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DETECTION AND DIFFERENTIATION OF FILARIAL PARASITES BY UNIVERSAL PRIMERS AND POLYMERASE CHAIN REACTION–RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

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Abstract. Filarial nematode parasites are a serious cause of morbidity in humans and animals. Identification of filarial infection using traditional morphologic criteria can be difficult and lead to misdiagnosis. We report on a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP)–based method to detect and differentiate a broad range of filarial species in a single PCR. The first internal transcribed spacer 1 (ITS1) along with the flanking 18S and 5.8S ribosomal DNA (rDNA) were isolated and cloned from *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia pahangi*. Sequence analysis identified conserved sites in the 18S and 5.8S rDNA sequence that could be used as universal priming sites to generate ITS1-distinctive PCR products that were useful for distinguishing filariae at the genus level. The addition of a digestion of the ITS1 PCR product with the restriction endonuclease *Ase* I generated a fragment profile that allowed differentiation down to the species level for *W. bancrofti*, *B. malayi*, *B. pahangi*, *Dirofilaria immitis*, and *D. repens*. The PCR-RFLP of ITS1 rDNA will be useful in diagnosing and differentiating filarial parasites in human, animal reservoir hosts, and mosquito vectors in disease-endemic areas.

INTRODUCTION

Lymphatic filariasis is a mosquito-borne disease.[1] The major symptoms of Bancroftian and Malayan filariasis are related to damaged lymphatics. It is estimated that 1.1 billion people (20% of the world population) in more than 83 countries are at risk of acquiring the infection, while more than 120 million individuals have already been infected.[2] Lymphatic filariasis is ranked by the World Health Organization as the second leading cause of permanent and long-term disability[3] and has been targeted for elimination by 2020.[4]

Approximately 90% of lymphatic filariasis worldwide is caused by *Wuchereria bancrofti*, with a majority of the remaining 10% caused by *Brugia malayi*.[3] There are two forms of *B. malayi* that infect humans. The nocturnally subperiodic form is found in swamp and forest areas of southeast Asia, including Thailand, and the nocturnally periodic form is found in India, Malaysia, and other parts of southeast Asia.[5–7] Domestic cats and monkeys are animal reservoir hosts for the subperiodic *B. malayi*.[8] The fact that domestic cats also carry *B. pahangi* makes the diagnosis difficult due to similarities in morphology.[9] Control of Brugian filariasis will be complicated because animal-to-human transmission continues even after the infection in humans has been greatly reduced.[8] Therefore, in addition to chemotherapy and vector control, a successful lymphatic filariasis control program should also consider the control of reservoir hosts.

Zoonotic filariasis is caused by infection with mosquito-transmitted *Dirofilaria immitis*, *D. repens*, or *Dipetalonema reconditum*. Humans are dead-end hosts for these filarial parasites of dogs and cats,[8] but the developing parasites can cause pathologic changes. Human pulmonary dirofilariasis caused by *D. immitis* has been reported in Brazil, Italy, France, Greece, Spain, Ukraine, Russia, the United States, Australia, Japan, and Thailand.[10–13] *Dirofilaria repens* causes subconjunctival and subcutaneous nodules in humans in Africa, Europe, India, Sri Lanka, and Thailand.[14–16] *D. reconditum* has been reported recently in a human eye.[17] Control of zoonotic filariae in the canine and feline reservoirs would be of great veterinary interest and could contribute to a decrease in human cases. However, it is difficult to distinguish *D. immitis* from *D. reconditum* in canine blood smears because of the similarity in their morphology.

Routinely, diagnosis is carried out through microscopic examination of the morphology of and/or cellular distribution in microfilariae isolated from blood or skin snips. However, when Giemsa is used to stain specimens, it is difficult to discriminate clearly between closely related species such as *B. malayi* and *B. pahangi* or *D. immitis*, *D. repens*, and *D. reconditum*. Histochemical staining to detect acid phosphatase activity could overcome this problem,[18] but this technique requires fresh samples to yield optimal results.[19] Besides being time-consuming and labor-intensive, both staining methods require expertise to identify and confirm the species.[20] DNA technology has provided an alternative approach for identification of the filarial parasites.[19–24] Our laboratory has demonstrated that semi-nested, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of the internal transcribed spacer 1 (ITS1) could differentiate *B. malayi*, *B. pahangi*, and *D. immitis*.[19] However, due to the primers chosen, the assay could not be used to test for a wider spectrum of filarial species. In this study, we report on an assay that uses universal primers and a single PCR and RFLP of ITS1 to diagnose a wide range of filarial species.

