Clinical Infectious Diseases

BRIEF REPORT

Deep Sequencing Reveals Occult Mansonellosis Coinfections in Residents From the Brazilian Amazon Village of São Gabriel da Cachoeira

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Mansonella ozzardi and *Mansonella perstans* infections both cause mansonellosis but are usually treated differently. Using a real-time polymerase chain reaction assay and deep sequencing, we reveal the presence of mansonellosis coinfections that were undetectable by standard diagnostic methods. Our results confirm mansonellosis coinfections and have important implications for the disease's treatment and diagnosis.

Keywords. *Mansonella perstans*; *Mansonella ozzardi*; mansonellosis; coinfections; Brazil.

Typically, individuals found to be infected with *Mansonella ozzardi* are treated with ivermectin, whereas individuals infected with *Mansonella perstans* (which cannot effectively be treated with ivermectin) are treated with diethylcarbamazine (DEC) or mebendazole—neither of which is an effective treatment for *M. ozzardi* parasitemia [1]. How individuals who are coinfected with both parasites should be treated is therefore not immediately obvious. However, ever since *M. ozzardi* and *M. perstans* were first discovered to occur in the New World >100 years ago, the question as to whether the 2 parasites could coinfect the same human host has been controversial. As a consequence, no clinical guidelines on how such infections should be treated have yet been formulated [1–5].

After the publication of several molecular studies showing that Latin American *Mansonella* microfilariae reported to be "new species" or "atypical" forms of *M. ozzardi* were actually genetically identical to standard forms of *M. ozzardi*, some researchers began to question whether *M. perstans* even occurred in Latin America [1–3, 6]. In 2017, we recovered *M. perstans* CO1, 12S, and ITS-1 DNA sequences from the blood of São Gabriel da Cachoeira residents and in this way provided definitive evidence of the parasite's arrival in the New World [2]. To date, however, the age-old question as whether *M. ozzardi* and *M. perstans* coinfections actually occur and the related question as to how such infections ought to be best treated have both remained unanswered [1].

In this work we have utilized a recently developed ribosomal DNA ITS-1 real-time polymerase chain reaction (rt-PCR) assay, previously deployed to detected filarial parasites in Africa [7], to survey blood samples of individuals known to reside in an area where *M. ozzardi* and *M. perstans* occur sympatrically [2]. The rt-PCR assay contained *M. ozzardi* (GCAGCAACATATAGTTTTTTGC)– and *M. perstans* (CTATTCACTTTTATTTAGCAACATGC)–specific and fluorescently labeled probes that allow for the detection of both *M. ozzardi* and *M. perstans* monoinfection and coinfections. Applying this rt-PCR to blood samples obtained from residents of São Gabriel da Cachoeira, which microscopy and multilocus sequence typing (MLST) classified as being either monoinfected for *M. perstans* (n = 2) or *M. ozzardi* (n = 8), we were able to identify 2 putative occult coinfections (Table 1).

To further clarify the status of these putative coinfected samples, we performed a set of 4 separate Illumina HiSeq shot-gun deep sequencing runs (Table 1). Sequence reads were filtered to remove human DNA sequences and then pooled and used to assemble 2 mitogenome reference sequences, which have been annotated and deposited in GenBank following an approach described previously (the accession numbers referred to in this sentence are: MN432519; MN432520; MN432521; MN416134) [8]. Two large reference ribosomal DNA contigs representing 6397 nucleotides of the *M. ozzardi* ribosomal locus (MN432519) and 6976 nucleotides of the *M. perstans* locus (MN432520) were also assembled, annotated, and deposited in GenBank in a similar manner. The São Gabriel da Cachoeira *M. ozzardi* reference mitogenome sequence (MN416134) we have assembled for this study is >99% identical to that reported from the southern Amazon region of Tefé [8]; the *M. perstans* reference mitogenome (MN432521) that we have assembled is the first from the species to be published, however, the CO1 and 12S mitochondrial reference sequences contained within it share >99% identity to previously published reference sequences obtained from the region [2]. The 2 ribosomal reference sequences we have recovered also share high levels of identity (>99%) with

Received 1 October 2019; editorial decision 21 January 2020; accepted 24 January 2020; published online January 29, 2020.

