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Detection of *Brugia malayi* in laboratory and wild-caught *Mansonioides* mosquitoes (Diptera: Culicidae) using Hha I PCR assay

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Abstract

An Hha 1 based polymerase chain reaction (PCR) assay developed for the detection of *Brugia malayi*, the causative agent of Brugian lymphatic filariasis, was evaluated for its sensitivity in the laboratory and for its usefulness in measuring changes in transmission of the disease in the field. Laboratory studies showed that the new assay was highly sensitive in comparison with the standard dissection and microscopy technique. The assay can detect as little as 4 pg of parasite DNA or a single microfilaria in pools of up to 100 mosquitoes. The optimum pool size for convenience was found to be 50 mosquitoes per pool. The efficacy of PCR assay was evaluated in filariasis control programmes in operation in endemic areas of Kerala State, South India. The infection rates obtained by the Hha I PCR assay and the conventional dissection and microscopy technique were 1.2% and 1.7% respectively in operational areas and 8.3% and 4.4% respectively, in check areas, which were not significantly different ($P < 0.05$). Thus, the Hha I PCR assay was found to be as sensitive as the conventional technique and hence it can be used as a new epidemiological tool for assessing parasite infection in field-collected mosquitoes.

Introduction

Lymphatic filariasis is one of the major diseases of mankind in tropical and subtropical countries and a recent estimate of the world burden of this type of disease is around 119.1 million cases (WHO, 1998). Of these, 12.9 million are affected by Brugian filariasis. This form of filariasis, caused by *Brugia malayi* (Brug) (Nematoda: Onchocercidae), is prevalent in Asia and especially in South-east Asian countries such as India, Thailand, Indonesia, Malaysia and the Philippines (Patricia, 1993). In India, Brugian filariasis is endemic in parts of Kerala, Andhra Pradesh, Tamil Nadu, Orissa, West Bengal, Assam and Madhya Pradesh (Raina *et al.*, 1995). Recently, the World Health Organization has identified filariasis as one of the human diseases that is potentially eradicable (Ottesen *et al.*, 1997). The strategies to control and eradicate filariasis need to be planned on the basis of disease prevalence and its burden. Prevalence can be estimated using a range of parameters. Vector infection, for example, is used in the assessment of transmission and is conventionally determined by dissection and microscopic examination of vector mosquitoes for parasites. This technique is cumbersome and hence cannot be employed in large-scale surveillance and monitoring of control operations.

The advent of DNA based technologies, especially

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polymerase chain reaction (PCR) amplification, has provided an opportunity for improved diagnosis of a variety of human pathogens including filarial parasites. A PCR assay, based on the Hha-1 repeat family, has been developed to detect *B. malayi* (McReynolds *et al.*, 1986; Lizotte *et al.*, 1994). The tandemly repeated Hha-1 sequences, which are 70% AT-rich and 322 bp in length, make up 12% of the *Brugia* genome (30,000 copies per haploid genome). In the laboratory, the primers Hha-I F and Hha-I R used in the assay have been reported to be highly species-specific and amplified only the *B. malayi* DNA with a 500 fold preference (Williams *et al.*, 1988). In preliminary laboratory studies the PCR assay was able to detect a single microfilaria in a 50 µl blood sample (Poole & Williams, 1990) and one infective larva in a pool of a hundred mosquitoes (McReynolds *et al.*, 1993). However, this assay has not been tested extensively in the field. In this study, the sensitivity and applicability of the Hha I PCR assay for the detection of infection with *B. malayi* in vectors were evaluated both in the laboratory and in the field.

Materials and methods

Study area

Brugian filariasis transmitted by the mosquitoes, *Mansonia annulifera* (Theobald), *Mansonia uniformis* (Theobald) and *Mansonia indiana* Edwards (Diptera: Culicidae) is known to be endemic in Cherthala 'taluk' (an administrative subdivision of a district) in Alapuzha district, Kerala State (Iyengar, 1938; Rajgopalan *et al.*, 1989). In view of the persistence of Brugian filariasis in this area, a large-scale community-orientated programme, known as the Technology Mission Project (TMP) was launched in 1986 (Rajagopalan *et al.*, 1989). An integrated disease control strategy was followed incorporating a vector management strategy as well as anti-parasite measures using mass single dose diethylcarbamazine (DEC) therapy in the operational areas of Cherthala 'taluk'. Mararikulam South panchayat (an administrative sub-division of a taluk comprising of about 10–15 wards, each with 400–500 houses and a population of about 2000–3000 people) located in the adjacent Ambalapuzha 'taluk' was selected as a check zone. Subsequently, during 1996, a diethylcarbamazine fortified salt programme was also introduced in Mararikulam North panchayat (which remained as the barrier zone of the Technology Mission Project), with the Punnapra panchayat (Ambalapuzha 'taluk') as check zone. Mosquito samples were obtained from these areas during 1995–1997 for the present study.

