Experimental Parasitology 127 (2011) 282–286

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

Detection and discrimination of *Loa loa, Mansonella perstans* and *Wuchereria bancrofti* by PCR–RFLP and nested-PCR of ribosomal DNA ITS1 region

Maribel Jiménez [a,*], Luis Miguel González [a], Cristina Carranza [b], Begoña Bailo [a], Ana Pérez-Ayala [c], Antonio Muro [d], José Luis Pérez-Arellano [b], Teresa Gárate [a,**]

[a] Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain
[b] Departamento Ciencias Médicas y Quirúrgicas, Facultad de Ciencias de la Salud, Universidad de Las Palmas de Gran Canaria, 35080 Las Palmas de Gran Canaria, Spain
[c] Unidad de Medicina Tropical, Servicio de Enfermedades Infecciosas, Hospital Ramón y Cajal, Madrid, Spain
[d] Laboratorio de Inmunología Parasitaria y Molecular, Centro de Investigación de Enfermedades Tropicales de la Universidad de Salamanca, Campus Miguel de Unamuno s/n, 37007 Salamanca, Spain

ARTICLE INFO

Article history:
Received 22 March 2010
Received in revised form 4 June 2010
Accepted 15 June 2010
Available online 19 June 2010

Keywords:
Loa loa
Mansonella perstans
Wuchereria bancrofti
ITS1
PCR–RFLP
Nested-PCR
Differential detection

ABSTRACT

The ribosomal deoxyribonucleic acid (DNA) internal transcribed spacer region (ITS1) of two filarial nematodes, *Loa loa* and *Mansonella perstans*, was amplified and further sequenced to develop an species-specific polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) protocol for the differentiation of both species from *Wuchereria bancrofti*, three filarial nematodes with blood circulating microfilariae. The ITS1–PCR product digested with the restriction endonuclease Ase I generated an specific diagnostic pattern for each of the three species. Moreover, three new specific nested-PCRs, targeting the ITS1 region, for differential detection of *L. loa*, *M. perstans* and *W. bancrofti* were developed and used when the ITS1–PCR products were insufficient for the Ase I enzymatic digestion. These filarial species-specific molecular protocols were evaluated in forty blood samples from African adult immigrants attending in the Hospital Insular of Gran Canaria, Canarias, Spain.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Human filariases are restricted to the tropics and subtropics endemic areas where more than 120 million people are estimated to be infected (WHO, 2010). In the last years, there have been an increase of African immigrants to Spain from filariasis endemic areas where *Loa loa*, *Mansonella perstans* and *Wuchereria bancrofti* are co-endemic (Carrillo et al., 2004; Pardo et al., 2006). These demographic changes require for a better filariae species-specific molecular diagnosis tests to solve problems derived from the microscopical identification of these microfilariae in patients blood that needs a great expertise and is time consuming (Walther and Muller, 2003; Nuchprayoon, 2009). In addition, both specificity and sensitivity of traditional serological methods to detect anti-filarial antibodies are really poor. Furthermore, serological diagnosis of filariasis in immigrants from endemic areas is not appropriate as these individuals usually have anti-filarial antibodies without an active infection (Klion, 2008).

In a previous study, Nuchprayoon et al. (2005) reported on an assay system that uses a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP), targeted the internally-transcribed spacer 1 (ITS1) region of the ribosomal RNA gene, based on the Ase I restriction enzyme digestion that discriminates between five species of filarial nematodes: *W. bancrofti*, *Brugia malayi*, *Brugia pahangi*, *Dirofilaria inmitis* and *Dirofilaria repens*, suggesting the utility of this PCR in the differential detection of other filariae as *L. loa* and *M. perstans* that sympatrically co-exist in West and Central Africa.

