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Comparison of conventional versus real-time PCR detection of *Brugia malayi* DNA from dried blood spots from school children in a low endemic area

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Abstract. Microscopic detection of active phase of lymphatic filariasis is indicated by the presence of microfilaria in whole blood. This method is not sensitive and requires relatively large amount of blood sample. PCR allows very sensitive detection of the parasite DNA using a smaller amount of blood; and the use of dried blood spots facilitates sample transportation. Nevertheless, limited studies have been reported on PCR using dried blood spot for detection of *Brugia malayi*. In this study, we investigated the effects of concentrating whole blood genomic DNA sample and the amplification methods [conventional PCR (C-PCR) and real-time PCR] on the detection of *B. malayi* DNA from dried blood spots from a very low endemic area in Malaysia. Both C-PCR and real-time PCR detected 2 out of 18 (11%) samples as positive from non-concentrated genomic DNA preparations. After the DNA samples were pooled and concentrated, both C-PCR and real-time PCR detected *B. malayi* DNA amplifications in 7 out of 18 (39%) samples. However one sample which showed faint band in C-PCR was detected as highly positive in real-time PCR. In conclusion, both C-PCR and real-time PCR using dried blood spots from a low endemic area demonstrated equal sensitivity for detection of *B. malayi* DNA.

INTRODUCTION

Brugian filariasis, caused by the parasitic nematode *B. malayi*, is a mosquito-borne disease and accounts for about 10% of the 120 million people infected with lymphatic filariasis worldwide. This disease is an important cause of disability in Southeast Asia and it is endemic in some parts of Malaysia (Lim *et al.*, 2001).

The World Health Organization has identified lymphatic filariasis as a major public health problem that potentially can be eliminated (Ottensen *et al.*, 1997). However, an important element for the success of any elimination effort is the identification and assessment of filariasis endemicity, followed by monitoring and certification activities in the affected communities (Mishra *et al.*, 2007).

Several diagnostic tools for brugian filariasis detection are currently available, and each test has its own limitations. The microfilaria (mf) detection by microscopy has low sensitivity while antibody detection test is an indirect assessment of infection.

PCR-based assays have become the preferred methods for the diagnosis of many parasitic infections in reference laboratories for several reasons. First, these assays do not require large amount of sample compared to other diagnostic tools. Secondly, the equipment and practical skills required to perform PCR are becoming increasingly available in the developing world (Rao *et al.*, 2006). In lymphatic filariasis, it offers a direct indication of active infection. Several conventional PCR (C-PCR) and real-time PCR assays have been developed for the molecular diagnosis of

lymphatic filarial infections. These assays detect DNA of *Wuchereria bancrofti* or *B. malayi* and *B. timori* in human blood and mosquito vectors with high sensitivity and specificity (Lizotte *et al.*, 1994; Bockarie *et al.*, 2000; Kluber *et al.*, 2001; Fischer *et al.*, 2002; Goodman *et al.*, 2003; Helmy *et al.*, 2004; Rao *et al.*, 2006).

Although PCR detection has been used extensively for the molecular diagnosis of filarial infections, the recovery and concentration of filarial DNA from human blood spot has been less thoroughly investigated. There are several reports on the detection of Brugia DNA by PCR from blood spots (Fischer *et al.*, 2002, 2005; Supali *et al.*, 2006). However the issue of the dispersion of mf within the parameter of the spots was not addressed. Since the DNA extraction from blood spot usually requires 3-5 punches from the whole spot, there is a possibility of the mf missing from the punched areas, particularly from infected individuals with low mf or without mf.

In this study, we investigated the effects of concentrating DNA sample and the amplification method on the detection of *B. malayi* DNA from dried blood spots from a very low endemic area in Malaysia.

MATERIALS AND METHODS

Sample collection

As part of an effort to screen school population in an area with extremely low endemicity for brugian lymphatic filariasis (designated as 'green zone'), 973 school children of 6 to 10 years old in a state in north peninsular Malaysia was screened with Brugia Rapid™ test (MBDr, Malaysia) using finger-prick day-time blood. A total number of 21 children (2.15%) were found to be positive by the rapid test. Historically this area is known to harbour subperiodic nocturnal lymphatic filarial species, thus venous night blood samples (from ~ 8 pm to 11 pm) were collected from samples positive by Brugia Rapid™ test, and this was successfully obtained from 18/21 children. A volume of 60 µl was used to make thick blood smears and 60 µl of blood was spotted and dried on Whatman 3MM filter paper. The spots were then sent to the laboratory at USM for detection of the parasite DNA by PCR. The slides were stained with Giemsa and examined microscopically by the personnel of the Vector-borne Disease Control Unit, Health Department, Kangar, Perlis.

