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Moses J. Bockarie
Case Western Reserve University School of Medicine

Peter U. Fischer
Washington University School of Medicine

Steven A. Williams
Smith College, swilliam@smith.edu

Peter A. Zimmerman

Lysaght Griffin

See next page for additional authors

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Authors
Moses J. Bockarie, Peter U. Fischer, Steven A. Williams, Peter A. Zimmerman, Lysaght Griffin, Michael P. Alpers, and James W. Kazura

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APPLICATION OF A POLYMERASE CHAIN REACTION-ELISA TO DETECT *WUCHERERIA BANCROFTI* IN POOLS OF WILD-CAUGHT *ANOPHELES PUNCTULATUS* IN A FILARIASIS CONTROL AREA IN PAPUA NEW GUINEA

MOSES J. BOCKARIE, PETER FISCHER, STEVEN A. WILLIAMS, PETER A. ZIMMERMAN, LYSAGHT GRIFFIN, MICHAEL P. ALPERS, AND JAMES W. KAZURA

Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea; Clark Science Center, Department of Biological Sciences, Smith College, Northampton, Massachusetts; Molecular and Cellular Biology Program, University of Massachusetts, Amherst, Massachusetts; Division of Geographic Medicine, Case Western Reserve University School of Medicine and University Hospitals of Cleveland, Cleveland, Ohio

Abstract. Chemotherapy-based eradication programs are aimed at stopping transmission of *Wuchereria bancrofti* by its obligatory mosquito vector. This study compares one year post-treatment *W. bancrofti* infection rates of *Anopheles punctulatus,* the main vector of lymphatic filariasis in Papua New Guinea, using traditional dissection techniques and a polymerase chain reaction (PCR)-based ELISA of a parasite-specific *Ssp* I repeat. A total of 633 mosquitoes in 35 batches were dissected. Six batches contained *W. bancrofti*-infected mosquitoes, giving a minimum infection rate of 0.9%. This value was not different than the actual infection rate, which was 9 (1.4%) of 633 mosquitoes ($P = 0.48$). The DNA was extracted from 47 pools containing a mean of 13.2 mosquitoes per pool. A total of 621 mosquitoes were processed for the PCR-ELISA, including 486 caught by human bait and 135 by light trap, which included both dead and live mosquitoes. Of 23 pools of alcohol-preserved human-bait mosquitoes, seven were positive by the PCR-ELISA, giving an infection rate identical to that obtained by dissection of individual mosquitoes (1.4%). The minimum infection rates for pools of light-trap mosquitoes found dead and alive were 2.7% (2 of 74) and 4.9% (3 of 61), respectively. These values did not differ from each other ($P = 0.84$), but the overall infection rate of light-trap mosquitoes was greater than that of mosquitoes captured by human bait (3.7% versus 1.4%; $P = 0.09$). These data indicate that the PCR-ELISA of a *W. bancrofti Ssp* I repeat using pools of mosquitoes is comparable to traditional dissection techniques for monitoring transmission intensity following introduction of mass chemotherapy. This approach may also be useful for rapid and cost-effective assessment of transmission in endemic areas where the frequency of overt lymphatic pathology is low.

INTRODUCTION

Lymphatic filariasis continues to be a major source of morbidity and permanent disability in endemic populations, with more than 128 million people estimated to be infected currently.[1] Recent clinical trials have shown that two-drug treatment with diethylcarbamazine (DEC) in combination with ivermectin or albendazole is significantly more effective than treatment with a single drug alone, yielding up to 99% clearance in microfilaremia up to one year after treatment.[2,3] These findings raised the possibility that a two-drug treatment regimen might effectively interrupt transmission of *Wuchereria bancrofti,* and were primarily responsible for the resolution by the World Health Assembly to eliminate lymphatic filariasis as a public health problem.[4]

An elimination strategy based on once-yearly, two-drug treatment interventions, has been developed.[4] The strategy is based on the intent to reduce microfilaremia in the community to levels below which transmission cannot continue. It is implicit that this reduction be maintained for the duration of the fecundity of adult worms (4–6 years).

