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A polymerase chain reaction assay for the detection of *Wuchereria bancrofti* in blood samples from French Polynesia

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Abstract

A polymerase chain reaction (PCR) assay based on a highly repeated deoxyribonucleic acid (DNA) sequence found in *Wuchereria bancrofti* (the *Ssp*I repeat) has been developed to address the shortcomings of traditional diagnostic methods. In this field study in a *W. bancrofti* endemic region of French Polynesia, 373 human blood samples were collected and 100 µL of blood were screened by the *Ssp*I PCR assay and 1 µL by membrane filtration. The *Ssp*I PCR assay detected 99 of 113 blood samples in which microfilariae had been detected by filtration (sensitivity of 88%) with a specificity of 100%. All the samples missed by the *Ssp*I PCR assay had less than 8 microfilariae per mL of blood. To evaluate the efficacy of screening larger blood samples by PCR, both 100 µL and 500 µL samples from 50 patients with very low-level microfilaraemia were screened by the *Ssp*I PCR assay; the sensitivity increased from 60% to 84% when using the larger volume of blood. Finally, an enzyme-linked immunosorbent assay-based version of the *Ssp*I PCR assay was used to screen blood from 12 patients following treatment with diethylcarbamazine, ivermectin, or both. These results showed that the PCR assay closely paralleled the presence or absence of microfilariae in the blood and that no increase in the DNA level was seen immediately following drug treatment.

Keywords: filariasis, *Wuchereria bancrofti*, diagnosis, polymerase chain reaction

Introduction

The world burden of lymphatic filariasis is estimated to be at least 120 million people infected primarily by *Wuchereria bancrofti* and, to a lesser extent, by *Brugia malayi* and *B. timori* (see OTTESEN & RAMACHANDRAN, 1995). Traditional methods of diagnosis by blood film examination or membrane filtration of blood are inexpensive but tedious, time consuming, and confer little guarantee of species identification in areas where several filarial species coexist. Furthermore, in areas where microfilaraemia is nocturnally periodic, blood samples must be collected from subjects at night. The development of deoxyribonucleic acid (DNA) probes has provided the opportunity to improve the diagnosis of filiarial parasites. A DNA probe assay based on the *B. malayi Hha*I repeat was shown to be species-specific and sensitive (MCREYNOLDS *et al.*, 1986; WILLIAMS *et al.*, 1988, 1993; POOLE & WILLIAMS, 1990). Subsequently, a polymerase chain reaction (PCR) assay, also based on the *Hha*I repeat, was developed to detect *B. malayi* in blood samples (LIZOTTE *et al.*, 1994). A repeat sequence (the *Ssp*I repeat) has been identified from a *W. bancrofti* genomic library and used as the basis for a *Wuchereria*-specific PCR assay to detect *W. bancrofti* in blood samples (ZHONG *et al.*, 1996) and in mosquito vectors (CHANTEAU *et al.*, 1994; NICOLAS *et al.*, 1996).

We have refined the *Ssp*I PCR assay to make it more sensitive and have used it to detect *W. bancrofti* in blood samples collected in French Polynesia. This PCR assay was compared with the traditional filtration technique to identify microfilaraemic patients and was used to assess the persistence of DNA released from killed *W. bancrofti* microfilariae (mf) in the blood of patients following treatment with filaricidal drugs.

Materials and Methods

Extraction of microfilarial DNA from blood samples

One hundred µL of human blood were thoroughly mixed with 500 µL of TE buffer (10 mM Tris-HCl, pH8, 1 mM ethylenediaminetetraacetic acid [EDTA]) and centrifuged to pellet blood cells and microfilariae. The pellet was then washed with 500 µL of TE buffer, resuspended in 500 µL of red cell lysis buffer (RCLB; sucrose 35% w/v, 10 mM Tris-HCl, pH 7·6, 5mM $MgCl_2$, 1% Triton X100®), and incubated for 5 min at room temperature to digest the red blood cells. After centrifugation, the pellet was washed again in RCLB and then carefully resuspended in 200 µL of DSP buffer (20 mM Tris-HCl, pH 7·6, 2·5 mM $MgCl_2$, 50 mM KCl, 0·01% proteinase K, 0·5% Tween 20®). Incubation in DSP was performed at 60°C for 3 h to lyse the microfilariae and release the DNA. The proteinase K was then inactivated by incubating the samples at 90°C for 10 min. Following brief centrifugation to pellet debris, the supernatant was kept for PCR analysis. Extraction of some samples with low-level microfilaraemia was carried out using 500 µL of blood (as well as 100 µL) as described above, except that in these cases the blood was washed with 1 µL of TE instead of 500 µL.