DNA extraction

For each dried blood spot sample, 12 punches (each diameter of ~ 3 mm) from each blood spot were made and placed in four tubes (3 punches/tube) for genomic DNA extraction. The DNA extractions were performed by using QIAamp DNA Micro kit (Qiagen, Hilden, Germany) and DNA was eluted with 50 µl elution buffer. This kit was chosen based on its ability to extract DNA from a small volume of dried blood. For negative controls, DNA was extracted from six non-infected individuals which were classified into two groups namely healthy individuals from a non-endemic area and healthy persons from an endemic area (these individuals tested negative with Brugia Rapid™ test and microscopic examination). The positive control DNA sample was obtained from a microfilaraemic individual.

Five µl of the extracted DNA from one tube was used as template in the first round of each kind of PCR assay namely conventional PCR (C-PCR) and real-time PCR. Subsequently the remaining eluted DNA and the those from other four tubes were pooled and concentrated using a vacuum concentrator (Eppendorf, USA) for 30 minutes until the volume reached 30 µl (thus 6.5 X concentrated) and this was used for a second round of each C-PCR and real-time PCR. Negative and positive control samples were concurrently processed with the test samples during DNA extraction and concentration procedures. In the latter procedure, the tubes are well-separated when arranged in the vacuum concentrator. These steps were taken as a precaution and a quality check for any possible cross-contamination of DNA samples among the tubes.

Conventional and real-time PCR

The *Hha*I repeat (GenBank accession no. M12691 and AF499109 to AF499129) is a commonly used target sequence for amplification of Brugia DNA (Fischer *et al.*, 2002, 2003). Thus, this target was used for both C-PCR and real-time PCR. The C- PCR mixture used was as follows: 25 µl final volume comprising 0.1 µM *HhaI*F (5'-GCG CAT AAA TTC ATC AGC-3') and *HhaI*R (5'-GCG CAA AAC TTA ATT ACA AAA GC-3') primers (Lizotte *et al.*, 1994; Fischer *et al.*, 2002, 2005; Supali *et al.*, 2006; Rao *et al.*, 2006), 12.5 µl HotStarTaq mastermix (Qiagen, Hilden, Germany), 5 mM $MgCl_2$ and 5 µl of the DNA template. The thermocycler profile was set to allow for 12 min of activation at 95°C and 2 min of annealing at 55°C, then 35 cycles, each 30 s of extension at 72°C, 30 s of denaturation at 94°C and 30 s of annealing at 55°C, followed by a final extension of 72°C for 7 min. The products of C-PCR were analyzed using 1% agarose gel and the image was captured using gel documentation system (Alpha Innotech, USA). Samples showing DNA bands at expected size of 322-bp were considered as positive.

The primer MGB-HhaI-For (5' GCA ATA TAC GAC CAG CAC 3'), MGB-HhaI-Rev (5' ACA TTA GAC AAG GAA ATT GGT T 3') and probe MGB-HhaI-probe (FAM 5' TTT TTA GTA GTT TTG GC 3' MGB) for the real-time PCR were based on those reported by Rao *et al.* (2006). The PCR mixture included 12.5 µl of HotStarTaq mastermix (Qiagen, Hilden, Germany), 400 nM each primer and 50 nM probe, 5 mM $MgCl_2$ and 5 µl of the DNA template in a final volume of 25 µl. Cycling conditions included a 15 min for initial denaturation at 95°C, followed by 50 cycles of 10 s denaturation at 95°C, 30 s annealing at 58°C and 30 s extension at 76°C. The fluorescence data were measured using real-time thermocycler (Rotorgene-Q, Germany) during the annealing step.

RESULTS AND DICUSSION

The Giemsa stained slides examined by the Vector-borne Disease Control Unit showed no mf in the smears of all the 18 blood samples. Both C-PCR and real-time PCR detected the presence of *B. malayi* DNA in 2/18 (11%) of the dried blood spots. Figures 1 and 2 show results of the C-PCR amplifications, and figure 3 shows representative results of the real-time PCR. After the DNA from each blood spot was pooled and concentrated, the C-PCR detected positive results in 7/18 (39%) of the samples, thus 2.5 time greater detection than before DNA concentration. One of the samples showed very faint PCR band which was seen only under direct UV illumination