Collection of mosquito samples

For laboratory studies, the Liverpool strain of *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) was obtained from a standard culture maintained at the Vector Control Research Centre, Pondicherry. The mosquitoes were kept at 28 ± 2°C and 65–75% RH in isolated and infection-free chambers. For field studies, both resting and man-landing collections were made at monthly intervals to sample the vectors following WHO recommended methods (WHO, 1979). For each collection, 24 houses were randomly selected from each of the six panchayats and searched for mosquitoes resting inside using a torch light for a period of 1 h (09.00–10.00 h) and collected using an oral aspirator. Whole-night man landing mosquito collections were carried out inside the selected houses and all the mosquitoes that landed on the human baits were collected continuously from 18.00 to 06.00 h. The mosquitoes collected by both the methods from a house were held separately in a glass tube, brought to the laboratory and identified to the species level. All the female *Mansonioides* mosquitoes collected on a particular day were segregated into two subsamples. One subsample of the mosquitoes was used for assessing infection rate by the dissection and microscopy method. The other subsample was dispensed into microfuge tubes (1.5 ml), dried in an oven at 90°C for 3 h, sealed using parafilm and then transported to the central laboratory at Pondicherry by mail for assessment of infection by PCR assay.

Dissection and microscopy

The mosquitoes were dissected individually in normal saline and examined for filarial parasites. Numbers of parasites of each developmental stage were counted and recorded. The infection rate was expressed as the proportion of mosquitoes containing any of the developmental stages of the parasite (microfilaria/first, second or third stage larva).

Polymerase chain reaction assay

Extraction of DNA from the mosquitoes

DNA from the mosquitoes was extracted using the method of Chanteau *et al*. (1994). Mosquitoes were anaesthetized using ether, transferred either individually or in pools to microfuge tubes (1.5 ml) and then dried at 90°C for 3 h. The dried mosquitoes were crushed using a sterile polypropylene pestle, the resulting powder was suspended in 1 ml of NIB buffer (0.1 M NaCl, 30 mM Tris-HCl, pH 8.0, 30 mM EDTA, 10 mM β-mercaptoethanol and 0.5% NP-40) and washed twice in the same buffer solution. The pellet was then lysed by resuspending in 100 µl of 0.1 M NaOH and 0.2% SDS and incubated for 1 h. The suspension was neutralized with 5 µl of 2 N HCl and added with 1 ml of L6 buffer (4.5 M guanidine thiocyanate, 0.1 M Tris-HCl pH 6.4, 1.2 % Triton X-100 and 20 mM EDTA) and 40 µl silica. Then, it was vortexed thoroughly, incubated at room temperature for 10–20 min and spun for 1 min. The silica pellet was washed twice with L2 buffer (4.5 M guanidine thiocynate, 0.1 M Tris-HCl, pH 6.4) followed by two washes with 70% ethanol. The pellet was then dried at 56°C, resuspended in 100 µl TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and incubated for 10–20 min at 56°C for eluting the DNA. The eluted DNA solution was clarified by centrifugation and used for PCR assay.

PCR amplification

Forward and reverse PCR primers, Hha I F and Hha I R, were used for the specific amplification of *B. malayi* DNA. The sequences of these primers are: Hha I F (18-mer): 5′-GCGCATAAATTCATCAGC-3′, Hha I R (23-mer): 5′-GCGCAAAACTTAATTACAAAAGC-3′. The PCR reaction mix contained 1 µl of template DNA, 10 mM Tris-HCl pH 9.2, 2.0 mM $MgCl_2$, 25 mM KCl, 10 pmol of each primer (Hha 1 F and Hha 1 R), 1.25 mM each of dNTPs (Pharmacia) and two units of *Taq* polymerase (Pharmacia), in