The transmission intensity of *W. bancrofti* is measured by the annual transmission potential, a product of the biting and infective rates (L3) of mosquito vectors.[5,6] Traditionally, detection of *W. bancrofti* in mosquitoes requires time-consuming dissection and microscopic examination of individual mosquitoes. The number of mosquitoes that can be processed using this technique is about 35 per person-hour when dissected fresh. The process is slower if the mosquitoes are preserved in alcohol. In some endemic areas, mosquitoes carry more than one species of filarial parasite, and there are few people with adequate training to distinguish between different species by microscopy. Accordingly, molecular or biochemical techniques may be necessary. To monitor the success of an elimination program and to detect reestablishment of transmission in the post-intervention period, rapid detection tools that are economical are required. Such tools must be of sufficient sensitivity and specificity to detect infection rates that can lead to transmission, and of sufficient simplicity to be applied globally by laboratories with widely ranging capabilities and resources.

In recent years, it has been shown that the polymerase chain reaction (PCR) is a sensitive and specific method for the detection of *W. bancrofti* DNA.[7–11] Screening of pools of mosquitoes would also speed up the processing of large numbers of specimens. A PCR assay for pool screening targeting a repeated DNA segment (*Ssp* I repeat) in the *W. bancrofti* genome has been successfully applied to culicine mosquitoes including *Aedes polynesiensis*[8] and *Culex quinquifasciatus.*[11] However, this technique has not been used to detect the parasite in *Anopheles* mosquitoes, which are important vectors of filariasis in many parts of the world. Filarial larva detection for epidemiologic use must also be compatible with vector collection and storage methods commonly used in endemic areas. Polymerase chain reaction assays have thus far been applied to fresh specimens and are yet to be evaluated for alcohol-preserved or dead mosquitoes. The latter constitute a large proportion of light-trap catches.

We describe the applicability of a standard PCR and a PCR-based ELISA to *Anopheles* mosquitoes collected in a filariasis control area, using the light-trap and human-bait collection methods. Mosquitoes processed included those

found dead in light traps and those preserved in 70% ethanol. Infection rates determined from pool screening were compared with those determined by individual dissection.

MATERIALS AND METHODS

Sampling and processing of mosquitoes. Mosquitoes were caught in Warasikau village where once-a-year mass treatment with DEC in combination with ivermectin started in June 1997. Warasikau is located in the Dreikikir District of East Sepik Province of Papua New Guinea, previously described by Bockarie and others.[12] Lymphatic filariasis caused by *W. bancrofti* is highly endemic in the Dreikikir area where *Anopheles punctulatus* complex mosquitoes are the only known vectors.[13,14] Mosquitoes used in this study were collected during March 1998, nine months after first mass treatment. Collections were performed using the Centers for Disease Control and Prevention (CDC) (Atlanta, GA) light trap and the human-bait catch methods as described previously.[13] Night-landing catches were conducted outdoors between 6:00 PM and 6:00 AM. The CDC light-traps were operated inside houses between 6:00 PM and 6:00 AM. Collections were performed for eight consecutive nights. Mosquitoes collected by the night-landing method were preserved in 70% ethanol. Those caught in light traps were separated into dead and alive and stored in liquid nitrogen.

Mosquitoes caught in light traps during the eight nights were processed by PCR. Human-bait catches from nights 1–4 were individually dissected in batches of ~20 to determine parasite infection and infective rates. Alcohol-preserved mosquitoes were stained with hemalum and dissected in glycerol as described previously.[13] Light-trap mosquitoes collected during nights 5–8 were processed by the PCR-based assay for comparison with results from the previous four nights. Ethical approval for this study and the procedure for oral informed consent were obtained from the Medical Research and Advisory Council of Papua New Guinea and Case Western Reserve University/University Hospitals of Cleveland Human Investigation Committee.

Extraction of DNA and PCR amplification. The DNA was extracted from mosquitoes according to the method described by Chanteau and others.[15] The PCR amplification of the *W. bancrofti Ssp* I repeat was performed as described by Williams and others[9] with modifications introduced to facilitate a quantitative ELISA-based aspect. Each amplification reaction was performed in a final volume of 50 μl containing two oligonucleotide primers, NV-1, NV-2, and 100 fg of an internal plasmid-based control.[16] The sequences of these primers were NV-1: 5′-CGTGATGGCATCAAAGTAGCG-3′ (21-mer) and NV-2: 5′-CCCTCACTTACCATAAGA-CAAC-3′ (22-mer). The NV-2 primer (reverse) used was biotinylated at the 5′ end to obtain a PCR product that can bind to a streptavidin-coated microtiter plate. The PCR conditions were the same as described previously.[9] The PCR products (8 μl) were subjected to electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide to confirm amplification before the ELISA.

