LF is endemic in 250 districts in 20 states with about 610 million people residing in endemic areas and continues to be an important public health problem in India by contributing about 44.3% of the global burden (Raju *et al*., 2010; WHO, 2012). Although, mortality from lymphatic filariasis is low, the disease is the fourth leading cause of permanent disability-adjusted life years (WHO, 1996). Past and ongoing control measures, aimed at interrupting transmission by eliminating the reservoir of infection through mass drug administration (MDA) wherever possible, e.g., Global Program for the Elimination of Lymphatic Filariasis (GPELF) has led to substantial decreases in the prevalence of infection and the risk of disease (WHO, 2008). GPELF relies on diagnostic tests to identify and map LF-endemic areas and to monitor the impact of interventions such as MDA. The value of different diagnostic tests (detection of parasite DNA in vector mosquitoes and serum antigen & antibody assays) for different phases of LF elimination programs have been studied and well analysed (Weil *et al*., 2007). Success of the filariasis elimination programme depends on the careful monitoring of infection levels in human populations as well as vectors, as the life cycle of the filarial parasite requires an intermediate host, the mosquito vector and the human host. Molecular xenomonitoring (MX) only provides an indirect measurement of human infection, but PCR method which specifically targets the infective stage larva (L3) can assess ongoing transmission directly when measurements on vector biting rates are available. The expression of L3 stage-specific proteins starts at late L2 stage and peaks at L3 stage. These proteins are abundantly synthesized in the infective stages of the parasite and believed to play a major role in the transmission and infectivity of the filarial parasites (Gregory *et al*., 2000;

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Gomez-Escobar *et al*., 2005). Such potential infective stage specific gene targets were identified and *Wuchereria bancrofti* L3 stage specific PCR assay was developed at VCRC for detecting the presence or absence of infective stage in the vector, *Culex quinquefasciatus* (Vasuki *et al*., 2008). This simple reverse transcriptase-PCR (RT-PCR) assay was further modified and developed as a multiplex Reverse Transcriptase-PCR assay for simultaneous detection of the infective stage (L3) *W. bancrofti* as well as the vector, *Culex quinquefasciatus* bearing the parasites and the results of the study are reported here. Uninfected *Culex quinquefasciatus* mosquitoes were obtained from the laboratory colony maintained at rearing and colonization facility at Vector Control Research Centre (VCRC), Puducherry. Microfilariae (mf) of *W. bancrofti* (nocturnally periodic strain) were purified from blood of mf positive patients residing in and around Puducherry town, which is a known endemic area for bancroftian filariasis (Das, 1976). 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 five high-count (> 20 mf/20 ml) mf carriers after obtaining written consent as approved by the ethical committee of the Centre.

Infective (L3) stage larvae of *W. bancrofti* were raised in the vector mosquito, *Cx. quinquefasciatus*. Female *Cx. quinquefasciatus* mosquitoes were fed on high-count microfilaraemic blood by employing artificial membrane feeding technique and infective stage larvae (L3) were harvested using Bearman's funnel technique on Day 14 post-infection (Paily *et al*. 1995). Each L3 larva was stored individually in 20 µl Trizol^{R} in a microfuge tube under -80^{o}C. Reconstructed samples were prepared using the stored parasites and the laboratory reared vector mosquitoes. Pools containing 5 or 10, 15, 20, 25, 30, 40 & 50 mosquitoes each were prepared and spiked with a single infective larva of *W. bancrofti*. These samples were further subjected to RNA extraction, cDNA synthesis by Reverse Transcription reaction and amplification by PCR assay for determining the sensitivity of the optimum pool size in the detection of infective larva. Extraction of RNA from mosquito samples was carried out using Axyprep multisource total RNA miniprep Kit (Axygen, CA, USA) at room temperature as per the instructions of the manufacturer. The Reverse transcription Reaction for first-strand cDNA synthesis protocol was optimized for use with <50 ng RNA which corresponds to the entire amount of RNA present in pools of mosquito samples (Sensiscript, Qiagen) following the instructions.

