A SIMPLE AND RAPID NON-RADIOACTIVE OLIGONUCLEOTIDE BASED HYBRIDIZATION ASSAY FOR THE DETECTION OF *WUCHERERIA BANCROFTI*

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Abstract. Five biotin labeled oligonucleotides was designed based on a previously cloned and characterized repetitive DNA sequence specific for *Wuchereria bancrofti.* The oligonucleotide mix (containing five probes) when used in a hybridization assay, detected as little as 100 pg of purified *W. bancrofti,* microfilarial DNA, a single infective stage larva and a single microfilaria in 50µl blood sample. A simple protocol was followed for the hybridization assay. Blood samples lysed with sterile distilled water and digested with proteinase K in the presence of a detergent were directly applied on to nylon membranes for dot blot assays. The DNA extract of mosquitos carrying infective stage larvae was eluted through sephadex G-50 minicolumns prior to blotting. The assay was also able to detect DNA extracted from microfilariae infected samples stored over five days at room temperature (28°C). This simple and rapid oligonucleotide hybridization protocol with the highly sensitive chemiluminescent-based detection has significant potential for the development of a field kit to detect *W. bancrofti* infection.

INTRODUCTION

Over 128 million people in 96 endemic countries have been estimated to be infected with lymphatic filariasis (Michael and Bundy, 1997). *Wuchereria bancrofti,* alone accounts for over 90% of the human lymphatic filariasis in the world (WHO, 1992). At present, *W. bancrofti* is the only form of lymphatic filarial infection that is prevalent in Sri Lanka (Dissanaike, 1991). In 1991, around 3.5 million persons were at risk of infection while the extent of the infection has spread during a 30 year period (Dissanaike, 1991). The number of persons at risk has now increased to nine million (AFC, 1996). The percentage of new cases of filarial infection increased to 23.7 in 1996 compared with 18.3 in 1995. Furthermore the infection rate of the vector *Culex quinquefasciatus* mosquito increased from 0.64% in 1990 to 0.72% in 1996 (Ministry of Health, Sri Lanka, 1996). Therefore, the control of filariasis is important especially in the developing third world countries as it causes serious economic and social consequences by affecting many young working adults.

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The detection of the filarial parasite in the host and the vector is essential for the effective treatment of filariasis and evaluation of control programs. The accurate and specific diagnosis of filariasis in the field depends on the availability of simple, sensitive and rapid diagnostic procedures. Of the different methods available, light microscopy has traditionally been the most commonly used method for the detection of filarial parasites, especially in the field. Furthermore, the collection, dissection, and microscopic examination of vector mosquitos are the techniques currently used in epidemiological surveys to detect and characterize filarial parasites. However, these technics are not always reliable because mosquitos may carry both human and other animal filariae that cannot be distinguished either biochemically or morphologically (Sim *et al,* 1986 a,b). Although these methods are comparatively cheap they are time-consuming and labor-intensive. The use of recombinant DNA based detection strategies offer the promise of greater sensitivity, specificity and speed in demanding field situations (Rajan, 1990). Therefore, considerable effort has been expended in recent years, for the development of alternative methods using DNA probes.

DNA probes, both radioactive and non-radioactive as well as PCR based methods have been developed for the detection of lymphatic filarial parasites (Williams *et al,* 1988, 1996; Zhong *et al,*

1996; Nicolas, 1997). A radioactive DNA probe (Siridewa *et al,* 1994) and a PCR based technique (Siridewa *et al,* 1996) have been developed in our laboratory for the detection of *W. bancrofti.* Even though these technics have been reported to be very sensitive and specific, there are practical and logistical problems in using these under field conditions in developing countries. An ideal DNA based assay would be a cocktail kit that can be used in the field. As part of our own attempt to achieve this goal a simple and rapid hybridization assay to detect *W. bancrofti* using biotinylated oligonucleotide probes designed from a diagnostic repetitive DNA sequence of *W. bancrofti* (Siridewa *et al,* 1994) has been developed.

MATERIALS AND METHODS

Blood samples were collected at night by venipuncture into blood collecting bags (containing sodium citrate) from *W. bancrofti* infected individuals living in Sri Lanka. Blood samples were stored at -20°C until used. Infective third-stage larvae (L_3) of *W. bancrofti* were obtained from laboratory-bred and infected *Culex quinquefasciatus* mosquitos. *Cx. quinquefasciatus* mosquitos (uninfected) were obtained from the laboratory-bred colony of mosquitos maintained in the insectary of the Department of Biochemistry and Molecular Biology, University of Colombo, Sri Lanka. *Brugia malayi* microfilariae were kindly provided by Dr John W McCall (University of Georgia, Athens, GA).

