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Characterisation of nuclear ribosomal DNA sequences from *Onchocerca volvulus* and *Mansonella ozzardi* (Nematoda: Filarioidea) and development of a PCR-based method for their detection in skin biopsies ☆

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Abstract

The internal transcribed spacer region (ITS1, 5.8S gene and ITS2) of the two filarial nematodes *Onchocerca volvulus* and *Mansonella ozzardi* was sequenced, and two species-specific primers designed in the ITS2 to develop a PCR-based method for their specific detection and differentiation. When used with a universal reverse primer, the two species-specific primers gave amplification products of different size, which were readily separated in an agarose gel. The PCR was tested on skin biopsies from 51 people from three localities in Brazil where *M. ozzardi* is present, and results have been compared with those of parasitological examination of blood. The species-specific PCR gave a higher percentage of detection of infection by *M. ozzardi* than the parasitological examination of blood. No infection with *O. volvulus* was detected by PCR. This PCR-based assay may assist in determining the nature of infection in areas where both filarial species exist in sympatry.

Keywords: *Onchocerca volvulus*; *Mansonella ozzardi*; Ribosomal DNA; Internal transcribed spacers; Species diagnosis; Genetic markers

1. Introduction

Onchocerca volvulus is the filarial nematode responsible for the disease, river blindness, and also for skin lesions and itching, in large parts of Africa, Yemen, South and Central America. While the foci in Africa can be large, in South and Central America they are smaller and localised. *Mansonella ozzardi* is also a filarial nematode present in South and Central America and parts of the Caribbean. Infection is generally considered non-symptomatic (McNeeley et al., 1988). Its distribution is not clear but it is known that, in at least one area of Brazilian Amazonia, it is in sympatry with *O. volvulus* (Shelley et al., 1997). Both filarial species are transmitted in South America by species of Simuliidae, having the same vector species, *Simulium oyapockense* s.l., in parts of Amazonia (Yarzábal et al., 1985; Shelley et al., 1987), while in the Caribbean *M. ozzardi* is transmitted by culicoid vectors (Nathan, 1981).

It is important to be able to accurately detect and identify both species in order to study their systematics, distribution and epidemiology, and also to reliably determine the nature of infections for correct treatment and control. As adult females of *O. volvulus* produce skin dwelling microfilariae and adult females of *M. ozzardi* release microfilariae which are blood dwelling, the conventional method used for the detection of either parasite involves the parasitological examination of skin biopsies in the case of *O. volvulus* and the parasitological examination of peripheral blood in the case of *M. ozzardi*. However, this method is not entirely reliable since *M. ozzardi* microfilariae have been also detected in the skin (Moraes, 1976; Ewert et al., 1981; Moraes et al., 1983) and *O. volvulus* in blood (Fuglsang and Anderson, 1974; Anderson et al., 1975; Duke et al., 1975). This would make it difficult to detect a co-infection if, for example, a light *O. volvulus* infection occurred in a

☆ Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDJB databases under the accession number-sAF228559-AF228564, AF228565-AF228576, AF254904-AF254923. An alignment has also been submitted and has accession number DS42678.

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person heavily infected with *M. ozzardi*. Furthermore, morphological diagnostic characters to separate the microfilariae of both filarial species have not been adequately studied, which hinders specific identification. Another method for the detection of *O. volvulus* is based on the detection of serum antibodies using a cocktail of recombinant antigens (Bradley et al., 1993; Bradley and Unnasch, 1996), however, the cross-reactivity with *M. ozzardi* triggered antibodies has not been thoroughly tested, and it is not known for how long these antibodies remain in the blood circulation post-infection. The PCR has been used to develop techniques for the detection of several filarial nematodes, including *O. volvulus* (Meredith et al., 1991; Lizzote et al., 1994; Zimmerman et al., 1994a; McCarthy et al., 1996; Toure et al., 1997; Fischer et al., 1998; Vakalis et al., 1999). The method developed for *O. volvulus* is based on the amplification of a repetitive sequence of 150 nucleotides (designated O-150) by PCR and its detection with a specific probe using the southern blot technique (Meredith et al., 1991; Zimmerman et al., 1993; Zimmerman et al., 1994a).

