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DETECTION OF *BRUGIA MALAYI* IN MOSQUITOES BY THE POLYMERASE CHAIN REACTION

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ABSTRACT. Accurate identification of filarial parasites in mosquitoes poses a major problem for the coordination of filariasis control programs. Traditional methods are tedious, and some are not specific enough to give satisfactory results. Amplification of specific gene sequences by primer-directed polymerase chain reaction (PCR) has been increasingly utilized as a diagnostic tool. However, current protocols for the extraction of parasite DNA from mosquito samples are tedious and could lead to failure of PCR amplification. We demonstrate that the use of Chelex is an efficient method for DNA extraction from mosquitoes and the parasite and that PCR amplification with primers specific for *Brugia malayi* yields a band of the expected size. The PCR products were transferred to a nylon membrane with Southern blotting, and a *B. malayi*-specific digoxigenin-labeled probe confirmed the sequence similarity of the PCR-amplified fragment and increased the sensitivity of the PCR assay. Use of this probe enabled us to detect PCR-amplified product from *B. malayi* even when a product was not visible on an ethidium bromide-stained agarose gel. This increased sensitivity allowed us to detect the parasite in the heads of mosquitoes.

KEY WORDS *Brugia malayi,* PCR detection, *Aedes togoi*

INTRODUCTION

Brugia malayi is a parasitic nematode that is the causative agent of human filariasis. The parasite is endemic in many tropical and subtropical countries of Africa, Central and South America, and Asia (Chiang et al. 1991). The principal mosquito vectors vary by region and occur in the genera *Mansonia, Anopheles, Aedes,* and *Armigeres* (Mak 1983, Brown 1975). According to the WHO Expert Report (1992), approximately 3 billion persons live in areas where the disease is endemic, and 751 million persons live in areas where transmission is known to occur. Of these, 72.8 million are infected with *Wuchereria bancrofti* and 5.8 million with *B. malayi* or *Brugia timori.*

The success of a control program is determined in a large part by its ability to detect the parasite in the mosquito vector. Traditionally, this has involved dissection and microscopic examination of mosquitoes. The specific identification of infective larvae (L3) is of great importance because there are more than 37 described species of filarial parasites transmitted by mosquito vectors (Mak 1984). However, this dissection procedure is time consuming. In recent years, considerable effort has been focused on the development of species-specific DNA diagnostic methods to replace laborious conventional methods. Many of these methods use the polymerase chain reaction (PCR) to amplify species-specific markers. One problem with these methods is that previous research has demonstrated that mosquitoes can contain potent PCR inhibitors (Dissanayake et al. 1991) and cause underestimation of disease prevalence. A wide variety of different methods for extracting DNA from biological materials exist and include phenol-chloroform extraction, the use of proteinase K digestion, and the use of glass powder (Grimberg et al. 1989). The objective of this study was to compare these 3 methods of DNA extraction from mosquitoes and test the utility of these extractions in yielding DNA that can then be used to detect *B. malayi* in mosquitoes by PCR.

MATERIALS AND METHODS

Aedes togoi (Theobald) mosquitoes are maintained in the insectary at the Division of Medical Entomology, Institute for Medical Research, and were used for this study. Four-day-old mosquitoes were infected by allowing them to feed on an anesthetized cat infected with the subperiodic form of *B. malayi.* After feeding, the mosquitoes were transferred to 3 cups and provided with sugar solution. Mosquitoes were removed from each cup on days 4, 7, and 12 and stored at −70°C.