Isolation of microfilaria and extraction of DNA

Blood samples containing microfilariae (mf) were lysed by mixing with 4 volumes of 1% SDS. The mf pellet was obtained by centrifugation (9,500*g*/ 4°C/ 25 minutes). This process was repeated twice and the mf pellet was washed in TE (10:1, pH 8.0), centrifuged 9,500*g* and the mf pellet was stored at -20°C until used. DNA from *W. bancrofti* mf was extracted as follows; The mf pellet in TE (3 ml) containing N-lauryl sarcosine (4%) was digested with proteinase K (20mg/ml) (37°C, 4 hours), followed by phenol:chisam (Sambrook *et al,* 1989) extraction and alcohol precipitation of the DNA. The DNA was then stored at -20°C until used.

DNA was also extracted by the above method from microfilariae infected blood samples, stored (from the day of bleeding) for up to five days at different temperatures (28°C, 4°C and -20°C).

Differential processing of blood and mosquito samples

The mf positive blood (50µl) was lysed in sterile distilled water (1 ml), centrifuged (12,000 x rpm/5 minutes) and the supernatant discarded. This process was repeated and the pellet was resuspended in TE (100µl) and processed to different stages as follows. One set of samples was processed to the DNA precipitation stage as described above. Another set of samples was processed to the phenol: chisam extraction stage and the aqueous layer was recovered, without precipitating the DNA. The third set of samples was processed up to the proteinase K (20 mg/ml) and detergent (56°C for $1\frac{1}{2}$ hours and 37°C for 2 hours) treatment. The proteinase K was denatured by boiling at 100°C (10 minutes).

Mosquitos containing L_3 larvae and uninfected *Cx. quinquefasciatus* mosquitos were crushed in liquid nitrogen prior to processing up to the three different stages as above. One set of extracted samples was eluted through sephadex G-50 mini columns (200 µl micropippet tips, were packed with sterile cotton wool at the tip and then filled with swollen sephadex G-50).

The extracted blood samples and mosquito extracts (50 µl or 100 µl) were denatured and applied onto nylon membranes using a dot blot manifold (Biorad, Richmond, CA, USA). Immobilization of DNA was accomplished by baking the membranes (80°C in vaccuum/1 hour).

Oligonucleotide hybridization assay

Five oligonucleotide probes (listed below) each labeled with a biotin group at the 5′ hydroxyl end, were obtained from Genosys Biotechnologies Inc, UK. These oligonucleotide sequences were based on a previously cloned and characterized repetitive DNA sequence specific for *W. bancrofti* (Siridewa *et al*,1994).

WBB1-5′bGTCGTAATCGAGGATATCAAAGC
TGTCTGAGATGCCGGG3′
WBB2-5′bCACTGGTGGAACTTCATCGGAT
GGACGCATCACCTG3′
WBB3-5′bGCGATGCAAAGCAGAAGATTGA
GCCAAATGATGGGTG3′
WBB4-5′b GCAGTGAATATCCCGGTCTCAAA
TAGAGGAACAAC3′
WBB5-5′b GCTAGTTTGACCTTCGAGAAAT
CAGGCATATTGTAGGATGG3′

Prehybridization, hybridization and post hybridization washing conditions were optmized as described by Albretsen *et al* (1988) for the five, pooled oligonucleotide probes.

Chemiluminescence detection

The hybridized biotin labeled oligonucleotide probe were detected with avidin-alkaline phosphatase conjugate and the chemiluminescent substrate disodium 3-{4-methoxysprio [1,2-dioxetane-3, 2´-(5´-chloro)-tricyclo (3.3.1.1) decan] 4-yl} phenyl phosphate (CSPD) according to the manufacturer's instructions (Southern - Light™ Chemiluminescent detection system for Biotin-labeled Probes, Tropix, Inc, MA, USA). The light emission was imaged on to Kodak X-ray films (XAR-5) for $\frac{1}{2}$ hour - 2 hours at room temperature (28°C).

