DIFFERENTIATION OF BRUGIA MALAYI AND BRUGIA PAHANGI BY PCR-RFLP OF ITS1 AND ITS2

S Nuchprayoon¹, S Sangprakarn¹, A Junpee¹, S Nithiuthai². S Chungpivat² and Y Poovorawan³

¹Department of Parasitology, ³Department of Pediatrics, Chula Medical Research Center, Faculty of Medicine, and ²Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn, University, Bangkok, Thailand

Abstract. Lymphatic filariasis has been targeted by the World Health Organization for elimination by the year 2020. Malayan filariasis, caused by *Brugia malayi*, is endemic in southern Thailand where domestic cats serve as a major reservoir host. However, in nature, domestic cats also carry *B. pahangi* infection. In addition to chemotherapy and vector control, control in reservoir hosts is necessary to achieve the elimination of the disease. Therefore, differentiation between *B. malayi* and *B. pahangi* in the cat reservoir will help the lymphatic control program to monitor and evaluate the real disease situation. It is difficult to differentiate these two *Brugia* species by microscopic examination. The technique is also time-consuming and requires expertise. We employed the polymerase chain reaction-linked restriction fragment length polymorphism (PCR-RFLP) technique of internal transcribed spacer regions, ITS1 and ITS2, of ribosomal DNA (rDNA) to differentiate *B. malayi* from *B. pahangi* species. Among the restriction enzymes tested, only the PCR product of ITS1 digested with *Ase* I could differentiate *B. malayi* from *B. pahangi*. This PCR-RFLP technique will be useful for lymphatic filariasis control programs for monitoring and evaluating animal reservoirs.

INTRODUCTION

Lymphatic filariasis, mainly caused by the filarial nematodes Wuchereria bancrofti and Brugia malavi. is a debilitating and disfiguring disease. It is estimated that 1.1 billion people, 20% of the world's population. in more than 80 countries, are at risk of acquiring the infection, while over 120 million have already been infected (WEO, 2000). Although Bancroftian filariasis has been controlled to a low level, 0.99 cases/100,000 population (Filariasis Division, 1999), Malayan filariasis is still endemic in southern Thailand. Lymphatic filariasis, caused by B. malayi, nocturnal subperiodic type, is prevalent mainly in Narathiwat Province. This is due to the many suitable mosquito breeding sites, large swamp areas, and the existence of animal reservoir-hosts. In Thailand, Mansonia sp are the main mosquito vectors of B. malayi (Filariasis Division, 1998). Domestic cats are important reservoir hosts for B. malayi (Palmieri et al, 1985; Phantana et al, 1987: Kanjanopas et al, 2001; Chansiri et al, 2002). Therefore, besides chemotherapy and vector control, the successful elimination of lymphatic filariasis should include the control of reservoir hosts. Not only B. malayi can infect domestic cats, but also another filarial

Correspondence: Dr Surang Nuchprayoon, Department of Parasitology. Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. Tel: 66 (0) 2256-4387; Fax: 66 (0) 2252-4963 E-mail: fmedstt@md2.md.chula.ac.th species, such as *B. pahangi* (Nithiuthai and Chungpivat, 1992; Chungpivat and Sucharit, 1993). Co-infections of both *Brugia* species in domestic cats is not uncommon in Thailand. This raises the possibility of misdiagnosis due to the difficulty in differentiating both species by the conventional microscopic method. Misdiagnosis would jeopardize the lymphatic filariasis control program.

In endemic areas, the routine method for identifying microfilaria species is microscopic examination, based on the delineation of particular morphological features using Giemsa stain, and geographic location, that is, where the specimens have come from. Nevertheless, using this technique, it is difficult to discriminate clearly between closely related species such as B. malayi and B. pahangi. Histochemical staining, to detect acid phosphatase activity, could overcome the problem (Yen and Mak, 1978; Chungpivat et al, 1990; Nithiuthai and Chungpivat, 1992; Chungpivat and Sucharit, 1993). However, this technique needs fresh samples to yield the best results. Furthermore, both staining methods require expertise to identify and confirm the species. Molecular analysis has been introduced as a new tool to distinguish parasite species. Ribosomal DNA (rDNA) has been a valuable tool to discriminate closely related species among several eukaryotic organisms. including nematodes. The application of internal transcribed spacers (ITS) to identify the organism has received the most attention by nematologists during the past decade (Gasser et al, 1994; 1996; Zhu et al.