All the above-mentioned techniques are time consuming and aim to detect only *O. volvulus*, and a negative result does not provide any information about the presence of *M. ozzardi*. Hence, a new PCR-based technique easy to implement and which can positively detect both parasites and at the same time differentiate them would be advantageous. Fischer et al. (1998) developed a nested PCR for the specific detection of *M. streptocerca* in skin biopsies by using a species-specific primer for the amplification of the 5S rDNA spacer. This method was demonstrated not to amplify DNA from the other skin-dwelling filarial nematode of Africa, *O. volvulus*. However, the internal transcribed spacers (ITS) of the rDNA have been extensively used to define genetic markers for different species of nematodes (Gasser et al., 1996; Gasser et al., 1999; Cherry et al., 1997; Powers et al., 1997; Zhu et al., 1998; Zhu et al., 1999; Heise et al., 1999; Hung et al., 1999) and therefore had potential for the differentiation of *O. volvulus* and *M. ozzardi*.

2. Materials and methods

2.1. Parasite material

Samples used to amplify and sequence the ITS region and 5.8S gene of the rDNA were three *O. volvulus* nodules kept in absolute ethanol, one taken from a Yanomami indian at the Indian Hospital in Boa Vista, Roraima state, Brazil, and two from Bolo in West Cameroon, which is the locality where the forest form of the *Onchocerca-Simulium* complexes was first described (Duke et al., 1966). A pool of *M. ozzardi* microfilariae obtained from blood of a heavily infected person from the Jujuy province in North Argentina, and kept in isopropanol was also used. In addition, human skin biopsies ($n = 51$) from three different localities in Brazilian Amazonia (Antimari on the river Acre, Labrea and Pauini on the river Purus), taken from the shoulder and/or buttock of individuals and preserved in absolute ethanol, were used to validate the diagnostic test developed.

2.2. DNA extraction

DNA was extracted from a portion of each of the three *O. volvulus* nodules, 51 human skin biopsies and two aliquots of the *M. ozzardi* microfilariae pool. The skin biopsies and pieces of nodules containing parasites were shredded with a scalpel, and 500 μl of the *M. ozzardi* microfilariae pool were sedimented by centrifugation and the supernatant removed prior to DNA extraction. Then, samples were rehydrated in 500 μl of 50 mM EDTA. After 5 min, 5 μl of 14 mg/ml Proteinase K and 5 μl of 10% SDS were added to the samples which were first incubated at 56°C for 1 h and then at 100°C for 30 min to inactivate the enzyme. NaCl was added to a final concentration of 0.2 M, and the samples were then centrifuged for 5 min at 14 000 rev./min to pellet debris. The supernatant was transferred to a new tube and 1 ml of 100% EtOH was added. Samples were then incubated at −20°C for 1 h to precipitate the DNA. The DNA was pelleted by centrifuging at 14 000 rev./min for 20 min, dried and redissolved in 25 μl of autoclaved water.

2.3. PCR amplification, sequencing and characterisation of ITS sequences

The ITS region (comprising the ITS1, 5.8S gene and ITS2) of the rDNA was amplified by PCR using as forward primer rDNA2 5′TTGATTACGTCCCTGCCCTTT-3′ (Vrain et al., 1992) situated in the 3′ end of the 18S, and as reverse primer NC2 5′-TTAGTTTCTTTTCCTCCGCT-3′, situated at the 5′end of the 28S rDNA (Newton et al., 1998). PCRs were performed in a total volume of 25 μl containing 1 × Buffer (Promega), 2 mM $MgCl_2$, 60 μM of each dNTP, 5 pmol of each primer and 0.5 U of *Taq* Polymerase (Promega). Usually, 1 μl of the extracted genomic DNA was added to the reaction. On top of the reaction two drops of mineral oil were added to avoid evaporation. The cycling conditions included an initial denaturation at 94°C for 2 min, after which the enzyme was added. This was followed by 30 cycles of 94°C for 30 s (denaturation), 55°C (*M. ozzardi*) or 53°C (*O. volvulus*) for 30 s (annealing), 72°C for 90 s (extension), and a final extension for 10 min at 72°C. A no-DNA and a human DNA reaction (Sigma's human placenta DNA which consists of a pool of DNA from 100 to 200 people) were included as negative controls. PCR products were electrophoretically separated in a 1% (w/v) agarose gel in 1 × TBE buffer. Gels were stained with ethidium bromide and visualised on a UV light transilluminator. Bands were excised from the gel and DNA was purified using the Geneclean kit (Anachem).