MATERIALS AND METHODS

Parasites. Ten milliliters of venous blood from *W. bancrofti*–infected Myanmar migrants were collected under sterile technique and universal precautions between 8:00 PM and midnight as previously described.[19,20,25] *B. malayi*–infected blood samples were collected from humans and domestic cats from Narathiwas Province in southern Thailand. *Brugia pahangi* and *D. repens* were obtained from experimentally infected cats at the Parasitology Unit, Department of Pathology, Faculty of Veterinary Sciences, Chulalongkorn Univer-

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sity (Bangkok, Thailand). *Dirofilaria immitis*–infected blood samples were isolated from infected random source dogs in Bangkok. Blood from healthy volunteers and uninfected domestic cats and dogs were used as negative controls. This study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (Bangkok, Thailand). The objective of the study was disclosed to patients and written consent were obtained before blood was obtained. All filarial parasite species were identified and confirmed by staining with Giemsa[26] and special staining for acid phosphatase activity.[19,20]

Semi-nested PCR amplification. DNA extraction and semi-nested PCR amplification were performed as previously described.[19] Briefly, FL1-F (5′-TTCCGTAGGTGAACCTGC-3′) and FL2-R (5′-ATATGCTTAAATTCAGCGGG-3′) primers, in 18S and 28S ribosomal DNA (rDNA), respectively, were used in the first-round PCR to amplify the ITS1/5.8S/ITS2 fragment from total nematode DNA (Figure 1). The primers FL1-F and Di5.8S 660-R (5′-ACCCTCAACCA-GACGTAC-3′) were used in the second-round PCR to amplify the ITS1 fragment that contained 38 basepairs from the 18S rDNA and 153 basepairs from the 5.8S rDNA on the 5′ and 3′ ends, respectively (Figure 1). All oligonucleotide primers were obtained from the Bioservice Unit, National Science and Technology Development Agency (Bangkok, Thailand).

Cloning the PCR products. The PCR products from the second round of PCR amplification from the *W. bancrofti*, *B. malayi*, and *B. pahangi* DNA templates (Figure 1) were ligated into the pGEM-T vector (Promega, Madison, WI) using T4 ligase (Promega) according to the protocol described by the manufacturer. The competent cells (*Escherichia coli* JM 109) were used for transformation using the manufacturer's protocol (Promega). The colonies were selected, cultivated, and screened for recombinant plasmids.

DNA sequencing and sequence analysis. Recombinant plasmids containing second-round PCR products from *W. bancrofti*, *B. malayi*, and *B. pahangi* were purified and sequenced in both directions using M13 forward and reverse primers by an automated DNA sequencer (Applied Biosystems, Foster City, CA). Alignments of the ITS1 and the flanking region sequences were made using the CLUSTAL X multiple alignment program.[27]

Single PCR of ITS1 with ITS1-F and ITS1-R primers. The PCRs were performed in a 50-μL reaction containing PCR buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl) (Amersham Phamacia, Freiburg, Germany), 200 μM each of dATP, dCTP, dGTP, and dTTP (Promega), 0.625 units of *Taq* DNA polymerase (Amersham Phamacia), 5 pmol of each primer (ITS1-F; 5′-GGTGAACCTGCGGAAGGATC-3′ and ITS1-R; 5′-GCGAATTGCAGACGCATTGAG-3′), and 10–20 ng of parasite DNA template. After incubation at 94°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters: denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds. The final amplification cycle included an additional extension at 72°C for 10 minutes.

Restriction fragment length polymorphism. The PCR products were digested with five units of *Ase* I according to the manufacturer's protocols (New England Biolabs, Beverly, MA). Analysis of DNA fragments was performed by submarine agarose gel electrophoresis, staining with ethidium bromide, and visualization under ultraviolet light.