1998; Almeyda-Artigas *et al*, 2000; Conole *et al*, 2001). Studies of PCR-linked restriction fragment length polymorphism (PCR-RFLP) profiles of the nematodes' ITS regions have provided data on nematode diversity, as well as the critical taxonomic character useful for species comparison and identification (Gasser *et al*, 1994, 1996). We report here the results of PCR-RFLP of ITS1 and ITS2 to differentiate *B. malayi* from *B. pahangi*.

MATERIALS AND METHODS

Specimen collection

In co-operation with the Filariasis Division, Department of Communicable Diseases Control, Ministry of Public Health, Thailand, collection of human blood specimens was performed as previously described (Triteeraprapab and Songtrus, 1999; Triteeraprapab et al, 2000; 2001; Nuchprayoon et al, 2001). Two milliliters of venous blood from domestic cats infected with B. malavi or B. pahangi were obtained from experimental cats at the Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. Dirofilaria immitis microfilariae were obtained from infected stray dogs' blood. Blood from healthy volunteers, noninfected domestic cats and dogs were used as the negative control. This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

All filarial parasites were identified and the species confirmed by Giemsa staining, and special staining for acid phosphatase activity (Yen and Mak, 1978; Chungpivat *et al*, 1990; Nithiuthai and Chungpivat, 1992; Chungpivat and Sucharit, 1993).

DNA extraction

The extraction method was modified from a previously reported protocol (William et al, 1996). Two hundred and fifty microliters of each blood sample were added to a 1.5 ml microfuge tube and mixed with 750 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The mixture was centrifuged and the supernatant was discarded. The pellet was washed with 750 µl of TE buffer, pH 8.0 and resuspended in 500 µl of red cell lysis buffer (RCLB; 1 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM MgCl,, 1% Triton X-100) twice. After centrifugation, the supernatant was discarded, 400 µl of DSP buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 0.5% Tween 20, 150 µg/ml Proteinase K) were added and incubated at 65°C for 3 hours. Enzyme activity was inactivated by incubation at 90°C for 10 minutes.

Primers design

The forward primer for the ITS1 (FL1-F) was designed from the conserved sequence of filarial parasites reported in the Genbank database (Fig 1). The reverse primer for the ITS1 (Di5.8S 660-R) and the forward primer for the ITS2 (Di5.8S 558-F) were designed from the reported 5.8S rDNA sequence of *D. immitis* (AF217800). The reverse primer for ITS2 (FL2-R) was designed from the 28S rDNA sequence of *D. immitis* (AF217800). All oligonucleotide primers were purchased from the Bioservice Unit, NSTDA, Bangkok.

Polymerase chain reactions

In order to obtain sufficient DNA for RFLP study, semi-nested PCR for ITS1 and ITS2 were performed.

First PCR ITS1-5.8S-ITS2 region. The entire ITS1-5.8S-ITS2 region (Fig 1) was amplified by PCR using FL1-F and FL2-R oligonucleotide primers. The hot-start PCR reaction was performed in a 25 ul reaction containing PCR buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, 50 mM KCl) (Amersham Pharmacia, Freiburg, Germany); 200 µM each of dATP. dCTP, dGTP and dTTP (Promega, Wisconsin, USA); 0.625 units Taq DNA polymerase (Amersham Pharmacia); 5 pmol of each primer (FL1-F and FL2-R); and 1 µl DNA template. After incubation at 94°C for 5 minutes, amplification was carried out for 5 cycles with the following temperature cycling parameters: 94°C for 30 seconds of denaturation; 58°C for 30 seconds of annealing; and 72°C for 90 seconds of extension; followed by 30 cycles of temperature cycling parameters at 94°C for 30 seconds of denaturation; 55°C for 30 seconds of annealing, and 72°C for 90 seconds of extension. The final amplification cycle included an additional 10 minutes' extension at 72°C.