PCR products were cloned into a pCR®2.1-TOPO® vector and transformed into TOP10 competent cells using

the TOPO TA cloning kit (Invitrogen) following the manufacturer's recommendations. Four to six clones of each sample were grown in 15 ml LB cultures, and recombinant plasmids were recovered using Hybaid's Plasmid Midi Prep Recovery Kit. The ITS was cycle sequenced using the Big Dye (ABI) chemistry in a Techne thermocycler, the sequencing cycle consisting of 2 min at 94°C, followed by 35 cycles of 95°C for 15 s; 50°C for 15 s; and 60°C for 4 min. The entire ITS was sequenced in both directions using a primer walking strategy.

Sequences were aligned using CLUSTAL W (Thompson, J.D., Higgins, D.G., Gibson, T.J., 1997. CLUSTAL W Multiple Sequence Alignment Program. Version 1.7. EMBL, Heidelberg) and then corrected by eye. The 3′ and 5′ ends of the 18S and 28S rDNA respectively, were determined by comparison with the GenBank sequences of the nematodes *Meloidogyne javanica* and *Bursaphelenchus mucronatus* (accession numbers U96305 and U93554 respectively), and the 5′ and 3′ ends of the 5.8S rDNA by comparison with the sequences of anisakid nematodes and the nematode *Ascaris* (Zhu et al., 1998; Zhu et al., 1999). P-distances were calculated for the ITS1 and ITS2 within and between species, in the latter case using the consensus sequences for the different clones, with the programme MEGA (Kumar, S., Tamura, K., Nei, M., 1993. MEGA: molecular evolutionary genetics analysis, version 1.01. The Pennsylvania State University, University Park, PA 16802, USA).

2.4. Design of species-specific primers and optimisation of species-specific PCR

Species-specific primers were designed using the programme OLIGO 4.0 (Rychlik, 1992) in specific regions of the ITS2. The regions were chosen to maximise the interspecific differences while minimising the intraspecific variation. The objective was to design two forward species-specific primers, which would be used with primer NC2 as the reverse primer. The PCR products for each species would have to be of a different enough size to allow separation in a 1% (w/v) agarose gel.

Species-specific primers were first optimised individually with primer NC2. Controls were used in each reaction to investigate the possibility of amplification of one species DNA by the other species' specific primer, and of human and vector DNA (*Simulium sanctipauli* larvae from the Sutri rapids in the Western region of Ghana and *Simulium oyapockense* adult males from the Brazilian Amazonia) by any of the primers. A negative control with sterile water as template was also run. In order to optimise the reaction when the three primers were combined in a single PCR, different primer concentrations were examined using different $MgCl_2$ concentrations and annealling temperatures. As template either 1 μl of DNA of one species or 0.5 μl of each was added.

2.5. Preliminary validation of the species-specific PCR on field samples

The species-specific PCR was partially validated by comparing the PCR results for skin biopsies from 51 people from Brazilian Amazonia (see Table 2) with the results for those same individuals using parasitological examination of blood. For the Antimari samples, where PCR and blood results were inconsistent, additional skin biopsies were parasitologically examined to corroborate one or the other method. Two different techniques were used: (1) detection of living microfilariae that emerge from skin biopsies after incubation in water (Prost and Prod'hon, 1978), and (2) a modification of the method by Schulz Key (1978) in which microfilariae are detected after the alcohol-preserved skin biopsy has been digested overnight with collagenase and stained with acetic orcein.

Amplification products resulting from the species-specific PCR of samples 103, 107, 111, 127 and 137 (five positives for *M. ozzardi* from Antimari) were cloned and four clones of each were completely sequenced in both directions. This was carried out to verify that the amplified products were species-specific and not contaminants or non-specific amplification products. The resultant sequences were aligned and compared with those of *M. ozzardi* and *O. volvulus*.