RESULTS

Optimization of assay conditions

The optimal conditions for the oligonucleotide based hybridization assay, were determined for the oligonucleotide mix, WBB 1-5 (WBBl, WBB2, WBB3, WBB4 and WBB5) by varying the assay conditions. The results of these experiments are given in Table 1. The optimal assay conditions for the oligonucleotide probes (WBB 1-5) were prehybridization in 6 x SSC at 42°C for 2 hours, hybridization in 6 x SSC containing formamide (50%) at 42°C for 2 hours with a total probe concentration of 25 pmol/ml (5 pmol/ml of each probe). Non-specific binding to human DNA could only be removed when the blots were washed in 0.5 x SSC at 42°C (3 x 15 mintes).

Sensitivity of the assay

To evaluate the sensitivity of the assay, dot blots containing serial dilutions of *W. bancrofti* microfilarial DNA (10, 5, 2, 1, 0.5, 0.25, and 0.1 ng), DNA extracted from L3 larvae (3, 2 and 1) and DNA extracted from microfilariae (20, 10, 5, 2 and 1 mf) in 50µl of blood were probed with the oligonucleotide mix under the optimal assay conditions. The oligonucleotides mix was able to detect as little as 100 pg of *W. bancrofti* microfilarial DNA, a single L3 larva and a single microfilaria (Fig 1a, b and c).

Effect of storage of blood samples on signal detection

Microfilariae infected blood samples were stored at different temperatures (28°C, 4°C and -20°C) for up to five days from the time of bleeding. DNA was extracted from the samples after 24 hours, 2 days, 3 days, 4 days and 5 days. Blood samples stored at -20°C (all five days) gave positive signals. However, false negative results were observed in some samples stored for more than 24 hours at 28°C and 4°C (Fig 2a). It was also noted that DNA

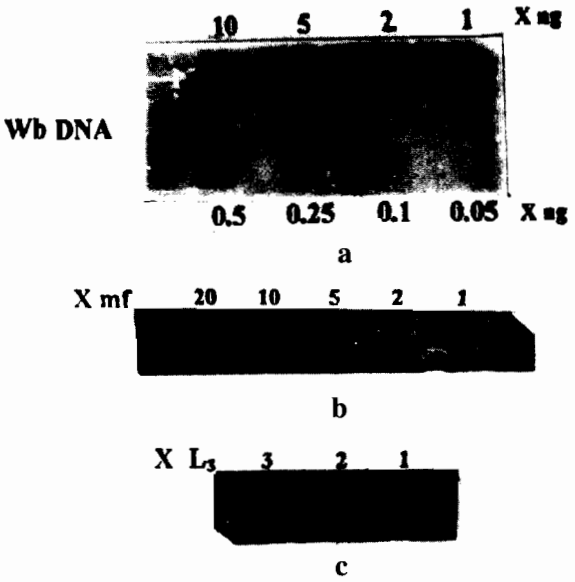

Fig 1–Chemiluminescent detection of dot blots of dilutions of (a) *W. bancrofti* DNA [Wb DNA] (10 ng, 5 ng, 2 ng, 1 ng, 0.5 ng, 0.25 ng, 0.1 ng and 0.05 ng), (b) DNA extracted from dilutions of 20, 10, 5, 2 and 1 microfilariae and (c) DNA from 3, 2 and 1 L_3 larvae, probed with the oligonucleotide mix, WBB1-5.

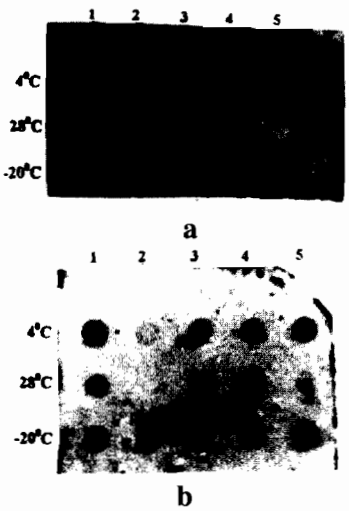

Fig 2–(a) Chemiluminescent detection of dot blot containing microfilariae positive blood samples stored at different temperatures (4°C/ 28°C/-20°C) for different duration (1 - 5 days), without passing through sephadex G-50, probed with the oligonucleotide mix, WBB1-5.

