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DETECTION OF DNA OF NOCTURNALLY PERIODIC *BRUGIA MALAYI* IN NIGHT AND DAY BLOOD SAMPLES BY A POLYMERASE CHAIN REACTION–ELISA–BASED METHOD USING AN INTERNAL CONTROL DNA

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Abstract. An internal control was used in a polymerase chain reaction (PCR)–ELISA–based technique to detect the *Hha* I repeat of the filarial parasite *Brugia malayi*. A single microfilaria added to 200 μl of blood was reliably detected. The assay was evaluated on field samples from persons living in an area endemic for *Anopheles*-transmitted, nocturnally periodic *B. malayi* in central Sulawesi, Indonesia. Examination of night blood of 138 individuals for the presence of microfilariae by filtration revealed 44 microfilaria carriers. All microfilaria carriers were also positive in the PCR-ELISA and, in addition, 14 more samples were proven to contain parasite DNA. The sensitivity of both methods was compared on night and on day blood samples collected from 113 persons. Whereas 37 microfilaria carriers were identified by filtration of night blood, no microfilariae were observed in the corresponding day blood samples. The PCR-ELISA result was positive in all 37 night blood samples of microfilaria carriers and in an additional 13 night blood samples without microfilariae. Parasite DNA was detected in 31 day blood samples of microfilaria carriers and in 3 day blood samples of amicrofilaremic persons. Assuming a sensitivity of the PCR-ELISA on night blood of 100%, the sensitivity of night blood filtration is 74% and that of the PCR-ELISA on day blood is 68%. These data suggest that the described PCR-ELISA method is capable of detecting infections with nocturnally periodic *B. malayi* in day blood samples. Therefore, this method may facilitate both the identification of endemic areas and the monitoring of control programs.

Lymphatic filariasis, caused by *Wuchereria bancrofti* and *Brugia malayi*, has been targeted by the World Health Organization for elimination as a public health problem by the year 2020.[1] Therefore, control programs have been or will be launched world-wide with an emphasis on community-wide treatment with diethylcarbamazine (DEC) and ivermectin or albendazole.[2] To monitor the success of such intervention programs, sensitive and cost effective diagnostic tools are required. It is estimated that 115 million people are infected with *W. bancrofti* and about 13 million with *B. malayi*.[3] In Asia, 1 of 5 filarial infections is due to *B. malayi* and in some countries this parasite is responsible for the majority of infections.[4,5]

Various sensitive polymerase chain reaction (PCR) assays have been established to detect *W. bancrofti* or *B. malayi* DNA in the human host or the mosquito vector.[6–15] Inhibition of a PCR, which may occur in both samples obtained from the mosquito vector and samples from the human host, is a common problem during DNA amplification and makes the standardization of PCR tests difficult.[16] Therefore, DNA constructs were used as internal standards (controls) in the PCR to avoid false-negative results in a diagnostic PCR.[17] For the detection of *W. bancrofti*, such internal control was developed and used in a PCR-ELISA-based method.[18] For traditional reasons, the assay was called a quantitative, competitive PCR-ELISA (QC-PCR-ELISA) method, although the method was rather semiquantitative than quantitative. However, the major application for the *W. bancrofti* PCR will be the detection of parasite DNA in the vector, since a simple, sensitive, and specific card test for the detection of circulating *W. bancrofti* antigen in human blood samples is now commercially available.[19] In contrast, for *B. malayi* there is no antigen test and the PCR is one of the most promising molecular approaches to detect the infection in both humans as well as in the vector.

In the present study we developed a PCR-ELISA method based on the *Hha* I repeat of *B. malayi*,[20] which involves the coamplification of an internal control. This method was evaluated on blood samples from persons living in an area endemic for nocturnally periodic *B. malayi* in central Sulawesi, Indonesia. To determine the sensitivity of the PCR-ELISA, we compared the results of the assay on blood samples collected during the night with those from samples collected during the day. We were able to demonstrate that the described method can be used to detect parasite DNA in day blood samples, which contained no microfilariae.