Polymerase chain reaction conditions

Forward and reverse PCR primers designated NV-1 and NV-2 were designed based on the consensus sequence of the *Ssp*I repeat (ZHONG *et al.*, 1996). The sequences of these primers, which allow amplification of a 188 bp DNA fragment from *Wuchereria*, are as follows: primer NV-1 (21-mer) 5′-CGTGATGGCATCAAAGTAGTAGCG-3′and primer NV-2 (22-mer) 5′-CCCTCACTTACCATAAGAGACAAC-3′. Additionally, a 17-mer species-specific probe that hybridizes only to the *Ssp*I repeat of *W. bancrofti* (between the 2 PCR primers) was also synthesized. This hybridization probe has the sequence 5′GTTTGCTTGGTATAACC-3′. Reagents for PCR were obtained from Perkin-Elmer in the USA and Promega in French Polynesia.

PCR reactions were performed using 2 µL of the DNA extracts prepared from blood samples as described above. Reagents were used at the following concentrations in a 50 µL total reaction volume: 10 mM Tris-HCl, pH 9·2, 1·5 mM $MgCl_2$, 75 mM KCl, 0·1 µM of each primer, 0·2 mM of each deoxynucleotide triphosphate, and 2 units of *Taq* polymerase. The temperature programme for the PCR was 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C and a final extension of 10 min at 72°C. Ten µL of the PCR product were loaded on to a 1·5% agarose gel and a unique band of 188 bp was visualized by ethidium bromide staining. A negative control for the PCR assay using water instead of DNA extract in the reaction mixture was included with all runs of PCR. A positive control was also included using 1 or 0·1 pg of *W. bancrofti* genomic DNA.

Human blood samples and patients

Human blood samples were collected from 373 subjects during the day on the island of Raiatea in the Soci-

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ety Archipelago, French Polynesia, where *W. bancrofti* is endemic and aperiodic and where *B. malayi* is absent. Informed consent was obtained from all blood donors. Microfilaraemia was determined by filtration of 1 mL of venous blood through a Nuclepore® membrane (OTTESEN, 1990); 113 of the 373 individuals were microfilaraemic (1–7500 mf/mL). Aliquots of each blood sample were kept frozen at –20°C in 0·1 M EDTA until they were processed for PCR analysis. In addition, 23 blood samples were collected at night in Sulawesi, Indonesia from an area endemic for nocturnally periodic *B. malayi* and used to confirm the species-specificity of the PCR assay. Blood was also obtained from a European volunteer living in a non-endemic area. These additional blood samples were processed and amplified by PCR exactly as described for the Polynesian samples.

PCR analysis was also conducted on an additional 50 human blood samples from the island of Tahaa, French Polynesia containing 1–50 mf/mL to determine the sensitivity of the PCR assay on patients with low-level microfilaraemia. Both 100 µL and 500 µL aliquots of blood were processed from these patients.

Sera were separated from all of the Polynesian blood samples and the presence of *W. bancrofti* adult worm antigen was determined using the Trop-Ag *W. bancrofti* enzyme-linked immunosorbent assay (ELISA) kit (JCU Tropical Biotechnology Pty Ltd, Queensland, Australia). This assay is based on the ELISA detection of the Og4C3 antigen (MORE & COPEMAN, 1990).

Detection of DNA in the blood of microfilaraemic patients following drug treatment

The 12 patients used in this study were adult men (>20 years old) enrolled in a chemotherapy trial to compare efficacy of a single annual dose of diethylcarbamazine at 6 mg/kg (DEC), ivermectin at 400 µg/kg (IVR), or a combination of both drugs (IVR + DEC), on microfilaraemia and adult worm antigenaemia (MOULIA-PELAT *et al.*, 1995). Blood samples were taken by venepuncture before drug treatment and at 2 h, 1 d, 7 d, and 1, 3, 6, 9 and 12 months after treatment. Microfilaraemia was determined in all samples as described above and separate aliquots of each blood sample were stored frozen in 0·1 M EDTA until being thawed for processing and PCR analysis. Adult worm antigenaemia was determined in sera collected before drug treatment. Five of the 12 individuals were amicrofilaraemic; 2 of them were treated with DEC, while the other 3 were treated with IVR + DEC. The other 7 patients in the study were microfilaraemic (mean 86·8 mf/mL; range 10–476). Two of these individuals were treated with DEC, 3 with IVR, and 2 with IVR + DEC.