RESULTS

Nucleotide sequence alignment and primer design. The nucleotide sequences of *W. bancrofti* (GenBank accession no. AY621473), *B. malayi* (GenBank accession no. AY621464), and *B. pahangi* (GenBank accession no. AY621469) 18S-ITS1-5.8S rDNAs were obtained by cloning and DNA sequencing. The sequences of the 18S-ITS1-5.8S rDNAs from *D. immitis* (GenBank accession no. AF217800), *Onchocerca volvulus* (GenBank accession no. AF228565), *Mansonella ozzardi* (GenBank accession no. AF228560), and *D. reconditum* (GenBank accession no. AF217801) were obtained from GenBank. The seven filarial 18S-ITS1-5.8S rDNAs sequences were aligned using the CLUSTAL X multiple alignment program (European Molecular Biology Laboratory, Heidelberg, Germany) (Figure 2).

The sizes of ITS1 of *W. bancrofti*, *B. malayi*, and *B. pahangi* were 363, 385, and 391 basepairs, respectively. Sequence comparison showed a substantial degree of variation in the ITS1 sequence near the 18S rDNA. There was a higher level of identity in the ITS1 sequence near the 5.8S rDNA. The 18S and 5.8S rDNA sequences from all of the filariae contained invariant regions at the 3′ end of the 18S rDNA and at the 5′ end of the 5.8S rDNA (Figures 1 and 2) that could be exploited to produce the universal primer pairs ITS1-F and ITS1-R. The single-step PCR using ITS1-F/ITS1-R primer set was very sensitive because we could adjust the PCR to detect as little as 1 pg of parasite DNA, which allows even the presence of a single microfilaria to be detected. The ITS1-F/ITS1-R primer set showed specificity in

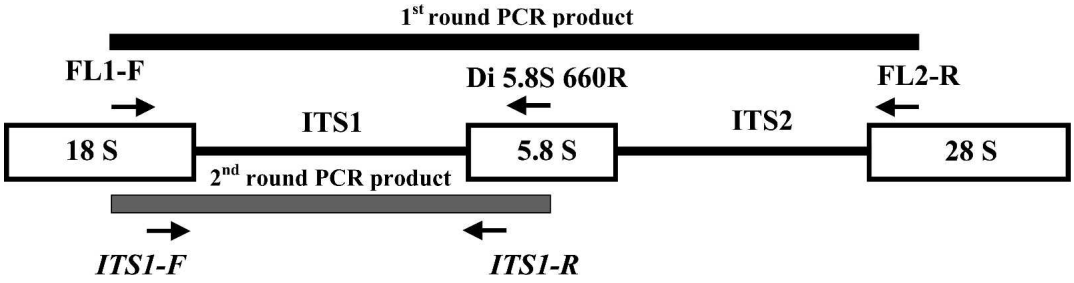

FIGURE 1. Forward and reverse primers for semi-nested and a single filarial internal transcribed spacer 1 (ITS1) polymerase chain reaction (PCR). FL1-F and FL2-R were the primers used in the first-round PCR, and FL1-F and Di5.8S 660-R were the primers used in the second-round PCR. ITS1-F and ITS1-R were the primers designed for a single PCR. The black bar indicates the first-round PCR product regions. The gray bar indicates the second-round PCR product regions, which were cloned and sequence (see Figure 2).