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a final volume of 50 µl. Amplification was done in a thermal cycler (Model DNA Engine, MJ Research Inc., Watertown, Massachusetts, USA) using the programme: 1 min at 94°C for denaturation, further 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C and followed by a final extension cycle of 10 min at 72°C. The PCR amplified products were electrophoresed on a 2% agarose gel with 100 bp marker DNA, stained with ethidium bromide and observed for the *B. malayi* specific band at 322 bp. A positive control (uninfected *A. aegypti* mosquitoes spiked with *B. malayi* microfilariae) and a negative control (uninfected mosquitoes) were always included in each assay. Uninfected mosquitoes were obtained from the standard culture and microfilariae of *B. malayi* (subperiodic strain) were obtained from the peritoneal cavity of infected Mongolian gerbils *Meriones unguiculatus* (Paily *et al.*, 1995) maintained at the Vector Control Research Centre.

Data analysis

The percentage infection (infection rate) obtained by PCR assay was calculated from the 'estimated probability of infection', which in turn was computed from pool positivity data, using the Pool Screen™ computer program developed by Drs Charles Katholi and Thomas R. Unnasch (Katholi *et al.*, 1995) of the University of Alabama at Birmingham, USA. All computations in this regard were made at the 95% confidence interval.

Statistical analysis

Comparison of infection rates obtained by both techniques was analysed using either the Fisher's exact test or the Chi-square test with Yate's correction. A P value of < 0.05 was considered statistically significant. Positive and negative predictive values were calculated using the odds ratio technique from a bi-variate table (Charles, 1987).

Results

Laboratory studies

Optimization of the PCR assay

In order to optimize the PCR assay, various concentrations of ingredients of PCR buffer ($MgCl_2$ and KCl) and primer concentrations were tested. Optimum amplification was obtained when 5 µl of 10X buffer (1.5 mM MgCl2, 10 mM Tris-HCl pH 9.2, 25 mM KCl) and 2 µl (10 pM) each of Hha I F and Hha I R were used in a 50 µl reaction mix.

Sensitivity of the PCR assay

In order to estimate the minimum number of parasites that could be detected by PCR assay in a pool of ten mosquitoes, samples with different numbers (one, two, four, eight and ten) of *B. malayi* microfilariae (mf) were prepared and tested. The minimum number of parasites that could be detected in a pool of ten mosquitoes was found to be one (fig. 1).

To determine the optimum mosquito sample size for assay, pooled samples of ten, 25, 50, 75 and 100 mosquitoes were prepared, a single microfilaria of *B. malayi* added to

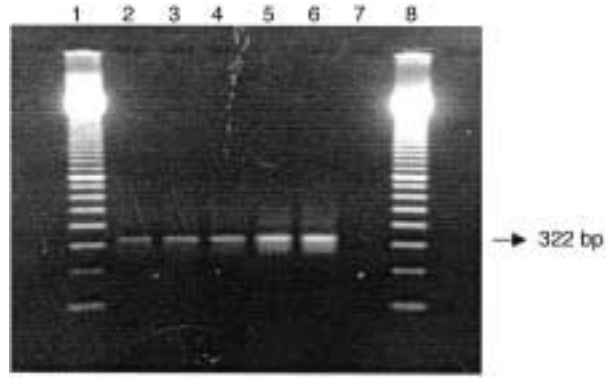

Fig. 1. Polymerase chain reaction (PCR) amplification of minimum number of *Brugia malayi* DNA detectable in a pool of ten mosquitoes. The mosquito pools were spiked with one, two, four, eight and ten microfilaria. The DNA was extracted and assayed by PCR. Lanes 1 and 8, 100 bp DNA molecular weight marker; lane 2, one microfilaria; lane 3, two microfilariae; lane 4, four microfilariae; lane 5, eight microfilariae; lane 6, ten microfilariae; lane 7, PCR negative control (pool of ten uninfected mosquitoes).

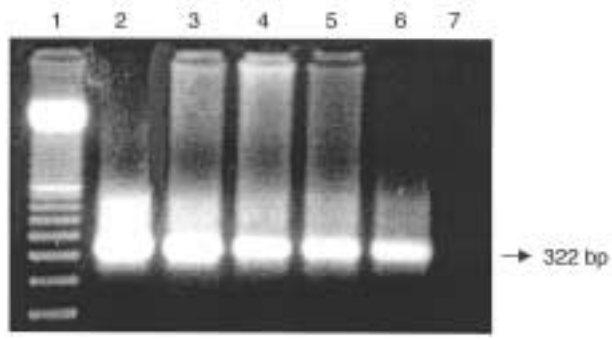

Fig. 2. Polymerase chain reaction amplification of *Brugia malayi* DNA in pools consisting of ten, 25, 50, 75 and 100 uninfected mosquitoes spiked with one microfilaria in each pool. Lane 1, 100 bp DNA molecular weight marker; lanes 2–6, pools of ten, 25, 50, 75 and 100 mosquitoes respectively; lane 7, negative control (pool of ten uninfected mosquitoes).