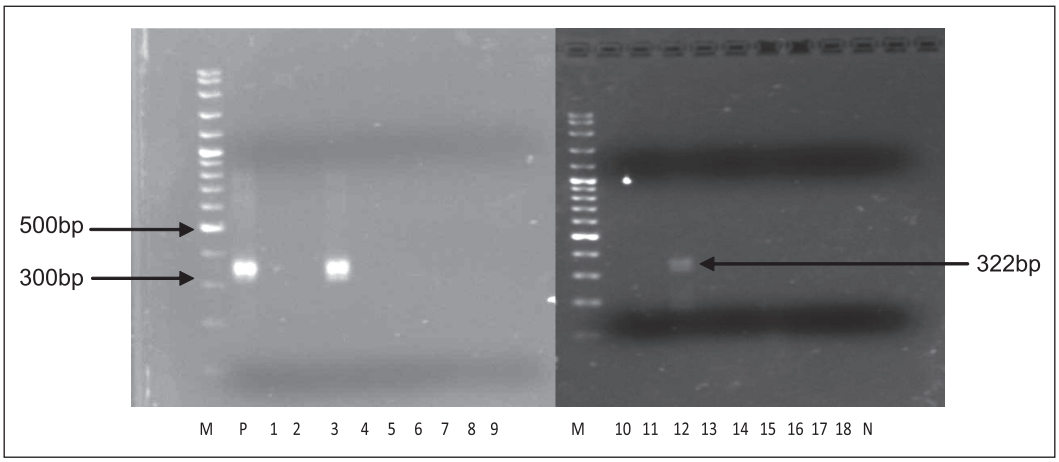

Figure 1. PCR products using DNA templates before DNA samples were pooled and concentrated. Lanes M: 100bp ladder (Fermentas); lane P: *B.malayi* positive control; lane N: negative control; lanes 1-18: samples 1-18.

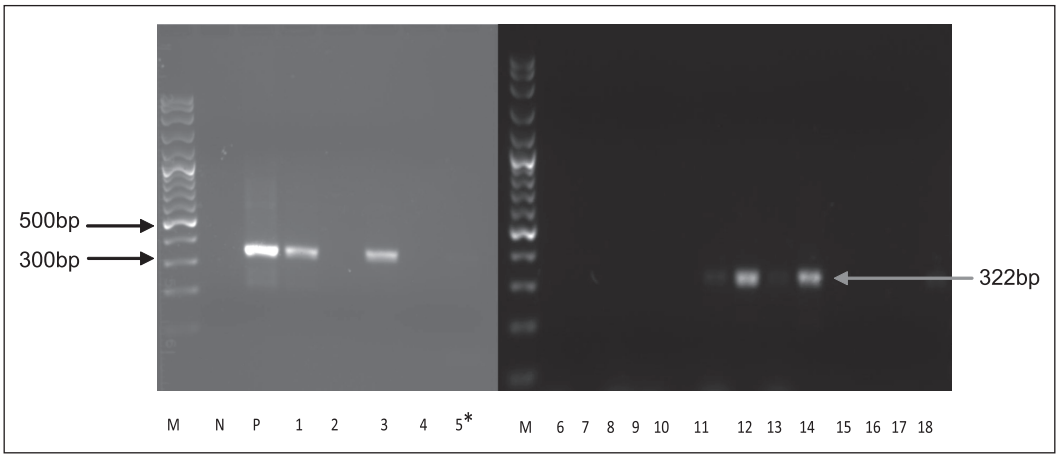

Figure 2. PCR products using DNA templates after DNA samples were pooled and concentrated. Lanes M: 100bp ladder (Fermentas); lane P: *B.malayi* positive control; lane N: negative control; lanes 1-18: samples 1-18.

*Note: Lane 5 has a very faint band at 322 bp which can only be seen when directly visualized under the UV-illumination, but is not apparent (above) when captured by the image analyzer.

