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Development of a quantitative, competitive polymerase chain reaction–enzyme-linked immunosorbent assay for the detection of *Wuchereria bancrofti* DNA

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Abstract A quantitative, competitive polymerase chain reaction (QC-PCR) assay for the sensitive detection of *Wuchereria bancrofti* DNA was developed. A competitor sequence was constructed by an exchange of nucleotides in the *Wuchereria*-specific *Ssp* I repeat. The PCR products were hybridized to specific DNA probes and their amounts, determined by an enzyme-linked immunosorbent assay (ELISA). In laboratory-prepared samples the QC-PCR-ELISA assay was capable of detecting the amount of DNA equivalent to 0.1 microfilaria (mf) added to 200 µl of blood lysate. The assay was also tested on 78 blood samples collected in endemic areas in Egypt. All 28 samples that were positive both for mf and for circulating antigen were also QC-PCR-ELISA-positive. In addition, one mf-negative but antigen-positive sample was also positive as determined by QC-PCR-ELISA. A positive correlation of mf density with the QC-PCR-ELISA was observed. Samples containing 10 or fewer mf/ml had a mean relative amount of *Ssp* I PCR product of 19.7 units, whereas samples with 11–100 mf/ml had a mean of 36.3 units and those with more than 100 mf/ml had a mean of 84.6 units. Because of the high standard deviation within each group, estimates of worm burdens in infected individuals using the QC-PCR-ELISA are not recommended. However, we present data indicating that the *W. bancrofti* QC-PCR-ELISA is a powerful new tool for evaluation of parasitic loads for community-based diagnosis of bancroftian filariasis.

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Introduction

The worldwide eradication of the human parasite *Wuchereria bancrofti*, which infects about 115 million people in tropical regions of Asia, Africa, the Americas, and the Pacific (Michael and Bundy 1997), is a declared goal of the World Health Organization (WHO). The comparison of DNA-based diagnostic techniques with classic parasitology techniques was recommended as an operational research need by the WHO (WHO 1994; Ottesen and Ramachandran 1995). Extensive control programs involving mass treatment of the human population using diethylcarbamazine and ivermectin or a combination of both have been initiated. In some regions, vector control as a part of general mosquito control is also performed. For monitoring the success of these procedures, specific and sensitive diagnostic tools are required. Since there is a quantitative correlation between transmission intensity, prevalence, microfilaria (mf) density, and morbidity in the community (Kazura et al. 1997), there is a special need to quantify the parasite load in human populations.

DNA-based diagnostic tests for *W. bancrofti* infections have been shown to overcome some deficiencies of parasitological and serological diagnosis, and specific and sensitive polymerase chain reaction (PCR)-based assays have been reported (Chanteau et al. 1994; Abbasi et al. 1996; McCarthy et al. 1996; Nicolas et al. 1996; Williams et al. 1996; Zhong et al. 1996; Furtado et al. 1997; Ramzy et al. 1997). However, these assays are not quantitative, primarily because blood samples or samples from insect vectors contain different amounts of PCR inhibitors (Tirasophon et al. 1994; Fischer et al. 1997), and within-sample tube-to-tube variations may occur (Schnell and Mendoza 1997). The most precise quantitation of DNA can be obtained by competitive coamplification of a specific target sequence together with known concentrations of an internal standard in one reaction tube (Zimmerman and Mannhalter 1996; Schnell and Mendoza 1997).

For the development of a quantitative, competitive PCR (QC-PCR) assay for the sensitive detection of *W. bancrofti* the *Ssp* I repeat was chosen as the target sequence. This dispersed DNA repeat exists in about 300 copies per haploid genome and has been used to detect sensitively *W. bancrofti* in blood (Williams et al. 1996) and mosquito samples (Chanteau et al. 1994). In the present study an internal control was constructed by an exchange of nucleotides in the wild-type *Ssp* I repeat. Following competitive PCR the wild-type *Ssp* I repeat and the internal control were identified by differential hybridization with two DNA probes, one specific for the wild-type *Ssp* I repeat and one specific for the internal control. The amounts of the hybridized PCR products were determined by an enzyme-linked immunosorbent assay (ELISA). This QC-PCR-ELISA was tested on blood samples with different numbers of *W. bancrofti* mf from an endemic area in Egypt.