3. Results

3.1. Characterisation of ITS sequences

The PCR products obtained with rDNA2 and NC2 were 1.2 and 1.1 kbp in size for *O. volvulus* and *M. ozzardi*, respectively. Sequences have been submitted to GenBank and they have been given accession numbers (see article footnote). The 3′ end of the 18S rDNA is situated at position 175 of the sequence, and the 5′ end of the 28S rDNA is 48 bases from the end of the sequence. The 5.8S rDNA is 156 bp long for both species. The length of the ITS1 in *O. volvulus* ranged from 400–420 bp and in *M. ozzardi* had a length of 368–369 bp (Table 1). The length of ITS2 sequence in *O. volvulus* was 340–352 bp, and in *M. ozzardi* it was 273–310 bp. Insertions/deletions of one or more bases were the causes of such length variation. For instance, in the ITS2 of *O. volvulus* a microsatellite (CAT)n was found in position 1038–1055 of the aligned sequences. This microsatellite was partly responsible for the variation in length of the ITS2. The ITS2 sequences from Brazilian samples (*Ov-its-9* to *Ov-its-12*) had six (CAT) repeats, while the African ones (*Ov-its-1* to *Ov-its-8*) presented three, five or six. The ITS regions are very A + T rich, being only 24.2 and 22.7% G + C for the ITS1 of *O. volvulus* and *M. ozzardi*, respectively, and 20.6 and 14.3% for the ITS2 (Table 1). The intraspecific p-distance for the ITS1 and ITS2 of *O. volvulus* ranged from 0 to 2% and 0 to 5.92%, respectively. The

Table 1
Length, and G + C content (in %) of the ITS 1, ITS 2 and 5.8S gene of *Onchocerca volvulus* and *Mansonella ozzardi*

Species	ITS1	5.8s	ITS2	G + C ITS1 (%)	G + C ITS2 (%)
Onchocerca volvulus	400–420 bp	156 bp	340–352 bp	24.2	20.6
Mansonella ozzardi	368–369 bp	156 bp	273–310 bp	22.7	14.3

sequence diversity was higher in the ITS2 than in the ITS1, but this may have been overestimated because the genetic divergence of a single ITS2 sequence, Liberian *Ov-its-3* ITS2, was high (4.73–5.92%) compared with the others. P-distances when *Ov-its-3* was not included ranged from 0 to 1.26% in the ITS1 and from 0 to 2% in the ITS2. The p-distances between African (Liberian) and Brazilian sequences (*Ov-its-1* to *Ov-its-8* and *Ov-its-9* to *Ov-its-12*, respectively) ranged from 0.25 to 1.25% and 0.28 to 2% for the ITS1 and ITS2 (sequence *Ov-its-3* not included). In *M. ozzardi*, the intraspecific p-distance ranged from 0.54 to 2.72% for the ITS1 and 0.32–2.26% for the ITS2. Between the two species, the p-distances in the ITS1 and ITS2, calculated from the alignment of the consensus sequences, are 21.51 and 30.45%, respectively.

3.2. Species-specific primers and optimum species-specific PCR

The designed species-specific primers were OvITS2 5′-TTCATACATATATAAATGTAGC-3′ for *O. volvulus*, situated 127 bp upstream the 3′ end of the ITS2, and MoITS2 5′-CTTATCATCAGGTGATATTAAT-3′ for *M. ozzardi*, situated 227 bp upstream the 3′ end of the ITS2.

Each primer used individually with NC2 gave PCR products of the expected size (295 bp for *M. ozzardi* and 195 bp for *O. volvulus*). When used in combination with NC2 the optimum condition for the species-specific PCR was 1.5 mM $MgCl_2$, 60 μM of each dNTP, 5 pmol of NC2, 10 pmol of MoITS2 and 2.5 pmol of OvITS2 in a reaction volume of 25 μl. And the optimum reaction cycle consisted of a first denaturation at 94°C for 2 min after which *Taq* polymerase was added to each reaction. This was then followed by 35 cycles of 94°C for 15 s (denaturation), 52°C for 30 s (annealling), and 72°C for 30 s (extension), with a final extension of 10 min at 72°C. The resultant products were of the expected size and could be easily distinguished in a 1% (w/v) agarose gel (Fig. 1). None of the species-specific primers amplified DNA of the other parasite species, human or vector, indicating specificity.

