Evaluation of a Polymerase Chain Reaction–Based Assay for Diagnosis of
Wuchereria bancrofti Infection

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To assess the utility of a polymerase chain reaction (PCR)–based method for diagnosis of Wuchereria bancrofti infection, blood, plasma, and paraffin-embedded tissue samples were tested using a PCR-based assay that detects a W. bancrofti–specific repetitive DNA sequence. The assay was positive in 100 μL of blood from 40 of 42 microfilaria-positive subjects, the 2 subjects with negative assays having microfilarial counts of 1. Samples from 127 uninfected subjects were PCR-negative. The assay was also positive in 7 of 10 daytime samples in regions where infection is nocturnally periodic; PCR amplification from paraffin-embedded sections established the diagnosis of W. bancrofti infection in another 2 cases. A microtiter ELISA plate–based method was developed for rapid evaluation of large numbers of samples. These results suggest that this PCR-based assay will be useful in diagnosis of W. bancrofti infection in a variety of clinical settings.

Infection with the lymphatic-dwelling filarial parasites Wuchereria bancrofti, Brugia malayi, and Brugia timori occurs throughout the tropics, with an estimated 120 million persons infected and a further 1.1 billion at risk of acquiring infection. The principal manifestations of infection are well-recognized and result from lymphatic damage leading to lymphedema, hydrocele, and elephantiasis.

Diagnosis is generally confirmed by demonstration of microfilariae in the circulation of an infected person. However, this generally necessitates collection of blood samples late at night on account of the nocturnal periodicity of microfilaremia in most regions, an inconvenient and frequently unacceptable requirement. Further, amicrofilaremic infection may be missed, and it may be difficult for the inexpert observer to distinguish microfilariae of W. bancrofti, B. malayi, Loa loa, and Mansonella species, some of which occur together. Serodiagnostic tests for parasite-specific antibodies are sensitive but relatively nonspecific, fail to distinguish active from past infection, and it may be positive in persons infected with nonfilarial helminth parasites. The recent development of assays capable of detecting circulating filarial antigen in the blood of infected persons [1, 2] has been a major advance in diagnosis of infection with W. bancrofti, although they may be negative in some subjects with low levels of circulating microfilariae [3].

The development of polymerase chain reaction (PCR)–based assays for the diagnosis of many parasitic and nonparasitic infections offers the possibility of improved sensitivity and specificity. These assays were recently developed for diagnosis of infection with the filarial parasites Onchocerca volvulus [4] and B. malayi [5] and are more sensitive than parasitologic diagnosis. The recent identification of a W. bancrofti repeated DNA sequence [6] has enabled the development of a PCR-based assay capable of detecting W. bancrofti genomic DNA in human blood [6] and in mosquito vectors [7]. To evaluate the sensitivity and specificity of this assay in diagnosis of infection with W. bancrofti, blood samples from persons with microfilarial counts ranging from 1500 to 0 were tested. In addition, blood samples from subjects with cryptic (amicrofilaremic) infection and daytime blood samples from persons with nocturnally periodic infection were tested to evaluate the diagnostic utility in these settings. Further, paraffin-embedded tissue biopsy samples from persons with suspected W. bancrofti infection were tested by PCR. To simplify the assay, several methods of preparation of the sample for PCR were evaluated, and an ELISA-based method was developed to allow for simultaneous screening of large numbers of samples after PCR amplification.

Methods

Study subjects and specimen collection. Blood samples were collected from 42 infected subjects and 127 uninfected subjects (table 1). Blood samples were collected from infected subjects at the time of peak microfilaremia (between 8 A.M. and 12 noon in the Cook Islands and between 10 P.M. and 2 A.M. in Brazil and India). All study subjects from areas in which these infections are
Table 1. Sensitivity and specificity of SspI PCR assay.

<table>
<thead>
<tr>
<th>Group</th>
<th>mf count*</th>
<th>Country of origin</th>
<th>No. tested</th>
<th>No. PCR-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected (n = 42)</td>
<td>1-1550 (70)</td>
<td>Cook Islands</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brazil</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>India</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Uninfected (n = 127)</td>
<td>0</td>
<td>Cook Islands</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>India</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>North America</td>
<td>54</td>
<td>0</td>
</tr>
</tbody>
</table>

* Range (median) microfilaria count.

