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Short Report: Genetic Characterization of Atypical *Mansonella* (*Mansonella*) *ozzardi* Microfilariae in Human Blood Samples from Northeastern Peru

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Abstract. DNA sequence comparisons are useful for characterizing proposed new parasite species or strains. Microfilariae with an atypical arrangement of nuclei behind the cephalic space have been recently described in human blood samples from the Amazon region of Peru. Three blood specimens containing atypical microfilariae were genetically characterized using three DNA markers (5S ribosomal DNA, 12S ribosomal DNA, and cytochrome oxidase I). All atypical microfilariae were clustered into the *Mansonella* group and indistinguishable from *M. ozzardi* based on these DNA markers.

The main filarial species that infect humans in Latin America are *Onchocerca volvulus*, *Wuchereria bancrofti*, and two species of *Mansonella*, *M. ozzardi*, and *M. perstans*. The latter are placed in different subgenera, *Mansonella* and *Esslingeria*, respectively.[1] *M. ozzardi* is limited to Latin America, whereas the other species also occur in sub-Saharan Africa and were introduced to the new world by the slave trade.[2] Atypical microfilariae (Mf) similar to *M. ozzardi* have been occasionally reported in Amerindian people from tropical South America. These Mf include *Microfilaria bolivarensis* in Venezuela,[3] a microfilaria sharing characteristics with *M. ozzardi* and *O. volvulus* in Brazil,[4] and an atypical Mf recently reported from Peru.[5] The atypical Mf reported from Brazil and Peru were morphologically similar, with two nuclei followed by a single nucleus just caudal to the cephalic space. This finding is in contrast to Mf of *M. ozzardi*, which have a single nucleus in this position.[4,5] The geographic distance between the communities where these atypical Mf were detected is approximately 850 km, and both areas are within *M. ozzardi*-endemic zones.[6] Additional studies were warranted to investigate whether the atypical Mf are from a novel filarial parasite species. Therefore, the objective of this study was to use molecular markers to characterize atypical Mf found during a malaria screening (Institutional Review Board approved under Instituto Nacional de Salud and Center for Diseases Control) in Peru.

Three frozen (ethylenedinitrilo)tetraacetic acid (EDTA) blood samples (two with *M. ozzardi* and one with atypical Mf) and three blood smears with only atypical Mf were sent to Washington University, St. Louis, MO, from the Instituto Nacional de Salud, Lima, Peru. The samples were collected in La Union (7°28′ S, 74°58′ W), Santa Clara (3°37′ S, 73°12′ W), and Cahuapanas (5°14′ S, 77°05′ W) villages in northeast Peru (Loreto Department). *M. ozzardi* was identified in Peru by morphological characteristics.[7] The difference between Mf of *M. ozzardi* and the atypical Mf is shown in Figure 1. Total genomic DNA was extracted from blood samples and dried blood smears using QIAamp DNA extraction kits (QIAGEN, Germantown, MD). Polymerase chain reaction (PCR) amplification was performed using a set of primers that is highly conserved among nematodes species. This set included primers for the 5S ribosomal gene spacer (5SF: 5′-GTTAAGCAACGTTGGGCCTGG-3′; 5SR: 5′-TTGACAGA-TCGGACGAGATG-3′),[7] a ribosomal RNA (rRNA) subunit gene of the mitochondrion 12S rDNA (12SF: 5′-GTTCCAGAATAATCGGCTA-3′, 12SR: 5′-ATTGACGGATGRTTTGTACC-3′),[8] and cytochrome oxidase I (COI; COIF: 5′-TGATTGGTGGTTTTGGTAA-3′, COIR: 5′-ATAAGTACGAGTATCAATATC-3′).[9] PCR was performed with 5 μL 10× High Fidelity PCR Buffer (Invitrogen, Carlsbad, CA), 1 μL 10 mM 2′-deoxynucleoside 5′-triphosphate (dNTP) mixture, 3 μL 50 mM MgSO4, 10 pmol each primer, 5 μL template DNA, 0.2 μL (1 unit) Platinum Taq High Fidelity polymerase (Invitrogen), and ddH_2O to make a total volume of 50 μL. Temperatures for PCR with the 5S and 12S primers were 94°C for 30 seconds, 55°C for 45 seconds, and 68°C for 1 minute for 40 cycles, and temperatures for PCR with COI were 94°C for 30 seconds, 52°C for 45 seconds, and 68°C for 1 minute for 40 cycles. PCR amplification products were analyzed by 2% agarose gel electrophoresis. PCR products corresponding to the expected sizes (380 bp for 5S, 450 bp for 12S, and 680 bp for COI) were cloned into TOPO-TA plasmid (Invitrogen) according to the manufacturer's protocol. Three clones were sequenced for each PCR product. Plasmid DNA was prepared with the QIAprep Miniprep kit (Qiagen) and sequenced with M13 primers in both directions. DNA sequences were analyzed by basic local alignment search tool (BLAST). Sequence alignment was performed by ClustalW method using MEGA 5.01 software.[10] Sequences from other filarial species were included for comparison, and only portions of the sequences that were well-aligned were selected for phylogenetic reconstruction using neighbor-joining[11] and bootstrap analysis (2,000 replicates).[12] Evolutionary distances were computed using the maximum composite likelihood method.[13] All novel sequences were submitted to GenBank (accession numbers JF412305–JF412347).

