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Research Brief

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

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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.

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.

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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.