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Atypical *Mansonella ozzardi* Microfilariae from an Endemic Area of Brazilian Amazonia

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Abstract. Mansonellosis is endemic in several regions of Africa, the Caribbean, and Latin America. *Mansonella ozzardi* and *Mansonella perstans* have been reported in Latin America, including the Amazon region. A morphological and molecular microfilariae study was performed in Pauini (Brazil). Blood samples were collected from 40 individuals, and were analyzed by Giemsa-stained blood film and by two different nested polymerase chain reactions which detect internal transcribed spacer-1 and the major sperm protein gene. By microscopy, 14 of 40 were positive: 11 as *M. ozzardi* and three as *M. perstans*–like infections. Both molecular methods detected 19 positive cases as *M. ozzardi*, including those 14 individuals detected by microscopy, without detectable genetic differences among any of the 19 positive samples. Molecular techniques showed an improvement of mansonellosis diagnosis and may become an effective tool to evaluate the present status of *M. ozzardi* and *M. perstans* in Latin America.

INTRODUCTION

Mansonellosis is a filarial disease caused by nematodes from the genus *Mansonella* that is transmitted by the bite of blood-sucking insects (*Simulium* in the Amazonia and *Culicoides* in the Caribbean regions, in Central and South America and in Africa).[1] The mansonellosis disease is generally considered innocuous, with few symptoms, such as fever, headache, articular pain, and erythematous cutaneous plaques with pruritus.[1] Microscopic examination of thick and thin peripheral blood smears stained with Giemsa or other appropriate stains is often regarded as the "gold standard" method for microfilariae diagnosis.[1,2] Two species of *Mansonella* are described in Latin America, *Mansonella ozzardi* and *Mansonella perstans*, the latter being restricted to the Amazon regions of Venezuela, Colombia, and Guyana.[3–7] In the last decades, a number of unidentified/atypical microfilariae have been described in the Amazonia areas of Brazil, Peru, and Venezuela based on their morphological characteristics, which suggest *M. perstans*-like.[8–10] Morphologically, the most important features to identify microfilariae species are size, shape, and space of the tail, presence or absence of a sheath, and terminal arrangement of nuclei of the tail. However, all these morphological characteristics are not enough to identify these filaria species, and therefore, molecular methods are necessary.[1,2,11]

The aim of this study was to evaluate the possible presence of *M. perstans* in the Brazilian Amazonia and to characterize unidentified/atypical microfilariae using molecular characterization approaches.

MATERIALS AND METHODS

For the molecular characterization of the atypical microfilaria in the Brazilian Amazonia, a study of the inhabitants of Pauini was performed. This area was chosen because in a previous study to validate the filaria nested polymerase chain reaction (FnPCR),[12] it had the highest prevalence of mansonellosis in the studied communities (Ta-Tang T-H, Rubio JM, de Moura Abrahim CM, and others, unpublished data).

Forty individuals were included in the study. Samples were obtained as part of a study approved by the Research Ethical Committee of the Amazon Hematology and Hemotherapy Foundation, Brazil. Blood samples were analyzed by Giemsa-stained blood film and by different molecular methods. Morphological identification was performed by six expert microscopists, to whom the origin of the samples was unknown, in line with published guidelines.[11] In the case of discrepancies, blood films were reexamined by the same microscopists. The molecular methods included a FnPCR that targets the internal transcribed spacer-1 (ITS-1) region of the ribosomal gene, which differentiates filarial species by the amplified fragment size and by gene sequence,[12] and a nested PCR which detects the major sperm protein (MSP) gene. This nested PCR uses, as first amplification process, the MSP-PCR, developed by Hojas and Post[13] and as the nested one, an in-house method, using primers MSP-1F (5′-ACCTGGTGACATCCACACAC AAC-3′) and MSP-2R (5′-CCAGGACACCGCATGGTGG ATC-3′) at 55°C for annealing.

PCR products from FnPCR were purified and sequenced in both directions by an automated sequencer ABI PRISM® 3700 DNA Analyzer (Foster City, CA). The sequences of the ITS-1 were aligned using CLUSTAL W (Cambridge, United Kingdom).[14] After alignment, a phylogenetic tree was performed with Treecon software (Antwerpen, Belgium)[15] by the neighbor-joining method.