Materials and methods

Construction of the internal control

The *Ssp* I repeat of *Wuchereria bancrofti* was amplified using the NV-1 and NV-2 primers (Fig. 1; Williams et al. 1996; Zhong et al. 1996), and the PCR product was ligated into the pCR 2.1 TA plasmid cloning vector according to instructions given by the manufacturer (Invitrogen, Carlsbad, Calif., USA). One clone (WbT14B) was selected for construction of the internal control. Two primers were designed that flanked the hybridization region of the *Ssp* I repeat (Zhong et al. 1996) and possessed 5′ overhangs of 7 and 9 nucleotides. All oligonucleotides used in the present study were purchased from Oligo Etc./Oligotherapeutics (Wilsonville, Ore., USA). These forward (5′ATCAGACTAAAAAAAAAAAT-AATTCAAAT3′) and reverse (5′GCTGTATCTTATTTTTT-AATCTTTTTTAAT3′) primers were phosphorylated using T4 polynucleotide kinase (Stratagene, La Jolla, Calif., USA). Subsequently, an inverse PCR using *Pfu* polymerase (Stratagene) was performed using the WbT14B plasmid as a template.

The linear product of the inverse PCR was then phosphorylated and ligated overnight in the same reaction tube using T4 polynucleotide kinase and T4 DNA ligase (Stratagene). This new internal control plasmid (Fig. 1) was then transformed into InvαF-competent *Escherichia coli* cells (Invitrogen) and plated on LB agar plates containing ampicillin and X-gal. Following overnight growth, ten colonies were picked and the inserts were directly amplified by PCR using T7 forward and M13 reverse primers.

DNA sequencing

The ten PCR products were purified using silica-gel membrane spin-columns (Qiaquick PCR Purification Kit; Qiagen, Hilden, Germany) and sequenced by direct cycle sequencing using the ABI Prism DNA Sequencing Kit (Applied Biosystems Division, Perkin Elmer, Foster City, Calif., USA). The products of the sequencing reactions were purified using Centri-Sep columns (Princeton Separation, Adelphia, N.J., USA) and were run on an ABI 373A automated DNA Sequencer (Perkin Elmer).

Selection and preparation of the internal control

The results of the DNA sequencing reactions were analyzed using the Seq Ed v 1.0.3 computer program (ABD, Perkin Elmer). One plasmid clone that was identical to the *Ssp* I wild-type repeat except for the desired nucleotides substituted in the hybridization region (Fig. 1) was selected for use as the internal control. This clone was cultured for 10 h in LBA medium and plasmid purification was performed using the QIAprep Plasmid Purification Kit (Qiagen). For use of the internal control plasmid as a competitor in the QC-PCR, it was linearized to make it as identical as possible to the wild-type *Ssp* I repeat. Therefore, it was digested using the restriction enzyme *Bgl* II (New England Biolabs, Beverly, Mass., USA). The concentration of the linearized internal control plasmid was determined with a Lambda 3 UV/VIS spectrophotometer (Perkin Elmer).

Blood samples and DNA preparation

For experiments to test the sensitivity of the QC-PCR, *W. bancrofti* mf were diluted in 1 X phosphate-buffered saline (PBS) to obtain solutions containing known numbers of mf. These mf were added to 100 µl of human blood [containing 1 m*M* ethylenediaminetetraacetic acid (EDTA)] collected from noninfected North Americans. Blood samples from areas endemic for *W. bancrofti* were collected in a rural village located 40 km northeast of Cairo, Egypt (Ramzy et al. 1997). The samples were examined for mf by filtration of 1 ml of blood through a membrane (5-µm pore size;

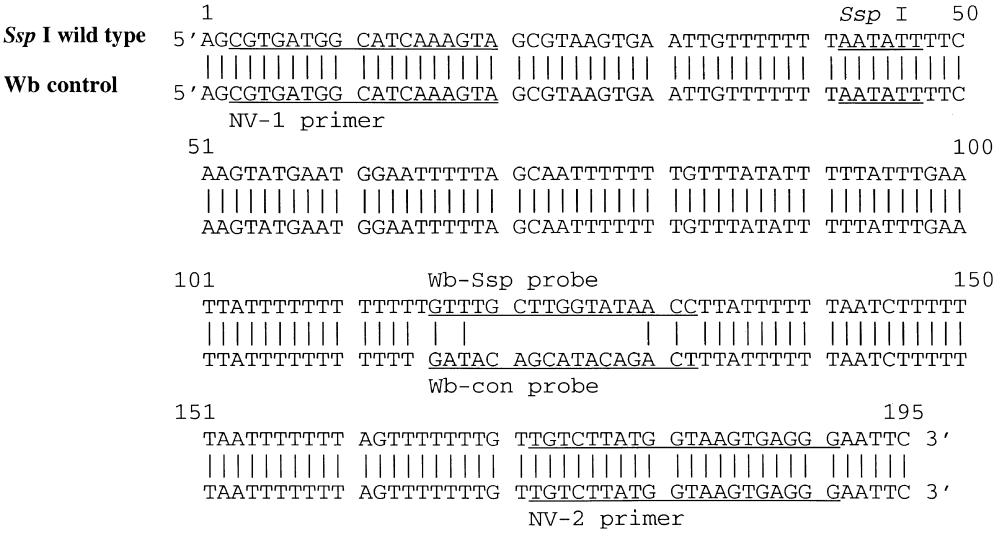

Fig. 1 Comparison of the *Ssp* I wild-type repeat of *Wuchereria bancrofti* and the modified *Ssp* I repeat used as the internal PCR control. The figure shows the location of the PCR primers NV-1 and NV-2, the *Ssp* I restriction site, and the hybridization region of the two different DNA probes (Wb-ssp, Wb-con)