DNA extraction: Three different techniques were used to extract *B. malayi* DNA from infected mosquitoes for PCR. First, infected mosquitoes frozen at day 12 were homogenized lightly in 20 µl of 0.9% NaCl, and 140 µl of 20% 100–200-mesh Chelex (BioRad, Hercules, CA) was added to the homogenate, vortexed, and boiled at 100°C for 10 min. The mixture was then centrifuged, and the supernatant was transferred to a fresh tube and stored at −20°C. Second, the infected mosquitoes were lightly homogenized in 25 µl of 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 µg of salmon sperm DNA was added during extraction to improve overall DNA recovery (Dissanayake et al. 1991). Proteinase K (2 µl of 20 mg/ml) and N-laurylsarcosine (2 µl of a 20% [w/v] solution) were added and the mixture was incubated for 20 min at 52°C. The supernatant was then transferred to a new tube, and the Geneclean kit (Bio 101, La Jolla,

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CA) was used for the extraction following the manufacturer's instructions. The DNA template was stored at −20°C. Third, the whole mosquito was homogenized in 120 µl of DEB (10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.2 M NaCl, and 0.5% sodium dodecyl sulfate [SDS]). Proteinase K (3 µl of 20 mg/ml) was added, and the homogenate was incubated for 1 h at 52°C followed by a phenol chloroform extraction (Sambrook et al. 1989).

PCR amplification: The PCR was carried out in a Perkin-Elmer 480 thermal cycler (Perkin-Elmer Corp., Norwalk, CT). The 100-µl reaction mixtures consisted of 10 µl 10× reaction buffer containing $MgCl_2$ (1.5 mM), deoxynucleoside triphosphate to a final concentration of 200 µM, and 10 pmol of each primer. The primer sequences were 5′-GCG CAT AAA TTC CAT CAG C-3′ and 5′-GCG CAA AAC TTA ATT ACA AAA GC-3′ (Michelle et al. 1994). The mixture was denatured for 5 min at 94°C and chilled on ice. Taq polymerase (2.5 units) (Perkin-Elmer) was added, and the mixture was overlaid with mineral oil. Thirty amplification cycles were completed with denaturation at 94°C (1 min), annealing at 55°C (1 min), and extension at 72°C (3 min). The PCR product (10 µl) was loaded on a 3% Nu Seive agarose gel (FMC Bioproducts, Rockland, MA) in 1× Tris-acetate buffer to test for the appropriate size target fragment. Gels were stained with ethidium bromide and amplified DNA bands were visualized by ultraviolet (UV) light illumination.

Detection of different larval stages of B. malayi *by PCR and Southern blot:* At 4, 7, and 12 days postinfection, mosquitoes were separated into heads, thoraces, and abdomens, and the Chelex method was used to extract DNA. Uninfected mosquitoes were used for negative controls and *B. malayi* worms were used as positive controls. The PCR amplification was then carried out as described above, followed by gel electrophoresis on a 1.5% agarose gel. The gels were stained with ethidium bromide and visualized by UV light illumination.

The DNA in the gel was denatured in 1.5 M NaCl and 0.5 M NaOH for 30 min and neutralized for 45 min in 1.5 M NaCl, 0.5 M Tris, pH 7.2, and 1 mM EDTA. This DNA was transferred to the nylon membrane (Amersham, Amersham International, Buckinghamshire, United Kingdom) with Southern transfer (Sambrook et al. 1989) and then UV crossed-linked. A *B. malayi* probe from the amplified sequence was labeled with digoxigenin with the DIG tailing kit (Boehringer Mannheim, Roche Bioscience, formerly Boehringer Mannheim, Palo Alto, CA) following the manufacturer's instructions. The probe sequence was 5′-ACG TGA ATT GTA CCA GTG CTG GTC G-3′ (Nutman et al. 1994). The nylon membrane was prehybridized with 20 ml of prehybridization solution consisting of 50% formamide, 5× sodium chloride–sodium citrate (SSC), 0.5 % SDS, 0.1% N-lauroylsarcosine, 3% skimmed milk, and 100 µg herring sperm DNA at 37°C for 2 h. At the end of 2 h, most of the prehybridization solution was discarded, leaving about 2.5 ml to which 10 µl probe (1–10 pmol) was added, and hybridization was carried out overnight at 37°C in a hybridization oven. The membrane was washed twice at room temperature in 50 ml 2× SSC, 0.1% SDS, and 0.02% N-laurylsarcosine with 10 min/wash and then washed 3 times at 37°C in 50 ml 0.01× SSC, 0.01% SDS, and 0.02% N-laurylsarcosine with 10 min/wash (Sambrook et al. 1989) and blocked with 3% skimmed milk and 1× Tris-buffered saline (TBS) (10× TBS stock = 1 M Tris, pH 7.5, 1.5 M NaCl). The membrane was then incubated with anti-digoxigenin alkaline phosphatase (3% skimmed milk, 1× TBS, anti-digoxigenin alkaline phosphatase) and washed 6 times at room temperature in 50 ml 1× TBS and 0.5% (v/v) Tween 20 with 5 min/wash. Colorimetric detection was carried out with a 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) tablet (Sigma, St. Louis, MO).