The phylogenetic reconstruction using 5srDNA (Figure 2) shows that the atypical Mf sequences are clustered into the *Mansonella* group and indistinguishable from *M. ozzardi* from Bolivia and Peru. The same clustering was obtained using primers for 12rDNA and COI. Unfortunately, no homologous *M. ozzardi* sequences from other regions were

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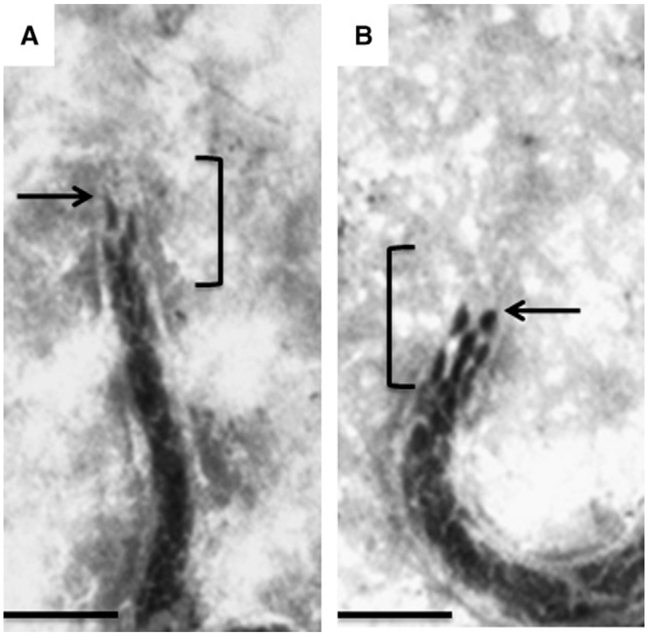

FIGURE 1. This figure shows the difference in the distribution of nuclei in the cephalic space (brackets) between (**A**) Mf of *M. ozzardi* and (**B**) atypical Mf from Peru. (Magnification: 1,000×; scale bar: 5 μm.) The atypical Mf have two nuclei followed by a single nucleus just caudal to the cephalic space (arrow), whereas the typical *M. ozzardi* Mf has a single nucleus at this location.