3.3. Preliminary validation of the species-specific PCR on field samples

The results of the validation of this method in skin biopsies from 51 people are shown in Table 2. The PCR detected 26 individuals positive for *M. ozzardi* (50.9% infection), while using blood examination 24 individuals were found positive (47% infection). No *O. volvulus* infection was detected. Samples 3, 8, 103, 127 and VAT1 were negative according to the blood microscopy but gave positive using the PCR method. Results for samples 103, 127 and VAT1 could be corroborated with the results obtained by parasitological examination of skin biopsies. In sample 103 three microfilariae were found when a skin biopsy was subsequently treated with collagenase. In individual 127 one microfilaria was found after treating a skin biopsy with collagenase. In VAT1 three microfilariae were found in a skin biopsy using the conventional method. All microfilariae found in these additional skin biopsies were identified as *M. ozzardi*. Thus, in all three cases PCR results were corroborated. Samples 32, 123 and 124 were blood-positive and PCR-negative. These results for individuals 123 and 124 were compared with those obtained by parasitological examination of skin biopsies. In individual 123 one microfilaria was found and identified as *M. ozzardi* when treating a skin biopsy with collagenase. For individual 124 no microfilariae were found in the skin biopsies. In individual 32 only one microfilariae was found in the blood. The remaining 43 samples gave concordant results with both techniques.

The sequenced PCR products representing individuals 103, 107, 111, 127 and 137 were 295 bp long, and the p-distance when aligned with the *M. ozzardi* ITS2 ranged from 0 to 1.70%, which is within the intraspecific variation found

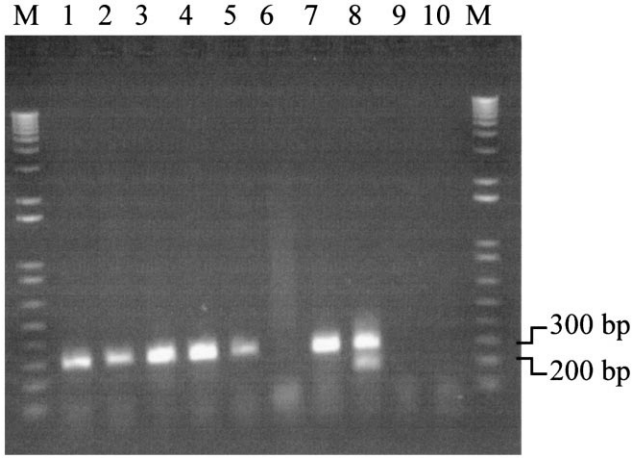

Fig. 1. Agarose gel showing the PCR results for some skin biopsies tested from the localities Pauini and Antimari. Samples 35 Pauini, 37 Pauni, 38 Pauini, 41 Pauini, 42 Pauini, and 137 Antimari, are positive for *M. ozzardi* (lanes 1–5 and 7, respectively) and sample 123 Antimari (lane 6) is negative. Lane 8 is a positive control with mixed DNA from *O. volvulus* and *M. ozzardi* as template. Lanes 9 and 10 are negative controls, the former with human DNA as template and the latter with sterile water as template. M represents the 1 Kb Plus size marker (bp) on the agarose gel.