Nucleopore, Pleasanton, Calif., USA) followed by microscopic examination of the Giemsa-stained filters. Most mf-positive samples contained only low numbers of mf. In addition, the blood samples were tested for the presence of circulating *W. bancrofti* antigen using an ELISA (Ramzy et al. 1991). Finally, lysates were prepared from these blood samples as described in detail elsewhere (Williams et al. 1996), and 2 µl of the lysate was used for the diagnostic PCR.

QC-PCR procedure

The PCR conditions were the same as those described previously (Williams et al. 1996). A 5′ biotinylated NV-2 (reverse) primer was used to obtain a biotinylated PCR product, which can easily be bound to a streptavidin-coated microtiter plate. For each sample, 100 fg of the internal control was added to the PCR master mix. The internal control was diluted in 0.1 X TRIS-EDTA buffer containing 10 ng fish-testes DNA/µl (Amresco, Solon, Ohio, USA) as a carrier and was stored in aliquots at −20 °C. Next, 8 µl of each PCR product was run on a 1.5% agarose gel and visualized by ethidium bromide staining. For comparison, the samples from Egypt were also tested in the conventional *W. bancrofti* PCR assay using a nonbiotinylated reverse primer (Williams et al. 1996).

PCR-ELISA procedure

This procedure was performed according to Nutman and coworkers (1994) with some modifications. Microtiter plates (Labsystems, Needham Heights, Mass., USA) were coated overnight at 4 °C using 1 µg streptavidin/ml (Sigma, St. Louis, Mo., USA) in coating buffer (0.1 *M* Na_2CO_3-$NaHCO_3$, pH 9.6). Before use the plates were washed twice with 2 X PBS. For duplicate testing of each sample (once with the control hybridization probe and once with the wild-type *Ssp* I specific hybridization probe), 40 µl of the biotinylated PCR product was mixed with 360 µl of hybridization buffer [6 X SSPE, 5 X Denhardt's solution, 0.1% sodium sarcosine, 0.02% sodium dodecyl sulfate (SDS), 0.05% NaN_3] and 100 µl was added to each of four wells and incubated for 30 min at room temperature. The samples were denaturated for 10 min at room temperature by the addition of 100 µl of 0.3 *M* NaOH. Subsequently the wells were washed once with 1 X PBS and once with hybridization buffer.

For each sample, two of the wells were hybridized with the wild-type *Ssp* I-specific DNA probe (Wb-ssp) and two were hybridized with the internal-control-specific DNA probe (Wb-con). Since only the reverse primer is biotinylated, probes were selected that hybridized to this strand of the PCR product (Fig. 1). Each oligo probe was fluorescein-labeled at both the 5′ and 3′ ends during synthesis (Oligo Etc./Oligotherapeutics). The probes were diluted to 50 (Wb-ssp) and 10 pg/µl (Wb-con) in hybridization buffer and were denaturated by heating for 5 min at 95 °C, and then 100 µl was added to the appropriate wells. Following hybridization for 30 min at 55 °C the wells were washed twice with 1 X PBS and once with 1 X PBS/0.1% bovine serum albumin (BSA) for 5 min at 55 °C. Thereafter, anti-fluorescein-AP, Fab fragments (Boehringer Mannheim, Germany) were diluted 1:3000 in 1 X 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 using 1 X PBS/0.5% Tween-20 and two times using 1% diethanolamine/20 m*M* $MgCl_2$ (pH 10.0). Finally, 100 µl of alkaline phosphatase substrate was added according to the instructions of the manufacturer (AP substrate Tablets 104, Sigma). Typically, color development started immediately after the addition of the substrate. ELISA reading was performed at 405 nm following 1 h of incubation at 37 °C using a Vmax microplate reader (Molecular Devices, Sunnyvale, Calif., USA). For the detection of ultralow mf densities the plate reading was repeated after an additional 1 h period. Unless otherwise noted, all washing steps were performed using 100 µl of solution at room temperature and all reagents were purchased from Sigma or Fisher (Fair Lawn, N.J., USA).