(b) Chemiluminescent detection of dot blot containing microfilariae positive blood samples stored at different temperatures (4°C/ 28°C-20°C) for different duration (1- 5 days), after passing through sephadex G-50, probed with the oligonucleotide mix, WBBl-5.

extracted from samples stored at 28°C and 40°C appeared to contain a pigment. This problem was overcome by eluting the DNA extracts through sephadex G-50 prior to blotting on to nylon membranes, these blood samples (stored at 28°C and 4°C) then gave positive signals for all five days (Fig 2b).

Effect of differential processing of blood samples on signal detection

Dot blots containing DNA extracts of blood samples positive for microfilariae processed up to different stages (proteinase K digests, chisam extracts and precipitated DNA) were hybridized under the optimal assay conditions. Positive signals were observed only in extracts processed up to the DNA precipitation stage (Fig 3a). Infected blood samples (n=26) processed upto proteinase K digestion, yielded positive results, only if they were lysed several times (4-5) prior to proteinase K treatment (Fig 3b).

Detection of L_3 larvae

The results of the hybridization assay for the dot blots containing L_3 larvae and vector mosquitos, processed up to different stages of extraction are given in Table 2. It was observed that true positive signals were observed for L_3 larvae in infected mosquitos, only when these samples were processed to the DNA precipitation stage and eluted through sephadex G-50, prior to dot blotting (Table 2).

Specificity of hybridization assay

To evaluate the specificity of the oligonucleotide mix WBB1-5; dot blots containing *W. bancrofti* DNA (0.5 and 0.25 ng), *Bugia malayi* DNA (100, 50, 10, 0.3 and 0.1 ng) and hurnan DNA (1 µg), were hybridized under the optimal assay conditions. The oligonucleotide mix WBB1-5; was observed to cross hybridize with purified *B. malayi* microfilarial DNA at concentrations > 10 ng (Fig 4) but there was no significant cross hybridization with human DNA.

Table 1
Hybridization conditions.

Prehybridization conditions 6 x SSC	Hybridization conditions 6 x SSC	Total probe concentration	Washing conditions 1%SDS	Sensitivity of detection (+)/non-specific binding to human DNA(NSB)
		5 pmol/ml		NSB / +
42°C/2h	40%FA/42°C/2h	10 pmol/ml	2xSSC/42°C	NSB / ++
		12.5pmol/ml	3 x 15 min	NSB / +++
		25 pmol/ml		NSB / ++++++
	40% FA42°C/1h		2 x SSC/42°C	NSB / +++
42°C/2h	40% FA42°C/2h	25 pmol/ml	3 x 15 min	NSB / ++++++
			1 x SSC/42°C	NSB / +++++
	40%FA/42°C/2h		3 x 15min	
			0.5x SSC/42°C	NSB / ++++
			3x15min	
42°C/2h		25 pmol/ml	1 x SSC/42°C	NSB / ++++
	S0%FA/42°C/2h		3 x 15min	
			0.5x SSC/42°C	+++
			3x15min	
42°C/ 1/2h				NSB /++
42°C/2 h ,	50%FA/42°C/2h	25 pmol/ml	0.5x SSC/42°C	+++
42 C/ 4 h			3 x 15min	+++/dark filter back ground
2 h/28°C(RT)	50%FA/42°C/2h	25 pmol/ml	0.5xSSC/42°C	NSB / ++++
2 h/42°C			3 x 15min	+++

+ - Intensity of the signal
FA - Formamide
NSB - Non specific binding to human DNA

Table 2
Detection of L_3 larvae and mosquitos.

Extract	Stage of extraction	Signal
	Proteinase K	+ve
L_3 +Mosq	Chisam	- ve
	DNA	- ve
	Proteinase K	+ve
L_3 + Mosq	Chisam	+ve
(sephadex G-50)	DNA	+ve
	ProteinaseK	+ve
Mosq	Chisam	- ve
	DNA	- ve
	Proteinase K	+ve
Mosq	Chisam	+ve
(sephadex G-50)	DNA	- ve

L_3 = one L_3 larva
Mosq = 10 Mosquitos
sephadex G-50 = extracts eluted through sephadex G-50

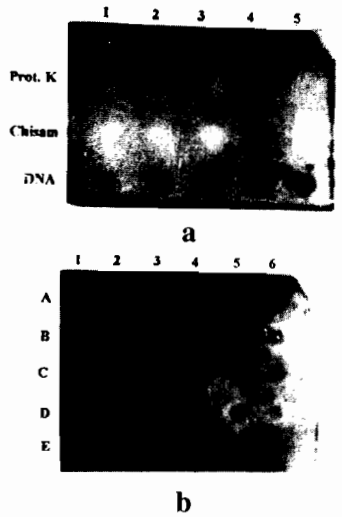

Fig 3–(a) Chemiluminescent detection of dot blot of microfilariae positive blood samples. Proteinase K digested extracts (lanes 1- 5), Chisam extracts (lanes 1 - 5) and precipitated DNA (lanes 1 - 5). These blots were probed with the oligonucleotide mix, WBB1-5.