Table 2
Results of the species-specific PCR in skin biopsies and of the parasitological examination of blood samples[a]

	O. volvulus PCR test		*M. ozzardi* PCR test		Blood microscopy			Locality
Samples	C	N	C	N	D	B	O	
1	−	−	−	−	0	0	0	Labrea
12	−	−	+	+	1748	45	1950	Labrea
40	−	−	−	−	0	0	0	Labrea
41	−	−	+	+	41	47	115	Labrea
42	−	−	+	+	2	4	6	Labrea
1	−	−	+	+	54	85	136	Pauini
3	−	−	−	+	0	0	0	Pauini
8	−	−	−	+	0	0	0	Pauini
13	−	−	+	+	9	2	326	Pauini
14	−	−	−	+	0		2	Pauini
15	−	−	+	−	1	1	16	Pauini
16	−	−	+	+	0	0	2	Pauini
17	−	−	+	+	0	0	2	Pauini
19	−	−	−	+	0	3	64	Pauini
22	−	−	+	+	67	208	154	Pauini
23	−	−	−	−	0	0	0	Pauini
24	−	−	+	+			2	Pauini
27	−	−	+	+	49	23	273	Pauini
29	−	−	+	+	8	14	2	Pauini
32	−	−	−	−	0	0	1	Pauini
33	−	−	−	−	0	0	0	Pauini
34	−	−	−	−	0	0	0	Pauini
35	−	−	+	+	34		17	Pauini
36	−	−	−	−	0	0	0	Pauini
37	−	−	+	+	1	1	4	Pauini
38	−	−	+	+	163	98	140	Pauini
39	−	−	−	−	0		0	Pauini
100	−	−	−	−	−			Antimari
102	−	−	−	−	−			Antimari
103	−	−	−	+	−			Antimari
107	−	−	+	+	+			Antimari
111	−	−	−	+	+			Antimari
113	−	−	−	−	−			Antimari
121	−	−		−	−			Antimari
123	−	−	−	−	+			Antimari
124	−	−	−		+			Antimari
127	−	−	+	+	−			Antimari
136	−	−	+	−	+			Antimari
137	−	−	−	+	+			Antimari
VAT1		−		+	−			Antimari
VAT2	−		−		−			Antimari
VAT3		−	−	−				Antimari
VAT4		−	−	−				Antimari
VAT5		−	−	−				Antimari
VAT6		−	−	−				Antimari
VAT7		−	−	−				Antimari
VAT8		−	−	−				Antimari
VAT9		−	−	−				Antimari
VAT10	−		−		−			Antimari
VAT11		−	−	−				Antimari
VAT12		−	−	−				Antimari

[a] Positive PCR results are indicated by +, and negative results by −. Numbers of microfilariae are given for blood samples from Labrea and Pauini; and microfilariae found (+) or not found (−) for blood samples from Antimari. Skin biopsies taken from the shoulder and from the buttock are indicated by C and N, respectively; blood samples taken from the finger, arm and ear are indicated by D, B and O, respectively.

in the pool of microfilariae from North Argentina. Thus, it was demonstrated that the PCR products were species-specific and that there was no evidence of non-specific amplification. These sequences have been deposited in the GenBank and have been given accession numbers (see article footnote). The alignment with the *M. ozzardi* sequences from Argentina has been submitted to EMBL and has the following accession number: DS42678.

4. Discussion

Although *O. volvulus* is a filarial nematode of medical importance, no previous studies of its rDNA and ITS as genetic markers have been published. Also, *M. ozzardi* has been similarly overlooked. The 5S rDNA spacer region has been sequenced, and a nested PCR-based detection method for *M. streptocerca* in skin biopsies was developed in this region (Fischer et al., 1998). Gasser et al. (1996) published a study in which PCR-RFLP was used to characterise closely related species of *Litomosoides*, however their ITS sequences have not been published. Even though the filarial nematodes are a group of medical importance, only four ITS sequences of three filarial nematodes have been sequenced before this study and are available in the data base.

