UNIVERSITI SAINS MALAYSIA
SCHOOL OF MEDICAL SCIENCES
PUSAT PENGAJIAN SAINS PERUBATAN
KAMPUS CAWANGAN KELANTAN

1969 - 1999

M E M O R A N D U M

Rujukan Fail : USM/PPSP/Pent/P9/b/98 Jld. VI

Tarikh : 27 Mac 1999

Puan Mazula Sabudin
Penolong Pendaftar
Bahagian Penyelidikan dan Pembangunan
Jabatan Canselori
Universiti Sains Malaysia
11800 Minden
Pulau Pinang

Puan,

Per:

1. **Permohonan Baru Projek Jangka Pendek**
   Prof. Xuancai S.T.Wan
   Prof. Madya Rabindarjeet Singh
   Prof. Madya Rosli Nordin
   Dr. S.C. Reddy
2. **Permohonan Baru IRPA RM 7 (Bersama Disket)**
   Prof. Madya Faridah Abdul Rashid
3. **Laporan Kemajuan USM Jangka Pendek**
   Dr. Wan Asim Wan Adnan
4. **Laporan Akhir Projek Jangka Pendek**
   Dr. Balbir Singh
   Dr. Sunil Gurtu

Merujuk kepada perkara di atas, bersama-sama ini disertakan empat permohonan baru projek Jangka Pendek, satu permohonan baru IRPA RM7, satu Laporan Kemajuan Jangka Pendek dan dua Laporan Akhir Projek Jangka Pendek yang telah dikemukakan melalui Jawatankuasa Penyelidikan dan Etika PPSP untuk tindakan puan selanjutnya.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"
"CINTAILAH BAHASA KITA"

Yang menjalankan tugas,

(AJINDAR KAUR) (Puan)
Penolong Pendaftar (Akademik)

s.k Pn. Nik Asmak Nik Idris } *salinan laporan akhir disertakan*
Penolong Pustakawan
Bahagian Bersiri dan Dokumentasi
Perpustakaan Perubatan
Kampus Cawangan Kelantan

16150, KUBANG KERIAN, KELANTAN, MALAYSIA.
TEL: 09-7651700 TELEX: MA53300 USMKEL. FAX: 09-7653370
http://www.ppsp.usm.my
Email: dekan@kb.usm.my

(S-PHI/dB-ku mae 25/na

Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di pusat pengajian

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN

CANSELORI

UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) **Nama Penyelidik:** DR.BALBIR SINGH

Nama Penyelidik-Penyelidik Lain *(Jika berkaitan)* : DR.JANET COX-SINGH

2) **Pusat Pengajian/Pusat/Unit** : SAINS PERUBATAN

3) **Tajuk Projek:** POLYMERASE CHAIN REACTION OF DRIED BLOOD SPOTS TO DETECT PARASITE DNA IN INDIVIDUALS WITH LYMPHATIC FILARIASIS

4) (a) **Penemuan Projek/Abstrak**

*(Perlu disediakan makluman di antara 100 - 200 perkataan di dalam **Bahasa Malaysia dan Bahasa Inggeris** Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).*

Project title Polymerase chain reaction of dried blood spots to detect parasite DNA in individuals with lymphatic filariasis.

Abstract

Lymphatic filariasis caused by *Brugia malayi* has traditionally been detected in the blood of infected individuals by microscopy. Screening for blood-stage microfilaria (mf) by microscopy is labour intensive with user fatigue and poor specimen handling responsible for false negative results. Recently a method to detect the DNA from circulating microfilaria using the polymerase chain reaction (PCR) has been described (Lizotte *et al.*, 1994). However, the specimen collection method described was unsuitable for routine screening in field situations. The aim of the study reported here was to adapt the PCR method to a simple blood spot sampling and DNA extraction method suitable for remote areas without compromising the sensitivity of PCR. Blood spots were collected from individuals in Kelantan and Terengganu to optimise the technique. A one tube DNA extraction method was developed and coupled to a nested PCR assay that was field tested on an endemic community in Sabah. There was 100% sensitivity when comparing PCR to microscopy but only 70% sensitivity when comparing microscopy to PCR. The increased sensitivity of PCR coupled with simple sample collection and DNA extraction provides a valuable alternative to microscopy for detecting *B. malayi* positive individuals in endemic regions of the world.

.....

.....

.....

.....

(b) **Senaraikan Kata Kunci yang digunakan di dalam abstrak:**

Bahasa Malaysia	**Bahasa Inggeris**
TINDAK BALAS	POLYMERASE CHAIN
RANTAIAN POLIMERASE	REACTION.
FILARIASIS.	FILARIASIS.
PARASIT .	PARASITE .