RESULTS

Amplification of *B. malayi* DNA was demonstrated by the presence of a 322-bp PCR product (Fig. 1) in infected mosquitoes treated with Chelex and Geneclean BIO 101. No DNA of the predicted size was detected in uninfected mosquitoes or in DNA extracted by the phenol-chloroform method. This band was also detected in DNA extracted from 4-, 7-, and 12-day-old infected mosquitoes, indicating that our assay detects all stages of the microfilarial worm.

We isolated DNA from and PCR amplification was performed on the head, thorax, and abdomen of mosquitoes (Fig. 2A). The head region was negative for *B. malayi* on days 4, 7, and 12. Thorax and abdomen analyses were positive on all 3 days. *Brugia malayi* was detected in the head of day-12 mosquitoes by Southern blotting, hybridization with the probe, and colorimetric detection.

DISCUSSION

Amplification of specific gene sequences by primer-directed PCR has become increasingly used as a diagnostic tool. However, protocols that are currently in use for the extraction of parasite DNA from mosquito samples are tedious. We found that the use of Chelex 100 was fast, simple, and gave consistent results. The reduction in the number of steps in sample preparation helps to reduce the chance of DNA contamination. Chanteau et al. (1994) and Nicolas et al. (1996) amplified target DNA for *W. bancrofti* by PCR. However, their DNA extraction methods were tedious and involved numerous steps.

The role of Chelex in DNA extraction for PCR is not well understood. Walsh et al. (1991) had sub-

