

Research note

## Sensitivity of the nested-polymerase chain reaction (PCR) assay for *Brugia malayi* and significance of ‘free’ DNA in PCR-based assays

Janet Cox-Singh<sup>a,\*</sup>, Andrea S. Pomrehn<sup>b</sup>, Nathan D. Wolfe<sup>c</sup>, Hasan A. Rahman<sup>d</sup>,  
Huong-Ying Lu<sup>a</sup>, Balbir Singh<sup>a</sup>

<sup>a</sup>Faculty of Medicine and Health Sciences, University Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia

<sup>b</sup>Department of Developmental Biology, Beckman Center, Stanford University School of Medicine, Stanford, CA 94035, USA

<sup>c</sup>Emerging Infections in Cameroon Project (EICAM), Johns Hopkins School of Hygiene and Public Health, 624N Broadway, Baltimore, MD 21205, USA

<sup>d</sup>Ministry of Health Malaysia, Bukit Damansara, Jalan Dungun, 50490 Kuala Lumpur, Malaysia

Received 7 August 2000; received in revised form 14 August 2000; accepted 14 August 2000

### Abstract

The blood filtration method was used as the gold standard to determine the detection level of simple blood-spot sampling and nested-polymerase chain reaction (PCR) for *Brugia malayi*. Of 100 samples, 48 were filtration-positive. Of these, 26 had microfilaria counts that were low enough (<1–29 microfilariae/ml) to accurately assess the limit of detection by nested-PCR. Nested-PCR consistently detected *B. malayi* DNA in samples with  $\geq 10$  microfilariae/ml. Post-filtration, microfilaria-depleted, blood-spots from microfilaria-positive samples were screened by nested-PCR and *B. malayi* specific ‘free’ DNA was detected in 51.7% of these samples. There was no evidence for ‘free’ DNA in microfilaria-negative individuals from this endemic community. © 2000 Published by Elsevier Science Ltd. on behalf of the Australian Society for Parasitology.

**Keywords:** *Brugia malayi*; Nested-polymerase chain reaction; Sensitivity; Free DNA; Filtration

*Brugia malayi* is one of the causative organisms of lymphatic filariasis and is restricted to the Asian region [1]. Recently we described a simple blood-spot sampling method coupled with nested-polymerase chain reaction (PCR) to detect circulating *B. malayi* microfilariae in individuals living in remote endemic areas [2]. The assay was tested on 145 field samples and was positive for all 30 microscopy-positive samples. Additionally, 13 microscopy-negative samples tested positive by the blood-spot assay, potentially indicating that the nested-PCR assay was more sensitive than microscopy. Here we report a study designed to determine the detection level of the nested-PCR assay using the blood filtration assay as the gold standard.

Previous studies on PCR methods for *B. malayi* have suggested that some infected individuals have circulating *B. malayi* DNA in the blood in the absence of circulating microfilariae [3,4]. The presence of ‘free’ DNA, if confirmed in our population, would theoretically overcome the problem of night sampling to coincide with the nocturnal microfilaria shedding characteristic of *B. malayi* infection

[5]. PCR analysis of the collected samples both before and after filtration in this study were tested for the presence of ‘free’ DNA in individuals with circulating microfilariae.

The filtration concentration method for microfilaria enumeration per ml of blood was used as the reference for accurate determination of microfilaria density [6]. For this method 1.5–10.0 (mean 4.1 ml) of venous blood, from 100 consenting individuals living in a remote *B. malayi* endemic area of Sabah, were collected into sodium citrate. The blood samples were collected in conjunction with a routine *B. malayi* screening exercise conducted by officers of the Vector-Borne Diseases Control Program, Sabah. Sample collection was complicated by the need to include a proportion of individuals with microfilaria counts below the level of detection by Giemsa-stained thick blood films. Studies have shown familial clustering of infected individuals and, by collecting samples from individuals who were microfilaria-negative by microscopy but residing with microfilaria-positive family members, we were able to collect blood samples with microfilaria levels below the detection level of microscopy [7]. Of the 100 specimens collected from family members, 26 samples met this criterion. All of the samples collected ( $n = 100$ ) were included in the study to determine the detection level of the nested-PCR method.

\* Corresponding author. Tel.: +60-82-671-000; fax: +60-82-671-975.

E-mail address: cox@tm.net.my (J. Cox-Singh).