Table 1. List of primers used for the multiplex RT-PCR assay

Primer specific to	Code	Sequence	References
Infectivity	WbL3-1F	5'- TGG TGT TTC GAT TGT CCA GA - 3'	
	WbL3-1R	5'- ATC ACA GTT CCA GGC ACT CC - 3'	Vasuki *et al.,* 2008
	WbL3-2F	5'- GAG TCG TTT GGT TGG GGA TA-3'	
	WbL3-2R	5'-TCT TCT TGC CCA GTA CAG CA -3'	Vasuki *et al*., 2008
Mosquito	Cq 1F	5'- GGT CGT ATC AAC AAA GTC CA -3'	
	Cq 1R	5'- ATG GAT ACG GAC TAC ACT GG -3'	Hettiaratchi *et al*., 2000
Infection	NV1F	5'- CGTGATGGCATCAAAGTAGCG-3'	
	NV2R	5'- CCCTCACTTACCATAAGACAAC-3'	Zhong *et al*., 1996

Table 2. Sensitivity and specificity of the multiplex RT-PCR assay for the detection of infective stage larvae of *W. bancrofti* and the vector using two different stage specific primers

L3 specific primer used in the assay	Number of positive pools	Number detected as positive (Sensitivity%)	Number of negative pools	Number detected as negative (Specificity)	Concordant result*
WbL3-1	10	7 (70%)	10	10 (100%)	85%
WbL3-2	10	9 (90%)	10	10 (100%)	95%

*- P value 0.747.

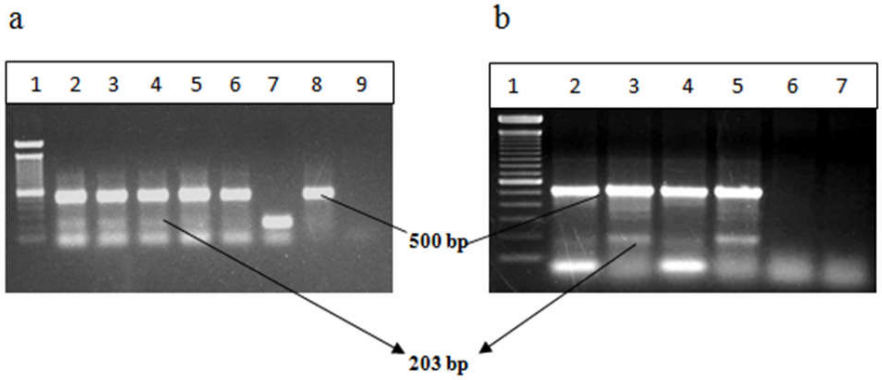

Fig 1. Simultaneous detection of *W. bancrofti* L3 stage and the vector *Cx. quinquefasciatus* by multiplex RT-PCR assay using WbL3-1 primer

a)In pools of vector mosquitoes ranging from 5-25
Lane 1: Molecular weight marker; Lane 2- 6: Mosquito pools of 5, 10, 15, 20 and 25 respectively with 1L3 each;
Lane 7: Positive control (L3 only); Lane 8: Negative control (Mosquitoes only); Lane 9: PCR control
b)In pools of vector mosquitoes ranging from 25-40
Lane 1: Molecular weight marker; Lane 2- 5: Mosquito pools of 25, 30, 35 and 40 with 1L3 each; Lane 6 & 7: PCR control