Thus, the objective of the present work was to develop and to evaluate this PCR–RFLP targeted the ITS1 from *L. loa*, *M. perstans* and *W. bancrofti*. The subsequent digestion of amplification products with Ase I restriction enzyme yielded species-specific fragments that allowed the differential identification of the three filarial species. Additionally, an specific nested-PCR for each filarial species was developed with primers based on their ITS1 regions to increase the diagnosis sensitivity when poor or negative ITS1–PCR products were observed. These molecular protocols were tested in 40 blood samples from African adult immigrants attending in the Hospital Insular of Gran Canaria, Canarias, Spain, for physical examination and routinely laboratory tests.

* Corresponding author. Fax: +34 91 5097034.
** Corresponding author. Fax: +34 91 5097034.
E-mail addresses: mjimenez@isciii.es (M. Jiménez), tgarate@isciii.es (T. Gárate).

0014-4894/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2010.06.019

2. Materials and methods

2.1. Blood samples and genomic DNA extraction

Peripheral blood samples were collected from 40 adult immigrants of different African geographic origin attending in the Hospital Insular of Gran Canaria, Canarias, Spain. The Hospital Insular of Gran Canaria Ethics Review Committee approved the protocols for obtaining blood samples from patients enrolled in the present study. After their written consent for parasitological diagnosis, Knott's test for microfilariae identification was carried out (Knott, 1939) (Table 1).

Whole blood with EDTA ($n = 28$) and blood in Whatman paper ($n = 12$) was used for DNA extraction. DNA was purified using the QIAamp® DNA Blood Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantification and purity of the DNA samples was determined by spectrophotometry with a NanoDrop ND-1000 spectrophotometer (Nucliber, Madrid, Spain), and the samples stored at −20 °C until use.

2.2. PCR amplification of ITS1 from L. loa and M. perstans

Genomic DNA obtained from samples No. 4 and No. 10 (Table 1) was used to amplify the ITS1 region of the ribosomal RNA gene from *L. loa* and *M. perstans*, respectively, as previously described by Nuchprayoon et al. (2005) with slight modifications. Universal primers ITS1-F (5′-GGTGAACCTGCGGAAGGATC-3′) and ITS1-R (5′-CTCAATGCGTCTGCAATTCGC-3′) situated in conserved regions of 18S and 5.8S subunits of ribosomal RNA gene were used (Fig. 1). Individual PCRs were carried out in a final volume of 50 μl. The amount of DNA in each reaction was 200 ng. The master mix for both PCRs consisted of 5 μl of 10× PCR buffer for Ampli-Taq Gold polymerase (Applied Biosystem, UK), 1 μl Ampli-Taq-Gold polymerase (5 U/μl) (Applied Biosystem), 0.2 mM of each deoxynucleoside triphosphate (dNTPs) (Amersham Pharmacia Biotech, Sweden), 1.68 mM of $MgCl_2$ (Applied Biosystem), 4 μl of BSA DNAse Free (0.8 μg/μl) (Amersham Pharmacia Biotech) and 200 ng/μl of each primer ITS1-F and ITS1-R. Working conditions were 1 cycle 95 °C 9 min then 35 cycles (94 °C 30 s, 58 °C 30 s, 72 °C 45 s) followed by 1 cycle 72 °C for 10 min. The PCR products were separated on ethidium bromide 2% agarose gel (Conda, Spain), visualized under UV light and photographed.

2.3. Cloning and sequence analysis of the ITS1–PCR products

The ITS1–PCR products obtained from *L. loa* and *M. perstans* were removed from the gel under UV exposure and purified by a QIAquick Gel Extraction Kit (Qiagen). Afterwards, the samples were subcloned into the plasmid PCR®4-TOPO® (Invitrogen, UK) and three of the clones sequenced two times each with ABI 3700 DNA sequencer (Applied Biosystems). Nucleotide sequences obtained for *M. perstans* and *L. loa* were aligned with the *W. bancrofti*

Table 1
Results obtained by microscopic diagnosis and the different PCR methods used in the study.