5) **Output Dan Faedah Projek**

(a) Penerbitan *(termasuk laporan/kertas seminar)*
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

KERTAS YANG TELAH DI KEMUKAKAN UNTUK PENERBITAN:

BRUGIA MALAYI: SIMPLE BLOOD SAMPLING AND NESTED POLYMERASE CHAIN REACTION DETECTION ASSAY FOR EPIDEMIOLOGICAL STUDIES(1998) COX-SINGH J., POMREHN A.S.,RAHMAN H. A., ZAKARIA R,MILLER A. O. AND SINGH,B.INTERNATIONAL JOURNAL FOR PARASITOLOGY

(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten:
(Jika ada dan jika perlu, sila gunakan kertas berasingan)

.....

.....

.....

.....

.....

.....

.....

.....

(c) Latihan Gunatenaga Manusia

i) Pelajar Siswazah .....

.....

.....

.....

ii) Pelajar Prasiswazah: .....

.....

.....

.....

iii) Lain-Lain : FULLBRIGHT STUDENT DARI AMERIKA SYARIKAT ANDREA POMREHN.

.....

.....

.....

6. **Peralatan Yang Telah Dibeli:**

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

.....

.....

.....

.....

.....

.....

T/TANGAN PENGERUSI
J/K PENYELIDIKAN
PUSAT PENGAJIAN

Project title Polymerase chain reaction of dried blood spots to detect parasite DNA in individuals with lymphatic filariasis.

Introduction

Lymphatic filariasis remains one of the major vector borne parasites causing debilitation and morbidity in those with pathology (World Health Organisation,1994). In Malaysia the predominant causative agent of lymphatic filariasis is the filarial nematode *Brugia malayi*. Adult filarial worms live in the lymph nodes and microfilaria (mfs), the first larval stage, are found in th circulation. Lymphatic filariasis is transmitted from person to person by the mosquito vector. The female mosquito takes up mf's, from the infected host during a blood meal. In the mosquito microfilaria develop into 3rd stage larvae which migrate to the salivary glands. The 3rd stage larvae are infective to the human host and are transmitted during subsequent blood meals. Considerable effort has been made to control the spread of lymphatic filariasis. A significant control measure relies on active case detection involving screening communities during the time when mf levels are estimated to be high. In Malaysia *B. malayi* is nocturnally periodic and subperiodic, with mf counts reaching a peak at 23.00 hours (Chang *et al.*, 1992). Consequently, filariasis case detection surveys are carried out at night as close to the peak period as is socially acceptable (19.00 - 22.00 hours). Thick blood films are prepared from 60 ul capillary blood from each individual and microfilaria are detected by microscopy.

Microscopy is the time-honoured method of choice for screening populations for filariasis detection. However, microscopy is laborious with false negativity resulting from operator fatigue, poor staining and, in addition, low levels of microfilaria may be missed as they are prone to lift off the slide during staining. In an effort to improve detection of circulating microfilaria a sensitive polymerase chain reaction (PCR) method was described to detect *B. malayi* DNA in the blood of infected individuals (Lizotte *et al.*, 1994). PCR was reported to be more sensitive than microscopy however, the blood collection method described relied on collecting whole blood and required refrigerated transport to the research facility. In addition to cold transport requirement, the DNA extraction method was time consuming and therefore unsuitable for screening endemic populations, that are frequently found in the more remote regions on the world.

A simple blood spot sampling and DNA extraction method coupled with PCR has been developed for detecting malaria parasites for epidemiology studies (Singh *et al.*, 1996 and Cox-Singh *et al.*, 1997). It was the aim of this study to couple PCR for *B. malayi* with a simple sampling and DNA extraction method. The simplicity of blood spot sampling combined with the sensitivity and specificity of PCR would provide a powerful epidemiological tool to screen and monitor the incidence of lymphatic filariasis particularly in remote communities. The advantages of the simple sampling and DNA extraction coupled with PCR, as a screening tool for active case-detection of Brugian filariasis will be discussed.

Materials and methods

Blood specimen collection
Blood was obtained from individuals living in filariasis endemic areas in Kelantan, Terengganu and Sabah. Capillary blood was spotted onto Whatman 3M filter paper, approximately 50UL per spot, and air-dried before storing in individual plastic bags. The blood spots were transported to the research facility at Universiti Sains Malaysia immediately following collection or forwarded by post.