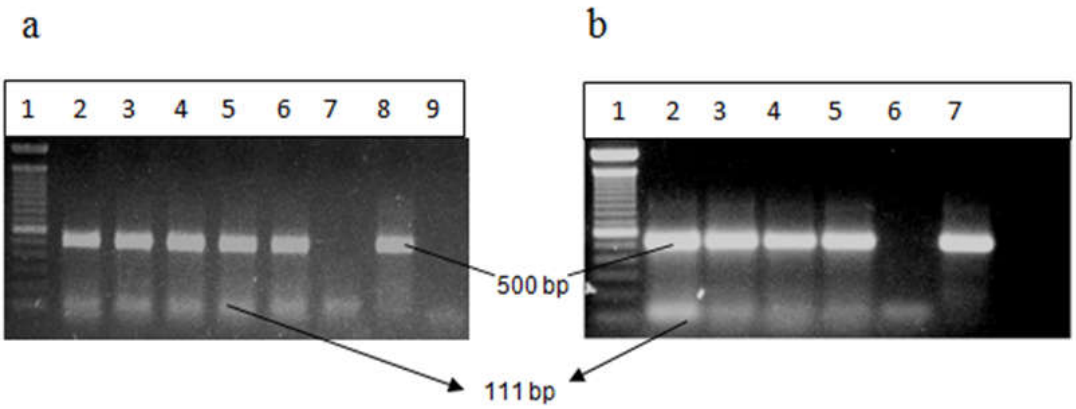

Fig 2. Simultaneous detection of *W. bancrofti* L3 stage and the vector *Cx. quinquefasciatus* by multiplex RT-PCR assay using WbL3-2 primer

a)In pools of vector mosquitoes ranging from 5-25
Lane 1: Molecular weight marker; Lane 2- 6: Mosquito pools of 5, 10, 15, 20 and 25 respectively with 1L3 each;
Lane 7: Positive control (L3 only); Lane 8: Negative control (Mosquitoes only); Lane 9: PCR control.
b)In pools of vector mosquitoes ranging from 25-40
Lane 1: Molecular weight marker; Lane 2- 5: Mosquito pools of 25, 30, 35 and 40 with 1L3 each;
Lane 6: Positive control (L3 only); Lane 7: Negative control (Mosquitoes only)

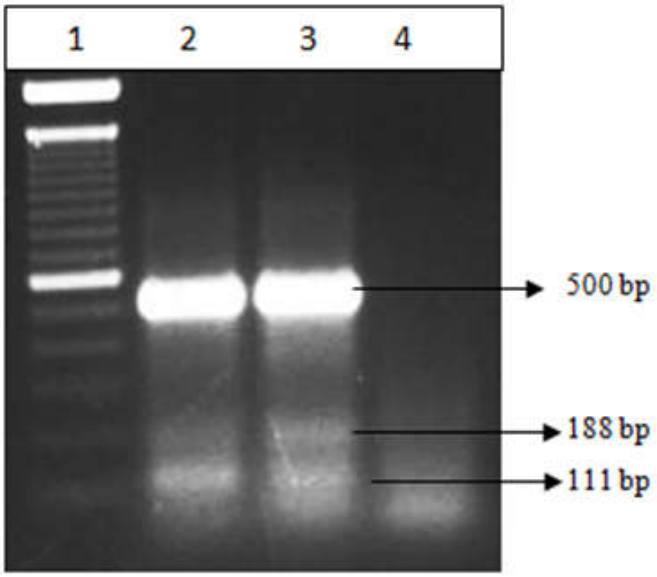

Fig 3. Simultaneous detection of *W. bancrofti* infection, infectivity and the species of the vector *Cx. quinquefasciatus* by multiplex RT-PCR assay using NV1 &NV2, WbL3-2F & R and Cq 1&2 primers

Lane 1: Molecular weight marker; Lane 2 & 3: Mosquito pools of 25 with 1 L3 indicating infectivity (111bp), infection (188 bp) and the species of the vector (500 bp); Lane 4: PCR control