MATERIALS AND METHODS

Blood samples and DNA preparation. Blood samples were collected in July 1998 in the villages of Rogo and Mahoni of the Palu area in central Sulawesi, which is known to be endemic for nocturnally periodic *B. malayi*.[21] The principal vector species in this area is *Anopheles barbirostris*. In these villages, blood was collected from all inhabitants who consented to participate in the study. The collection of samples was approved by the Indonesian Ministry of Health. Following blood collection, treatment with DEC was offered. To obtain an overview regarding the prevalence of *B. malayi*, capillary night blood from 329 individuals in Rogo and from 326 individuals in Mahoni was examined in the field for microfilariae by a thick blood smear. The overall prevalence of infection in the examined children and adults was 8.5% (Rogo) and 6.1% (Mahoni). To study the detection of *B. malayi* DNA by the novel PCR-ELISA method, 10 ml of venous blood was collected into EDTA-coated vacutainers (Becton Dickinson, Inc., Heidelberg, Germany) from 113 randomly selected adults >14 years of age both during the daytime (between 10:00 AM and 1:00 PM) and during the night (between 8:00 PM and 11:00 PM). Only night blood

samples were available from 25 adults. Individuals who participated in the study were registered, examined for clinical signs of filariasis, and interviewed for previous signs of the disease. During the stay in the field, the samples were kept refrigerated for up to one week and subsequently transported to a central laboratory at the Department of Parasitology at the University of Indonesia in Jakarta. All samples were examined for the presence of microfilariae by filtration of 1 ml blood through a membrane (5 μm pore size; Nucleopore, Pleasanton, CA), followed by microscopic examination of the Giemsa-stained filter.

Microfilariae of *B. malayi* were obtained from a zoophilic strain that was reared in jirds for generations at the Department of Parasitology in Jakarta. Microfilariae from *B. pahangi* were obtained from TRS Laboratories (Athens, GA). As negative controls, 10 blood samples from Ugandan patients living in an area non-endemic for *B. malayi*, but endemic for the filarial parasites *Onchocerca volvulus* and *Mansonella perstans*, were used. To investigate the sensitivity of the PCR-ELISA, *B. malayi* microfilariae were examined under a stereomicroscope and individually transferred to 200 μl of human blood (containing 1 mM EDTA) collected from noninfected North Americans.

Lysates of 200 μl of blood from all samples prepared as previously described in detail[15] were used as PCR templates. Two microliters of the lysate were used in the PCR. If a high level of PCR inhibition was detected, the blood lysate was diluted 1:5 in sterile water and the PCR was repeated.

Polymerase chain reaction. The PCR conditions were the same as described previously,[12] but *Taq* Gold polymerase (PE Applied Biosystems, Foster City, CA, USA) was used with an initial activation step of 10 min at 95°C before starting DNA amplification. When 0.2-ml ultra-thin PCR tubes and a GeneAmp PCR System 9700 thermocycler (PE Applied Biosytems) were used, the periods of denaturation, annealing, and extension was decreased to 30 sec each. By using this thermocyler, the duration of the PCR was reduced to less than 2 hr and the amount of *Taq* polymerase could be reduced to 1.5 units per 50-μl reaction. A 5′ biotinylated *Hha* I-reverse primer was used to obtain a biotinylated PCR product, which can easily be bound to a streptavidin-coated microtiter plate. For each sample, 10 pg of the internal control was added to the PCR master mixture. The internal control is available free of charge from the authors. The internal control was constructed using an inverse PCR on the *Hha* I repeat with primers that changed the hybridization target sequence. The product of the inverse PCR was cloned in *Escherichia coli* and one clone was selected that had in addition to the expected nucleotide exchange in the hybridization region a small deletion of 12 basepairs. Eight microliters of each PCR product were run on a 1.5% agarose gel and visualized by staining with ethidium bromide.

The PCR-ELISA. The PCR-ELISA was performed as described previously for the detection of *W. bancrofti*,[18,22] with a few *B. malayi*-specific modifications. For each sample, two of the wells were hybridized with the wild type *Hha* I-specific DNA probe (Bm-Hha) and two with the internal control-specific DNA probe (Bm-con). The oligonucleotide probes Bm-Hha (5′-ACGTGAATTGTACCAGTGCTGGTCG-3′) and Bm-con (5′-TTACGTCGCCCTTCG CTAGTCTCT-3′) were fluorescein-labeled at both the 5′ and 3′ ends during synthesis (Oligo Etc./Oligotherapeutics, Wilsonville, OR). Each probe was diluted to 50 pg/μl in hybridization buffer and later detected using an anti-fluorescein-alkaline phosphatase-conjugated antibody (Boehringer Mannheim, Mannheim, Germany). Alkaline phosphatase substrate was added according to the instructions of the manufacturer (AP substrate tablets 104; Sigma, St. Louis, MO). The ELISA reading was performed at 405 nm following incubation for 1 hr at 37°C using a Vmax microplate reader (Molecular Devices, Sunnyvale, CA).