RESULTS

Fourteen of 40 blood films analyzed by microscopy showed microfilariae (35%). Eleven were characterized as *M. ozzardi* (78.6%) and the other three (21.4%) were characterized as *M. perstans*-like by five of the six microscopists and as *M. ozzardi* by the sixth. The morphological description of these atypical microfilariae included no sheath, short cephalic space, and tail shape with nuclei until the end which resemble the morphology of *M. perstans* (Table 1, Figure 1).

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TABLE 1
Comparison of morphological characteristics among *Mansonella ozzardi*, *Mansonella perstans*, and atypical microfilariae

Characteristics species (type)	Length (μm) (average μm) (Ref.)	Width (μm) (average μm) (Ref.)	Sheath (Ref.)	Cephalic space (Ref.)	Tail and tail nuclei (Ref.)
M. perstans	190–200 (195) ([11])	4–5 ([11])	Absent ([11])	Morphological feature not described in standard textbooks for microfilariae species diagnosis.	Tapered and bluntly. Nuclei to end of tail. ([11])
M. ozzardi	163–203 (183) ([11])	3–5 ([11])	Absent ([11])	After cephalic space, a single nucleus. ([8]–[10])	Long and slender, with seven to nine nuclei that do not reach the tail end. ([11])
Brazilian Amazon atypical microfilariae	Measures close to *M. ozzardi* ([8])	Measures close to *M. ozzardi* ([8])	Absent ([8])	Two paired nuclei after the cephalic space, followed by a single one, and afterwards begin the proceeding characteristic column of nucleus ([8]).	The posterior extremity, where a nuclear column with 7–8 nuclei are present in a regular and aligned arrangement and a detachment from cuticle is very perceptible ([8])
Peruvian Amazon atypical microfilariae	120–130 (125) ([9])	ND	Absent ([9])	Two nuclei followed by a single nucleus just caudal to the cephalic space ([9])	ND
Venezuelan Amazon atypical microfilariae: named as *Microfilaria bolivarensis* by the authors	220–280 (250) ([10])	7–8 (7.5) ([10])	Absent ([10])	The cephalic space is short; typically, its length is only slightly greater than its width. ([11])	Posterior one-fifth tapered rather sharply. Tail is shorter, less attenuated tail than *M. ozzardi*, is devoid of nuclei. ([10])
Atypical microfilariae described in this work	126–180 (153)	3–3.6 (3.4)	Absent	Short cephalic space with two nuclei followed by single nucleus, just caudal to the cephalic space.	Nuclei reach the tip of the tail and short, blunt, and less attenuated tail.

ND = no data.

The FnPCR detected 19 filariae-positive cases (47.5%), five cases more than that detected by microscopy, showing higher sensitivity. The 19 cases yield a fragment of 305 base pairs (bp) corresponding to the expected size for *M. ozzardi*. The Basic Local Alignment Search Tool searches confirmed highest similarity with *M. ozzardi* sequences from GenBank. Furthermore, a phylogenetic tree performed by neighbor-joining method comparing the sequences from the atypical filariae with others from GenBank showed that atypical filarial sequences formed a monophyletic clade with *M. ozzardi* (Figure 2). The nested PCR detecting MSP gene showed that the same 19 samples were positive, showing the expected fragment of 406 bp which belonged to *M. ozzardi*; it was also used in proven *M. perstans* samples, giving a fragment size of