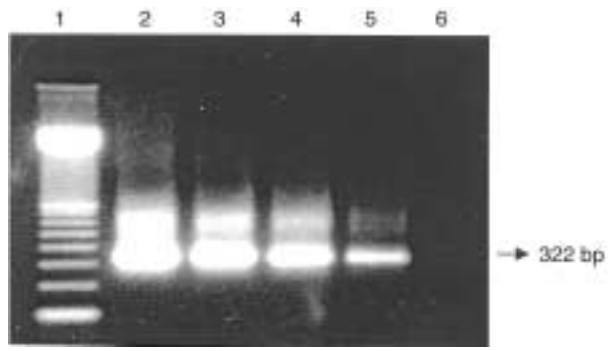

Fig. 3. Polymerase chain reaction amplification of *Brugia malayi* genomic DNA from infective stage (L3) larvae at different concentrations. Lane 1, 100 bp DNA molecular weight marker; lane 2, 200 pg; lane 3, 20 pg; lane 4, 8 pg; lane 5, 4 pg of template DNA; lane 6, negative control without parasite DNA.

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each, and the DNA extracted and subjected to PCR amplification. The results showed that all the pool sizes were amenable for the PCR assay (fig. 2). Pool sizes exceeding 50 mosquitoes, however, were not desirable as they were found to be inconvenient to handle while extracting the DNA. Hence, the optimum pool size for convenience can be taken as 50 mosquitoes.

To determine the minimum amount of parasite DNA required for obtaining good amplification and positive signals, DNA was extracted from the infective stage of the parasite. It was serially diluted and subjected to PCR amplification with different template DNA concentrations, viz. 200 pg (Neat), 20 pg (1\10), 8 pg (1\25) and 4 pg (1\50) (fig. 3). The results showed that as little as 4 pg (i.e. equivalent to 1/50th of a single parasite) DNA could be detected by the PCR technique.

Specificity of the PCR assay

To determine the specificity of the PCR assay using pooled samples of mosquitoes, 50 samples of ten mosquitoes each of *A. aegypti* were prepared. Out of these, 25 were spiked with a single microfilaria of *B. malayi* and the rest were left without addition of parasites. Of the 25 containing a filarial parasite, 24 pools gave positive signals in the PCR assay. One pooled sample failed to give a positive signal, possibly due to the failure of not adding a microfilaria to the sample. None of the negative pooled samples gave a positive reaction, indicating the high specificity for the assay.

Field evaluation of the PCR assay

Mansonia annulifera, M. uniformis and *M. indiana* are known vectors of Brugian filariasis in the study areas of Kerala state (Sabesan *et al*., 1991). Among the indoor resting mosquitoes, *M. annulifera* constituted 85.9%, *M. uniformis* 12.7% and *M. indiana* 1.4%, with average per man hour densities (PMD) of 3.3, 0.25 and 0.01%, respectively (Pradeep Kumar *et al*., 1992). The infection rate of these species with *B. malayi* was 1.61%, 1.64% and 1.00% respectively in Cherthala 'taluk' (Pradeep Kumar, 1990). A survey conducted in the *B. malayi* endemic belt across the State revealed that the prevalence of human infection (microfilarial rate) ranged between 0.28% and 4.7%, and the vector infection rate ranged from 0 to 1.43% in different panchayats (Sabesan *et al*., 2000).

The data on the infection of *Mansonioides* spp. with *B. malayi* assessed by both the dissection and microscopy (conventional) technique and PCR assay are presented in table 1. Generally, the density of these mosquitoes in the study areas was very low and *Mansonioides* mosquitoes constituted 39.8% of the total population. The total number of mosquitoes that were collected on a particular day was therefore pooled for analysis purposes. The estimated probability of infection was calculated taking the pool size as eight, since most of the batches of mosquitoes collected (30 out of 47) consisted of eight mosquitoes.