Data analysis. Data analysis was performed using the computer program Epi-Info version 6.02 (Centers for Disease Control and Prevention, Atlanta, GA) as described previously.[18] We found in a few cases too much PCR inhibition for accurate detection of positive samples. Therefore, if the optical density reading of the Bm-Hha wild-type probe ($OD_{Bm\text{-}Hha}$) or the Bm-con probe ($OD_{Bm\text{-}con}$) was <1, the assay was repeated as mentioned above. A positive $OD_{Bm\text{-}Hha}$ reading for a blood sample was defined as the $OD_{Bm\text{-}Hha}$ of the negative controls plus 5 times the standard deviation of the negative controls. The cut-off value varied between 0.11 and 0.253. For about 80% of the field-collected blood samples, positive $OD_{Bm\text{-}Hha}$ readings were >0.253. Negative controls contained PCR products from 10 pg of the internal control plasmid DNA and DNA extracts of blood samples from 10 uninfected persons from Uganda or from 3 persons from North America. Because 10 pg of the internal control plasmid were used as the competitor DNA in all samples, an index for the amount of *B. malayi* DNA present in positive samples was calculated as 10 times the relation of the $OD_{Bm\text{-}Hha}$ of the sample to the $OD_{Bm\text{-}con}$ of the sample.

RESULTS

Development of the novel PCR-ELISA. The amplification of the internal control was very efficient and as little as 1 fg of DNA was sufficient as PCR template in a 50-μl reaction. However, the wild-type *Hha* I sequence is highly repeated and comprises about 13% of the entire genome of *B. malayi*. Therefore, in most samples containing moderate or high numbers of microfilariae the amount of wild-type target sequence was in such an excess that almost no internal-control PCR product was obtained that could be detected by the PCR-ELISA. To more accurately quantify the wild-type PCR product, the amount of internal control was increased to 10 pg per reaction. This was sufficient template to obtain detectable internal control PCR products even in the presence of an abundance of wild-type *Hha* I repeat, while, on the other hand, this amount of competitor DNA allowed the detection of low microfilaria densities or circulating DNA in microfilariae-negative samples.

The internal control was 12 basepairs shorter than the *B. malayi* wild-type *Hha* I repeat. This size difference was visible on a 1.5% agarose gel. In addition, since the wild-type *Hha* I repeat is organized as a tandem repeat, in some samples not only was the monomer amplified but also dimers and trimers of the wild-type repeat were amplified (Figure 1). However, to quantify the amount of wild-type PCR product and of internal control PCR product and to increase the sensitivity, an ELISA detection was necessary.

Sensitvity of the PCR-ELISA. In a series of experiments,

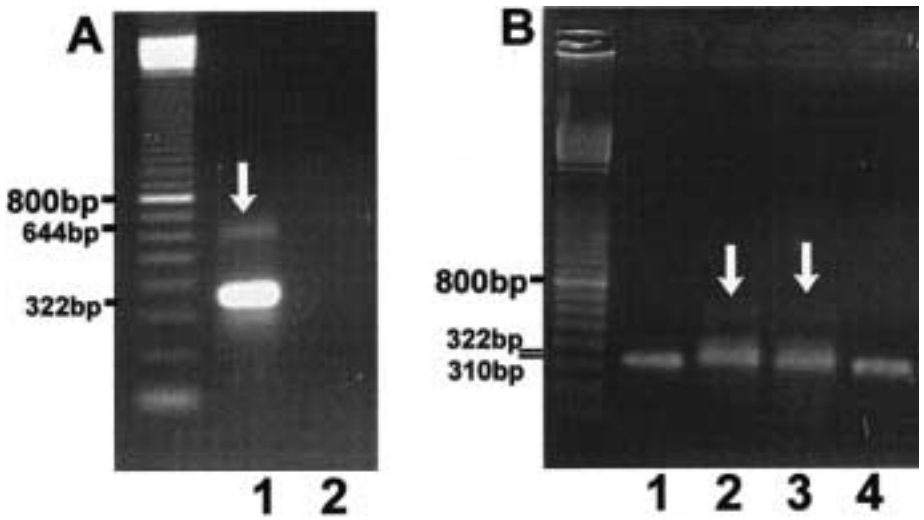

FIGURE 1. **A,** amplification of the *Hha* I wild-type repeat resulted in monomers of 322 basepairs (bp) and dimers of 644 bp (**arrow**; lane 1, 1 ng of genomic *Brugia malayi* DNA; lane 2, negative control). **B,** polymerase chain reaction (PCR) products from 4 blood samples collected central Sulawesi, Indonesia. The samples in lanes 1 and 4 represent microfilariae and PCR-ELISA-negative samples and only PCR products of 310 bp of the internal control BM-con were obtained. The samples in lanes 2 and 3 were from microfilaria carriers and positive in the PCR-ELISA and their vast amount of PCR product is the *Hha* I wild-type repeat of 322 bp (**arrows** indicate dimers).