Endemic were evaluated for infection by both hemofiltration and circulating antigen assay [3]; both tests were negative in all uninfected subjects. Ten paired day and night blood samples were collected from microfilaremia-positive subjects. An additional 7 blood samples from subjects who were circulating filarial antigen (CFA)-positive but microfilaria-negative were tested. Plasma samples for PCR-based diagnosis were obtained from 18 microfilaria-positive subjects by immediate centrifugation and separation of EDTA-anticoagulated fresh blood samples. For storage of blood and plasma before PCR, Na2-EDTA was added to 1 mL of blood to a final concentration of 100 mM and stored at -20°C. Microfilarial counts were determined by standard membrane filtration of 1-mL samples, except for the daytime samples, for which up to 10 mL of blood was filtered to ensure that low levels of microfilaria were not missed. Paraffin-embedded tissue samples were obtained from 2 patients after diagnostic excisional biopsy: a breast mass in a 51-year-old woman and a scrotal mass in a 25-year-old man.

**Extraction of parasite DNA from blood, plasma, and paraffin-embedded tissue samples.** Several methods of extraction were evaluated. Standard techniques of DNA extraction involving proteinase K digestion and phenol-chloroform extraction followed by ethanol precipitation, drop dialysis, or ultrafiltration were all found to allow for amplification of the SspI repeat from blood samples from persons with low levels of microfilaria (counts as low as 1 microfilaria [mf/mL]) [6]. Plasma samples were prepared for PCR in a fashion identical to that for whole blood.

To simplify extraction techniques, to avoid the use of organic solvents, and to reduce the time required to process the samples, two additional methods were evaluated. The QIAamp blood method (Qiagen, Chatsworth, CA) was evaluated using the manufacturer’s protocol. A method involving red cell lysis and differential centrifugation of nuclei [8] was also evaluated.

DNA was extracted from paraffin-embedded tissue specimens using the QIAamp tissue kit (Qiagen) following the protocol provided by the manufacturer.

**PCR amplification.** PCR amplification was done as described [6] using the following primers to amplify the 188-bp SspI repeat of W. bancrofti: NV-1, 5'-CGTGATGGCATCAAAGTAGGG-3', and NV-2, 5'-CCCTCACTTACCATAAGACAAC-3'.

**Detection of PCR products.** PCR products were detected by standard techniques of agarose gel electrophoresis and Southern blot hybridization using an internal probe. The sequence of the SspI probe is 5'-FL-GTTTGCTTGGTATAACC-3' (where FL = fluorescein). Membranes were hybridized overnight at 42°C, washed in 1x saline sodium citrate plus 0.1% SDS at 42°C, and detected using the ECL detection system (Amersham, Arlington Heights, IL).

**PCR-ELISA.** For some samples, detection was done using a PCR-ELISA. The PCR reaction was done as above, except that the NV-2 primer was biotinylated at the 5' end. Ninety-six-well microtiter plates (U-bottom Immulon 2; Dynatech, Chantilly, VA) were coated overnight with 50 µL of strepavidin (1 µg/mL; New England Biolabs, Beverly, MA) in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6). After being washed six times in TBS/ Tween (TRIS-buffered saline/0.05% Tween 20), 5 µL of biotin-labeled PCR product was added to each well with 20 µL of hybridization buffer (6X sodium chloride, sodium phosphate, EDTA [SSPE]/5X Denhardt’s solution/0.1% sodium sarcosine/0.02% SDS), and incubated for 30 min at room temperature. After being washed in TBS/Tween, the plate-bound double-stranded PCR products were denatured by adding 100 µL of 1 M NaOH for 1 min at room temperature. After six washes in PBS/Tween, 50 µL of a solution containing the fluorescein-labeled SspI probe was added at 50 ng/mL in hybridization buffer and incubated at room temperature for 30 min. After another six washes, a high-stringency wash was done by adding 100 µL of wash buffer containing 0.1X SSPE/0.1% SDS (prewarmed to 42°C) and incubated at 42°C for 5 min. After being washed, 50 µL of an alkaline phosphatase-labeled anti-fluorescein antibody (Fab fragment [Boehringer-Mannheim; Indianapolis] diluted 1/10,000 in TBS/1% bovine serum albumin) was added and incubated for 30 min at room temperature. After a wash in TBS/Tween, plates were developed using the ELISA Amplification System (Life Technologies GIBCO BRL, Gaithersburg, MD). Color development was stopped with 0.3 M H2SO4, and the optical density was read at 490 nm using a multwell ELISA reader. Values 3 SD above the net mean optical density of 6 PCR samples from unexposed North American subjects with no travel to regions in which W. bancrofti is endemic were considered positive.

**CFA.** CFA was detected as described [3].

**Results.**

**Evaluation of DNA extraction methods.** While all methods enabled PCR amplification of the SspI repeat from blood samples from subjects with low-level microfilaria, the red cell lysis/differential centrifugation method [8] proved to be the
most convenient method and, further, gave the most reproducible results (data not shown).