Data analysis

Since each sample was tested twice, the arithmetic mean of the optical density (OD) of both samples was used for further calculation. First, the OD readings of the blanks were subtracted from the OD values recorded for all samples and controls. During the ELISA, at least four wells were used as blanks and were treated like samples but contained no PCR product. $OD_{Wb\text{-}ssp}$ indicates the OD of a sample hybridized with the Wb-ssp probe, whereas $OD_{Wb\text{-}con}$ indicates the OD of a sample hybridized with the control probe. A positive $OD_{Wb\text{-}ssp}$ reading for a blood sample was defined as being 5 times the $OD_{Wb\text{-}ssp}$ recorded for the negative controls (in most cases, positive OD readings were > 0.059). Negative controls contained PCR product from 100 fg of the internal control plasmid DNA and DNA extracts of blood samples from five uninfected North Americans. Later in the study, water was used as the negative control because no difference in the $OD_{Wb\text{-}ssp}$ reading of PCR products obtained from uninfected blood samples or from water was found.

Because 100 fg of the internal control plasmid was used as the competitor DNA in all samples, an index for the amount of *Wuchereria* DNA present in positive samples was calculated as follows:

$$X = 100 \times \frac{(OD_{Wb\text{-}ssp} \text{ of sample})}{(OD_{Wb\text{-}con} \text{of sample})} \quad .$$

For more accurate comparison of the results of different ELISA detections, an additional factor, *F*, was defined, which describes the relationship of the maximal extinction of the ELISA reader (3.000) with the $OD_{Wb\text{-}con}$ value recorded for the negative control of each ELISA detection. This factor approached 1.0 if the blank readings were low and the maximal reading capacity of the ELISA reader was reached with the control probe Wb-con hybridized to a negative-control PCR product:

$$F = \frac{3.000}{OD_{Wb-con} \text{ of negative control}} \quad .$$

Finally, the product of *X* and *F* was used to define the relative amount of *Wuchereria* DNA in wild-type *Ssp* I-positive samples.

Results

Construction and amplification of the internal standard Wb control

The sequence of the *Ssp* I wild-type repeat and that of the internal control are compared in Fig. 1. With the exception of the hybridization region between nucleotides 115 and 132, both sequences are identical. As little as 10 fg of the internal control could be detected by PCR and agarose-gel electrophoresis with ethidium bromide staining (Fig. 2). When the PCR-ELISA was used for detection of the PCR product, 1 fg of the internal control was detectable by PCR, suggesting a 10-fold higher sensitivity for the ELISA detection as compared with conventional gel electrophoresis. No difference was found in the sensitivity of the PCR assay when one of the primers was biotinylated. These results showed that the internal control was amplified at least as efficiently as the wild-type *Ssp* I repeat. Therefore, this linearized plasmid containing the nucleotide exchange at the Wb-ssp hybridization region was judged suitable as a competitor for the wild-type *Ssp* I repeat in the QC-PCR.

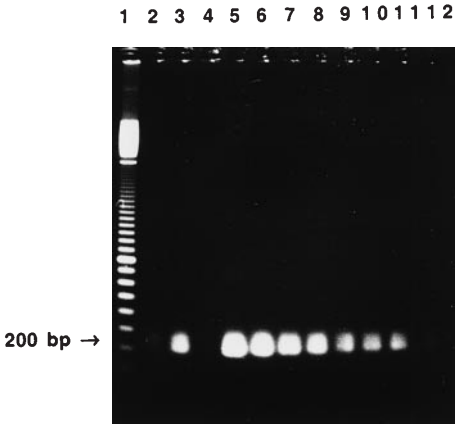

Fig. 2 Sensitivity of the amplification of the internal PCR control plasmid. The PCR products were run on a 1.5% agarose gel and stained by ethidium bromide (*Lane 1* 100-bpair ladder; *lane 2* 1 pg genomic *W. bancrofti* DNA; *lane 3* 100 pg genomic *W. bancrofti* DNA; *lane 4* negative control – buffer; *lanes 5–12* linearized internal control plasmid – 10 ng, 1 ng , 10 pg, 1 pg, 100 fg, 50 fg, 10 fg, and 1 fg, respectively)

QC-PCR findings

It was found that use of the correct amount of competitor DNA is a crucial factor for the accuracy of the assay. Too much of the internal control can lead to an excess of control PCR product and too little wild-type *Ssp* I PCR product for efficient ELISA detection. A concentration of 100 fg/50 µl of the internal control for the competitive PCR was determined to be most suitable for high mf densities as well as for low mf densities. This amount corresponds to the DNA equivalent of 0.1 mf in 200 µl of blood lysate.