distinct numbers of *B. malayi* microfilariae were added to human blood samples and following lysis the PCR-ELISA was performed. The method was sensitive enough to detect a single microfilaria in 200 μl of human blood (Table 1). All 31 samples containing microfilariae were positive and the 6 samples without microfilariae were negative in the assay. The microfilariae used in this experiment belonged to a zoophilic strain of the parasite that was reared in jirds. When microfilariae of the closely related filarial parasite *B. pahangi* were added to blood samples, the PCR-ELISA gave negative results. The results showed that a single *B. malayi* microfilaria contained sufficient DNA to be reliably detected in 200 μl of human blood that was free of any circulating *B. malayi* DNA.

To determine the sensitivity of the assay to detect DNA of anthropophilic, nocturnally periodic *B. malayi* in naturally infected individuals, 138 blood samples were collected in central Sulawesi during the night and examined by the described PCR-ELISA method. Microfilariae were detected in 44 (31.9%) blood samples by filtration of 1 ml of night blood. Forty-one (29.7%) persons were also positive by the examination of a thick smear of capillary blood. The geometric mean microfilaria density in microfilaria carriers was 194 microfilariae/ml, ranging from 1 to 3,242 microfilariae/ml blood. All samples from microfilaria carriers were positive by the PCR-ELISA, suggesting a sensitivity of 100%. In addition, 14 samples from amicrofilaremic were also found to be PCR-ELISA positive. The filtration of an additional ml blood of 7 of these 14 samples showed that 6 samples were again microfilaria negative, but 1 sample contained 3 microfilariae. Fifty-eight (42%) samples were found to be positive by the PCR-ELISA. All negative controls from African patients were negative in the assay. These results demonstrate that the described PCR-ELISA method has a higher sensitivity compared with the blood filtration.

TABLE 1

Sensitivity of the polymerase chain reaction (PCR)–ELISA on laboratory produced samples: *Brugia malayi* microfilariae (mf) were added to 200 μl of human blood containing 1 mM EDTA, the optical density was measured at 405 nm, and the relative number of units of wild-type *Hha* I PCR product was calculated

No. of mf added	No. of samples	PCR-ELISA-positive samples	
		No. (%)	Mean units ± SD*
0	6	0 (0)	†
1*	15	15 (100)	1.687 ± 1.462
25	9	9 (100)	6.747 ± 1.101
100	7	7 (100)	8.831 ± 1.109

* Differences between the groups were significant ($P = 0.000001$ by chi-square test).
† The theoretical mean was 0.025 ± 0.017, but in these experiments the cut-off value was calculated as 0.11.

The PCR-ELISA for diagnosis of clinical filariasis. Clinical signs associated with brugian filariasis were observed in only a few examined individuals from central Sulawesi. Three patients had elephantiasis on their legs. They were all microfilaria and PCR-ELISA negative. Two of them had received DEC treatment before 1991. Forty-three persons claimed a history of one or more acute filariasis attacks during the last year. Of these persons, 26 (60.5%) were microfilaria carriers, 28 (65.1%) were PCR-ELISA positive, and 6 (14%) reported previous malaria attacks. In contrast, among 88 persons without acute filariasis attacks 12 (13.6%) were microfilaria carriers, 25 (28.4%) were positive in the PCR-ELISA, and 17 (19.3%) had a history of malaria.

Detection of DNA of nocturnally periodic *B. malayi* in day blood. The collection of blood samples during the night involves serious logistical and social problems. Therefore, we examined the sensitivity of the novel PCR-ELISA method on 113 blood samples collected during daytime and compared their results with those from the corresponding night blood samples of the same individuals. Whereas 37 (32.7%) of these persons had microfilariae in their night blood as determined by blood filtration, all blood samples were microfilariae negative during the day. This finding supports the strong nocturnal periodicity of this strain of *B. malayi*. How-

TABLE 2

Comparison of the sensitivity of the polymerase chain reaction (PCR)–ELISA for the detection of nocturnally periodic *Brugia malayi* on night and day blood samples from 113 individuals living in an endemic area in central Sulawesi, Indonesia

	PCR result			
	Night blood samples		Day blood samples	
Microfilariae (Mf) status*	Positive	Negative	Positive	Negative
Mf positive (n = 37)	37	0	31	6
Mf negative (n = 76)	13	63	3	73
Total	50	63	34	79

* Microfilaria density was determined by filtration of 1 ml of night blood. All samples were microfilaria negative by filtration of blood collected during the daytime.