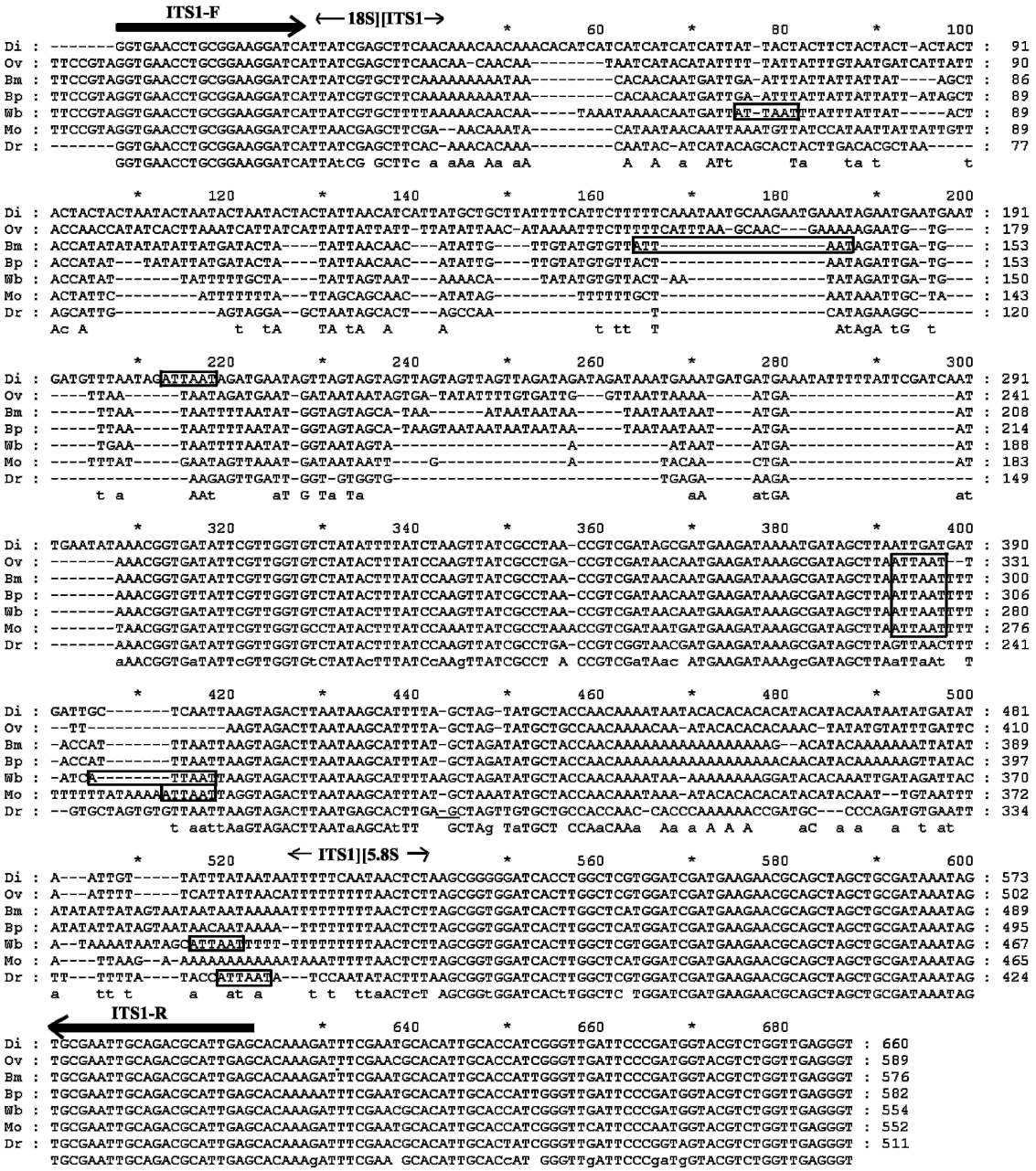

FIGURE 2. Alignment of ribosomal DNA (rDNA) from *Dirofilaria immitis* (Di), *Onchocerca volvulus* (Ov), *Brugia malayi* (Bm), *B. pahangi* (Bp), *Wuchereria bancrofti* (Wb), *Mansonella ozzardi* (Mo), and *Dipetalonema reconditum* (Dr). The internal transcribed sequence 1 (ITS1) forward primer (ITS1-F; **right arrow**) and reverse primer (ITS1-R; **left arrow**) are located in the conserved regions of 18S rDNA and 5.8S rDNA, respectively. Boxes indicate the predicted *Ase* I restriction sites (5′..AT^TAAT..3′).

that no amplicons were produced in control reactions that contained human DNA only.

Analysis of filarial ITS1 digested with *Ase* I by PCR-RFLP. The ITS1-F/ITS1-R primer set yielded PCR products of 482, 504, 510, 595, and 602 basepairs from *W. bancrofti*, *B. malayi*, *B. pahangi*, *D. immitis*, and *D. repens*, respectively (Figure 3A). Analysis of the undigested ITS1 PCR products by electrophoresis on agarose gels demonstrated that although it was relatively easy to distinguish parasites at the genus level, it was difficult to differentiate between species within the same genus.