The PCR-ELISA. This procedure was performed according to the detailed protocol developed by Fischer and others.[16] Microtiter plates (Lab-Systems, Needham Heights, MA) were coated overnight at 4°C using 1 μg/ml of streptavidin (Sigma, St. Louis, MO) in coating buffer (0.1 M Na_2C0_3-$NaHCO_3$, pH 9.6). For duplicate testing of each sample (once with control hybridization probe and once with the wild-type *Ssp* I-specific hybridization probe), 40 μl of the biotinylated PCR product from each pool was mixed with 360 μl of hybridization buffer (6× SSPE [0.8 M sodium phosphate, pH 8.3, 5 mM EDTA]), 5× Denhardt's solution (0.01% ficoll, 0.01% polyvinylpyrrolidone, 0.01% bovine serum albumin), 0.1% sodium sarcosine, 0.02% sodium dodecyl sulfate, 0.05% NaN_3). 100 μl of the solution was then added to each of four wells. Samples were left to incubate for 30 min at room temperature before adding 100 μl of 0.3 M NaOH to denature the DNA. The wells were subsequently washed once with 1× phosphate-buffered saline (PBS) and hybridization buffer.

For each sample, two of the wells were hybridized with the wild-type *Ssp* I-specific DNA probe and two were hybridized with the internal-control–specific DNA probe. Following hybridization for 30 min at 55°C, the wells were washed twice with 1× PBS and once with 1× PBS/0.1% bovine serum albumin (BSA) for 5 min at 55°C. Thereafter, anti-fluorescein alkaline phosphate Fab fragment (Boehringer Mannheim, Mannheim, Germany) was diluted 1:3,000 in 1× PBS/0.1% BSA and 100 μl was added to each well. After incubation for 30 min at 37°C, the wells were washed three times with 1× PBS/0.5% Tween 20 and two times with 1% diethanolamine/20 mM $MgCl_2$ (pH 10.0). Finally, 100 μl of alkaline phosphate substrate was added according to the instructions of the manufacturer (AP substrate Tablets 104: Sigma). Typical color development started immediately after addition of the substrate. The ELISA reading was made at 405 nm following a 1-hr incubation at 37°C using a Vmax microplate reader (Molecular Devices, Sunnyvale, CA). A positive reading was defined as five times the optical density of the negative controls (PCR product from 100 fg of the internal control plasmid DNA and water instead of mosquito genomic DNA).

To compare the sensitivity of the PCR-ELISA with a standard PCR assay, all samples were retested using conditions similar to those described above except that the internal control was omitted. The PCR products (8 μl) were subjected to electrophoresis on a 1.5% agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, OR), and visualized with a STORM 860 optical scanner (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Dissections. Table 1 shows the infection rates of mosquitoes determined by dissection. A total of 633 *An. punctulatus* mosquitoes were dissected in 35 batches, 28 of which contained 20 mosquitoes. The other 7 batches contained 7–10 mosquitoes. Infected mosquitoes were detected in 6 batches. Four of the infected batches contained only one infected mosquito; the other two had two and three, bringing the total number of infected mosquitoes to nine. The infection rate based on one infected mosquito per batch (0.9%) was lower than the true infection rate (1.4%) but the difference was not statistically different (χ^2 = 0.49, degrees of freedom [df] = 1, P = 0.484). None of the mosquitoes contained L3.

The PCR-ELISA. The DNA was extracted from 47 pools

TABLE 1

Comparison of *Wuchereria bancrofti* infection rates of *Anopheles punctulatus* mosquitoes determined by dissection and polymerase chain reaction (PCR)–ELISA

	Dissection Human bait Alive	PCR-ELISA			
		Human bait Alive	Light trap		
			Alive	Dead	Total
No. of mosquitoes	633	486	61	74	135
No. of pools processed	35	23	11	13	24
No. of pools positive	6	7	3	2	5
Minimum infection rate* (%)	0.9	1.4	4.9	2.7	3.7

* Assuming one infected mosquito per pool.

