Lymphatic filariasis (LF) caused by the parasitic nematode *Wuchereria bancrofti*, continues to be a major source of morbidity and permanent disability in endemic populations. LF is targeted for elimination as >120 million people are infected worldwide and 1.1 billion are at risk of acquiring infection\(^1\). The elimination strategy, based on annual mass drug administration (MDA) of single dose of diethylcarbamazine (DEC) or ivermectin and albendazole for at least five years with an effective coverage of > 65%, is anticipated to reduce microfilaria rate in the community to below 1% at which transmission can not continue\(^2\). In India, MDA has been introduced in 255 endemic districts of 20 states in line with global programme to eliminate lymphatic filariasis (GPELF) as a public health problem by the year 2015\(^3\). Rapid detection tools are required to monitor the success of this programme and to detect reestablishment of transmission in the post-intervention period.

Molecular tools such as polymerase chain reaction (PCR) are now available to detect *W. bancrofti* infection. PCR assays are more sensitive and specific, and less cumbersome than traditional methods of dissection and microscopy to assess changes in parasite prevalence rates in endemic populations after MDA\(^4\). In post-MDA scenario, assays with high-throughput capacity and more precision are required to monitor the success of this programme and to detect reestablishment of transmission in the post-intervention period.

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Recently, real-time PCR assay has been exploited for detecting and quantifying the filarial DNA for monitoring large-scale filariasis elimination programme\(^6\). Real-time PCR assay offers good sensitivity and eliminates the need to visualize the amplicons by gel electrophoresis thereby greatly reducing the risk of contamination and the inclusion of false positive\(^7\). In spite of the potential advantages of this technology, the major constraint in its widespread adoption is the cost involved in the extraction of genomic DNA, consumables and equipment for the analysis. Additional research is, therefore, needed to further optimize methods to reduce expenditure incurred in real-time PCR assay. In an attempt to cut down the cost and processing time, recently we have standardized a simple method of genomic DNA extraction and reported its utility in high-throughput detection of *W. bancrofti* infection in reconstructed laboratory mosquito samples by real-time PCR assay\(^8\). We further extended the study with pools of wild caught mosquitoes from an endemic area, Ammapettai Primary Health Centre (PHC) of Thanjavur district, Tamil Nadu, India, and demonstrated the detection of *W. bancrofti* infection by real-time PCR assay by employing the simple TE (Tris-EDTA) based method of DNA extraction in comparison with gold standard (Qiagen method). The results are presented in this communication.

Ammapettai, a PHC situated (latitude 10° 77’ and longitude 79° 34’) in the Thanjavur district of Tamil Nadu, India, was selected for the study to develop and validate sampling strategies for mosquito collection and assessment of infection rates for monitoring and evaluation of the LF elimination programme. This PHC has 15 wards and 18 villages (a total of 33 sites) with a population of 19,147 inhabited in 5910 households (HH). Gravid traps were set outdoors in the selected houses at least 1 h prior to sunset (1700 hrs) and the mosquito collection cages were removed from the traps next morning (0630 hrs). A sample of at least 5500 *Culex* gravid females is necessary to assess the vector infection rate of 0.25% by each of the
DNA extraction method. The sample size was calculated by assuming a margin of error of 0.05% and a power of 75%. Since, a pool size of 25 *Culex* gravid females has been found optimum, with 100% sensitivity and specificity, it was decided to collect 231 pools from all the 33 sites in the PHC with an average of seven pools per site which could result in a maximum of 5775 gravidas. Mosquitoes collected using gravid traps from each of 231 randomly selected SHs were sorted by species and gravid *Culex quinquefasciatus* mosquitoes were divided into two separate pools of 25, each in a 2 ml microfuge tubes. Mosquitoes in microfuge tubes were dried on a dry bath at 92℃ for 3–6 h, appropriately coded (indicating the collection date, time, trap and location) and preserved as two sets of samples for assessing infection using the two methods of DNA extraction, viz. gold standard method (Qiagen, DNeasy blood and tissue kit) and TE method of VCRC respectively.

Of the two sets of coded samples, first set of samples was subjected to DNA extraction using commercial DNeasy blood and tissue kit (Qiagen, Hilden, Germany) as standard method following the manufacturer’s instructions. The second set of samples was utilized for extracting DNA by TE method as described previously using bead beating (BB) for grinding the mosquitoes. The DNA samples obtained from both the methods were coded accordingly and analyzed by real-time PCR.

A total of 10,937 *Cx. quinquefasciatus* mosquitoes were collected from 231 randomly selected HHs from 33 sites of the study area. Parasite DNA can be detected in female mosquitoes only after they have taken at least one blood meal. Since, collections were made using gravid traps which target ovipositing females, it was ensured that a larger proportion of the female mosquitoes captured had at least one blood meal. Female mosquitoes collected were sorted into pools of 25 each and a total of 462 pools were obtained.

Real-time PCR reactions were performed using 12.5 µl of TaqMan mastermix (Applied Biosystems, USA) along with 450 nmol/l of each primer: LDR1-5’ATTTTGAT-CATCTGGGAACGTATAA-3’; LDR2-5’CGACTGT-CTAATCCATTACAGTA-3’ and 125 nmol/l probe (6 FAM-ATCTGCCCATAG AAATAACT ACGGTGG-ATCTG-TAMRA) in a final volume of 25 µl in 96-well MicroAmpoptical plates (Applied Biosystems, USA). One microlitre of the extracted DNA (by Qiagen or TE method) was used as template in real-time PCR as described earlier along with 1, 100 and 10 pg of purified genomic DNA samples as positive controls and water controls. All real-time PCR reactions were run in duplicate and cycle threshold (Ct) values for each sample were determined by the following manufacturer’s protocol. Thermal cycling parameters used were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Thermal cycling and data analysis were done with an ABI Prism 7000 instrument using sequence detection system (SDS) software (Applied Biosystems, USA). Cycle threshold (Ct) values of samples ranging from 1–39 were considered positive, and samples that failed to reach the fluorescence threshold beyond 39 were considered indeterminate and repeated to confirm the negativity or positivity of those samples as described by Rao et al. Poolscreen V2.0 software developed by Katholi was used to obtain the maximum likelihood estimate of the vector infection (prevalence) rate and its 95% CI from the data generated by qPCR assays.