Microscopy
Thick blood films were made with 60UL capillary blood and stained with Giemsa stain. Microfilaria (mf) were counted and reported as mf/60UL of blood. All microscopy was performed by experienced Officers of the Vector Borne Diseases Control Programme Offices.

DNA extraction
Method 1. One spot of blood was carefully cut from the filter paper and placed in 300UL pre-boiled Chelex ™ 5%w/vol (Bio-Rad Laboratories). The preparation was boiled for 10 minutes before freeze thawing (-70°C 5 minutes to boiling water bath X 3). The tube was vortexed and then centrifuged at 12,000 RPM for 2 minutes. The supernantant containing the DNA template was collected taking care to avoid Chelex™ resin carryover and stored at 4°C. Two UL of template was used per 50UL PCR reaction.
Method 2. Using a 6mm diameter paper punch, 5 blood soaked discs equivalent to 50 UL of blood were removed from each sample to a 1.5 ml microcentrifuge tube containing 100 UL of digestion buffer (20mM Tris pH 8.0, 50mM KCL, 2.5mM MgCl2, 0.5%Tween 20, 0.015%w/v Proteinase K). The discs were incubated at 65°C for 2 hours with occasional vortexing during the incubation before boiling for 15minutes. Three hundred UL of InstaGene™ was added to each tube and vortexed. The tubes were incubated at 56°C for 30 minutes with vortexing after 15 minutes and at the end of the incubation. The tubes were then boiled for 15 minutes and the supernatant recovered following centrifugation at 12,000rpm for 3 minutes. Care was taken to avoid resin carryover and the template containing supernantant was used immediately for PCR or stored at -20°C. Ten UL of template was included in 50UL PCR reactions.

PCR reaction conditions
Nest 1 PCR
PCR conditions and oligonucleotide sequences as reported by Lizotte et al., were used except that the $MgCl_2$ concentration was increased to 4mM and the thermocycler was programmed for 35 cycles and included a 94°C for 4 minutes hot start and 72°C for 5 minutes at the end of the programme. Boehringer Manheim reagents were used for all PCR reactions.

Nest 2 PCR
Two UL of the N1 PCR reaction was amplified in a 20UL nest 2 reaction with internal oligonucleotides. The reagent mix was as follows: 1X PCR buffer, 2.5 mM MgCl2, 1.25mM each dNTP, 0.5 units Taq polymerase. The reaction

conditions were 94°C for 4 minutes; 94°C for 1 minute, 60°C 1 minute, 72°C 1 minute (35 cycles); 72°C 7 minutes. PCR products were visualised on 3% agarose gels by UV transillumination and ethidium bromide staining.

Results

Optimisation of DNA extraction

Filarial DNA was extracted from blood spots using Chelex™ extraction (method 1) and amplified using the conditions described for Nest 1. The PCR results were compared to microscopy. (Table 1). The sensitivity of PCR compared to microscopy was 55% with little correlation between mf density and PCR detection. The Chelex™ method was inhibitory to PCR when template volumes greater that 5UL were added and together with poor disruption of cuticle the PCR was not sensitive. An extraction method using Proteinase K to disrupt the cuticle and InstaGene™ to remove inhibitors of PCR(method 2) was developed. Ten UL of template was amplified using nest 1 conditions. The PCR results were compared to microscopy (Table 2). Four of the Nest 1 PCR reactions gave inconsistent results and the sensitivity of N1 was 64% when compared to microscopy.

In order to improve the sensitivity of the reaction the products of the nest 1 reaction were amplified under nest 2 conditions Table 2. The sensitivity of PCR compared to microscopy was 91%.

Field trial using extraction method 2 and nested PCR.

One hundred and forty five individuals living in an endemic region of Sabah were screened by microscopy and nested PCR using DNA extraction method 2. The sensitivity of the PCR method was compared to microscopy, using the microscopy results obtained by the officers of the Vector-Borne Diseases Control Programme Office, Sabah (Table 3). Thirty individuals were found mf positive by microscopy and 16 of these were detected by nest 1 PCR. The sensitivity of nest 1 PCR to microscopy was 53%. The Nest 1 reactions were re-amplified in a nest 2 reaction. All of the microscopy positives were detected by PCR giving 100% sensitivity when comparing PCR to microscopy. A further 13 individuals were found positive by nested PCR which were negative by microscopy (Table 3). The sensitivity of microscopy compared to nested PCR and DNA extraction method 2 was 70%.