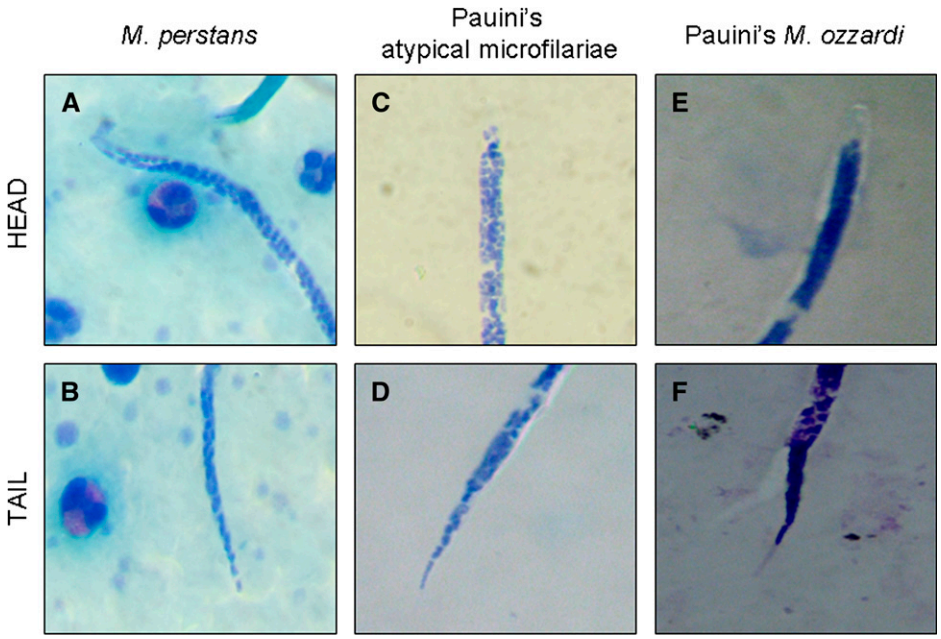

FIGURE 1. Giemsa-stained blood film. (**A**, **B**) Comparison of head and tail of *Mansonella perstans* microfilaria from Africa, (**C**, **D**) atypical microfilaria, and (**E**, **F**) *Mansonella ozzardi* microfilaria.