Different numbers of PCR cycles ranging up to 35 were tested. A smaller cycle number kept the PCR in the exponential phase over a broader range and a more accurate quantitative analysis was achieved. However, cycle numbers between 25 and 32 resulted in poor sensitivity of the PCR assay. Therefore, 35 cycles were used for the QC-PCR.

Hybridization of the internal control and the wild-type *Ssp* I repeat

No cross-hybridization between the internal control and the DNA probe specific for the wild-type *Ssp* I repeat (Wb-ssp) was found. When 100 pg of genomic *Wuchereria bancrofti* DNA was used as a PCR template the OD of the hybridization with the *Ssp* I wild-type-specific probe (Wb-ssp) was 1.675 ± 0.916 ($n = 18$, arithmetic mean value ± SD), whereas the OD of the hybridization with the internal-control-specific probe (Wb-con) was 0.047 ± 0.054. In contrast, when 1 pg of the linearized internal control plasmid was used as a template the OD of the hybridization with the *Ssp* I wild-type-specific probe Wb-ssp was 0.204 ± 0.131, whereas the OD of the hybridization with the internal-control-specific probe Wb-con was 2.527 ± 0.642 ($n = 18$, arithmetic mean value ± SD).

Sensitivity of the QC-PCR-ELISA

In a series of experiments the following numbers of mf were added to 100 µl of blood containing 1 m*M* EDTA: 0 mf to each of 5 samples; 1 mf to each of 15 samples; 2 mf to each of 5 samples; and 10, 100, 150, 200, 500, and 1000 mf to each of 2 samples. All 5 samples containing 0 mf and 7 of the 15 samples with 1 mf were negative in the PCR-ELISA, whereas all other samples were positive, showing increasing amounts of wild-type *Ssp* I PCR product (Table 1). The negative results recorded for 7 of the 15 samples containing 1 mf could be explained by the failure to add a single mf to the samples. Because of the difficulty in adding a single mf to 100 µl of blood, many of the 15 samples may not have had one mf added, whereas some may have had two added. To prove this hypothesis we prepared DNA from mf and mixed it with 200 µl of blood lysate from noninfected individuals. When an amount of DNA equivalent to 0.1, 1, 10, and 20 mf was added to each of 3 samples, all 12 samples were positive, showing relative amounts of wild-type PCR product of 20.2 ± 6.6 (arithmetic mean value ± SD), 26.1 ± 2.5, 61.4 ± 5.5, and 106.6 ± 20.3 units, respectively.

To test the assay on field samples from an area hypoendemic for *W. bancrofti* we tested 78 blood samples from Egypt. All 28 samples that were mf-positive as determined by parasitological examination of 1 ml of blood were also positive in the PCR-ELISA (Table 2). In addition, one of the samples thought to be mf-negative was shown to contain *W. bancrofti* DNA.

Table 1 Sensitivity of the competitive PCR-ELISA tested on blood samples with a known number of mf. Mf were added to 100 µl of human blood containing 1 m*M* EDTA. The optical density was measured at 405 nm. The relative number of units of wild-type *Ssp* I PCR product was calculated as described shown in Materials and methods

Number of mf added	Number of samples	PCR-ELISA-positive samples	
		n	Mean units ± SD
0	5	0	–
1[a]	15	8	10.2 ± 5.2
2	5	5	12.6 ± 7.2
10	2	2	6.9
100–200	6	6	19.1 ± 7.4
500	2	2	25.0
1000	2	2	58.7

[a] Because of the difficulty in adding a single mf to 100 µl of liquid, many of these samples may not have had one mf added, whereas some may have had two added

Table 2 Comparison of the parasitological assessment of mf using circulating antigen detection (Ag assay), the conventional PCR assay, and the QC-PCR-ELISA for the detection of *Wuchereria bancrofti* infection using blood samples from patients living in an endemic area in upper Egypt

Number of mf per1 ml blood	Number of samples	Number of positive samples		
		Ag assay	Conventional PCR	QC-PCR-ELISA
0	50[a]	6	1	1
1–5	3	3	3	3
6–100	21	21	21	21
>100	4	4	4	4

[a] 3 Samples were not tested in the Ag assay

Comparison of the QC-PCR-ELISA with conventional PCR and antigen detection

All 3 assays were positive in all 28 mf-positive samples from Egypt (Table 2). When the conventional PCR assay was used, 1 of the 50 mf-negative samples was identified as being infected with *W. bancrofti*. This same sample was also found to be positive by the QC-PCR-ELISA assay and by the detection of circulating *W. bancrofti* antigen.