No.	Sample type	Geographical origin	Knott technique	ITS1–PCR	ITS1–RFLP	Nested-ITS1
1	Wp	Conakry Guinea	*M. perstans*	Positive	n.d.	*M. perstans*
2	Wp	Mali	*M. perstans*	Negative	n.d.	*M. perstans*
3	Wp	Cameroon	*M. perstans*	Negative	n.d.	*M. perstans*
4	Wp	Cameroon	*L. loa*	Positive	*L. loa*	n.d.
5	Wp	Cameroon	*M. perstans*	Positive	*M. perstans*	n.d.
6	Wp	Ghana	*M. perstans*	Positive	*M. perstans*	n.d.
7	Wp	Equatorial Guinea	*L. loa*	Positive	n.d.	*L. loa*
8	Blood	Equatorial Guinea	*L. loa/ M. perstans*	Positive	*L. loa/ M. perstans*	*L. loa / M. perstans*
9	Blood	Guinea Bissau	*W. bancrofti/ M. perstans*	Positive	*W. bancrofti/ M. perstans*	*W. bancrofti/M. perstans*
10	Blood	Guinea Bissau	*M. perstans*	Positive	*M. perstans*	n.d.
11	Blood	Aiun	Negative	Negative	n.d.	Negative
12	Blood	Morocco	Negative	Negative	n.d.	Negative
13	Blood	Mauritania	Negative	Negative	n.d.	Negative
14	Blood	Mauritania	Negative	Negative	n.d.	Negative
15	Blood	Mauritania	Negative	Negative	n.d.	Negative
16	Wp	Mali	*M. perstans*	Negative	n.d.	*M. perstans*
17	Wp	Sierra Leone	*M. perstans*	Positive	*M. perstans*	n.d.
18	Wp	Sierra Leone	*W. bancrofti*	Negative	n.d.	*W. bancrofti*
19	Wp	Equatorial Guinea	Negative	Negative	n.d.	Negative
20	Wp	Equatorial Guinea	*M. perstans*	Negative	n.d.	*M. perstans*
21	Blood	Guinea Bissau	*M. perstans*	Positive	*M. perstans*	n.d.
22	Blood	Guinea Bissau	*M. perstans*	Positive	*M. perstans*	n.d.
23	Blood	Equatorial Guinea	*M. perstans*	Positive	*M. perstans*	n.d.
24	Blood	Equatorial Guinea	*M. perstans*	Positive	*M. perstans*	n.d.
25	Blood	Equatorial Guinea	*M. perstans*	Positive	*M. perstans*	n.d.
26	Blood	Nigeria	*M. perstans*	Positive	*M. perstans*	n.d.
27	Blood	Morocco	Negative	Negative	n.d.	Negative
28	Blood	Morocco	Negative	Negative	n.d.	Negative
29	Blood	Morocco	Negative	Negative	n.d.	Negative
30	Blood	Equatorial Guinea	Negative	Negative	n.d.	Negative
31	Blood	Equatorial Guinea	Negative	Negative	n.d.	Negative
32	Blood	Nigeria	*M. perstans*	Positive	*M. perstans*	n.d.
33	Blood	Equatorial Guinea	Negative	Negative	n.d.	Negative
34	Blood	Mali	Negative	Negative	n.d.	Negative
35	Blood	Equatorial Guinea	Negative	Negative	n.d.	Negative
36	Blood	Equatorial Guinea	Negative	Negative	n.d.	Negative
37	Blood	Morocco	Negative	Negative	n.d.	Negative
38	Blood	Morocco	Negative	Negative	n.d.	Negative
39	Blood	Morocco	Negative	Negative	n.d.	Negative
40	Blood	Morocco	Negative	Negative	n.d.	Negative

Wp, Whatman paper; n.d., not done.