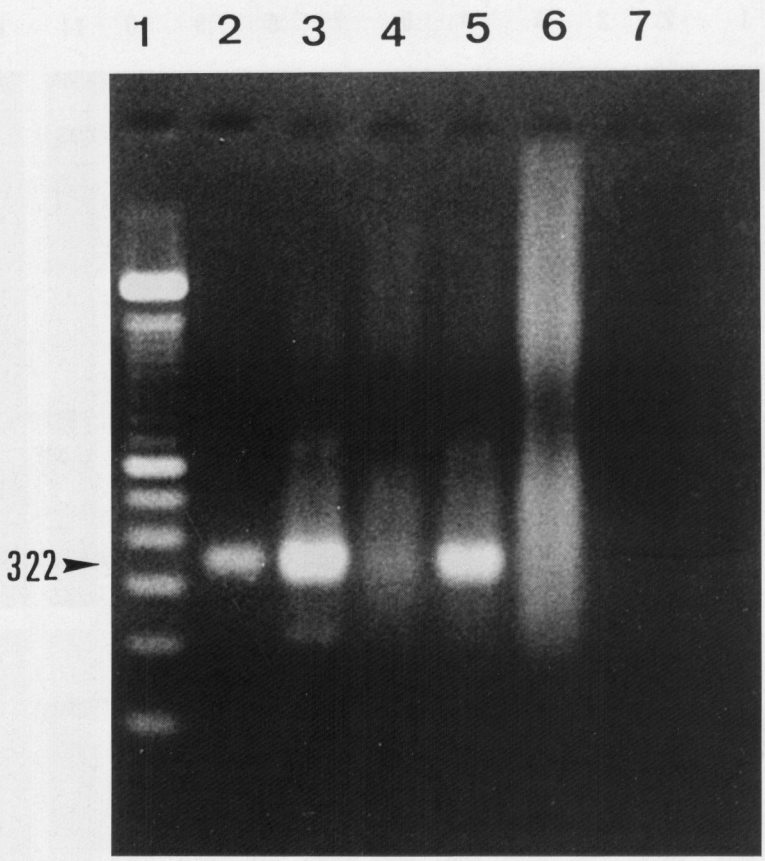

Fig. 1. Polymerase chain reaction amplification of *Brugia malayi* in infected *Aedes togoi* mosquitoes demonstrated by gel electrophoresis. Three different methods were used to extract DNA of *B. malayi* in mosquitoes. L1 = marker; L2, L3, L5 = Chelex method; L4 = Geneclean method; L6 = phenol/chloroform; L7 = negative control.

jected purified DNA to boiling distilled water alone and in the presence of Chelex. They found that DNA treated by boiling without Chelex became inactive in PCR. If the DNA is boiled in 0.01 M Tris-HCl, pH 8.0, with 0.1 mM EDTA, it is also inactivated as template for PCR (Singer-Sam et al. 1989). The boiling treatment is useful for releasing DNA from low numbers of cells, and Chelex protects DNA from the effects of boiling (Walsh et al. 1991). Schriefer et al. (1991) had used Chelex for extraction of DNA from mosquitoes infected with *Plasmodium falciparum* and they were successful in PCR amplification. Chelex has also been used by other workers for the extraction of DNA from forensic material (Walsh et al. 1991).

The phenol-chloroform method of extraction with proteinase K pretreatment did not work, perhaps because mosquitoes contain some protein inhibitors that prevent PCR amplification. This method has also been demonstrated in PCR of *W. bancrofti* in mosquitoes (Dissanayake et al. 1991). Isolation of parasite DNA from mosquito extracts with a DNA binding slurry (Geneclean) also seems to eliminate the inhibitory effects of the mosquito components. However, less PCR-amplified product was obtained than when using template purified with Chelex. Also, the procedure for extraction is tedious when compared with the Chelex method. Mosquitoes that were 4, 7, and 12 days postinfection were all positive.

One major disadvantage of using PCR for the detection of parasites (*B. malayi*) is that one cannot differentiate between infective and infected mosquitoes. In order to overcome this problem, the head, thorax, and abdomen were tested separately. Those mosquitoes with positive heads will be infective with L3 larvae. In our study, no PCR band was visible on agarose gels when the heads of 12-day old mosquitoes were used. However, probing of Southern blots of these agarose gels indicated the presence of amplified L3 DNA in the head. Thus, use of a labeled probe increased sensitivity of the PCR assay. Perhaps there is something in the head of mosquitoes that inhibits PCR.

Figure 2 shows that multiple bands are amplified in infected mosquitoes. The primers were designed from a *Hha*I repeat sequence in *Brugia*. It is likely that the multiple bands represent dimers and trimers