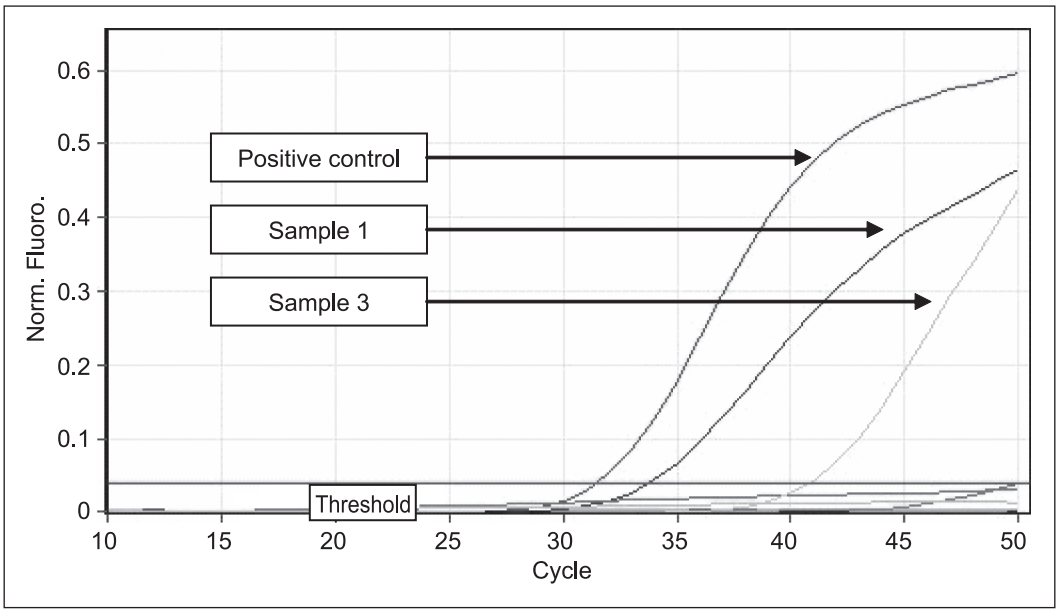

Figure 3. Real-time PCR results after DNA from samples 1 and 3 were each pooled and concentrated.

and not apparent when the gel image was captured by the image analyser (lane 5 in figure 2); this was probably due to the low copy of amplifiable DNA. Using real-time PCR, *B. malayi* DNA amplification was detected in the same 7 samples as the second round of C-PCR; with median Ct value 30.8 and range $29.0 < Ct < 34.0$. The sample with the very faint band in C-PCR was detected as highly positive by the real-time PCR (Ct value of 30.8). All the negative samples with C-PCR were also negative with the real-time PCR. DNA was successfully amplified in the positive control, and no amplifiable DNA was

obtained from the both types of negative controls. Since negative and positive control DNA samples were concurrently extracted and concentrated with the test samples, and the negative controls showed no false positives, thus the extraction and concentration procedure did not cause cross-contamination of the DNA among samples.

In a previous study conducted in a highly endemic area in Indonesia, C-PCR was performed on 30 µl of dried blood spots. Out of 37 mf negative but Brugia Rapid™ positive samples, 9 (24.3%) were found to be PCR-positive (Fischer *et al*., 2005). Thus it is anticipated that greater rate of detection may be obtained if the extracted DNA was concentrated prior to performing the PCR. In a study conducted by Supali *et al.* (2006) in a medium to high endemic area, five to ten spots (30 µl blood spots) were pooled in a microfuge tube, followed by addition of 1 ml water and 5% chelex to bind the inhibitors. By using this pool screen method, the ten pools gave better PCR results whereby 17 out of 24 pools (70.8%) were positive. However it is difficult to compare the present method with the above due to the differences in methodologies.

In the study by Rao *et al.* (2006), the investigators noted that using mf+ blood diluted to the point that no mf is expected to be present in one ml of blood, both C-PCR and Eclipse MGB real-time PCR detected 2 out of 21 (10%) of the samples. Thus, this is consistent with the result of this study which was conducted in a very low endemic area whereby the real-time PCR demonstrated the same level of detection as C-PCR. However, the real-time PCR could readily detect one sample with low DNA copy number, which was detected as only a faint band using C-PCR. One explanation for the similar detection of the two kinds of PCR assays is the fact that the primers and probes were designed from *Hha*1 sequence which is a tandem repeat sequence, and found as several thousand copies per haploid genome (Ghedin *et al*., 2004). If the PCR primers were based on genes with low number of repeats, the two kinds of PCR assays may not demonstrate similar sensitivity rates.

Surprisingly, an area which has been considered as 'green' (i.e. 'non-endemic') during the mapping process of the National Filariasis Elimination Programme, has been shown in this study to have primary school children that were infected with *B. malayi*. Thus, a larger scale study of prevalence of the infection in this area needs to be conducted to determine the true endemicity of brugian filarisis. Since 7/21 (33%) of the rapid test positive children were found to have circulating parasite DNA, the study also clearly demonstrated the usefulness of Brugia Rapid™ test as a screening tool to detect infection in school children. The PCR method still requires night blood sampling to achieve high sensitivity; thus, the rapid test which can be performed at any time of the day is more convenient and practical for large scale testing.

The results of this study concluded that in settings with low filarial endemicity such as Malaysia or in countries where the Lymphatic Filariasis Elimination Programme has reached the certification and surveillance phases, it may be important that the DNA is extracted from a relatively large spot of blood sample (such as from 60 µl), then concentrated before the PCR is performed. Further studies will need to be performed to determine the minimum volume of blood spot sample that needs to be used, and the degree of concentration that should be performed. The detection assays can then be performed by either using C-PCR or real-time PCR since both the assays showed the same level of detection.

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