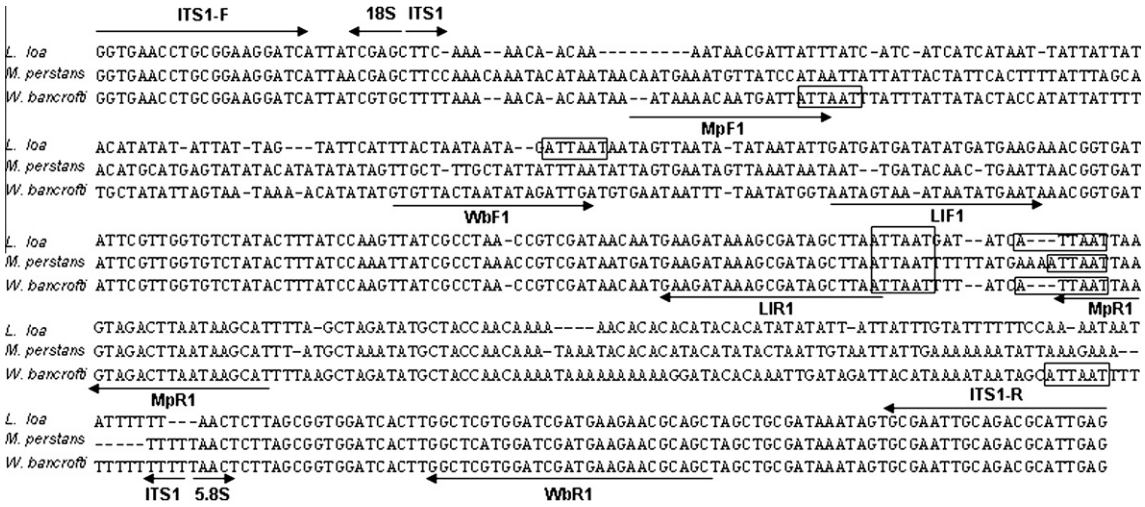

Fig. 1. Alignment of the ITS1 from *L. loa* (457 bp; GenBank Accession No. DQ995497), *M. perstans* (484 bp; GenBank Accession No. DQ995498) and *W. bancrofti* (482 bp; GenBank Accession No. AY621473). Primers ITS1-F and ITS1-R are located in conserved regions of 18S and 5.8S ribosomal DNA subunits, respectively (Nuchprayoon et al., 2005). For the nested-PCRs specific forward primers for *L. loa* (LIF1), *M. perstans* (MpF1) and *W. bancrofti* (WbF1) were designed into the ITS1 region. Reverse primer for *L. loa* (LIR1) designed into the ITS1 region and reverse primer for *W. bancrofti* (WbR1) designed into the 5.8S region were common in all the three species. Specific reverse primer for *M. perstans* (MpR1) was designed into the ITS1 region. Underlined bases correspond to the primers. Predicted Ase I restriction sites are indicated in boxes.

482 bp sequence (GenBank™ Accession No. AY621473). Multiple sequence alignments were made using ClustalW method. The predicted Ase I restriction sites were determined with the DNASTAR Lasergene v7.1 program.

2.4. Polymerase chain reaction–restriction fragment length polymorphism

The ITS1–PCR protocol was applied to genomic DNA obtained from the 40 samples, as described above. After amplification, 25 μl of each PCR sample were electrophoresed on ethidium bromide 2% agarose D5 (Conda) gel in 1× TAE buffer, visualized under UV light and photographed.

Each sample was tested twice with the primers sets (see above). Negative controls without DNA were included in each PCR. Subsequently, 25 μl of each PCR product were digested with 5 U of Ase I at 37 °C in 50 μl of reaction volume according to the manufacturer's protocols (New England Biolabs, UK). The digested PCR products were then fractionated in 3% agarose gel (Conda), visualized under UV light, after ethidium bromide staining, and photographed.