Second PCR for ITS1 region. The ITS1 region was amplified by using 1 µl of PCR product from the first PCR as a DNA template. The PCR reaction was performed in a 50 µl reaction containing PCR buffer. as described above, with 5 pmol of each primer of FL1-F and Di5.8S 660-R. Amplification was carried out for 5 cycles with the following temperature cycling parameters: 94°C for 30 seconds of denaturation; 58°C for 30 seconds of annealing; and 72°C for 45 seconds of extension. followed by 30 cycles of temperature cycling parameters: 94°C for 30 seconds of denaturation, 55°C for 30 seconds of annealing; and 72°C for 45 seconds of extension. The final amplification cycle included an additional 10 minutes extension at 72°C.

Second PCR for ITS2 region. The ITS2 region

was amplified by using 1 µl of PCR product from the first PCR as a DNA template. The PCR reaction was performed in a 50 µl reaction containing PCR buffer with 5 pmol of each primer, Di5.8S 558-F and FL2-R. After incubation at 94°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 30 seconds of denaturation; 55°C for 30 seconds of annealing; and 72°C for 45 seconds of extension. The final amplification cycle included an additional 10 minutes' extension at 72°C.

Restriction fragment length polymorphism (RFLP)

After precipitation of each PCR product and

resuspension in 10 μ l sterile distilled water, 1 μ l of PCR product was digested with 5 units of each restriction endonuclease, according to the manufacturer's protocols (New England Biolabs, Massachusetts, USA). The digestion was incubated at 37°C for 3 hours. The following enzymes were evaluated; *Ase* I, *Acc* I, *Hinf* I and *Rsa* I. Analysis of DNA fragments was performed by 2-2.5% submarine agarose gel electrophoresis.

RESULTS

Oligonucleotide primers were designed based on the reported conserved sequences of 18S, 5.8S and 28S

	FLI-F	•				
5′1	ggtgaacctg	cggaaggatc	attatcgagc	ttcaacaaac	aacaaacaca	tcatcatcat
61	catcattatt	actacttcta	ctactactac	tactactact	aatactaata	ctaatactac
121	tattaacatc	attatgctgc	ttattttcat	tetttteaa	ataatgcaag	aatgaaatag
181	aatgaatgaa	tgatgtttaa	tagattaata	gatgaatagt	tagtagtagt	tagtagttag
241	ttagatagat	agataaatga	aatgatgatg	aaatattttt	attcgatcaa	ttgaatataa
301	acggtgatat	tcgttggtgt	ctatattta	tctaagttat	cgcctaaccg	tcgatagcga
361	tgaagataaa	atgatagett	agaanaa	tgetcaa	ttaagtagac	ttaataagca
421	ttttagctag	tatgctacca	▲ Di 5.8S 558	8-F cacacac	atacatacaa	taatatgata
481	taattyttat	ttataataat	tt	t_aagcgg	gggatcacct	ggctcgtgga
541	tcgatgaaga	acgcagctag	ctgcgataaa	tagtgcgaat	tgcagacgca	ttgagcacaa
601	agatttcgaa	tgcacattgc	accatcgggt	tgattcccga	tggtacgtct	ggttgagggt
661	caatatcgat	cagattatca	aataatatac	caaaattgat	acatat	ptg
721	atat ^{*+} gttt	tggatatatt	atttgttaaa	attaattcat	caggtg Di5.	85 660-R 1ta
781	atcattgaaa	aatgacaaaa	atcccctgat	taagtataat	aaaaathaa	-agoaaaadag
841	gcaaattttt	acttacaaaa	tattacatat	tggaataaat	aatttaatct	ttcatatata
901	taactgtgat	acatcataaa	tatatata	gcaaaagaaa	gatataaatt	attatcattt
961	tttttgatca	ttattgctat	aattattatc	ctgcttgttt	aactataata	atagcaatga
1021	atgaatcaaa	atttaaatga	tacgttaaaa	tccaaccaat	atgttataaa	ttttcttca
1081	tttttgacct	caactcagtc	gtgattacgc	gctgaattta	agcatataac	ta 3'
				[





Fig 1- Forward and reverse primers for ITS1 and ITS2. The FL1-F primer is conserved in filarial parasites. All other primers were designed from the *D. immitis* 5.8S and 28S rDNA sequence (AF217800). FL1-F and Di5.8S 660-R are primers for the amplification of ITS1; Di 5.8S 558-F and FL2-R primers are for ITS2.