The intraspecific variation observed in the ITS1 and ITS2 of *O. volvulus* (not including *Ov-its-3*) and *M. ozzardi* is higher than the 0.3% found for anisakid nematodes (Zhu et al., 1998) and for equine strongyloids (Hung et al., 1999), but is similar to that found in the ITS2 of some species of gastrointestinal nematodes of ruminants such as *Haemonchus contortus*, which had an intra-specific variation of 2.6% (Heise et al., 1999). The variation observed in the ITS sequences of both filarial species can be explained by insertion/deletion events and substitutions. The former are likely to be the result of DNA slippage mechanisms as a result of the very high A + T content (Tautz et al., 1987). The microsatellite found in the ITS2 of *O. volvulus* is another source of length variation within the species, and the different number of tandem repeats observed may have arisen by means of slippage mechanisms (Schlötterer and Tautz, 1992). Few microsatellites have been described for nematodes (e.g. Herder et al., 1994; Fisher and Viney, 1996; Zarlenga et al., 1996; Hoekstra et al., 1997), and although Herder et al. (1994) studied dinucleotide microsatellites in *O. volvulus*, no descriptions of the repeated sequences or primers were given. It may be the case that this trinucleotide microsatellite could be useful for studies of molecular ecology and epidemiology in *O. volvulus* (Bruford and Wayne, 1993). In the present study, one ITS2 sequence, *Ov-its-3*, showed a higher genetic distance with respect to all other *O. volvulus* sequences than that amongst these other sequences. The high divergence of *Ov-its-3* has several possible explanations. First, this sequence could represent a non-functional copy that mutates at a higher rate. Indeed, in *Drosophila* up to 60% of rDNA copies are not transcribed, and are non-functional due to the presence of insertions in the 28S gene (John and Miklos, 1988). Although there is no information relating to non-functional copies of rDNA in nematodes, there are species in which a large transposable element has been found inserted in the 28S rDNA of some of their rDNA repeat units and this could make them non-functional (Burke et al., 1995). Such elements may prevent mechanisms of gain and loss of copies by obstructing pairing with adjacent functional repeats (Anderson et al., 1998). However, this explanation is not consistent with the observation that the divergence between the 5.8S rDNA and the ITS1 of *Ov-its-3* and the other sequences is not as high as in the ITS2. If *Ov-its-3* was a non-functional copy, we would expect to find a higher level of variation in the other parts, which are also transcribed sequences. A second explanation could be that the *Ov-its-3* sequence belongs to another species. However, even though other filariae are known in the geographical area where the nodule was collected (for example *Loa loa* and *Wuchereria bancrofti*), this is unlikely because the sample was taken directly from a nodule. A third possible explanation is that it belongs to a second set of rDNA copies within the genome with homogenisation occurring at a lower rate between the two rDNA loci (Dover, 1982; Elder and Turner, 1995).

The differences between *O. volvulus* from Brazil and from Africa are not higher than among African or Brazilian samples, and these findings suggest that these populations have not been long separated. This supports the theory that *O. volvulus* was introduced to the Americas with the African slaves taken there by the Europeans within the last 500 years (Dalmat, 1955; Zimmerman et al., 1994b), and opposes the independent evolutionary origin of the species in America (Marroquín, 1963). Similarly, in *M. ozzardi* the differences between the ITS2 sequences from the pool of microfilariae from North Argentina and the incomplete ITS2 from the Brazilian skin biopsies are similar to the divergences found within each location. Two types of vectors transmit *M. ozzardi*, *Culicoides* spp. in the Caribbean region (Nathan, 1981), *Simulium* spp. in Amazonia (Yarzábal et al., 1985; Shelley et al., 1987), and both vectors in Argentina (where culicoids are the most efficient vectors, (Shelley and Coscarón, 2001, in press). It has been suggested that there could be two different species of *M. ozzardi*, one for each vector (Nelson and Pester, 1962). However, morphological studies show that microfilariae from Colombia (Simuliid-transmitted) and Haiti (Culicoid-transmitted) are almost identical (Kozek and Raccurt, 1983). Our results, although rather preliminary because of the small sample number, give no support to the separation into two species but are consistent with the proposal that they are a single species sharing a common gene pool.

The difference between the consensus sequences of *O. volvulus* and *M. ozzardi* is much higher than the level of sequence variability within each taxon. This demonstrates the usefulness of the region for discriminating between these species of filarial nematodes. The degree of difference between them is similar to that found among other nematode species at the order, family and genus levels (Newton et al., 1998; Zhu et al., 1998; Gasser et al., 1999; Heise et al., 1999; Hung et al., 1999).