Quantitation of the *Ssp* I PCR product

It was found that the higher the number of mf in a sample, the higher the relative amount of *Ssp* I PCR product. When 1–10 mf were added to 100 µl of uninfected blood the average relative amount of wild-type *Ssp* I PCR product was 10.6 units, whereas when more than 100 mf were added it was 28.2 units. This difference was statistically significant (Mann-Whitney *U*-test, $P = 0.0006$). For these samples a positive correlation was found between the number of mf added and the relative amount of *Ssp* I PCR product ($r = 0.6976$, *df* 17; Fig. 3). In the field samples from Egypt with fewer than 11 mf/ml the relative amount of *Ssp* I PCR product was an average of 19.7 units, in samples containing 11–100 mf/ml it was an average of 36.3 units, and in the four samples with more than 100 mf/ml it was 84.6 units. These differences were also statistically significant (Mann-Whitney *U*-test, $P = 0.013$). Additionally, for the field samples a positive correlation between mf density and the relative amount of *Ssp* I PCR product was detected ($r = 0.3964$, *df* 27; Fig. 4), although it was somewhat weaker as compared with the laboratory-produced samples.

When the results recorded for PCR-ELISA-positive samples of the laboratory and the field study were compared, it was found that the relative amount of wild-type *Ssp* I PCR product was significantly higher in samples collected from naturally infected persons (Mann-Whitney *U*-test, $P = 0.0012$). Since the mf density in the field-collected samples was lower, this observation may indicate the presence of a source of *W. bancrofti* DNA other than intact mf in these samples.

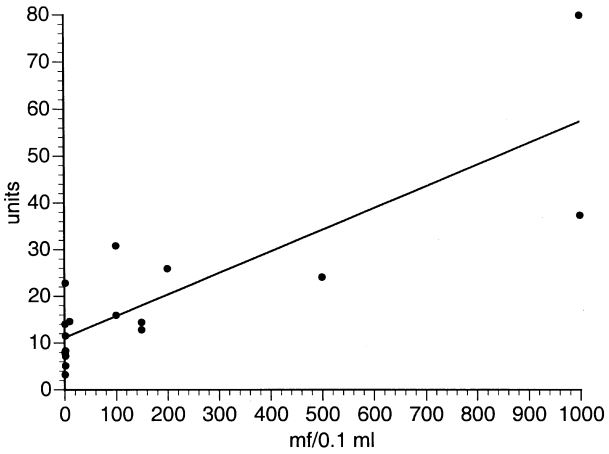

Fig. 3 Comparison of the relative amount of wild-type *Ssp* I PCR product (units) in relation to the number of *W. bancrofti* mf added to 100 µl of blood

For investigation of the reliability of the QC-PCR-ELISA, one sample containing 233 mf/ml and one with 10 mf/ml were repeatedly tested. For each sample, nine different PCR reactions were performed. Although the sample with the high mf density produced only a moderate amount of PCR product as visualized on agarose gels (Fig. 5), the ELISA results revealed an average relative amount of 33.5 units of wild-type *Ssp* I PCR product (SD 7.8 units, range 22.2–47.3 units). The sample with the low mf density yielded a much higher amount of PCR product on the agarose gel (Fig. 5), but the differential hybridization and ELISA detection showed that most of the product was amplified internal control, with the average relative amount of wild-type *Ssp* I PCR product being only 4.7 units (SD 0.6 units, range 3.5–5.4 units). These results display some degree of variability for individual PCR reactions using the

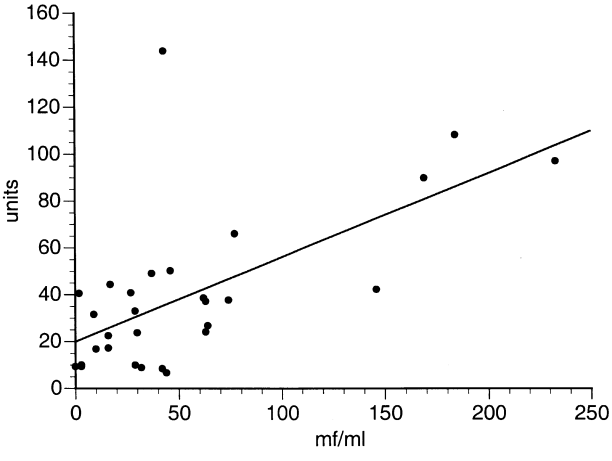

Fig. 4 Comparison of the relative amount of wild-type *Ssp* I PCR product (units) in relation to the number of mf present in 1 ml of blood collected from persons living in an area hypoendemic for *W. bancrofti* in Egypt