Sensitivity and specificity of the SspI PCR for detection of microfilaremia. PCR-based diagnosis was done on samples drawn from 42 infected subjects at the time of peak microfilaremia (27 from the Cook Islands, 14 from India, and 1 from Brazil), as well as from 127 uninfected subjects, including 73 from areas in which W. bancrofti is endemic (64 from the Cook Islands and 9 from India) and 54 unexposed North Americans. Microfilarial counts in infected subjects ranged from 1 to 1500/mL (median, 70; table 1). After extraction of DNA from 100 µL of blood, PCR amplification was done and products were visualized first by ethidium staining of PCR products separated by agarose gel electrophoresis. The results were validated in a subset by Southern hybridization (figure 1). The PCR was positive in 40 of 42 microfilaria-positive subjects; the 2 negative subjects both had counts of 1 mf/mL. None of 73 endemic and 44 nonendemic control subjects were found to be PCR-positive, leading to a calculated sensitivity of 95% (40/42) and specificity of 100% (127/127).

Detection of parasite DNA in day blood samples in areas where microfilaremia is nocturnally periodic. To evaluate the utility of PCR-based diagnosis in regions in which the infection is nocturnally periodic, paired day and night blood samples were collected from 10 study subjects from South India. Nighttime blood microfilarial counts were 12–1500/mL (median, 700). When daytime blood samples were carefully screened, with filtration of up to 10 mL of blood, 7 of 10 subjects were microfilaria-positive during the day, with microfilarial counts of 3–90/mL (median, 11); the nighttime counts of the 3 subjects microfilaria-negative during the day were 400, 40, and 12 mf/mL. When 100 µL of blood was tested with PCR, all 10 subjects had a positive PCR-based assay on their night blood; similarly, all 7 microfilaria-positive daytime blood samples were positive, while the assay was negative in the 3 samples from subjects in whom microfilariae could not be detected.

Detection of parasite DNA in plasma. To evaluate plasma or serum as a source of parasite DNA for PCR-based diagnosis, DNA was prepared from plasma samples from a subset of those studied for diagnosis using whole blood. Of 18 microfilaria-positive subjects with a positive PCR-based assay for whole blood, only 5 had positive results when plasma was used in place of whole blood, suggesting that parasite DNA is most likely cell-associated or not generally circulating freely in the plasma.

Detection of cryptic infection. Within endemic areas, there are persons who are microfilaria-negative but have positive assays for CFA in their blood. Since the antigen is released from adult worms, it is presumed that such persons harbor adult worms. To investigate the correlation between the PCR-based assay and a positive assay for CFA in microfilaria-negative subjects, 100 µL of blood from 7 subjects who had a positive test for CFA but who were microfilaria-negative were tested with the PCR assay; 3 of the 7 subjects tested were found to be PCR-positive (figure 1).

Detection of parasite DNA in a paraffin-embedded tissue sample. To assess the utility of the PCR-based assay in making a definitive diagnosis from stored biopsy material, DNA was extracted from paraffin-embedded samples from 2 subjects who had undergone surgical excision of tissue masses for diagnostic purposes. In 1 case, a breast lump had been removed from a 51-year-old woman, and in the other, a spermatic cord...
mass had been removed from a 25-year-old man. In both cases, histopathologic evaluation of the lesions had suggested a diagnosis of \textit{W. bancrofti} infection, but accurate speciation of the organisms required specialist assistance in the breast lesion and had not been possible from the spermatic cord lesion because of degeneration of the nematode in the tissue. After DNA extraction from the paraffin blocks, a diagnosis of \textit{W. bancrofti} infection was confirmed in both cases by PCR amplification of the \textit{W. bancrofti}--specific repeat.

Detection of \textit{SspI} PCR product using an ELISA-based methodology. To simplify detection of \textit{SspI} PCR products and to allow for rapid screening of large numbers of samples, an ELISA-based format for detection of PCR product was developed. Samples from 23 of the 42 microfilaria-positive subjects originally studied were retested using this technique, and all 23 were positive. In addition, 34 of the 53 uninfected subjects from areas in which \textit{W. bancrofti} is not endemic, as well as all 73 microfilaria-negative subjects from areas in which the organism is endemic, were retested using this method. None of the uninfected subjects from areas in which the organism is not endemic were positive, while 3 of the 74 microfilaria-negative subjects from areas in which the organism is endemic were positive. All 3 were CFA-positive.

Discussion

The standard technique for making a diagnosis of bancroftian filariasis has been examination of blood for the presence of microfilariae. The finding of a 95% sensitivity of the PCR-based assay in subjects with microfilaria is comparable to that observed in antigen-based assays [1]. Further, the ability to detect parasite DNA in some microfilaria-negative persons suggests that this technique may in some cases be more sensitive than other techniques. The 2 subjects with a false-negative PCR result both had microfilarial counts of 1 mf/mL. Since only 100 \(\mu\)L was assayed by PCR, it is probable that DNA extraction from larger quantities of blood would lead to a further increase in sensitivity.