rDNA of filarial worms (B. malavi, W. bancrofti and D. immitis). Nevertheless, not all rDNA sequences of the selected filarial worms were completely reported. The available filarial rDNA sequences from the online database (Genbank) were as follows: 18S rDNA from B. malavi (AF036588), W. bancrofti (AF227234), and D. immitis (AF217800); ITS1, 5.8S and ITS2 sequence from D. immitis (AF217800); partial 28S rDNA sequence from D. immitis (AF217800) and B. malavi (AF499130). The FL1-F primer was designed from the conserved region at the 3' end of B. malavi, W. bancrofti and D. immitis 18S rDNA (Fig 1). The FL2-R primer was selected from the beginning 5' end of D. immitis 28S rDNA, which was also conserved among the reported 28S rDNA from other nematodes. After the first round of PCR, very faint DNA bands of ITS1-5.8S-ITS2 were observed. Subsequently, semi-nested PCR was performed to obtain a high vield of PCR products. Two internal primers, Di5.8S 660-R and Di5.8S 558-F, were designed from the D. immitis 5.8S rDNA region (AF217800) in order to amplify the ITS1 and ITS2 regions, respectively.

PCR-RFLP patterns of ITS1 and ITS2

The 667 bp band of D. immitis ITS1 was amplified as expected (Fig 2A). However, both B. malavi and B. pahangi ITS1 had smaller ITS1 sizes of about 590 bp. In contrast, the ITS2 from D. immitis was 570 bp, and for B. malayi and B. pahangi ITS2 about 650 bp (Fig 2B). Both B. malavi from humans and domestic cats showed the same size of PCR products and similar digested patterns for ITS1 and ITS2 (Fig 2A, B). There were 2 bands of about 290 bp and 300 bp from the Ase I digested B. pahangi ITS1 (Fig 2A), while about 140 bp, 160 bp and 290 bp bands were from the B. malavi digested ITS1. PCR-RFLP of ITS1 with Acc I and with Hinf I did not differentiate B. malayi from B. pahangi. Similary, the PCR-RFLP of ITS2 with Rsa I and with Ase I did not discriminate either Brugia species.

PCR-RFLP of *D. immitis* ITS1 by *Ase* I showed the 455 bp and 212 bp predicted products. All other digested ITS1 and ITS2 showed the predicted PCR-RFLP patterns. We demonstrated that the semi-nested PCR-RFLP of ITS1 with *Ase* I could differentiate *B. malayi*, *B. pahangi* and *D. immitis*.

DISCUSSION

The conventional method used for the detection and species differentiation of lymphatic filarial parasites is the identification of microfilariae by Giemsa stain. Although this method is not expensive,

it requires skill and experience to differentiate among closely-related species. Histochemical stain of acid phosphatase activity is another useful technique for species identification. It clearly discriminates different filarial nematode species (Yen and Mak, 1978. Chungpivat et al. 1990: Nithiuthai and Chungpivat 1992; Chungpivat and Sucharit, 1993). However, the enzymatic activity is rather sensitive to light and requires fresh specimens. As a result, PCR-RFLP of the ITS regions may be an alternative method to differentiate closely-related species, which are indistinguishable by morphology (Gasser et al. 1998) Furthermore, PCR-RFLP could be used to identify parasites in developmental stages (Gasser and Chilton 1995; Newton et al, 1998; Almeyda-Artigas et al, 2000) in different geographic distributions (Gasser and Chilton, 1995; Ramachandran et al, 1997).

The ITS1 and ITS2 regions of rDNA are useful for investigating some variations among closely-related species (Gasser et al, 1996; Ramachandran et al, 1997). The ITS regions are flanked by conserved rDNA genes, and the sequences can be used to design primers to amplify the intervening regions by PCR. Moreover, the presence of multiple copies provides a large number of target sequences for PCR in most organisms (Long and Dawid, 1980). As a result, the ITS regions of filarial nematodes are suitable targets for amplification and detection, even in mildly infected individuals. Although the first round PCR was inadequate to provide a detectable amount of PCR products, a subsequent semi-nested PCR has been utilized to increase the sensitivity for the detection of the ITS1 and ITS2 regions. Since the filarial DNA was extracted from whole blood specimens of infected individuals. host genomic DNA might possibly interfere with the PCR reaction. However, the bases at the 3' end of both internal primers designed for semi-nested PCR of ITS1 and ITS2 were not complementary to human rDNA (U13369). Therefore, no ITS1 or ITS2 products were amplified from the semi-nested PCR of the noninfected human blood samples (data not shown). There was no reported sequence of ITS1 and ITS2 from domestic cats and dogs. Nevertheless, no PCR products of ITS1 and ITS2 were amplified from the non-infected blood samples of domestic cats and dogs (data not shown).