2.5. Primer design and nested-PCRs

For the new nested-PCRs, specific forward primers for *L. loa* (LIF1: 5′-GATGATGATATATGATGAAG-3′), *M. perstans* (MpF1: 5′-CAATG AAATGTTATCCATA-3′) and *W. bancrofti* (WbF1: 5′-GTGTTACTAATA TAGATTG-3′) were designed to encompass the ITS1 region. Reverse primer for *L. loa* (LIR1: 5′-TTAAGCTATCGCTTTATCTTC-3′) was also designed into the ITS1 region. Reverse primer for *W. bancrofti* (WbR1: 5′-GCTGCGTTCTTCATCGATCCACGAGCC-3′) was designed into the 5.8S region. Both reverse primers were common in all the three species. Specific reverse primer for *M. perstans* (MpR1: 5′-AA ATGCTTATTAAGTCTACTTAATTAAT-3′) was designed into the ITS1 region (Fig. 1). The nested-PCRs were carried out with 1 μl of DNA template from the first PCR reaction (ITS1–PCR protocol), diluted 4:1000 in DNase-free water (Promega). The working conditions were 1 cycle 95 °C 9 min then 35 cycles (94 °C 30 s, 58 °C 30 s, 72 °C 45 s) followed by 1 cycle 72 °C for 10 min. The nested-PCRs samples were electrophoresed through a 2% agarose D1 (Conda) gel in 1× TAE buffer and visualized by trans-illumination with ultra-violet light. Each sample was tested twice with each set of primers. Negative controls without DNA were included in each PCR. To avoid PCR contamination, sample preparation, reactions set-up and PCR amplifications were carried out in separate rooms, with different lab coats and gloves.

3. Results and discussion

The present study reports a PCR–RFLP and three nested-PCRs for differential species-specific diagnosis of *L. loa*, *M. perstans* and *W. bancrofti*, based on the ITS1 region. The ITS1 regions of *L. loa and M. perstans* were amplified and their sequences compared to the *W. bancrofti* ITS1. Thus, PCR products were visualized and found to be 457 bp for *L. loa*, 484 bp for *M. perstans* and 482 bp for *W. bancrofti* (Fig. 2). The 457 bp-ITS1 from *L. loa* and the 484 bp-ITS1 from *M. perstans* were deposited in the GenBank™ (Accession Nos. DQ995497 and Q995498, respectively). These sequences together with the *W. bancrofti* 482 bp sequence, GenBank™ Accession No. AY621473, were aligned by ClustalW Multiple alignment (Fig. 1). Analysis of each individual sequence data provided the predicted Ase I restriction endonuclease pattern, with an specific diagnosis pattern for each of the three filarial species (Table 2). In addition, the study of the multiple alignments allowed us to design three different set of primers and used them in three specific and independent nested-PCR protocols to distinguish the filarial species (Fig. 1), more efficiently than the PCR–RFLP system described above.

To evaluate the ITS1–RFLP as a differential diagnosis method in the specific identification of the three filarial species, the ITS1–RFLP was carried out in genomic DNA obtained from 40 blood samples collected from adult immigrants of different African geographic origin (Table 1). The PCR products were visualized and found to be 457 bp for *L. loa*, 484 bp for *M. perstans* and 482 bp for *W. bancrofti* (Fig. 2A, Table 2). Digestion of the ITS1–PCR products yielded an exclusive specific RFLP pattern for each species. The digested *L. loa* PCR product generated fragments of 12 bp, 122 bp, 129 bp and 194 bp (Fig. 2B, lanes 7 and 8, Tables 1 and 2), *M. perstans* yielded fragments of 17, 195 and 272 bp (Fig. 2B, lanes 1–6, 8 and 9, Tables 1 and 2) and *W. bancrofti* produced fragments of 10, 64, 100, 104 and 202 bp (Fig. 2B, lane 1, Tables 1 and 2). Mixed infection detected by microscopic analysis in samples No. 8 (*L. loa*/*M. perstans*) and No. 9 (*W. bancrofti*/*M. perstans*) were also