TABLE 3

Identification of *Brugia malayi* infection in 113 adults from 2 villages in central Sulawesi by different parasitologic and polymerase chain reaction (PCR)–ELISA procedures*

	Infected persons			
	Rogo (n = 33)		Mahoni (n = 80)	
	No.	%	No.	%
Thick smear/capillary night blood	14	42.4	18	22.5
Filtration/night blood	14	42.4	23	28.8
Filtration/day blood	0	0	0	0
QC-PCR-ELISA/night blood	15	45.5	35	43.8
QC-PCR-ELISA/day blood	10	30.3	24	30.0

* QC = quantitative, competitive.

ever, *B. malayi* DNA was detected by PCR-ELISA in 34 (30.1%) of these day blood samples (Table 2). The 6 PCR-ELISA-negative day blood samples of infected individuals were from persons with low microfilaria densities in their night blood: 2 of them had only 1 microfilaria/ml, and the others had 10, 59, 66, and 126 microfilariae/ml, respectively. Control samples from areas proven to be non-endemic for *B. malayi* were all negative by the PCR-ELISA. As summarized in Table 3, the PCR-ELISA performed on night blood samples has a higher sensitivity than performed on day blood samples. The results above demonstrate the ability of the assay identify villages endemic for nocturnally periodic *B. malayi* examination of day blood samples.

Quantitation of the *Hha* I PCR product. When increasing numbers of microfilariae were added to 200 μl of blood, an increasing relative amount of *Hha* I wild-type PCR product was detected by the PCR-ELISA (Table 1). This positive correlation was statistically significant (r = 0.84, degrees of freedom [df] = 30). A similar, but statistically nonsignificant correlation was observed for the PCR products of the 58 PCR-ELISA positive field collected night blood samples from Sulawesi. The comparison of the relative amount of wild-type *Hha* I PCR product obtained from PCR-ELISA positive night blood and day blood samples revealed a statistically significant difference, with an average relative amount of 9,106 units in night blood samples and an average relative amount of 1,061 units in day blood samples (P = 0.0006, by Mann-Whitney U test). Although no significant correlation between the microfilaria density and the number of acute filariasis attacks could be shown for PCR-ELISA-positive samples, a weak correlation was found between the relative amount of *Hha* I PCR product and the number of filariasis attacks (r = 0.28, df = 51). These results showed that the relative amount of *Hha* I PCR product may be both an indicator for the microfilarial load and for the occurrence of acute filariasis attacks in an endemic community.

DISCUSSION

The present study demonstrates that the described PCR-ELISA is an excellent method for detecting *B. malayi* DNA in blood samples. Using this method, it is possible to detect both zoophilic *B. malayi* strains that were reared in jirds as well as the anthropophilic, *A. barbirostris*-transmitted, nocturnally periodic strain from central Sulawesi. So far no sequence consistent differences of the *Hha* I repeat in different strains of *B. malayi* have been found and it was reported that the *Hha* I repeat of *B. malayi* is species specific and distinct from that of the closely related species *B. pahangi*.[20] Since the *Hha* I sequence is highly repeated and comprises about 13% of the whole *B. malayi* genome, this sequence appears to be preordained as PCR target for the development of specific and sensitive detection assays for this parasite.