For epidemiological and population studies, as well as for the correct diagnosis of infections, it is important to have a detection method that not only is easy to implement but that is also specific and sensitive. The specificity of the method was tested by PCR using as template DNA from *S. sancti-*

pauli and *S. oyapockense* (vector species in West Africa and in Brazilian Amazonia) and human DNA from a pool of 100–200 people (the host species). No amplification of DNA from either the vector species or from the host was observed with the species-specific primers. Amplification of known *O. volvulus* and *M. ozzardi* DNA using the converse species-specific primer always gave negative results. Thus, we can conclude that the test will be species-specific given the absence of other *Onchocerca* or *Mansonella* spp. Other human filariae are known in South America, including *Mansonella perstans* in Venezuela. However, this species has not been found in Brazil and, unlike *M. ozzardi*, there is no evidence of its microfilariae being present in skin biopsies. Therefore, the probability of confounding the two *Mansonella* spp. is remote.

The sensitivity of the method was tested using field samples and comparing the results with those obtained by parasitological examination of blood. The PCR method was positive for *M. ozzardi* in 50.9% of the individuals and the parasitological examination of blood in 47%. No *O. volvulus* was detected in the skin-snips. This was expected as there is no report of *O. volvulus* in the area where the skin biopsies were collected (localities of Pauini, Labrea and Antimari), although high levels of eye lesions have been reported and associated with the presence of *M. ozzardi* (Chamon et al., 1999). We have found five individuals (samples 3, 8, 103, 127 and VAT1) who were amicrofilaraemic in the blood but were positive with the PCR of their skin biopsy samples (Table 2). They may be false positives, but the fact that *M. ozzardi* microfilariae were found in samples 103, 127 and VAT1 when additional skin biopsies were examined parasitologically suggested that the blood examination by microscopy had simply failed to detect them initially (no additional skin biopsies were available for samples 3 and 8). As it was demonstrated that the PCR achieved species-specific amplification, we concluded that in these cases the PCR was more sensitive than the blood microscopy. However, in three other cases (samples 32, 123 and 124) the opposite occurred, they were positive by parasitological examination of blood while the PCR did not detect microfilariae (Table 2). This finding might be explained because the detection by PCR of *M. ozzardi* infections presumably depends on the presence of microfilariae in the skin biopsies, which in turn depends on the level of infection. Sometimes, it would be expected that if the level of infection is very low, no or very few microfilariae would be present in the skin (Moraes et al., 1983). Of these three cases, we know that in individual 32 only one microfilaria was found in the blood. The consequent parasitological examination of additional skin biopsies found only one microfilaria in sample 123 and none in sample 124. These findings indicate a very low parasitaemia in these individuals, with the possibility that there were no microfilariae in the skin-snips used in the PCR.

In a study in which four parasitological methods of detection of *M. ozzardi* in humans were compared, Raccurt et al. (1982) found that the analysis of three samples of blood taken from the finger could detect 95% of the infections, while analysis of one sample from the finger could detect around 80%, and the analysis of blood from the earlobe 85% of the cases. If the number of blood microscopy and PCR positive cases represented 100% of the infections (29 positives of 51 samples), the PCR then detects about 90% and the parasitological analysis of the blood 83%. The PCR of skin biopsies therefore gives a higher percentage of detection of *M. ozzardi* infections than the parasitological examination of blood, and it is able to reveal cases where no microfilariae are detected in the blood or where there is a very low level of microfilaraemia (Table 2). However, parasitological examination of blood samples may still be necessary to detect those few cases which present no microfilariae in the skin. Raccurt et al. (1982) gave a figure of only 35% of *M. ozzardi* infections detected by examination of skin biopsies. Our figure with the PCR is much higher than that, even though PCR amplification depends on the presence of microfilariae in the skin. Thus, the PCR is a more sensitive method for the detection of *M. ozzardi* infection than the microscopic examination of the skin biopsies, in which only a small fraction of the microfilariae in the skin are released.

In this study validation of the method with skin-snip samples infected with *O. volvulus* has not been accomplished, but there would be no a priori reason why the PCR method should not work in the same way as it has done with *M. ozzardi*. Complete validation of the method developed will come when it has been more extensively used and compared with other diagnostic methods.

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