When real-time PCR assay was performed on DNA samples of 231 pools extracted by Qiagen method, 53 [23.2%; Ct value: range (mean±SD): 25.8–38.8 (33.4±2.96)] and 175 (76.8%) were detected as positive and negative respectively. The corresponding values by TE method were 54 [23.6%; Ct value: range (mean±SD): 15.5–38.5 (32.5±5.9)], and 175 (76.4%). The results were inconclusive for three and two of the pools by Qiagen and TE methods respectively. Among the 53 positives by Qiagen method 18 and 35 were positive and negative by TE method. Among the 175 negatives by Qiagen 36 were positive, 137 negative and two were inconclusive by TE method. Among the 175 negatives by Qiagen 36 were positive, 137 negative and two were inconclusive by TE method. The percentage of overall concordance (95% CI) was 67.7% (62–74.5%). When data were subjected to the Poolscreen software, the infection rates estimated for TE and Qiagen methods were found to be 1.09% (0.79–1.46) and 1.13% (0.82–1.51) respectively (Fig. 1) and there was no significant difference between the two estimates (p>0.05).

![Fig. 1: Vector infection rate (%) based on DNA extraction by TE method and Qiagen method (95% CI); Qiagen—Commercially available DNeasy blood and tissue kit from Germany; VCRC-TE—Tris-EDTA buffer method developed at Vector Control Research Centre (ICMR), Puducherry, India.](image-url)
In the present study, real-time PCR protocol provides an alternative to the classic detection of *W. bancrofti* and has increasingly superseded conventional-PCR (c-PCR) due to its greatly improved molecular detection efficacy. Similarly, real-time PCR-based method has been successfully used for the detection of several parasites, including *Brugia malayi*, *Dirofilaria immitis* and *Plasmodium falciparum*. In an earlier study long DNA repeat (LDR) of *W. bancrofti* amplified by real-time PCR assay using *W. bancrofti* specific primers and probes, was found to be more sensitive in detecting filarial DNA in large panels of mosquito DNA extracts from Egypt and Papua New Guinea than the conventional PCR with Ssp I as target sequence. The better sensitivity and efficiency most probably could be due to the constant number of LDR repeats in *W. bancrofti* genomic DNA throughout the parasite’s life cycle.

More recently, MX was used to map LF after MDA in American Samoa and the results of the study suggested that sensitive molecular methods such as quantitative PCR (qPCR) detecting parasite DNA in pools of mosquitoes can help to identify sites of continuing LF transmission that may require further treatment after MDA for interrupting transmission. In another study on a comprehensive assessment of LF in Sri Lanka six years after cessation of MDA, *W. bancrofti* DNA was detected in pools of *Cx. quinquefasciatus* mosquitoes by real-time PCR assay and the use of MX was strongly supported as a tool to complement transmission assessment survey (TAS) for post-MDA surveillance. In all the above mentioned studies, template DNA extraction from mosquitoes was carried out using a commercial DNeasy blood and tissue kit (Qiagen), which is expensive and time-consuming. MX by real-time PCR can be considered as a potential tool for monitoring the impact of MDA in filariasis elimination programme, however, the costs of the instrument and the reagents for DNA extraction and amplification are of concern for its use in filariasis endemic countries.

In this study, we have demonstrated for the first time the performance of LDR real-time PCR assay in amplifying the genomic DNA of *W. bancrofti* extracted by a simple TE based method in comparison with Qiagen (standard) method for detecting filarial infection in large number of field collected mosquito pools from an endemic area in a cost-effective manner. TE based protocol is beneficial in many ways— (i) The method is simple as the processing time taken for extraction of 40 pools is 1–1.50 h when compared to 4–5 h by Qiagen method, (ii) In TE based method of extraction, dried mosquitoes are ground and homogenized using zinc plated ball beads to a fine powder which results in complete lysis of tissues. Boiling the homogenate in TE (at 100 °C), denatures the proteins and kills most of the PCR inhibitors. The extracted DNA is diluted (1:20) which further minimizes the inhibitors, if any, when 1 µl of the diluted template is added to the reaction mixture. In Qiagen method, protein lysis involves longer incubation (1 h) and several washing steps, and (iii) Further, the cost involved in DNA extraction of a single pool of 25 mosquitoes is ₹25/- for TE based method as against ₹310/- for Qiagen protocol. The TE based method is safer and cheaper as it utilizes TE buffer only and does not involve any hazardous chemicals or expensive purification columns for isolation. Our findings prove that the DNA extracted by TE based method is as good as the purified DNA extracted by Qiagen method for high-throughput detection of *W. bancrofti* by LDR real-time PCR assay. There was no significant difference observed between the two estimates.

Cost of the real-time PCR instrument and the reagents is declining over time with competitive prices. Cost of a real-time PCR assay is almost comparable to c-PCR when considering the cost and time involved in the agarose gel electrophoresis. Cost comparison in our laboratory showed that the TE based extraction of genomic DNA saves the cost (12 times) and the time (75%)8. Therefore, TE based extraction of DNA could be used as an alternative method as it could be less expensive and time saving while processing large number of mosquito samples by real-time PCR during post-MDA surveillance and monitoring.

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