Post-filtration blood was collected for nested-PCR analysis to establish the presence of ‘free’ *B. malayi* DNA in microfilaria-depleted blood from microfilaria-positive individuals. Nested-PCR results from 29 post-filtration samples contributed to the ‘free’ DNA study.

From the venous blood samples collected, three spots of approximately 50  $\mu$ l each were immediately dropped onto filter paper to avoid the accumulation of microfilariae and labeled ‘pre-filtration’. The blood-spots were thoroughly air-dried before storing in plastic bags, one sample per bag. The volume of venous blood remaining was recorded before passing the blood through a polycarbonate filter with 5  $\mu$ M diameter pore size [6]. The filtered blood was collected and blood-spots were made as above and labeled ‘post-filtration’. The filters were Giemsa-stained and microfilariae per filter counted. The counts were performed at the field site and then repeated ‘blind’ on returning to the research facility. The results were adjusted to microfilariae/ml and reported as the mean microfilariae/ml of two counts. Giemsa-stained thick blood film results for individuals in this study were collected and analysed by the officers conducting the routine filariasis survey on the same date as our study. DNA was extracted from the pre- and post-filtration blood-spots and nested-PCR performed as previously described [2].

Of the 100 samples collected, 52 were negative by filtration. The range of microfilaria counts in the remaining 48 samples was <1–517 microfilariae/ml. Samples with  $\geq$ 10 microfilariae/ml were consistently positive by the nested-PCR method with 80% of samples with 5–9 microfilariae/ml also detected by nested-PCR. We consider that the detection level of the method can be conservatively reported as 10 microfilariae/ml (Table 1). The sensitivity of nested-PCR was 71% (34/48 positive by PCR) overall when compared with filtration. However, when taking the number of filtration-positive samples at or above the level of detection by nested-PCR ( $\geq$ 10 microfilariae/ml) the sensitivity of nested-PCR becomes 96% (27/28 positive). The false negative result was a sample with 48 microfilariae/ml and was positive by repeat PCR. Of the 52 filtration-negative samples, one was found positive by nested-PCR. This sample was repeated using duplicate and freshly extracted template and found to be negative. The specificity of nested-PCR compared with blood filtration was 98% (51/52 nega-

Table 1  
Level of detection of blood-spot nested-PCR for *B. malayi*

Microfilariae/ml (filtration)	<i>n</i>	PCR positive (%)	Thick blood film positive (%)
0	52	1 (1.9)	1 (1.9)
< 1–4	15	2 (13.3)	2 (13.3)
5–9	5	4 (80.0)	3 (60.0)
10–29	6	6 (100)	2 (33.3)
30–517	22	21 (95.5)	20 (90.9)
Overall	100	34	28

Table 2

Assessment of pre- and post-filtration samples for the presence of ‘free’ *B. malayi* DNA

Microfilariae/ml (filtration)	<i>n</i>	PCR positive	
		Pre-filtration (%)	Post-filtration (%)
< 1–9	6	6 (100.0)	2 (33.3)
10–29	3	3 (100.0)	2 (66.6)
30–100	14	14 (100.0)	8 (57.1)
> 100	6	6 (100.0)	3 (50.0)
Total	29	29 (100.0)	15 (51.7)

tive by nested-PCR). The limit of detection for Giemsa-stained thick blood films during the study was 30 microfilariae/ml (Table 1). The cut-off for thick blood films reported here is a guide rather than an accurate determination of sensitivity. The presence of our research team and the brief to try to collect from individuals with low microfilaria counts may have influenced the accuracy of microscopy with some slides being double-checked and may have biased the level of sensitivity reported.

To test the hypothesis of ‘free’ DNA in microfilaria-positive samples, 29 post-filtration blood-spots were analysed from individuals with microfilaria counts ranging from <1 to >100 microfilariae/ml (Table 2). All (100%) of these samples were pre-filtration PCR-positive and 51.7% were post-filtration PCR-positive (Table 2). In this study there was no evidence for ‘free’ DNA in microfilaria-negative individuals as previously reported [3,4]. All of the filtration-negative samples in our study population were confirmed nested-PCR negative. Post-filtration PCR-positive results suggest the presence of detectable levels of *B. malayi* specific DNA in ‘free’ form that is dissociated from microfilariae. The source of the DNA in these samples is assumed to be from microfilaria damaged by shearing forces during filtration or DNA released from parasites in response to an event occurring in vivo during the period of microfilaria shedding - perhaps as a function of the host immune response. Slight damage to microfilariae would presumably be enough to release sufficient PCR target sequences from this highly repetitive part of the *B. malayi* genome to give a positive nested-PCR result [3,4]. The risk of shear damage during filtration may be expected to increase with microfilaria load, but there was no association between microfilaria load and the presence of post-filtration DNA except perhaps for samples with <1 microfilaria/ml. (Table 2).