The addition of a digestion of the ITS1 PCR products with the restriction enzyme *Ase* I, followed by gel electrophoresis, allowed for the unequivocal differentiation of the five species tested (Figure 3B and Table 1). The electrophoretic profiles observed were consistent with the size of *Ase* I restriction fragment predicted (Table 1), with the exception of *W. bancrofti* and *B. malayi*. For *W. bancrofti*, five restriction fragments were predicted, but in an ethidium bromide–stained agarose gel, only three bands could be seen, presumably because of co-migration of the 100-basepair and 104-basepair bands and the 12-basepair and 64-basepair bands. For *B. malayi*, three restriction fragments were predicted, but only two bands could be seen, presumably because of co-migration of

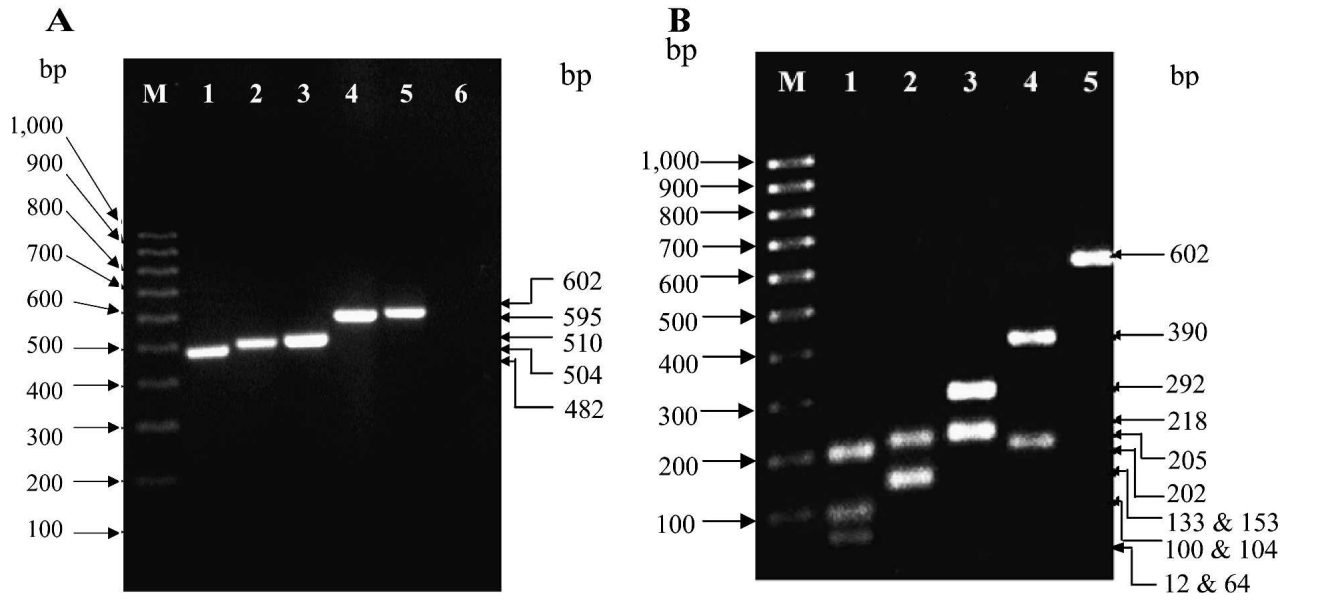

FIGURE 3. Restriction fragment length polymorphism (RFLP) analysis of internal transcribed sequence 1 (ITS1). **A**, Separation of the ITS 1 polymerase chain reaction (PCR) products using ITS1-F and ITS1-R primers after a single-round PCR. Lane M = 100-basepair (bp) ladder; lane 1 = *Wuchereria bancrofti*; lane 2 = *Brugia malayi*; lane 3 = *B. pahangi*; lane 4 = *Dirofilaria immitis*; lane 5 = *D. repens*; lane 6 = negative control. **B**, PCR-RFLP analysis of filarial ITS1 digested with *Ase* I. Lane M = 100-bp ladder; lane 1 = *Wuchereria bancrofti*; lane 2, *Brugia malayi*; lane 3 = *B. pahangi*; lane 4 = *Dirofilaria immitis*; lane 5 = *D. repens*.

the 133-basepair and 153-basepair bands. Digestion of the ITS1 PCR products with *Ase* I yielded two bands for *B. pahangi* and two bands for *D. immitis*. The ITS1 from *D. repens* does not contain an *Ase* I site.