Reverse–transcription reactions were stored on ice and continued directly with PCR for amplification of second strand. The PCR reaction mixture contained 12.5 µl of Go Taq mastermix (Promega, USA), 15-20 pmoles of primers [L3 specific, vector specific and infection specific (Table 1)] and 3 ul of template (RT-reaction) made up to a final volume of 25 ul with sterile water. For long-term storage, transcription reactions were stored at - 80°C. The PCR amplification cycles were: initial denaturation at 94°C for 5 min, followed by 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 under UV-illumination after staining with Ethidium bromide followed by destaining. PCR amplified DNA product of the respective diagnostic bands of 111 bp (for the L3 specific primer WbL3 2); 188 bp (for the primer NV1 & NV2 specific for infection) and 500 bp (specific for the vector, *Cx. quinquefasciatus*) were identified in the presence of the 100 bp molecular weight marker under UV trans-illumination and documented using GelDoc (UVP, UK). Comparison of the sensitivity of the two stage specific primers in the detection of L3 stage: Twenty coded samples containing ten positive samples each with 25 laboratory reared uninfected *Cx. quinquefasciatus* mosquitoes with single L3 stage *W. bancrofti* larva and ten negative samples each with 25 laboratory reared uninfected *Cx. quinquefasciatus* mosquitoes only were used for

assessing the sensitivity of the multiplex RT-PCR assay using each of the two sets of infective stage specific primers and the primers specific to the vector hosting the parasites. The detection potential of each of the two L3 specific primers was assessed individually on all the twenty samples. Results were analyzed and compared using Fisher test of significance using Epicalc 2000 software (http://www.brixtonhealth.com/epicalc.html). In the present study, molecular detection of infective (L3) stage *W. bancrofti* in vector mosquito *Cx. quinquefaciatus* was carried out using a recently developed multiplex RT-PCR assay. This multiplex RT-PCR assay can simultaneously detect the infective stage *W. bancrofti* and the species of the vector mosquito harboring the parasite from the same sample. Two sets of L3 specific primers designed based on the "Wb L3 activated transcripts" (Vasuki *et al*. 2008) and a mosquito species specific primer (Hettiaratchi *et al*. 2000) reported earlier were employed in the multiplex RT-PCR assay. The presence of L3 stage parasite in the vector was detected as an amplicon of 203 bp or 111 bp respectively when WbL3-1 or WbL3-2 primers were used along with a *Cx. quinquefasciatus* specific primer indicating a band of 500 bp for the vector.

The assay was optimized to detect the parasite and the mosquito simultaneously in pools of mosquitoes, each containing 5 or 10, 15, 20, 25, 30, 40 & 50 mosquitoes and a single L3 larva with each set of L3 specific primer. The assay was able to detect the infective stage in pools up to 20 mosquitoes. Pool size higher than 20 resulted in a smudge and hence, optimization with serial dilutions of the template (RT product) and varying the concentration of the corresponding primer (WbL3-1 or WbL3-2) were undertaken. Detection of infective stage parasite and the vector was optimized at 50% dilution of the template and 20 pmol concentration of the primer (WbL3-1 or WbL3-2) for pool size up to 40. Infective stage specific band of 203 bp (for primer WbL3-1) or 111 bp (for primer WbL3-2) and vector specific band of 500 bp were detectable and consistent up to the pool size of 25 (Fig 1 and Fig 2). Though bands were observed in pool size of 30 and 40, results were inconsistent and required further optimization of other parameters. In pool size of 50 no bands were detected. Twenty coded samples were processed for assessing the sensitivity of the multiplex RT-PCR assay in the detection of infective stage *W. bancrofti* and the vector by the multiplex RT-PCR assay. On decoding, out of twenty samples 7 and 9 samples were found positive for the presence of L3 stage and 13 and 11 samples were found negative respectively, when the primers WbL3-1 and WbL3-2 were used in the multiplex RT-PCR assay (Table 2). The detection potential in terms of sensitivity and specificity of the primer WbL3-1 was 85% and 100%, whereas the same for the primer WbL3-2 was 95% and 100% respectively (Table 2) and the difference in sensitivity between the two primers was found to be insignificant (Chi Square 0.10; P value 0.747).