available in GenBank for comparison, except for the *M. ozzardi* from Peru that we sequenced during this study. *M. ozzardi* 12rDNA sequences obtained from two different blood samples (JF412317 and JF412318) were 100% identical. Also, these *M. ozzardi* sequences had 99% homology to the atypical Mf from two different regions (~850 km apart): La Union/Santa Clara (JF412323) and Cahuapanas (JF412324). Other *Mansonella* species, such as *M. perstans* and *M.* (*Tetrapetalonema*) *atelensis amazonae*, had 84% and 87% homology, respectively, to the atypical Mf 12rDNA sequences. However, COI sequences of the atypical Mf from Cahuapanas (JF412336) and La Union/Santa Clara (JF412331) had 99% homology to *M.* (*Mansonella*) *ozzardi* (e.g., JF412344), 83% homology to *M.* (*Tetrapetalonema*) *atelensis amazonae*, and 80% homology to *M*. (*Cutifilaria*) *perforata*.

The length of the atypical Mf in dried blood smears examined in the present study was approximately 120–130 μm. For comparison, dried Giemsa-stained Mf of *M. ozzardi* from the same region in Peru had a length of about 120 μm. Of note, the atypical Mf previously reported from Brazil was described as being similar in size to *M. ozzardi*, with lengths of 198–247 μm.[4] Previous studies have reported variable lengths for *M. ozzardi* Mf from South America (between 149 and 228 μm).[14] More accurate measurements of Mf of *M.* (*Mansonella*) *ozzardi* were obtained from experimental infections of patas monkeys. Formalin-fixed and hematoxylin-stained Mf were 207–232 (mean = 220) μm long and 3–4 μm in diameter, whereas Mf in methanol-fixed hematoxylin or Giemsa-stained thick blood films were 185–214 (mean = 200) μm long and 4–5 μm in diameter.[7] Differences in specimen preservation and staining methods may explain some of the variation.

We found that the atypical Mf from Peru could not be differentiated from *M.* (*Mansonella*) *ozzardi* based on three different DNA markers that are commonly used for phylogenetic analysis of filarial parasites.[8,9,15] Limitations of our study include the lack of *M.* (*Mansonella*) *ozzardi* DNA sequences in Genbank for comparison (especially for 12S rDNA and COI sequences) and the small sample size.

Molecular identification is complimentary to microscopy for identifying filarial species. For example, a nested PCR-based assay easily differentiated between *M.* (*E.*) *streptocerca* and *O. volvulus*, which are difficult to distinguish by morphological examination.[16] Our results show that the atypical Mf from Peru are identical or very closely related to *M. ozzardi*. Minor morphological differences described in preliminary reports may reflect natural variation within the species[5] or can be artificially seen from stained fixed Mf in slides during the field study because of irregular contractures of the Mf body that can also modify the length as described above.

Molecular markers are useful tools for characterizing new nematode species together with other information, such as geographic location, morphological features, transmission patterns, hosts, and pathological effects.[17] Restricted access to remote areas, such as those areas in the rainforests of South America, has held back studies of *M.* (*Mansonella*) *ozzardi*, despite its discovery more than 100 years ago.[18] The origin, distribution, and transmission of *M.* (*Mansonella*) *ozzardi* in Latin America are poorly understood. Vectors for *M.* (*Mansonella*) *ozzardi* include various species of blackflies and ceratopogonid midges. Although the vector of *M.* (*Mansonella*) *ozzardi* in Peru has not been established, studies from the Brazilian Amazon implicate the blackfly species *Simulium amazonicum* and *Simulium n.s.p.* in the transmission[19]. In South America, the phylogenetic relationship of *M.* (*Mansonella*) *ozzardi* to animal filariae is not clear. However, in North America, previous studies have proposed that *M. ozzardi* is most closely related to *M.* (*Mansonella*) *llewellyni*, a parasite of raccoons, and *M.* (*Mansonella*) *interstitium*, which is from squirrels.[7] It is possible that Amerindians were first infected by *M.* (*Mansonella*) *ozzardi* from similar host animals in South America and that the parasite was introduced into islands such as Haiti and Trinidad when humans from South America colonized islands in the Caribbean.[20]