DNA was extracted from 500 µL aliquots of blood collected from each of the 12 individuals at each time point until month 3. PCR was carried out on 0·4% of each DNA extract using the procedure described above except that the NV-2 primer was 5′end-labelled with biotin. The PCR products were first analysed qualitatively by electrophoresis on 1·5% agarose gels stained with ethidium bromide. The PCR products were then quantified using a DNA ELISA. Briefly, biotin-labelled PCR products were immobilized on microtitre plates coated with streptavidin, hybridized with a fluoresceinated internal oligonucleotide probe, and quantified by detection with an enzyme-conjugated antibody to fluorescein using substrate-based colorimetric detection. The 17-mer internal hybridization probe was labelled using a 3′end oligolabelling system (Amersham). The DNA ELISA technique was modified from that of NUTMAN *et al.* (1994) as follows: (i) streptavidin was used to coat the microtitre plates in phosphate buffered saline (PBS) instead of carbonate buffer and (ii) following hybridization, the oligonucleotide probe was detected using horse-radish peroxidase conjugated to the antifluorescein antibody instead of an alkaline phosphatase conjugate.

To estimate the quantity of *W. bancrofti* DNA in the patient samples, a standard curve was established comparing the optical density obtained in the DNA ELISA and the concentration of *W. bancrofti* DNA (using two-fold dilutions of genomic DNA isolated from mf).

Testing for Ssp*I PCR product following drug treatment*

To test whether the DNA extraction protocol described above could be used to detect an increase in DNA released from dead or dying worms following drug treatment, blood samples were collected at various times following treatment of 12 Polynesian patients with IVR, DEC, or a combination of IVR and DEC; 500 µL aliquots of blood were extracted and amplified by PCR as described above.

Results

The *Ssp*I PCR assay on 100µL of blood detected 99 of the 113 samples shown to be microfilaraemic by filtration (Table 1). The 14 microfilaraemic samples that were

Table 1. Comparison of membrane filtration with the *Ssp*I PCR assay for the detection of microfilaraemia in 373 Polynesian blood samples

No. of microfilariae per mL	Number of subjects		
	Total	Positive by PCR	Antigenaemic[a]
Amicrofilaraemic subjects			
None	260	0 (0%)	105 (40·3%)
Microfilaraemic subjects			
1–10	17	3	11
11–50	7	7	7
51–200	11	11	11
201–1000	24	24	24
>1000	54	54	54
Total	113	99 (88%)	107 (95%)

[a]Og4C3 antigen detected by ELISA.

negative by PCR contained very low numbers of mf (<8/mL), and 6 of these samples also gave negative results when tested for adult worm antigen. All the samples with >9 mf/mL were positive by both PCR and antigen detection. All the 260 amicrofilaraemic samples were also negative by PCR, while antigen was detected in 105 of them (40·3%); 52 of these amicrofilaraemic PCR-negative samples were selected for further analysis, 26 because they gave a positive result for adult worm antigen; the other 26 were a random selection of antigen-negative samples (500 µL of blood were processed from these samples). The PCR products from these 52 samples were hybridized on a Southern blot with the internal 17-mer probe; all remained negative. In addition, all 26 of the human blood samples containing *B. malayi* from Indonesia were negative when tested using the *Ssp*I *Wuchereria*-specific PCR assay (all 26 of these samples were positive when tested using the *Hha*I *Brugia*-specific PCR assay).

When the PCR assay was performed on all of the Polynesian and Indonesian samples using 100 µL of blood, the sensitivity of the assay was 87·6% and the specificity was 100%. The positive predictive value was 100% and the negative predictive value was 94·9%.

Both 500 µL and 100 µL of blood from samples with low-level microfilaraemia (1–50 mf/mL, mean=14·9, SD=13·4) were subjected to the *Ssp*I PCR assay. The sensitivity was increased when DNA was extracted from 500 µL (Table 2). When 500 µL were tested, all the samples still negative by PCR, but which were microfilaraemic, contained <5 mf/mL.

Twelve patients were tested by the *Ssp*I PCR assay for *W. bancrofti* DNA in blood following drug treatment with DEC, IVR, or both. Five of these 12 patients were amicrofilaraemic before treatment and remained so for at least 3 months following treatment. All 12 patients

Table 2. *Ssp*I PCR assay of 50 blood samples with low-level microfilaraemia

No. of microfilariae per mL	No. of samples positive by PCR 100 μL	500 μL
1–5	2/19	11/19
6–10	7/8	8/8
11–20	7/8	8/8
21–50	14/15	15/15
Total	30/50 (60%)	42/50 (84%)

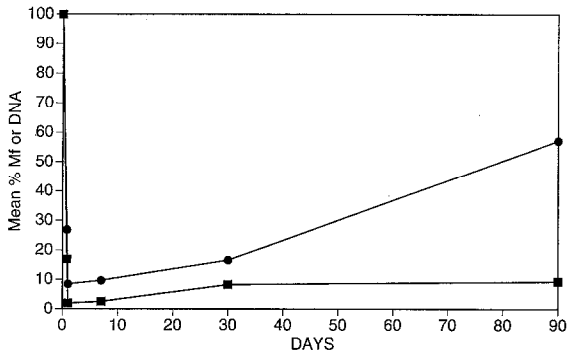

Figure. *Wuchereria bancrofti*: the amount of DNA (I) and the number of microfilariae (n) remaining following drug treatment plotted against time, calculated by averaging the amount of DNA or the number of microfilariae for all 7 patients listed in Table 3 and then converting to percentages of the mean value at time 0.