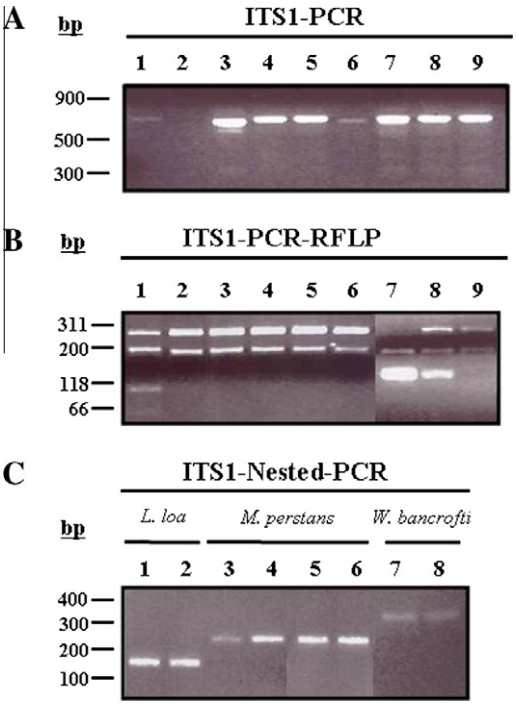

Fig. 2. Differential diagnosis of filarial species. (A) ITS1–PCR products of several blood samples included in the study. *L. loa*, 457 bp; *M. perstans*, 484 bp and *W. bancrofti*, 482 bp. Lanes 1, 4, 5, 7, 8, and 9 (samples Nos. 1, 5, 6, 8, 9 and 10 in Table 1); Lanes 3 and 6 (samples Nos. 4 and 7 in Table 1). (B) ITS–RFLP profiles of several samples used in the study. *L. loa* (12, 122, 129, 194 bp), *M. perstans* (17, 195, 272 bp), *W. bancrofti* (12, 64, 100 y 202 bp). 12 and 17 bp fragments are not visible on the gel. Lanes 2–6 and 9, *M. perstans*; Lane 1, mixed infection *M. perstans*/*W. bancrofti*; Lane 7, *L. loa*; Lane 8, mixed infection *M. perstans*/*L. loa*. (C) Nested-PCRs of several samples used in the study. *L. loa*, 143 bp, *M. perstans*, 225 bp and *W. bancrofti*, 324 bp. Lanes 1 and 2: *L. loa*; lanes 3, 4, 5 and 6, *M. perstans*; lanes 7 and 8, *W. bancrofti*.

successfully detected by ITS1–RFLP showing a characteristic pattern capable to distinguish two filarial species in one PCR reaction (Fig. 2B, lanes 8 and 1, respectively, Tables 1 and 2). However, negative results were notably obtained by ITS1–PCR in five samples that previously were positives under optical microscopy (samples 2, 3, 16, 18, 20). Moreover, scarce ITS1–PCR products were obtained in samples 1 and 7 (Fig. 2A, lanes 1 and 6, respectively).

Consequently with these results, three specific nested-PCRs were designed to encompass the ITS1 region. Each nested-PCR yielded specific PCR products for *L. loa* (143 bp), *M. perstans* (225 bp) and *W. bancrofti* (324 bp) (Table 2). The specificity of the new technique was evaluated using the nested-PCRs with the uncertain samples (1 and 7). The PCR products had 225 bp for sample No. 1, identified *M. perstans*, and 143 bp for No. 7 related to *L. loa* (Fig. 2C, lanes 4 and 1, Tables 1 and 2). The unidentified samples by ITS1–RFLP, or microscopy, were analysis by the new nested-PCR protocols. Thus, a band of 225 bp, corresponding to *M. perstans*, was observed in samples 2, 3, 16 and 20. Sample 18 showed a 324 bp band corresponding to *W. bancrofti*.

Table 2
PCR profiles of filarial species obtained with the different protocols.