(b) Chemiluminescent detection of dot blot containing proteinase K digested extracts (A1-A6, B1-B6, C1-C6 and D1-D6) of microfilariae positive blood samples (n = 26), passed through sephadex G-50, probed with the oligonucleotide mix, WBB1-5. *W. bancrofti* DNA (E1, 0.5 ng and E2, 0.25 ng) and of human DNA (E3, 2 µg and E4,1 µg).

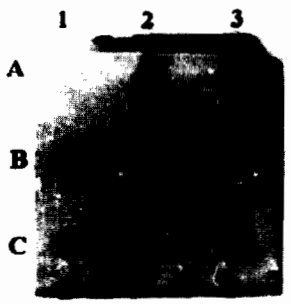

Fig 4–Chemiluminescent detection of dot blot containing dilutions of human DNA (A1, 1 µg), *W. bancrofti* DNA (A2, 0.25 ng and A3, 0.5 ng) and *B. malayi* DNA (B1, 100 ng; B2, 50 ng; B3, 10 ng; C1, 0.3 ng and C2, 0.1 ng), probed with the oligonucleotide mix, WBB1-5.

DISCUSSION

In recent years considerable effort has been expended for the development of alternative methods using DNA probes, for the detection of a wide variety of pathogenic organisms for clinical diagnosis. These methods offer considerable advantages over the conventional approaches for detecting pathogens (Wolcott, 1992). Bancroftian filariasis is a disease affecting the population in developing countries where the infrastructure facilities such as transport and laboratory technics are minimal. It is therefore essential that, for the successful application of DNA based diagnostics the technology should be simplified while retaining the sensitivity and specificity.

In the present assay, five oligonucleotides designed from a previously reported repetitive element for *W. bancrofti* (Siridewa *et al,* 1994), were used as probes. The assay detected as little as 100 pg of mf DNA, 1 mf and 1 L_3 and was found to be more sensitive than the cloned probe pWb12, from which they were derived (Siridewa *et al,* 1994). In the initial experiments proteinase K digests and the chisam extracts of microfilariae positive blood samples as well as microfilariae positive blood stored for up to five days at different temperatures gave false negative results. This can be attributed to the presence of the blood components (pigments) on the nylon filters following application of these extracts interfering with the hybridization and/or detection by chemiluminescence. It was possible to eliminate the interference caused by the pigment by either eluting the blood extracts through sephadex G-50 mini-columns or simply, lysing the blood samples

several times (4-5) with sterile distilled water prior to processing. It is no longer necessary for the blood samples to be processed up to the DNA precipitation stage for hybridization assay. Prompt processing of the blood samples may not be possible in the field due to lack of facilities. Hence the ability of this assay to detect blood samples stored for several days at ambient temperature, prior to processing could be an important advantage, especially for field surveys. Results indicated that mosquito samples need to be processed to the DNA precipitation stage and eluted through sephadex G-50 minicolumns to obtain consistent results. The oligonucleotide mix, WBBI-S; was found to cross hybridize with genomic DNA (>10 ng) of *Brugia malayi* (Fig 4). However, this will not hamper the detection as the lymphatic filariasis prevalent in Sri Lanka is caused by *W. bancrofti* only.

Microscopic detection of mf is a tedious and time-consuming process, and is disadvantageous especially when handling a large number of samples in the field. The PCR based assay developed in this laboratory for the detection of *W. bancrofti* (Siridewa *et al,* 1996) is more sensitive than the present hybridization assay. However, the method of detection reported in this paper is less costly than the PCR based assay (Rs 40.00/test, equivalent to 70 US cents) and does not require sophisticated and expensive laboratory equipment as for PCR. These biotinylated oligonucleotides due to their rapid, sensitive and simple hybridization protocol could form the basis for the future development of a specific and sensitive field kit for the identification of *W. bancrofti.*

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