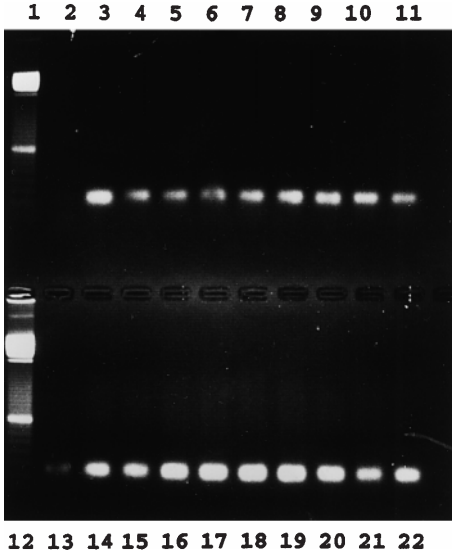

Fig. 5 Reliability of the QC-PCR for two samples with different numbers of mf as compared in independent PCR reactions. The PCR products were run on a 1.5% agarose gel and stained using ethidium bromide. (*Lanes 1, 12* 100-bp ladder; *lane 2* negative control – no sample, no internal control; *lane 3* wild-type *Ssp* I-negative control but with internal positive control; *lanes 4–11, 13* results of 9 individual PCR reactions using a sample containing 233 mf/ml as a template; *lanes 14–22* results of 9 individual PCR reactions using a sample containing only 10 mf/ml as a template)

same sample as a template. However, they indicate that the presence of PCR inhibitors in the sample with the high number of mf was detected reliably in each individual reaction tube.

Discussion

The objective of the present study was to develop a simple, fast, sensitive, and inexpensive PCR assay for estimation of the relative amount of *Wuchereria bancrofti* DNA in blood samples. The ELISA-based detection of PCR products of the *Ssp* I repeat was successfully employed to increase the sensitivity of the PCR assay, to process rapidly a large number of samples simultaneously, and to estimate the relative amount of *W. bancrofti* DNA present in a sample.

Our results demonstrated that the modified *Ssp* I internal control is a compatible PCR competitor for the wild-type *Ssp* I repeat. The efficiency of amplification and the sensitivity of detection of the internal control sequence was at least as good as that observed for the wild-type *Ssp* I repeat. In a few cases the PCR efficiency and the sensitivity of the ELISA detection of the internal control was even better than that noted for the wild-type *Ssp* I repeat (data not shown). This may be due to the observation that the internal control sequence comprises about 5% (200 bp of 4.1 kb) of the plasmid DNA control, whereas the wild-type *Ssp* I repeat comprises only about 0.06% of the *W. bancrofti* genome (Zhong et al. 1996). Furthermore, the nucleotide sequence of the wild-type *Ssp* I repeat in a population is somewhat heterogeneous, whereas the internal control sequence was completely homogeneous.

It has previously been reported that with the conventional *Ssp* I PCR assay, as little as 1 pg of *W. bancrofti* genomic DNA can be detected. This is about 1% of the DNA in one mf (Zhong et al. 1996). The results of the present study show that a similar degree of sensitivity can be achieved using one biotinylated primer; the DNA equivalent of 0.1 mf was detected when mf DNA was mixed with 200 µl of blood lysate. The results also show that the relative amount of *Ssp* I PCR product obtained by the QC-PCR-ELISA assay on samples from naturally infected persons is generally higher than that obtained using uninfected blood to which an equivalent number of mf have been added. In contrast to laboratory-produced samples, samples from naturally infected individuals may also harbor DNA released from dead mf or from cells of worms shed into the blood.

All mf-positive samples from persons living in an endemic area in Egypt were also QC-PCR-ELISA-positive, even if their mf density was low. Samples with as few as two or three mf per ml of blood gave positive results when only 100 µl of blood was examined. In addition, one mf-negative sample was identified as being positive by the QC-PCR-ELISA assay.

QC-PCR has been used extensively for a number of different purposes. Primarily, the method has been used to quantify mRNA, but it has also been used for the quantitation of DNA from slow-growing mycobacteria (Kolk et al. 1994) or from viruses (Piatak et al. 1993). In a few studies, QC-PCR has been used to detect protozoan parasites such as *Trypanosoma cruzi* (Centurion-Lara et al. 1994), *Toxoplasma gondii* (Luo et al. 1997), and *Plasmodium* sporozoites. In the *Plasmodium* studies, rRNA of a size that was different from that of the wild-type target sequence (Vernick et al. 1995, 1996) or rRNA of a related species was used as the competitor (Li et al. 1995). In contrast to the use of QC-PCR to detect viruses, prokaryotes, or eukaryotic protozoans, there is no published report of the application of QC-PCR to quantify genomic DNA of metazoan parasites in clinical samples. Recently an internal standard for the PCR detection of *W. bancrofti* was described (Nicolas and Plichart 1997). The authors used a plasmid that was about 30 bp longer than the wild-type *Ssp* I repeat, and no clinical sample was examined by the QC-PCR-ELISA test. However, our results using a different internal control show that it is possible to evaluate the efficiency of the *Ssp* I PCR in detecting *W. bancrofti* in one reaction tube without the use of standard curves or comparison with external controls.