The PCR-RFLP profiles of domestic cat *B. malayi* and human *B. malayi* ITS1 and ITS2 were similar for all restriction endonucleases used in our study. Our results supported the suggestion that domestic cats play an important role as animal reservoirs of *B. malayi*, as reported previously by studies of microfilarial morphology, periodicity, PCR-RFLP of *Hha* I repeat

A. ITS1



B. ITS2



Fig 2- PCR-RFLP of B. malayi and B. pahangi ITS1 (A) and ITS2 (B). M: 100 bp ladder molecular weight marker; hBm: human B. malayi; cBm: cat B. malayi; Bp: B. pahangi; Di: D. immitis; Ase I. Acc I. Hinf I and Rsa I are restriction enzymes used for the digestion of ITS1 and ITS2 PCR products.

genes and PCR of Trans-spliced Leader Exon I (SLX) (Chansiri *et al*, 2002).

In summary, we demonstrated that the PCR-RFLP of ITS1, digested with *Ase* I, could differentiate *B*. *malayi*, *B. pahangi* and *D. immitis*. Recently, it has been shown that domestic cats in Thailand can carry not only *B. pahangi* and *B. malayi*, but also *D. immitis* (Chansiri *et al*, 2002). Further study, for the field application of the PCR-RFLP method to monitor and evaluate infections in the reservoir hosts, would help the on-going lymphatic filariasis control programs.

ACKNOWLEDGEMENTS

This study was supported by the grants for graduate students in public universities, the Graduate School and Molecular Biology Funds, Research Affairs, Faculty of Medicine, Chulalongkorn University, Y Poovorawan was a Senior Research Scholar of the Thailand Research Fund. We would like to thank Dr. Saravudh Suvannadabba, Dr Suvith Thampaolo, Ms Kobkarn Kanjanopas, Ms Sumas Loymak, officers at the Filariasis Division, and regional officers, for their support during specimen collection. We are thankful to Ms Woraporn Sirivichayakul and all staff in the Parasitology Unit, Department of Pathology, Faculty of Veterinary Science and staff in the Department of Parasitology, Faculty of Medicine, Chulalongkorn University for their technical help. We also appreciate Dr Benjawan Sichnasaj and staff from the Rabies Control Section, Bangkok Metropolitan Area for their kind assistance.

REFERENCES

- Almeyda-Artigas RJ, Bargues MD, Mas-Coma S. ITS-2 rDNA sequencing of *Gnathostoma* species (Nematoda) and elucidation of the species causing human gnathostomiasis in the Americas. J Parasitol 2000:86:537-44.
- Chansiri K, Tejangkura T, Kwaosak P, Sarataphan N, Phantana S. Sukhumsirichart W. PCR based method for identification of zoonostic Brugia malayi microfilariae in domestic cats. Mol Cell Probes 2002;16:129-35.
- Chungpivat S, Nithiuthai N, Sichanasai B, Santiwatanatarm T. Histochemical diagnosis of Dirofilaria immitis microfilariae by acid phosphatase activity. J Thai Vet Pract 1990:2:195-203.
- Chungpivat S, Sucharit S. Microfilariae in cats in Bangkok. *Thai J Vet Med* 1993;23:75-87.
- Concle JC, Chilton NB, Jarvis T, Gasser RB. Mutation scanning analysis of microsatellite variability in the second internal transcribed spacer (precursor ribosomal RNA) for three species of *Metastrongylus* (Strongylida: Metastrongyloidea). *Parasitology* 2001;122:195-206.
- Filariasis Division, CDC Department, Ministry of Public Health, Bangkok, Thailand, Annual report, 1998 (in Thai).
- Filariasis Division, CDC Department, Ministry of Public Health, Bangkok, Thailand. Annual report.

1999 (in Thai).