of 1–28 mosquitoes (mean = 13.2). A total of 621 mosquitoes were processed, including 486 caught by human bait and 135 by light trap. The human-bait mosquitoes were all caught alive and anesthetized by exposure to chloroform before preservation in 70% ethanol. The light-trap mosquitoes included 74 (54.8%) that were already dead before they were preserved. All PCR products tested by the ELISA contained a 188 basepair (bp) internal control product. Visualization of amplified control products by gel electrophoresis confirmed a successful PCR from each of the 47 pools. Table 1 shows the infection status of pools of mosquitoes processed by the PCR-ELISA. Of 23 pools of alcohol-preserved human-bait mosquitoes, only seven were positive, giving a minimum infection rate of 1.4%. Dead and living mosquitoes found in light traps were placed in different pools. Of a total of 13 pools of dead light-trap mosquitoes, two were ELISA positive compared with three of 11 pools of live mosquitoes. The minimum infection rates for these two light-trap groups were 2.7% (2 of 74), and 4.9% (3 of 61), respectively (Yates' corrected $\chi^2 = 0.04$, df = 1, $P = 0.844$). The overall minimum infection rate for light-trap mosquitoes (3.7%) was greater than human-bait mosquitoes (1.4%), but the difference was not statistically significant ($\chi^2 = 3.52$, df = 1, $P = 0.06$).

Standard PCR. The reaction mixtures for the standard PCR assay did not contain the internal control used in the PCR-ELISA. Thus, the 188-bp product could only be visualized in the positive control and in pools containing *W. bancrofti* genomic DNA. Figure 1 shows the fluorescence scanned image of PCR products from 25 reaction mixtures, including two positive pools (lanes 6 and 8), a positive control (lane 14), and a negative control (lane 26). The 188-bp product indicating the presence of *W. bancrofti* was observed in 8 of the 47 pools processed. All pools positive by the standard PCR were also positive by the PCR-ELISA but four ELISA-positive products were negative by the standard PCR. There was no significant difference in the minimum infection rate determined for all 621 specimens by PCR-ELISA (1.8%) and standard PCR (1.2%) ($P > 0.05$).

DISCUSSION

As global programs aimed at eradication of Bancroftian filariasis are implemented, it will become increasingly important to evaluate their effectiveness in a timely and cost-effective manner. Evaluation of transmission potential of the local mosquito population represents an approach that is minimally intrusive to residents of endemic areas. In addition, because of the relatively long period of time between exposure and establishment of infection (months) or development of lymphatic disease (years) in humans, monitoring of mosquito infection rates is likely to detect changes in transmission in a more timely fashion. In this study, we used a specific, inexpensive PCR-based ELISA to detect *W. bancrofti* infection in pools of *An. punctulatus* in an area where mass chemotherapy with DEC plus ivermectin had been distributed one year earlier. The low post-treatment infection rate determined by traditional methods using individually dissected mosquitoes (1.4%) suggests that large numbers of mosquitoes will need to be evaluated to detect statistically significant changes brought about by further chemotherapy. Studies of L3 infection rates in Tanzania estimate that more than 40,000 mosquitoes need to be evaluated to detect changes in transmission intensity effected by more than one annual treatment with DEC.[17] Individual dissections of such large numbers of mosquitoes are not economically feasible in some endemic countries such as Papua New Guinea where labor costs are relatively high. When we carried out this

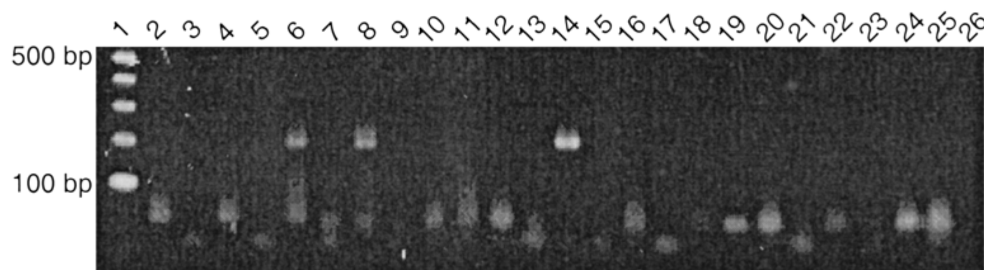

FIGURE 1. Detection of *Wuchereria bancrofti* by a polymerase chain reaction (PCR) from 23 pools of *Anopheles punctulatus.* The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, OR), and visualized by scanning with a STORM 860 optical scanner (Molecular Dynamics, Sunnyvale, CA). Lane 1, a 100-basepair (bp) ladder (molecular size marker), lanes 6 and 8, positive pools; lane 14, positive control; lane 26, negative control.

study, the cost of employing one trained technician to dissect this many mosquitoes was estimated to be US $3,000. In contrast, it is estimated that the cost of reagents and technician time for the PCR-ELISA described here is ~$1,880. The costs of thermocyclers have been going down and are now comparable to stereomicroscopes.