The PCR-ELISA is sensitive and reliable enough to detect as little as one *B. malayi* microfilaria added to 200 μl of blood. Therefore, this assay has at least the same or even a higher sensitivity than conventional PCR assays for the detection of filarial parasites.[7,9–12] It has been reported that a conventional PCR-ELISA without any internal control is a very sensitive tool for the detection of PCR products of the *B. malayi Hha* I repeat.[13] Our results confirmed this finding using a different labeling hapten and a different antibody-conjugate for the ELISA detection. In addition, we were able to show that an internal control can be used to avoid false-positive results and the ELISA can be used to quantify PCR products to provide semiquantitative data. When evaluated on field-collected blood samples, the results of the present study suggest that the *B. malayi* PCR-ELISA is more sensitive than the corresponding PCR-ELISA method for the detection of *W. bancrofti*.[18] This may again be due to the fact that the *Hha* I PCR target sequence is highly repeated in a tandem array in contrast to the *W. bancrofti* target sequence, which has only about 300 dispersed copies per haploid genome.[11] The high abundance of the *Hha* I repeat in *Brugia* parasites may facilitate the sensitive detection of this molecule at physiologic levels without PCR. Although signal amplification techniques such as ultra-sensitive branched DNA assays have some advantage compared with the PCR, they have been developed so far only for a limited number of viral and bacterial agents.[23] It remains an objective for further research whether signal amplification methods can be developed that are as sensitive and easy to perform as the described *B. malayi* PCR-ELISA.

Diagnostic procedures for brugian filariasis, which could be useful as a screening tool for large-scale intervention programs, should involve convenient sample collection procedures. The diagnosis of nocturnally periodic filariasis in day blood samples would facilitate the sample collection enormously. Therefore, the World Health Organization Expert Committee on Filariasis recommended the development and validation of diagnostic assays that can replace night blood examination for detection of microfilaremia.[24] Using the described PCR-ELISA on night blood samples as a reference method, the sensitivity of the PCR-ELISA method per-

formed on 200 μl of day blood revealed a similar sensitivity as the filtration of 1 ml of night blood. In agreement with these results, the detection of DNA of nocturnally periodic *W. bancrofti* by PCR in day blood samples has been recently reported.[10] In this study, a conventional PCR performed on DNA extracts from 500 μl of day blood plasma was compared with a thick blood film prepared from 60 μl of night blood. However, we were able to show that the novel PCR-ELISA method is capable of detecting free DNA of nocturnally periodic *B. malayi* in as little as 200 μl of day blood while still achieving a sensitivity comparable with the filtration of 1 ml of night blood.

On the basis of the *W. bancrofti* PCR-ELISA test results, it could not be recommended to estimate the worm burden in infected individuals because of its high standard deviation. However, on a community level, an excellent agreement between microfilariae density and the PCR-ELISA result was observed. Therefore, this method is suitable for the evaluation of parasitic loads in the community-based diagnosis of filariasis.[18] Our results from the *B. malayi* PCR-ELISA support this finding, although the standard deviation is even higher and no significant correlation could be proven. This may be due to the fact that individuals who are capable of killing microfilariae efficiently and have, therefore, low microfilariae densities, may have huge amounts of *Hha* I PCR target DNA in their blood, because up to 130 fg of the *Hha* I repeat can be released by a single dead microfilaria. Therefore, further studies are required to elucidate the relationship between microfilaria density and the amount of parasite DNA in blood samples of infected individuals with different immunologic status. This is also of relevance in exploring the dynamics of *B. malayi* DNA clearance following chemotherapy. Furthermore, a more standardized procedure for DNA extraction is desirable.

For the monitoring of filariasis control programs, specific and very sensitive diagnostic assays are required. However, it appears difficult to define how sensitive a method has to be and what are the thresholds for prevalence in humans and for the vector infection rate to interrupt transmission. In China, the threshold prevalence for transmission of *B. malayi* by *A. sinensis* is estimated to be between 1.55% and 2.23% of the human population, providing no individual has a higher microfilaria density than 12 microfilariae/60 μl of capillary night blood.[25] This low microfilaria density would correspond to 30 microfilariae/ml of venous night blood.[26] Depending on the mosquito biting rate, the threshold for the vector infection rate was in the Chinese study estimated to be between 0.23% and 0.024%. Our results from the blood screening experiments suggest that the described PCR-ELISA is a suitable method to detect such low prevalence even when performed on day blood. In addition, preliminary results indicate that the novel PCR-ELISA is also an excellent tool for mosquito pool screening to detect the low threshold vector infection rates (Supali T and others, unpublished data). Therefore, the PCR-ELISA method has the capacity for the monitoring of low endemicity areas and may help to avoid premature cessation of control programs and protect these areas from a resurgence of filariasis.

Taken together, the described PCR-ELISA test for the DNA detection of *B. malayi* is a powerful new diagnostic tool that can be applied not only on blood samples collected during the night, but also on day blood samples. In addition, the test appears most suitable for the rapid detection of *B. malayi* in individual or pools of vector mosquitoes. This test may complement existing diagnostic tools involved in intervention programs, which aim to eliminate lymphatic filariasis as a public health problem in the near future.

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