Discussion

During the development of the assay, the simple sampling and DNA extraction from *B. malayi* infected blood with method 1 failed to yield sufficient mf DNA template for efficient PCR amplification. In method 1 dried filarial infected blood spots, roughly equivalent to 50UL of capillary blood, were extracted by boiling with Chelex™ and then freeze/thawed to disrupt the mf cuticle (Meredith *et al.*, 1991). Only 2UL of the template rich supernatant was included in a single nest PCR reaction which limited the sensitivity of the Chelex™ extraction method. Volumes of more than 5UL of Chelex™ supernatant in the nest 1 reaction were inhibitory and there was no observed increase in sensitivity whether 2 or 5UL were included in the nest 1 reaction (results not shown). Microfilaria infected blood used for Chelex™ extraction

was collected from one patient and taken at 12 hourly intervals. The mf densities were low (between 1 to 15 mf/60UL blood) or absent and clearly demonstrate the variability of mf density and the need for a highly sensitive PCR method to detect low mf densities. The Chelex™ method described here was based on a simple sampling and DNA extraction developed for malaria epidemiology studies (Singh *et al.*, 1996) but was not efficient enough to detect low density mf levels. In contrast to the malaria parasite, mfs are enclosed in a highly cross-linked and resistant collagen cuticle. The success of a simple sampling and DNA extraction procedure for filariasis depends on adequate disruption of the cuticle and sufficient volume of sample included in the nest one reaction for amplification of small amounts of DNA. DNA extraction method 2 incorporated Proteinase K to disrupt the cuticle and release DNA from the mf and Chelex™ was replaced by InstaGene™. PCR inhibitors found in blood are more efficiently removed by InstaGene (Cox-Singh *et al.*,1997), therefore the volume of template in the nest 1 reaction was increased from 2UL using Chelex extraction to 10UL when using InstaGene™. Increasing the template volume increased the probability of the target sequence being included in the nest 1 reaction. Theoretically one microfilaria should be detected in 50UL of blood using one PCR reaction (Lizotte *et al.*, 1994). However, while PCR amplification is not inhibited by the InstaGene™ method the DNA template preparation is very crude compared to phenol chloroform extraction (Lizotte *et al.*, 1994). It is thought that the nest 1 reaction enriches the target sequence in the InstaGene™ supernatant leading to efficient amplification in the nest 2 reaction. Re-amplification of nest one products under nest 2 conditions was sensitive and reproducible in detecting *B. malayi* DNA in all of the microscopy positive samples (mf range 1 – 288 mf/60UL blood) during a field trial. Thirteen specimens, negative by microscopy, were PCR positive. The positives detected by PCR but not microscopy were thought to be true positives as there was extensive use of negative controls which were consistently negative by PCR. In addition, repeat DNA extraction and PCR of the positives confirmed the PCR results. Furthermore, PCR is reported to be more sensitive, not only than thick film microscopy where 60UL of blood is examined, but also by the filtration method where mf in 1 ml of blood are detected (Lizotte *et al.*, 1994 & Weil *et al.*,1997).

The World Health Organisation has set a target for global eradication of filariasis by the year 2020 (Ottesen, 1998). A major component of the exercise is to interrupt transmission of the disease. To interrupt transmission infected communities must first be identified. Until recently the most widely used tool to identify communities at risk was microscopy. There are many disadvantages to using microscopy, not the least being false negativity. PCR remains the most sensitive method for active case detection of *B. malayi*, however, using standard DNA protocols for extracting filarial DNA from infected blood prohibits the routine use of PCR to screen endemic communities (Lizotte *et al.*, 1994). Currently filariasis screening by microscopy imposes a considerable financial burden on health authorities. The simple sampling and DNA extraction method described here is suitable for sample collection, even in the most remote regions of the world, and facilitates rapid processing of multiple samples at the PCR facility. Air dried

blood spots are stable at room temperature for up to 2 years for sensitive *B. malayi* PCR detection (unpublished results). Collecting blood spots requires little skill and it is possible that blood spots, collected by health assistants in remote villages, would be posted to the PCR facility without the need for vector control teams to enter the study site. The sampling method described here is eminently suitable for community participation in self screening and monitoring the efficiency of filariasis control programmes aimed at achieving the WHO target of global eradication of lymphatic filariasis by the year 2020.

Acknowledgements

The assistance and contribution of the following was greatly appreciated: Dr Ranjit Kaur, Chief Medical Officer, Department of Health, Pasir Mas, Kelantan. Dr Mohd. Yusoff and Staff of the Department for the Control of Vector Borne Diseases, Kuala Terengganu, Terengganu. Dr Hasan Abdul Rahman, Sabah State Deputy Director for Health (Health). The officers of the Vector Borne Diseases Control Programme Offices in Kelantan, Terengganu and Sabah. Mr Andy O. Miller, Mr. Robaiza Zakaria and Mr. Kavinderjit Singh.