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Clinical Infectious Diseases® 2020;XX(XX):1–4

DOI: 10.1093/cid/ciaa082

Table 1. Classification of Filarial Parasite Infections From 11 Residents of Amazonas State

Sample	Microscope Classification	Microfilarial Load (mf/mL)	ITS-1 Classification	CO1 Classification	12S Classification	Real-time PCR Classification	Mitochondrial DNA Recovered From Our Deep Sequence Data Analysis	Ribosomal DNA Recovered From Our Deep Sequence Data Analysis	*Wolbachia* DNA Recovered From Our Deep Sequence Analysis
SGC-100	*Mansonella ozzardi*	100	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*			
SGC-136	*Mansonella perstans*	100	*M. perstans*	*M. perstans*	*M. perstans*	*M. ozzardi* + *M. perstans*	*M. perstans* 127.2 Kb covering 100% of the MN432521 mitogenome; *M. ozzardi* 305.9 Kb covering 99.96% of the MN416134 mitogenome	*M. perstans* 1.8 Kb covering 27.5% of the MN432520 ribosomal DNA reference sequence; *M. ozzardi* 2.3 Kb covering 30.18% of the MN432519 ribosomal DNA reference sequence	20 Kb of sequence data mapped to *Wolbachia* genome NZ_AP013028; SNP patterns consistent with the occurrence of 2 *Wolbachia* strains observed in the 16S gene sequence
SGC-157	*M. ozzardi*	250	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*			
SGC-190	*M. ozzardi*	9500	*M. ozzardi*	*M. ozzardi*		*M. ozzardi*	*M. ozzardi* 11 124 Kb covering 100% of the MN416134 mitogenome	*M. ozzardi* 38.9 Kb covering 89.49% of the MN432519 ribosomal DNA reference sequence	2910.9 Kb of sequence data mapped to *Wolbachia* genome NZ_AP013028
SGC-159	*M. ozzardi*	750	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	…	…	…
SGC-172	*M. ozzardi*	250	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	…	…	…
SGC-173	*M. ozzardi*	550	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	…	…	…
SGC-220	*M. ozzardi*	450	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	…	…	…
SGC-723	*M. ozzardi*	100	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	…	…	…
SGC-783	*M. perstans*	50	*M. perstans*	*M. perstans*	*M. perstans*	*M. ozzardi* + *M. perstans*	*M. perstans* 456.7 Kb covering 100% of the MN432521 mitogenome; *M. ozzardi* 86.3 Kb covering 97.4% of the MN416134 mitogenome	*M. perstans* 38.5 Kb covering 98.70% of the MN432520 ribosomal DNA reference sequence; *M. ozzardi* 30.6 Kb covering partial 89.7% of the MN432519 ribosomal DNA reference sequence	250 Kb sequence data mapped to the *Wolbachia* genome NZ_AP013028. SNP patterns consistent with the occurrence of 2 *Wolbachia* strains were observed in the 16S, coxB, IMP dehydrogenase, insulinase gene sequences
Tefe-466	*M. ozzardi*	≥50	*M. ozzardi*	*M. ozzardi*		*M. ozzardi*	*M. ozzardi* 302 Kb covering 99.2% of the MN416134 mitogenome	*M. ozzardi* 514.9 Kb covering 97.8% of the MN432519 ribosomal DNA reference sequence	474 kb of sequence data mapped to *Wolbachia* genome NZ_AP013028