In a study of PCR-based diagnosis in brugian filariasis [5], samples from all 66 microfilaria-positive subjects were PCR-positive, suggesting that the \textit{HhaI} PCR assay for brugian filariasis is more sensitive than the \textit{SspI} PCR assay for bancroftian filariasis. This may be because the \textit{HhaI} repeat is 100 times more abundant in \textit{B. malayi} than is the \textit{SspI} repeat in \textit{W. bancrofti} [6, 9]. Further, as no CFA assay has yet been developed for diagnosis of brugian filariasis, PCR-based diagnosis of this infection is likely to be especially useful.

Daytime diagnosis of nocturnally periodic infection has been a longstanding goal in the development of diagnostic assays in bancroftian filariasis. The recently developed assays for circulating filarial antigen are generally positive at times when microfilariae are absent from the peripheral circulation [10]. It was therefore important to evaluate this new test for its diagnostic potential in daytime specimens. When samples from 10 subjects from a region where infection is nocturnally periodic were evaluated with both day and night microfilarial counts (with up to 10 mL of blood) and PCR, 7 of 10 remained positive by both hemofiltration and PCR in daytime samples (from 100 \(\mu\)L of blood). While those with positive assays in both day and night samples tended to have much higher nocturnal levels (median of 700 mf/mL at night vs. 11 during the day), this was not universally so, with 1 subject having 120 mf/mL in a night sample compared with 50 in a day sample. However, it was necessary to filter up to 10 mL of daytime blood samples to confirm that 3 of the 10 study subjects were indeed microfilaria-negative. The fact that many subjects with nocturnal microfilaremia have microfilariae in daytime blood, albeit at lower levels [11], may be overlooked, and examination of daytime samples may be worthwhile in many clinical settings. Three of 10 subjects with nocturnal microfilaremia were PCR-negative in daytime samples, suggesting that this technique is less sensitive than sampling night blood. However, examination of larger numbers of samples, investigation of different methods of extraction of DNA, or extraction from larger volumes of blood (>100 \(\mu\)L) may lead to greater sensitivity of the assay. Further, the use of multiplex PCR to amplify other target sequences [12] may improve sensitivity.

The diagnosis of cryptic (amicrofilaremic) infection, in which adult worms are present but microfilariae are absent, may be difficult and generally can only be inferred from a positive assay for CFA or by detection of adult worms by ultrasound. The fact that 3 subjects with a positive assay for CFA were also PCR-positive for parasite DNA in their circulation provides further evidence that such persons do indeed harbor cryptic infection. Further, the fact that these subjects have circulating parasite DNA without microfilariae suggests that it may indeed be possible to improve the sensitivity of the assay. However, the fact that the sensitivity of the test was lower in plasma than blood, with only 5 microfilaria of 18 positive whole blood samples having PCR-positive plasma samples, indicates that the majority of parasite DNA in the blood of infected persons is either present within microfilariae or is cell-associated.

The development of an ELISA-based system to evaluate the results of the diagnostic PCR reaction will allow for the rapid and simultaneous evaluation of large numbers of samples. This technique is more sensitive than evaluation by ethidium staining of an agarose gel and of equal sensitivity to detection by Southern or dot-blot hybridization (unpublished data). Further, the technique obviates the need for the specialized reagents and equipment required for these other techniques, and the ability to undertake ELISA-based detection is almost universally available in diagnostic laboratories. In addition, because the results are numeric, it should be possible to quantitate the level of parasite DNA and relate it to the level of microfilaria present. However, the cost of such assays is likely to remain higher than parasitologic methods, particularly in regions in which the infection is endemic.

The ability to confirm the diagnosis of bancroftian filariasis from a tissue sample by amplification of parasite-specific DNA
is also likely to be of significant assistance when doubt exists regarding the species of parasite responsible for the infection. While a diagnosis may be confirmed with the assistance of a skilled histopathologist, such assistance may not be universally available. Further, the nematode tissues in biopsy specimens are frequently degraded, as in the spermatic cord nodule evaluated here, and a firm pathologic diagnosis may be impossible. Furthermore, as PCR-based diagnosis has been developed for the other common human filarial infections, *O. volvulus* [4], *B. malayi* [5], and *L. loa* (unpublished data), it should be possible to readily identify the species responsible for infection.

While the transfer of diagnostic techniques using the tools of molecular biology from the research setting to the clinical laboratory has been slow, the methodologies for diagnosis of widely divergent organisms are generally very similar. This fact may ultimately lead to the more widespread application of molecular techniques in the diagnosis of filarial infections along with other uncommon and difficult-to-diagnose infections.

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**References**