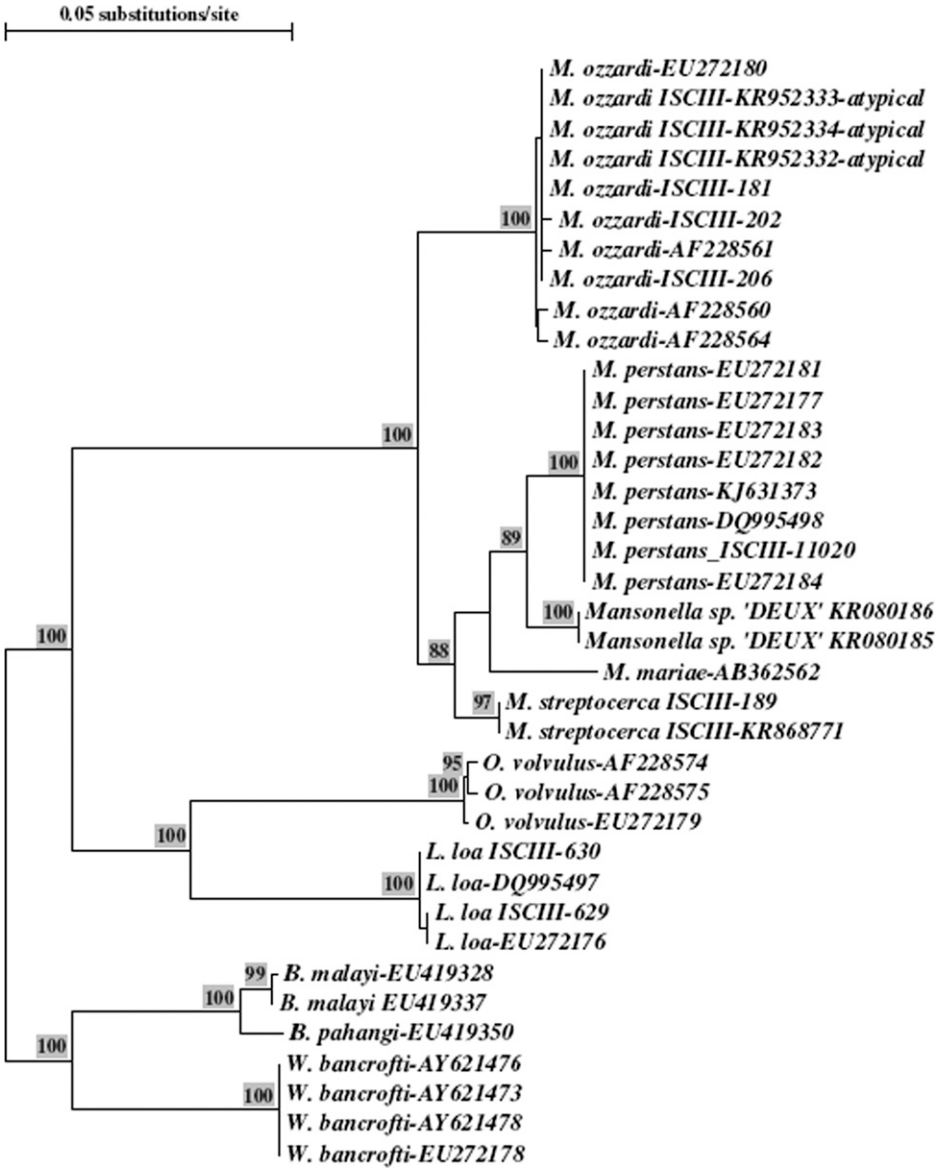

FIGURE 2. Phylogenetic tree of internal transcribed spacer-1 and flanking regions by neighbor-joining method with bootstrap analysis. The alignment file includes the *Mansonella ozzardi* atypical sequences, other filarial species identified in the laboratory named as ISCIII, plus the internal number and sequences from GenBank with the corresponding access number. The atypical microfilariae sequences cluster with the *M. ozzardi* group.

395 bp. Atypical microfilaria amplified fragments for ITS-1 and MSP were sequenced and *M. ozzardi* identification was confirmed (GenBank access no. for ITS-1: KR952332, KR952333, KR952334, *M. ozzardi*-MSP: KT224437, KT224438, KT224439 and *M. perstans*-MSP: KT224440).

DISCUSSION

More than 20% of microfilariae present in the Pauini inhabitants showed an atypical morphology, which several microscopists characterized as *M. perstans*-like. Using conventional parasitological techniques, differentiation between the microfilariae species is quite difficult, since there are no reliable morphological criteria to differentiate them.[1] It becomes more complicated when two or more species are in sympatry, which could have medical significance, that is, in the monitoring for onchocerciasis recrudescence.[16] In contrast with other microfilariae known to routinely infect humans in the Brazilian Amazon, the microfilariae of *M. ozzardi* are reported to be 163–203 × 3–5 μm, and to have no nuclei at the end of their tails; *M. perstans* are recorded as having dimensions of 190–200 × 4–5 μm and as having nuclei at the end of their tails (Table 1).[11] The observed atypical *Mansonella* microfilariae in this report had uncommon sizes (ranging from 126–180 × 3–3.6 μm), smaller than both *M. ozzardi* and *M. perstans*, although the average was smaller, overlapped with the size of *M. ozzardi.* The method of specimen preparation is known to have a large effect on microfilariae body size measurements, but all samples were treated in the same way and length measurements are comparable with those published by previous authors for normal and atypical microfilariae.[8–10] The cephalic space of these *M. perstans*-like had

two nuclei followed by single nucleus, which is very similar to other atypical microfilariae reported from Brazilian Amazon,[8] Peruvian Amazon,[9] and Venezuelan Amazon,[10] whereas in contrast, *M. ozzardi* has a single nucleus in this position. Furthermore, the posterior region has a blunt tail filled with nuclei similar to *M. perstans* (Table 1, Figure 1).

Molecular methods allow the differentiation of the species of filariae. In the present study, *M. ozzardi* was the unique species present, independent of the target used, ITS or MSP, with no detectable genetic differences among any of the 19 positive samples.

Records of distribution and prevalence of *M. perstans* in Latin America are restricted to Colombia, Guyana, and Venezuela, and all of them, except one,[4] are from the late sixties to the early eighties of last century.[3,5–7] Most recent reports describe atypical microfilariae besides *M. ozzardi*, but not *M. perstans*,[8–10] and in the cases where some molecular identification has been performed, as in this report and one other,[9] the unique species found was *M. ozzardi.*

The studies, in which *M. perstans*[3–7] was described, were done in laboratories either without molecular diagnostic techniques or where these were not easily available. It is possible that these old studies might have misinterpreted atypical *M. ozzardi* as *M. perstans* because of the reliance on the morphological analysis. It would be interesting to carry out new field studies to analyze the present status of *M. perstans* in those communities. Molecular techniques show an improvement of mansonellosis diagnosis and may become an effective tool to evaluate the present geographical distribution and prevalence of *M. ozzardi* and *M. perstans* in Latin America.

CONCLUSIONS

There is a need to evaluate the real situation of *M. perstans* in Latin America, since the previous descriptions from the sixties to the eighties of the last century may be an atypical morphological presentation of *M. ozzardi*. Molecular techniques can have a special value to provide additional data and support evidences to confirm and to determine whether *M. perstans* is actually present in Latin America and to characterize those unidentified/atypical microfilariae.

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