Percentage infection estimated by the conventional technique was 1.7% in operational areas and 4.4% in check areas. The corresponding infection rates estimated by PCR assay, were 1.2% and 8.3%, respectively (table 1). When the infection rates obtained by these two techniques were compared, there were no significant differences in operational areas ($P = 0.925$) or check areas ($P = 0.0587$), suggesting that the sensitivity of the two techniques showed a similar trend.

Discussion

The efficacy of Hha I PCR assay for the detection of *B. malayi* parasites in mosquito vectors has been examined in a few laboratory studies (McReynolds *et al.*, 1986; Williams *et al*., 1988,) but has not been tested in the field. In this study, the sensitivity and specificity of this assay was tested in the laboratory, while its potential as an epidemiological tool for the detection of *B. malayi* infection was evaluated in the field.

The laboratory studies showed that the Hha I PCR assay is highly sensitive and can detect as little as 4 pg of parasite DNA. Further, this assay detected a single microfilaria in pools of up to 100 mosquitoes. Earlier, the Hha I PCR assay has been reported to be highly sensitive, detecting a single microfilaria in a 50 μl blood sample (Poole & Williams, 1990) and one infective larva in a pool of one hundred mosquito heads (McReynolds *et al.*, 1993). Nicolas & Scoles (1997) have reported that a multiplex PCR assay could detect 1 and 10 pg of DNA of *Wuchereria bancrofti* (Cobbold) and *Dirofilaria immitis* (Leidy) (Nematoda: Onchocercidae) respectively in a pool of five adult *Aedes polynesiensis* Marks (Diptera: Culicidae). Our results show that whole mosquitoes can be pooled without separating the heads for the estimation of infection rate. As an earlier study indicated that infective stage larvae (L3) wander between thorax and head (Paily *et*

Table 1. Comparison of *Brugia malayi* infection in *Mansonioides* mosquitoes collected from Cherthala, Kerala State, by PCR assay and dissection and microscopy techniques.

Technique	Areas	
	Operational	Check
Dissection and microscopy		
No. of mosquitoes tested	232	383
No. +ve for infection	4	17
Percentage infection	1.72	4.44
PCR assay		
No. of mosquitoes tested	153	203
No. of pools tested	22	24
No. of pools +ve for infection	2	12
Percentage infection*	1.2	8.3
P value for percentage infection	0.925	0.0587

* Determined using 'Pool Screen' computer program.

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al., 1995), using heads alone for extracting parasite DNA would lead to an underestimation of infection rates. Hence, pools of whole mosquitoes were used for the estimation of infection rates. In the present study the optimum pool size for convenience was found to be 50. However, the pool size has to be decided based on the density of mosquitoes in the area that varies greatly according to the size of the area under investigation (and hence the proportion of mosquitoes that can be sampled effectively) and the season of collection.

The Hha I PCR assay, once standardized, was evaluated for its usefulness in transmission studies in a health programme currently in progress to control Brugian filariasis in Kerala State. The results of the evaluation under field conditions showed that this assay was at least as sensitive as the conventional dissection and microscopy technique. A PCR assay developed for detecting *W. bancrofti* (Zhong *et al.*, 1996) was tested in the field by Nicolas *et al*. (1996) who concluded that the Ssp I PCR assay was as sensitive as the conventional technique in detecting infection with *W. bancrofti* in *A. polynesiensis* collected from Tahaa Island, French Polynesia. Ramzy *et al*. (1997) also reported satisfactory sensitivity of this PCR assay when tested on *Culex pipiens* Linnaeus collected in a village in the Nile Delta of Egypt. In the present study, a comparison of infection rates in operational and check zones showed that the rate was lower in the former zone when determined by the conventional technique. This pattern was also observed when the infection rate was assessed by PCR assay, indicating that the trend in the infection rates as determined by the two techniques was similar.

The advantage of the PCR assay over the conventional technique is that the vectors can be assessed for infection in pooled samples, allowing the handling of large numbers of mosquito samples in a relatively short time at lower cost. In regions where effective control programmes reduce the number of infected mosquitoes to very low levels, the technique should allow the rapid screening of thousands of samples to assess very low rates of infection in a way not previously possible. Thus, the findings of this study indicate that Hha I based PCR system is a potential and useful epidemiological tool for assessing *B. malayi* infection in wild-caught mosquitoes under practical conditions.

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