Filarial species	ITS1 product (bp)	ITS1–RFLP with Ase I (bp)	Nested-ITS1 (bp)
Loa loa	457	12, 122, 129, 194	143
Mansonella perstans	484	17, 195, 272	225
Wuchereria bancrofti	482	12, 64, 100, 104, 202	324

Mixed infections diagnosed by microscopic analysis in samples No. 8 (*L. loa*/*M. perstans*) and No. 9 (*W. bancrofti*/*M. perstans*), were also detected by ITS1–RFLP (Fig. 2B, lanes 8 and 1, respectively, Tables 1 and 2). Furthermore, they were specifically identified by the new nested-PCRs (Fig. 2C, lanes 3, 4, 2, 5, 6, 7 and 8, Tables 1 and 2). Therefore, both nested-PCRs and ITS1–RFLP, were able to detect mixed infections as well as microscopic examination. In addition, the nested-PCRs showed more sensitivity in comparison to the ITS1–RFLP when the PCR products were imperceptible, or were very faint, to be digested with Ase I.

The samples 4, 5, 6, 10, 17, 21, 22, 23, 24, 25, 26 and 32 (Table 1) were not determined by the nested-PCR, as the ITS1–PCR followed by RFLP already yielded species-specific patterns for *M. perstans* (samples 5, 6, 10, 17, 21, 22, 23, 24, 25, 26, 32) and for *L. loa* (sample 4).

The PCR–RFLP and the three nested-PCRs can be carried out with DNA from blood samples with EDTA or in Whatman paper. The molecular protocols, with DNA extracted from whole blood with EDTA or in Whatman paper, provided concordant results with the microscopic diagnosis after Knott's concentration regardless to the form of DNA extraction from whole blood with EDTA or in Whatman paper (Table 1). Regarding blood preservation in Whatman paper, this could be useful in epidemiological surveys in the handing out of high number of samples, as paper is a simple preservation method and greater ease in transporting to reference laboratories (Sales et al., 2007).

On the other hand, it had been previously demonstrated that nocturnally periodic *W. bancrofti* infection can be detected by PCR in blood samples collected during the day (Furtado et al., 1997), and also the sensitivities were not significantly different whether samples collected during the day or night were used (Lucena et al., 1998). In our work, the blood from individuals positive to *W. bancrofti* (samples Nos. 9 and 18) were collected at night. Therefore, more samples will be needed to support these observations, which could allow the detection of the three filarial species using blood collected during the day.

In addition, the improved sensitivity of the nested-PCRs developed could help the diagnosis of *L. loa* and *W. bancrofti* in amicrofilaremic patients. For example, it is well known that many *L. loa* infected individuals in endemic regions do not have circulating microfilaria (occult loiasis), as it was confirmed by another molecular protocol (Touré et al., 1998a,b); also, a similar amicrofilaremic status was described for *W. bancrofti* patients (Hoerauf, 2008).

Finally, Nuchprayoon et al. (2005) suggested the possible intraspecies geographic variation in the Ase I digestion pattern. In this sense, our results with the 40 samples, with different geographic origins, showed the same molecular pattern with the Ase I restriction enzyme for each filarial species. Moreover, lack of polymorphisms was confirmed by sequencing twelve ITS1–PCR products from *M. perstans* and two from *L. loa* (data not shown). Therefore, more samples will be needed to check the hypothesis by Nuchprayoon and co-workers.

In conclusion, this paper describes for the first time the simultaneous PCR detection of *L. loa*, *M. perstans* and *W. bancrofti*. Both ITS1–RFLP and nested-PCRs protocols offer scope for a species-specific differentiation of the three filarial species. The PCR–RFLP and the three nested-PCRs can be carried out with DNA from blood samples in EDTA or in Whatman paper. The molecular protocols provided concordant results with the microscopic diagnosis after Knott's concentration technique regardless to the form of DNA extraction from whole blood with EDTA or in Whatman paper (Table 1). In addition the Whatman paper method of preservation could be useful in epidemiological surveys as it is a simple preservation, and greater ease, method to the blood samples transport to reference laboratories.