The data presented here show that PCR-ELISA detection of a *W. bancrofti*-specific *Ssp* I repeat using pools of human-bait- or light-trap-captured mosquitoes was similar to traditional dissection approaches in terms of its ability to detect minimal infection rates. These results are encouraging in terms of the potential precision and sensitivity of pool screening given the fact that more than one infected mosquito may be contained in pools of variable numbers. The higher proportion of infected mosquitoes detected in batches captured using light traps compared with human bait may in part be explained by the presence of infected blood meals in fresh mosquitoes caught by the former method. If human-bait catches are performed properly, they should not include blood-fed mosquitoes.

Anopheles mosquitoes are important vectors of *W. bancrofti* in Africa, Asia, and some areas of the Pacific. However, PCR-based techniques to detect infection have not been previously evaluated for these vectors. In addition, despite the fact that 70% ethanol is a widely used preservative for mosquitoes collected during the course of filariasis surveys, there are no reports of how ethanol affects the utility of PCR-based approaches. Previous studies of these assays have been limited to fresh or frozen culicine mosquitoes.[8,11] The results presented here indicate that ethanol-preserved *Anopheles* mosquitoes are suitable for use in PCR-based assays. In addition, results showing that the PCR method detects infection in dead mosquitoes collected in light traps suggest that these specimens may be suitable for pool screening. Filarial larvae rapidly degenerate in dead mosquitoes left at room temperature for more than 1 hr. In the present study, 54.8% of the mosquitoes collected from light traps were dead and dehydrated.

To evaluate the relative sensitivity of a standard PCR assay with gel electrophoresis and the PCR-ELISA, results obtained with the two approaches were compared. Despite using highly sensitive gel staining and imaging techniques to visualize the 188-bp product, four pools of mosquitoes that were positive by PCR-ELISA were negative by standard PCR. These results suggest that the PCR-ELISA may be more sensitive for detection of the *W. bancrofti Ssp* I repeat. This difference may be related to potent PCR inhibitors in mosquitoes.[18] The PCR-ELISA used here was designed to circumvent this problem (i.e., ascertain false-negative reactions) by using an internal control DNA template that is co-amplified with the target *W. bancrofti* sequence. The use of such an internal control in studies carried out by Fischer and others[16] demonstrated that the PCR-ELISA is 10-fold more sensitive than conventional electrophoresis when applied to known quantities of internal control template, as was done in the current study.

The utility of the PCR-ELISA and other DNA detection methods relative to other approaches for rapid and cost-effective determination of levels of filarial endemicity and the need for control measures remain to be established. Giapong and others[19] have reported that evaluation of the prevalence of lymphatic disease (hydrocele and/or lymphatic pathology of the extremities) may be useful for rapid assessment in these circumstances. Results of studies in which night blood surveys for microfilaremia, filarial antigenemia, mosquito infection rates, and human disease prevalence are directly compared should be helpful in deciding which strategies are most informative as well as cost-effective.

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Authors' addresses: Moses J. Bockarie, Lysaght Griffin, and Michael P. Alpers, Papua New Guinea Institute of Medical Research, PO Box 378, Madang, Papua New Guinea. Peter Fischer, Bernhard Nocht Institute for Tropical Medicine, vBernhard-Noct-Strasse 74, 20359 Hamburg, Germany. Steven A. Williams, Clark Science Center, Department of Biological Sciences, Smith College, Northampton, MA 01063 and Molecular and Cellular Biology Program, University of Massachusetts, Amherst, MA 01003. Peter A. Zimmerman and James W. Kazura, Division of Geographic Medicine, Case Western Reserve University School of Medicine and University Hospitals of Cleveland, 2109 Adelbert Road, Cleveland OH 44106.

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