The first documented human case of *M.* (*Mansonella*) *ozzardi* in Peru was reported more than 50 years ago in the same region where the atypical Mf studied in this paper were collected.[21] Additional human cases of *M.* (*Mansonella*) *ozzardi* infection have been reported from the same region (Loreto),[22] with Mf prevalence rates as high as 25–35%.[23,24] Other than *M.* (*Mansonella*) *ozzardi* infection, very few filarial infections have been reported from Peru. A zoonotic *Brugia* spp. infection was reported in a tourist who had been camping in the jungle near Loreto.[25] Two humans cases of *Dirofilaria* spp. and a possible infection with an *Onchocerca* spp. were recently reported.[26] Methods and new sequences from this study may be useful for characterizing unusual Mf that might be identified in the future in South America.

In conclusion, we found no genetic evidence to justify classification of the atypical Mf from Peru as novel species. The slightly different arrangement of the first nuclei behind the cephalic space is most likely caused by natural variation within the species *M. ozzardi*.

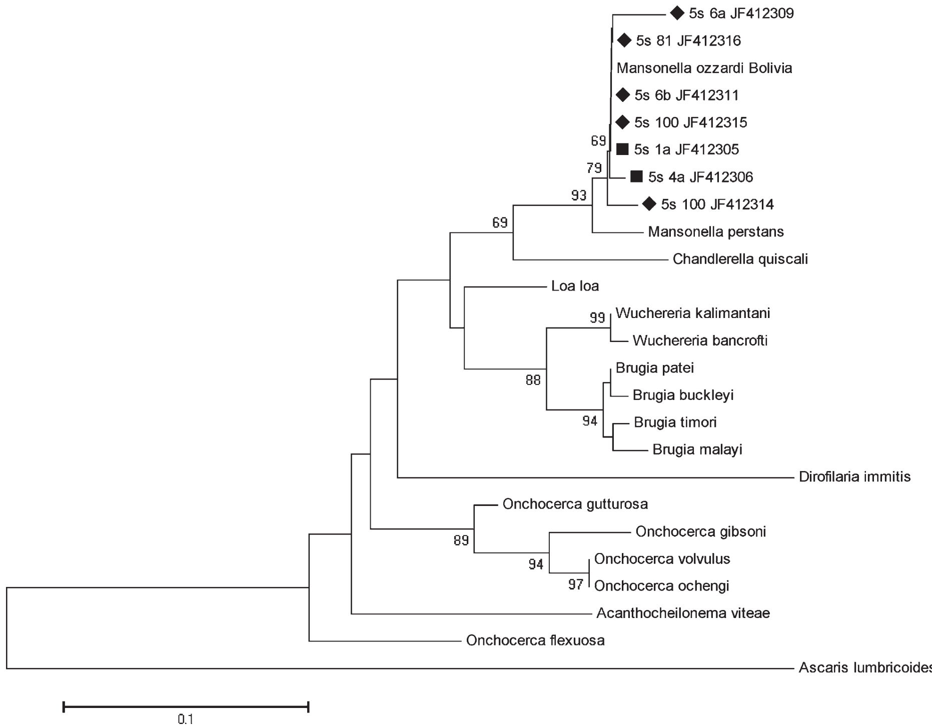

FIGURE 2. Phylogeny of the atypical Mf from Peru (◆ = JF412309 and JF412311 sequences are from La Union/Santa Clara and JF412315 and JF412316 sequences are from Cahuapanas) and *M. ozzardi* from Loreto, Peru (■ = JF412305 and JF412306 sequences are from La Union/Santa Clara) based on 5SrDNA gene sequence. *Ascaris lumbricoides* was used as an outgroup. Bootstrap confidence intervals after 2,000 replicates are shown at the nodes only for values higher than 50%, and the scale bar indicates distances based on substitutions per nucleotide. Tree reconstruction was inferred using the neighbor-joining method. All positions containing gaps and missing data were eliminated. There was a total of 147 positions in the final dataset. The only *M. ozzardi* sequence available for comparison was *M. ozzardi* from Bolivia (AJ279033).

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