In summary, the PCR methods developed are potential tools for daily routine laboratory detection and differentiation of the three

blood filarial species in clinical samples from individuals from endemic areas where the three filarial species are co-endemic.

Acknowledgments

This work was supported by projects RETIC-RICET (RD06/0021/0019 and RD06/0021/0009) and FIS PI06/1355. We thank to Dr. Estrella Montero from Servicio de Parasitología, ISCIII, Madrid, Spain for the review of the paper.

References

Carrillo, E., Iglesias, B., Gómez, J., Guinovart, C., Cabezos, J., 2004. Cribaje de microfilariasis sanguínea (*Loa loa*) en la población inmigrante de zonas endémicas. Revista Española de Salud Pública 78, 623–630.

Furtado, A.F., Abath, F.G.C., Regis, L., Gomes, Y.M., Lucena, W.A., Furtado, P.B., Dhalia, R., Miranda, J.C., Nicolas, L., 1997. Improvement and application of a polymerase chain reaction system for detection of *Wuchereria bancrofti* in *Culex quinquefasciatus* and human blood samples. Memorias do Instituto Oswaldo Cruz 92, 85–86.

Hoerauf, A., 2008. Filariasis: new drugs and new opportunities for lymphatic filariasis and onchocerciasis. Current Opinion in Infectious Diseases 21, 673–681.

Klion, A.D., 2008. Filarial infections in travelers and immigrants. Current Infectious Disease Reports 10, 50–57.

Knott, J., 1939. A method for making microfilarial surveys on day blood. Transactions of the Royal Society of Tropical Medicine and Hygiene 33, 191–196.

Lucena, W.A., Dhalia, R., Abath, F.G.C., Nicolas, L., Regis, L.N., Furtado, A.F., 1998. Diagnosis of *Wuchereria bancrofti* infection by polymerase chain reaction using urine and day blood samples from amicrofilaraemic patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 92, 290–293.

Nuchprayoon, S., 2009. DNA-based diagnosis of lymphatic filariasis. Southeast Asian Journal of Tropical Medicine and Public Health 40, 904–913.

Nuchprayoon, S., Junpee, A., Poovorawan, Y., Scott, A.L., 2005. Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction–restriction fragment length polymorphism analysis. The American Journal of Tropical Medicine and Hygiene 73, 895–900.

Pardo, J., Carranza, C., Muro, Moreno, A., Martín, A., Martín, T., Hernández-Cabrera, M., Pérez-Arellano, J.L., 2006. Helminto-related eosinophilia in african immigrants, Gran Canaria. Emerging Infectious Diseases 12, 1587–1588.

Sales, R., Descalzo, M.A., Muñoz, L., García Calleja, J.M., Abeso, C., Abeso, N., Malmierca, E., Molina, L., García-Saíz, A., Benito, A., 2007. Resultados de la encuesta nacional de la infección por el VIH en Guinea Ecuatorial en 2004. Enfermedades Emergentes 9, 61–67.

Touré, F.S., Kassambara, L., Williams, T., Millet, P., Bain, O., Georges, A.J., Egwang, T.G., 1998a. Human occult loiasis: improvement in diagnostic sensitivity by the use of a nested polymerase chain reaction. The American Journal of Tropical Medicine and Hygiene 59, 144–149.

Touré, F.S., Mavoungou, E., Kassambara, L., Williams, T., Wahl, G., Millet, P., Egwang, T.G., 1998b. Human occult loiasis: field evaluation of a nested polymerase chain reaction assay for the detection of occult infection. Tropical Medicine and International Health 3, 505–511.

Walther, M., Muller, R., 2003. Diagnosis of human filariases (Except Onchocerciasis). Advances in Parasitology 53, 149–193.

World Health Organization: Lymphatic filariasis. WHO, 2010. Available at: <http://www.who.int/lymphatic-filariasis/epidemiology>. Accesed 10 february 2010.