Data reported here are discordant with a recent report on a sensitive PCR-enzyme-linked immunosorbent assay (ELISA) technique capable of detecting 1 fg of target *B. malayi* sequence [4]. The authors were able to demonstrate the presence of *B. malayi*-specific ‘free’ DNA in filtration-negative blood samples. We suggest that the discrepancy in the reports may be the result of different sample treatment between the two studies. In the study presented here, blood-spot sampling and filtration were completed on-site

within minutes of venous blood sample collection. This is in contrast to the procedure used in the PCR-ELISA study in which 10 ml of venous blood was collected and stored in ethylene diamine tetra-acetic acid (EDTA) at 4°C for up to 1 week before filtration of a 1 ml sample and subsequent DNA template extraction. In the event of disruption of even one microfilaria in the 10 ml sample, sufficient template for PCR-ELISA detection would be released and a filtration-negative but PCR-positive result would be predicted. Our results, using nested-PCR and simple blood-spot sampling, suggest that ‘free’ DNA, from whatever source, would not improve the detection level for *B. malayi*-infected individuals in endemic areas of Sabah, Malaysian Borneo.

At this juncture blood-spot sampling and nested-PCR remains a highly sensitive and specific practical tool for the detection of circulating *B. malayi* microfilariae in remote endemic regions of Asia. The method requires the presence of circulating microfilariae and consequently will be most effective when blood is collected during the peak periods of microfilaria shedding [5]. However, the simplicity of sample collection and stability of DNA during storage and transport introduces the potential for self-monitoring at the community level. Community volunteers, trained in finger-prick blood collection onto filter papers, would co-ordinate collection and transport of blood-spots to the public health laboratory for nested-PCR. This system would not only increase the accuracy of assessment of microfilaria rate within communities but also greatly reduce the financial burden imposed on healthcare systems by teams of microscopists stationed for prolonged periods at remote field sites.

## Acknowledgements

We wish to extend our appreciation to Dr Gary Weil for donating the blood filters and filter units used in this study. We also wish to thank the officers of the Vector-Borne Diseases Control Programs of Sabah and Sarawak for their assistance and co-operation during this study. The study was funded by a Fulbright-Hays student-ship to A.S.P. and a short-term grant from The University of Malaysia Sarawak (UNIMAS). N.D.W. was funded by a Fulbright Fellowship, a National Science Foundation Graduate Research Fellowship and a Talpin Fellowship.

## References

- [1] Michael M, Bundy DAP. Global mapping of lymphatic filariasis. *Parasitol Today* 1997;13:472–6.
- [2] Cox-Singh J, Pomrehn AS, Rahman HA, Zakaria R, Miller AO, Singh B. Simple blood-spot sampling with nested polymerase chain reaction detection for epidemiology studies on *Brugia malayi*. *Int J Parasitol* 1999;29:717–21.
- [3] Lizotte MR, Supali T, Partono F, Williams SA. A polymerase chain reaction assay for the detection of *Brugia malayi* in blood. *Am J Trop Med Hyg* 1994;51:314–21.
- [4] Fischer P, Supali T, Wibowo H, Bonow I, Williams SA. 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. *Am J Trop Med Hyg* 2000;62:291–6.
- [5] Chang MS, Ho BC, Hardin S, Doraisingam P. Filariasis in Kota Samarahan district Sarawak. *Trop Biomed* 1992;9:39–46.
- [6] Cheeseborough M. *Medical laboratory manual for tropical countries*, vol. 1. Sevenoakes, Kent: Butterworth, 1987. pp. 360–95.
- [7] Das PK, Sirvidya A, Vanamail P, et al. *Wuchereria bancrofti* microfilaraemia in children in relation to parental infection status. *Trans R Soc Trop Med Hyg* 1997;91:677–9.