Further, an attempt was made to improve the above mentioned multiplex assay by incorporating the primers specific for infection (NV1 & NV2) of any stage of the parasite with minor modification. One of the L3 specific primers WbL3-2, *Cx. quinquefasciatus* specific primer and infection (any stage) specific primer were used in a single reaction containing 25 mosquitoes with 1 L3, for the amplification of the corresponding bands of 111 bp, 500 bp and 188 bp. Successful amplification was obtained with respective diagnostic bands indicating vector mosquito *Cx. quinquefasciatus,* infection and infectivity of *W. bancrofti* in the same sample (Fig 3). It was also observed that the *Cx. quinquefasciatus* specific band of 500 bp may serve as an internal control (as *Cx. quinquefasciatus* is the only vector of bancroftian filariasis in India), when isolated L3 larvae are not available as positive control. There is currently a global program attempting to eliminate this disease by administering drugs to affected communities with the goal of interrupting transmission of the parasite. Detection of filarial DNA in mosquitoes by PCR cannot differentiate infective mosquitoes from infected mosquitoes. New diagnostic tool/assay to evaluate transmission risk of filariasis is needed that can specifically detect infective L3 stage parasites. In response to this urgent need, simple conventional L3 stage specific RT-PCR assays have been developed for detecting infective stage *W. bancrofti* (Vasuki *et al*., 2008; Laney *et al*., 2010) and *B. malayi* (Laney *et al*., 2008).

An assay to detect filarial L3 in pools of mosquitoes requires 1) a method of mosquito sample preparation that preserves parasite RNA, 2) an effective RNA extraction method that isolates parasite RNA along with mosquito RNA, 3) stage specific primer based on the L3-activated gene to ensure only infective stage parasites are detected, and 4) sensitive and species-specific detection of the L3-stage parasite. The first three requirements, preservation of mosquitoes, extraction of parasite RNA in mosquitoes and development of stage specific primers, were successfully accomplished earlier and mentioned in the methods section. As an accomplishment of the fourth requirement, we now report the development of a multiplex RT-PCR assay that specifically detects the infective stage *W. bancrofti* and simultaneously detects the species of the vector harboring the parasite. Two different pairs of L3 stage specific primers viz. WbL3-1 & WbL3-2 were employed in this multiplex RT-PCR assay for detecting the infective stage *W. bancrofti* in pools of vector mosquitoes. The assay could detect a single L3 stage in pools of 25 mosquitoes and also the species of the vector while utilizing either of the above primers. Laney *et al*. (2010) reported a conventional multiplex RT-PCR assay that detects one infective mosquito in a pool of up to 20 mosquitoes and further indicated that the assay was tested with one infective mosquito (may harbor > I L3 and) but not tested at the level of single worm detection due to difficulty in obtaining isolated *W. bancrofti* L3 parasites.

Therefore, the assay developed by us is able to detect a single L3 in a pool containing more than 20 mosquitoes, which has not been reported so far. The detection potential of each of the two primers used was assessed in terms of the sensitivity and specificity of the multiplex RT-PCR assay. The sensitivity and specificity of the multiplex assay (using the L3 specific primers WbL3-1 or WbL3-2) were found to be 85-95% and 100% respectively based on the decoded results of the assessment of 20 coded samples and the variation in the sensitivity was insignificant (P>0.05). Since the detection assessment was done with limited reconstructed samples, further evaluation with field samples and/or larger sample size is needed. The attempt made for the simultaneous detection of *W. bancrofti*

infection, infectivity and the species of its vector in the same sample was successful and thus served as a proof of principle for developing such an assay. This assay may be further repeated with larger number of samples. Over the past few decades much progress has been made in advancing diagnosis of LF but not in monitoring transmission. GPELF currently uses indirect human measures to evaluate the success of its primary goal, the interruption of transmission. An L3-detection assay provides a more direct measure of transmission risk and may be useful as a sensitive and non-invasive method for monitoring GPELF programs particularly during post-MDA scenario. This multiplex L3 detection assay could also be a non-invasive surveillance tool for early detection of LF resurgence following suspension of MDA by detecting infection, infective (L3) stage parasite and also the vector mosquito which harbours it. This assay may also be useful for identifying the vector, *Cx. quinquefasciatus* in areas where this is not already known.

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