References

Chang, M.S., Ho, B.C., Hardin, S. and Doraisingam, P. (1992). Filariasis in Kota Samarahan district Sarawak. Trop. Biomed. **9**, 39 - 46.

Cox-Singh, J., Mahayet S., Abdullah, M.S. and Singh, B. (1997). Increases sensitivity of malaria detection by nested polymerase chain reaction using simple sampling and DNA extraction. Int. J. Parasitol. **27**, 1575-1577.

Lizotte,. M.R., Supaili, T., Partono, F. and Williams, S.A. (1994). Polymerase chain reaction assay for the detection of *Brugia malayi* in blood. Am. J. Trop. Med. Hyg. **51**, 314 - 321.

Ottesen, E. (1998). Lymphatic Filariasis, WHO Fact Sheet No.198.T.R. (1991).

Meredith, S.E.O., Lando, G., Gbikama, A.A., Zimmerman, P.A. and Unnasch, *Onchocerca volvulus*: Application of the polymerase chain reaction to identification and strain differentiation of the parasite. Exp. Parasitol. **73**, 335 - 344.

Singh, B., Cox-Singh, J., Miller, A.O., Abdullah, M.S., Snounou, G. and Rahman, H.A. (1996). Detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. Trans., Royal Soc. Trop. Med. Hyg. **90**, 519 - 521

Weil, G.J., Lammie, P.J. and Weiss, N. (1997). The ICT Filariasis Test: A rapid-format antigen test for diagnosis of Bancroftian filariasis. Parasitol. To-

day, **13**, 472 - 476.

World Health Organisation (1994). Lymphatic filariasis infection and Disease: control strategies. TDR/CTD/FIL/PENANG/94.1)

Specimen No.	mf/60µl blood	PCR	Repeat PCR
F278	13	+	+
F279	6	+	+
F280	15	+	ND
F281	0	-	ND
F282	1	+	ND
F283	1	-	-
F284	0	-	ND
F285	1	-	-
F286	2	+	ND
F287	0	-	ND
F288	0	+	+
F289	4	-	+
F290	0	-	ND
F291	1	-	-

Table 1
PCR using Chelex™ extracted template from blood spots collected in Pasir Mas, Kelantan, Malaysia. Nest 1 PCR conditions are compared with microscopy. '+' indicates PCR positive, '-' indicates PCR negative and ND indicates not determined.

Specimen No.	mf/60 µl blood	PCR Nest 1	PCR Nest 2
F306	2	+/-	+
F307	3	+/-	+
F308	5	+/-	+
F309	53	+	+
F310	4	+	+
F311	3	+/-	-
F312	78	+	+
F313	1	+	+
F314	200	+	+
F315	25	+	+
F316	77	+	+

Table 2
PCR using InstaGene extracted template from blood spots collected in Terengganu, Malaysia. Nest 1 and nest 2 PCR conditions were compared with microscopy. '+' indicates PCR positive, '-' indicates PCR negative, '+/-' indicates inconsistent results in repeat experiments and ND indicates not determined.

Sample Number	mf/ 60 µl blood	PCR N1	PCR N2
TA6	3	-	+
TA7	14	-	+
TA8	288	-	+
TA9*	0	-	+
TA10	19	-	+
TA11*	0	-	+
TA12	2	-	+
TA16*	0	-	+
TA22*	0	+	+
TA23	6	+	+
TA25	17	+	+
TA26	5	+	+
TA29*	0	+	+
TA35	9	-	+
TA38*	0	-	+
TA39	2	-	+
TA40*	0	-	+
TA43	17	-	+
TA44*	0	-	+
TA48	13	+	+
TA49	23	+	+
TA53	70	+	+
TA56*	0	-	+
TA57	8	+	+
TA58	11	+	+
TA59*	0	+	+
TA65*	0	-	+
TA73	20	-	+
MO5	2	-	+
MO8	4	-	+
MO9	4	-	+
MO16*	0	-	+
MO20	6	+	+
MO22	16	-	+
MO25*	0	-	+
MO35	1	+	+
MO37	8	+	+
MO39	27	+	+
MO40	4	+	+
MO48	18	-	+
MO61	8	+	+
MO62	1	+	+
MO63	3	+	+
MO64	28	-	+
Total	31	19	44

Table 3. Field trial of PCR using InstaGene extracted template on blood spots collected in Sabah. '+' indicates PCR positive reactions and '-' indicates negative PCR reactions.* Samples that were negative by microscopy but positive by PCR.