Our data showed a positive correlation between the relative amount of wild-type *Ssp* I PCR product as determined by the ELISA detection and the number of mf present in a blood sample. Therefore, we conclude that the described QC-PCR-ELISA assay allows the quanti-

tation of mf present in blood samples. However, we find that when individual samples are tested repeatedly, the estimate of mf density obtained using the QC-PCR-ELISA assay is too variable. One possible explanation is that some patients may be capable of killing mf very efficiently and may therefore have low mf densities but much parasite DNA in their blood. Another explanation for this high variance may be a differential efficiency of DNA extraction between individual samples. Furthermore, the conditions for the QC-PCR-ELISA assay were chosen primarily to achieve a high degree of sensitivity. Therefore, the number of PCR cycles was too high and the duration of color development in the ELISA was too long to keep both reactions in their exponential phase, which would be necessary for a more exact estimation of high numbers of mf. Therefore, our assay should be called semiquantitative. However, we prefer to use the term QC-PCR, because this term is traditionally used for this kind of PCR, which involves coamplification of an internal standard. Because anyone shown to be infected should be treated anyway and since an accurate parasitological measurement of individual mf density requires multiple blood collections, it is more relevant to use this QC-PCR-ELISA to evaluate the parasite burden at the community level. On the community level we found an excellent agreement between mf density and the result of the QC-PCR-ELISA assay. Field-collected samples with more than 100 mf/ml of blood had an average relative amount of wild-type *Ssp* I PCR product that was about 4 times higher than that found in samples containing fewer than 11 mf/ml. Therefore, the assay performed on blood samples could be extremely helpful as an indicator of the mf density and the morbidity due to *W. bancrofti* in a community.

The ELISA format simplifies the detection of *Ssp* I PCR products and enhances the rapid screening of large numbers of samples. The method appears most suitable for processing of large numbers of samples at a community level (e.g., for the monitoring of global intervention programs). ELISA readers are available in many diagnostic laboratories in endemic countries for serological detection of other pathogens such as human immunodeficiency virus (HIV) and should represent no additional cost factor. It has been estimated that the costs of the PCR and the ELISA detection are about U.S. $0.80 per sample (Nutman et al. 1994). The internal control plasmid that is needed for the QC-PCR-ELISA assay is available from our laboratories to the scientific community without charge. The QC-PCR can be performed as rapidly as the conventional PCR for the detection of *W. bancrofti* DNA. The ELISA detection of the PCR products requires about 3 h additionally but is more sensitive than agarose-gel electrophoresis and less time-consuming and less expensive than Southern-blot hybridization. Up to 46 samples can be processed simultaneously per ELISA plate and the results provide quantitative data. An increase in automation of the assay may further decrease the costs per sample significantly.

The high sensitivity of the QC-PCR-ELISA assay implies that the assay may improve the daytime diagnosis of nocturnally periodic *W. bancrofti* infection as well as the diagnosis of cryptic infections. The mf densities in the blood samples from Egypt examined in the present study were very low (see Table 2). These mf densities were in the same range as those previously detected in daytime blood samples from heavily infected persons from Tanzania with nocturnally periodic filariasis (Simonsen et al. 1997). However, in future studies we will compare the sensitivity of the assay on blood samples obtained from the same patients during the daytime and the nighttime so as to prove our hypothesis that patients with microfilaremia have parasite DNA in the blood, which can be detected during the day. In addition, it has been suggested that the use of larger quantities of blood for DNA extraction may increase the sensitivity of the conventional PCR assay (McCarthy et al. 1996; Williams et al. 1996). Another possibility for the achievement of higher sensitivity would be the application of PCR directly on unprocessed blood samples. Elimination of extraction procedures, which may lead to a loss of DNA, has been described for malaria diagnosis (Tirasophon et al. 1994). Unfortunately, the presence of PCR inhibitors in blood samples has limited the use of PCR assays on unprocessed blood. Since the QC-PCR-ELISA assay described in the present report uses an internal control for the detection of any PCR inhibition, it may be promising to develop a protocol for performance of the QC-PCR-ELISA assay for the detection of *W. bancrofti* directly on unprocessed blood samples.

The QC-PCR-ELISA assay for the detection of *W. bancrofti* is a powerful new tool for the evaluation of parasitic loads in the community diagnosis of bancroftian filariasis. The assay may also prove useful for the screening of individual or pools of vectors to evaluate ongoing transmission. Therefore, this new diagnostic procedure could significantly improve the monitoring of *W. bancrofti* control programs.

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