DISCUSSION

The most widely used method for diagnosis of filarial infections is microscopic examination of microfilariae from blood or skin samples. This approach has limited sensitivity and is not suitable for large-scale microfilaria screening in disease-endemic areas. Moreover, it requires considerable expertise to distinguish among filarial parasite species because of their rather similar morphology. The molecular techniques such as DNA hybridization assays,[28–32] and PCR-based techniques,[19–24,33–36] have been used in filarial parasite detection and differentiation. However, there is no report of a single technique that can detect and differentiate all filarial parasites.

TABLE 1
RFLP analysis of filarial ITS1 PCR products digested with *Ase* I*

Filarial species	Undigested ITS1 PCR product (bp)	Restriction bands (bp) predicted after *Ase* I digestion
Species tested		
Wuchereria bancrofti	482	12, 64, 100, 104, 202
Brugia malayi	504	133, 153, 218
Brugia pahangi	510	218, 292
Dirofilaria immitis	595	205, 390
Dirofilaria repens	602	602
Predicted from published sequences		
Onchocerca volvulus	513	198, 315
Mansonella ozzardi	480	20, 198, 262
Dipetalonema reconditum	446	99, 337

* RFLP = restriction fragment length polymorphism; ITS1 = internal transcribed spacer 1; PCR = polymerase chain reaction; bp = basepairs.

In this study, we report on an assay system that uses a single-step PCR followed by RFLP analysis that discriminates between filariae at the species level. Although the restriction fragment patterns between *B. pahangi* (218 and 292 basepairs) and *O. volvulus* (198 and 315 basepairs) are predicted to be rather similar, the two parasites have non-overlapping geographic distributions, so they are unlikely to be confused. However, electrophoresis using polyacrylamide or Metaphor agarose gels (FMC Bioproducts, Rockland, ME) could easily differentiate both species.

Based on analysis of sequence data, the predicted pattern of *Ase* I digestion of the ITS1 sequences from *O. volvulus*, *M. ozzardi*, and *D. reconditum* should yield two, three, and two fragments, respectively (Table 1), which would be diagnostic for these parasites. Because the primers were designed from highly conserved regions of filarial 18S and 5.8S rDNAs,[37] we anticipate that the primers will amplify the ITS1 sequence from other filarial parasites such as *B. timori*, *Loa loa*, *M. streptocerca*, and *M. perstans*. Further studies are required to address this issue and the issue of possible intra-species geographic variation in the *Ase* I digestion pattern.

The PCR-RFLP of ITS1 may have utility in the differential detection of filariae in situations where species are co-endemic. Examples include Cameroon, where *L. loa*, *M. perstans*, and *O. volvulus* are co-endemic in humans; Italy, where *D. immitis* and *D. repens* represent diagnostic challenges in clinical and veterinary infections; and Malaysia and Thailand, where *B. malayi*, *B. pahangi*, *D. repens*, and *D. immitis* present diagnostic problems.[9,11,38] The PCR-RFLP of ITS1 rDNA can be used to replace special stains that require experienced microscopists to differentiate filarial species. In ad-

dition, this PCR-RFLP technique can be an alternative for the standard skin snip technique to detect the two skin–dwelling filarial species *M. streptocerca* and *O. volvulus*. The rapid and reliable epidemiologic assessment and clear identification of filarial nematode species in both human and animal reservoir hosts are necessary for an accurate assessment of prevalence and incidence in intervention programs. This PCR-based approach to species identification is robust, simple to perform, and easy to interpret, which makes it suitable for use in reference laboratories. Treatment of infected animals is important to decrease the risk to humans in the vicinity of the infected animals when suitable mosquito vectors are present.[39] The PCR-RFLP of ITS1 has potential utility in monitoring lymphatic filariasis control programs, as well as in monitoring and evaluation of animal hosts.

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