- Gasser RB. Chilton NB, Hoste H, Stevenson LA. Species identification of trichostrongyle nematodes by PCR-linked RFLP. *Int J Parasitol* 1994:24:291-3.
- Gasser RB, Chilton NB. Characterisation of taeniid cestode species by PCR-RFLP of ITS2 ribosomal DNA. Acta Trop 1995;59:31-40.
- Gasser RB, LeGoff L, Petit G, Bain O. Rapid delineation of closely-related filarial parasites using genetic markers in spacer rDNA. *Acta Trop* 1996:62:143-50.
- Gasser RB, Monti JR, Bao-Zhen Q, Polderman AM, Nansen P, Chilton NB. A mutation scanning approach for the identification of hookworm species and analysis of population variation. *Mol Biochem Parasitol* 1998;92:303-12.
- Kanjanopas K, Choochote W, Jitpakdi A, et al. Brugia malayi in a naturally infected cat from Narathiwat Province, southern Thailand. Southeast Asian J Trop Med Public Health 2001;32:585-7.
- Long EO, Dawid IB. Repeated genes in eukaryotes. Annu Rev Biochem 1980; 49:727-64.
- Nithiuthai S, Chungpivat S. Lymphatic filariasis (*Brugia pahangi*) in dogs. *J Thai Vet Pract* 1992:4:122-33.
- Newton LA, Chilton NB, Beveridge I, Hoste H. Nansen P, Gasser RB. Genetic markers for strongylid nematodes of livestock defined by PCR-based restriction analysis of spacer rDNA. Acta Trop 1998:69:1-15.
- Nuchprayoon S. Yentakam S, Sangprakarn S, Junpee A. Endemic bancroftian filariasis in Thailand: detection by Og4C3 antigen capture ELISA and the polymerase chain reaction. J Med Assoc Thai 2001:84:1300-7.
- Palmieri JR, Ratiwayanto S, Masbar S, Tirtokusumo S, Rusch J, Marwoto HA. Evidence of possible natural infections of man with *Brugia pahangi* in South Kalimantan (Borneo), Indonesia. *Trop Geogr Med* 1985;37:239-44.
- Phantana S, Shutidamrong S, Chusattayanond W. Brugia malayi in a cat from southern Thailand. Trans R Soc Trop Med Hyg 1987;81:173-4.
- Ramachandran S. Gam AA. Neva FA. Molecular differences between several species of *Strongyloides* and comparison of selected isolates of *S. stercoralis* using a polymerase chain reaction-

PCR-RFLP OF ITS I AND ITS2

linked restriction fragment length polymorphism approach. *Am J Trop Med Hyg* 1997:56:61-5.

- Triteeraprapab S, Songtrus J. High prevalence of bancroftian filariasis in Myanmar-migrant workers: a study in Mae Sot district, Tak province, Thailand. J Med Assoc Thai 1999;82:735-9.
- Triteeraprapab S, Kanjanopas K, Suwannadabba S, Sangprakarn S, Poovorawan Y, Scott AL. Transmission of the nocturnal periodic strain of Wuchereria bancrofti by Culex quinquefasciatus: establishing the potential for urban filariasis in Thailand. Epidemiol Infect 2000;125:207-12.
- Triteeraprapab S, Karnjanopas K, Porksakorn C, Saingam A, Yentakan S, Loymak S. Lymphatic filariasis caused by *Brugia malayi* in an endemic area of Narathiwat Province, southern of Thailand. *J Med Assoc Thai* 2001;84 (suppl 1):S182-8.

Williams SA, Nicolas L, Lizotte-Waniewski M, et al.

A polymerase chain reaction assay for the detection of *Wuchereria bancrofti* in blood samples from French Polynesia. *Trans R Soc Trop Med Hyg* 1996;90:384-7.

- World Health Organization. Elimination filariasis: Attack poverty. The global alliance to eliminate lymphatic filariasis. Proceedings of the first meeting. Santiago de Compostela, Spain. 4-5 May 2000. A greenlight from the global alliance. Geneva: WHO, 2000.
- Yen PK, Mak JW. Histochemical differentiation of Brugia, Wuchereria, Dirofilaria and Breinlia microfilariae. Ann Trop Med Parasitol 1978; 72:157-62.
- Zhu X, Gasser RB, Podolska M, Chilton NB. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. *Int J Parasitol* 1998;28:1911-21.