PCR data and mf count data were in perfect agreement on which samples were positive and which samples were negative, except for patient no. 3. Both PCR product and mf count data decreased rapidly 2 h after treatment in all patients. In 2 patients (nos. 2 and 3), there was a sharp increase in DNA at 3 months although mf counts remained very low at this time. In these 2 individuals, microfilaraemia increased again in the following months (Table 3).

Discussion

In this field study, the *Ssp*I PCR assay proved efficacious in species-specific detection of *W. bancrofti* mf in human blood samples; it was positive only when mf were present in the blood. Of the 113 samples shown to be microfilaraemic by filtration of 1 mL of blood, 99 were positive when 100 mL of blood were processed for PCR. The 14 samples that were microfilaraemic but negative by PCR all had <8 mf/mL of blood. Since these samples would have contained, on average, fewer than 1 mf per 100 μL, it is not surprising that some of these samples with low-level microfilaraemia would prove to be negative by PCR. It seems logical that increasing the volume of blood processed for PCR would increase the likelihood of a positive PCR result with samples containing very low numbers of mf. To test this hypothesis, 50 blood samples with <50 mf/mL were collected and tested by processing 100 μL and 500 μL of blood for PCR. The sensitivity increased from 60% with 100 μL to 84% with 500 μL, suggesting that even better results might be obtained by processing 1 μL of blood for PCR.

Since treatment with DEC, IVR, or both is expected to lead to rapid killing of mf in the blood, it was thought that the *Ssp*I PCR-ELISA might detect a transient post-treatment

Table 3. Microfilaria counts and quantity of *W. bancrofti* DNA found in the blood of seven Polynesian patients following drug treatment

Treatment[a] and patient no.	Time after treatment[b] –	2 h	1 d	7 d	1 Month	3	6	9	12
Diethylcarbamazine 6 mg/kg									
3	289/>100	30/12	6/0	8/3	8/0	3/>100	17/–	98/–	170/–
23	51/40	4/0	2/8	8/0	28/>100	26/>100	34/–	34/–	40/–
Ivermectin 400 μg/kg									
26	22/>100	3/0	0/0	0/0	0/0	0/0	20/–	22/–	102/–
19	476/>100	238/>100	6/2	0/9	1/0	51/>100	221/–	340/–	527/–
2	204/>100	44/10	0/0	0/0	1/2	3/62	–/–	68/–	220/–
Both treatments									
8	118/>100	6/0	0/0	0/0	0/0	1/0	4/–	–/–	16/–
63	10/3	1/0	0/0	0/0	0/0	0/0	0/–	0/–	0/–

[a]Single annual dose.
[b]Results shown are no. of microfilariae per mL/ pg of DNA per mL of blood; – indicates that the DNA assay was not done.

gave negative results in the PCR assay throughout the study. The kinetics of microfilaraemia in the other 7 patients (Figure; data from Table 3) were derived from the observations of MOULIA-PELAT *et al.* (1994). There was a marked reduction in microfilaraemia 2h after treatment in all 7 individuals, regardless of which drug treatment was used. All patients treated with IVR alone or DEC + IVR were rapidly cleared of mf between days 1 and 7, in contrast with those treated with DEC, who remained microfilaraemic. Three to 6 months following treatment, mf counts increased in all patients except for one treated with IVR + DEC, who remained amicrofilaraemic for 12 months.

The standard curve (not shown) indicated that the minimum amount of DNA detected by the PCR assay was 3 pg/mL of blood. The amount of *W. bancrofti* PCR product revealed by the DNA ELISA closely paralleled the mf count at all time points (Table 3). At day 1, the increase in *W. bancrofti* DNA levels in the blood. No such increase was seen in any of the 7 patients examined, suggesting that the DNA released from dead or dying mf is rapidly degraded or cleared by the host immune system.

Thus, the *Ssp*I PCR assay seems ideal for monitoring blood samples for microfilaraemia since it is positive only when mf are present in the blood and can detect as few as one mf per mL of blood (ZHONG *et al.*, 1996). The filarial antigen assay, on the other hand, gave positive results with many amicrofilaraemic, as well as microfilaraemic, patients. The former patients were assumed to be harbouring adult worms, although this was impossible to verify. Therefore, the antigen assay may be a more sensitive indicator of infection, while the PCR assay can be used to determine microfilaraemia more efficiently and with greater sensitivity than blood film examination (ZHONG *et al.*, 1996). Since the antigen assay indicates infection and the PCR assay indicates micro-

filaraemia, the 2 assays may prove complementary in a variety of studies.

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