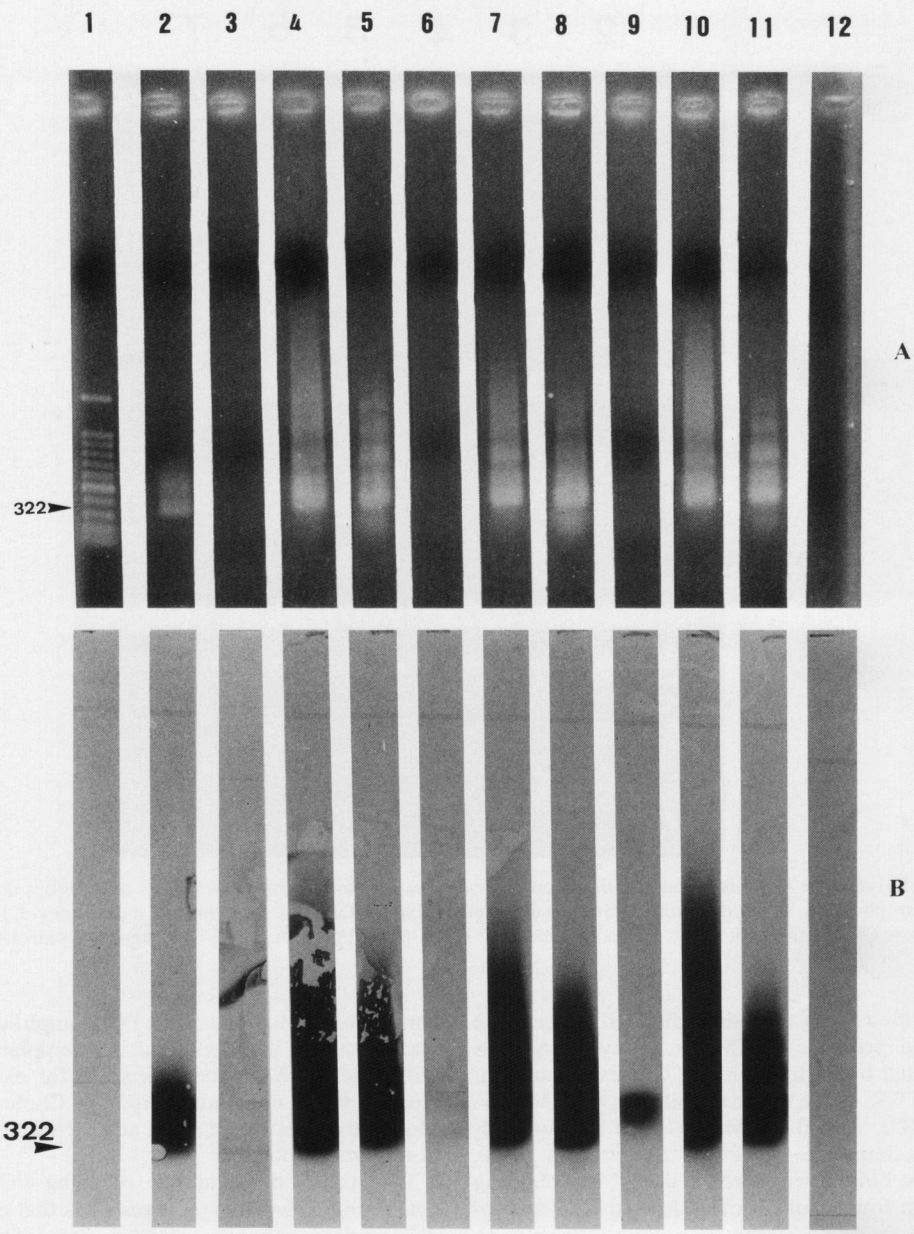

Fig. 2. Identification of *Brugia malayi* DNA in head, thorax, and abdomen of infected mosquitoes on days 4, 7, and 12 for different larval stage development. (A) Ethidium bromide staining pattern: L1 = marker (Promega); L2 = positive control (*B. malayi* worm); L3, L4, L5 = head, thorax, and abdomen, respectively, day 4; L6, L7, L8 = head, thorax, and abdomen, respectively, day 7; L9, L10, L11 = head, thorax, and abdomen, respectively, day 12; L12 = negative control (noninfected mosquito). (B) Colorimetric detection pattern panel shown on (A) probing with digoxigenin labeled *B. malayi* probe; L9 (head, day 12) is positive.

of the *Hha*I repeat. These multiple bands were not eliminated when PCR was carried out at higher annealing temperatures in the range of 60°C to 65°C.

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