The 10 blood samples taken from residents of our study area in São Gabriel do Cachoeira have an SGC prefix in their names and were collected and analyzed for a project that received ethical clearance from the Instituto Oswaldo Cruz (reference number 41678515.1.0000.5248). The blood sample from Tefé (where repeated blood smear surveys have failed to detect *M. perstans*) was collected and analyzed for a project with approval from the ethics committee of the Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (reference number 1504/10). The microscope classification and microfilariae counts shown in this table were made using thick blood smears and approximately 20 μL of blood. Multilocus sequence typing (MLST) and real-time PCR classifications were made using DNA extracts prepared with QIAGEN blood and tissue kits and 200 μL of ethylenediaminetetraacetic acid–preserved venous blood. Our MLST PCRs used the same filarial parasite identifier sequence targets, PCR primers, and protocols that have been used for previous analyses of this type [2, 8]. This table also shows the quantity of mitochondrial, ribosomal DNA, and *Wolbachia* contigs detected from 4 shotgun sequence libraries prepared from samples SGC-136, SGC-190, SGC-783, and Tefe-466. Not one sequence read (≥100 nucleotides) recovered from the *M. ozzardi*–monoinfected libraries of samples SGC-190 and Tefe-466 matched the *M. perstans* mitogenome reference sequence MN432521 or the variable regions of ribosomal DNA reference sequence MN432520 with ≥97% identity; therefore, no *M. perstans* data are shown for these samples in this table.

Abbreviations: IMP, inosine-5′-monophosphate dehydrogenase; mf, microfilariae; PCR, polymerase chain reaction; SGC, Sao Gabriel do Cachoeira; SNP, single-nucleotide polymorphism.

previously published species-specific taxonomic identifier reference sequences [2, 8]. Our deep sequence analysis recovered complete or near-complete *M. ozzardi* and *M. perstans* mitogenome sequences from both of our putative coinfected samples and also managed to detect substantial portions of both parasites' ribosomal DNA (Table 1).

We believe these results have 2 important implications for mansonellosis diagnosis and treatment. First, our results lend weight to the legitimacy of historic, light microscopy–based reports that mansonellosis coinfections are very common throughout northern Brazil, Venezuela, Colombia, Guyana, French Guiana, and Suriname [4, 5, 9]. The second related implication is that conventional diagnostic methods, both microscopy and/or standard PCR assays [1], can miss coinfections and this may lead to inappropriate and ineffective treatment selection. In our study both samples that were classified by traditional methods as *M. perstans* monoinfections were found, through rt-PCR and deep sequencing, to also have occult *M. ozzardi* infections.

At the end of the 19th century, Sir Patrick Manson reported the occurrence of Guyanese residents with both "sharp-tailed" and "blunt-tailed" microfilariae in their blood and proposed that these individuals might have *M. ozzardi* and *M. perstans* coinfection [4]. However, Daniels argued that if Manson's proposal was correct, he should be able to find blunt-tailed microfilariae (*M. perstans*) monoinfections in some Guyanese residents [5]. Reporting that whenever he found blunt-tailed microfilariae (*M. perstans*) he could invariably find sharp-tailed microfilariae in the same individual by examining enough blood, Daniels questioned the occurrence of *M. perstans* monoinfections in Guyana and indeed the occurrence of *M. perstans* and *Mansonella* coinfections in Latin America [5]. Daniels proposed instead that Guyanese residents with sharp- and blunt- tailed microfilariae in their blood might be infected with a novel species of filarial parasite with 2 different blood stages [5]. Given that we can now be confident that Manson's coinfection proposal was correct, Daniel's observation can be seen in a different light that fits with our data. It suggests that Latin American *M. perstans* infections are often or always accompanied with *M. ozzardi* infections and, thus, that Latin American *Mansonella* infections may never (or only rarely) be treated effectively with DEC and mebendazole.

The importance of these observations to Latin American mansonellosis epidemiology, treatment, and control can be illustrated by considering a typical mansonellosis epidemiological scenario from northeastern Colombia. In a thick blood smear survey conducted in Comisaria del Guainia, 42 of 152 (27.6%) individuals found with *Mansonella* parasitemia were recorded as having coinfections, with 35 of 152 (22.4%) recorded having *M. perstans* monoinfections. This means that >50% of *Mansonella* microfilaremia in Comisaria del Guainia would not be effectively treated with ivermectin, and only 0–24% would be effectively treated with DEC and mebendazole. Our results therefore highlight the potential utility of new diagnostic tools that can detect occult coinfections and in this way minimize the chance of an individual being misdiagnosed and mistreated. It is, however, important to note that while the rt-PCR assay we used for this study (and a recently developed field-friendly loop-mediated isothermal amplification assay [10]) has the potential to detect coinfections missed by MLST-based classification, no existing diagnostic method can reliably detect latent infections. Thus, there is still a need for new diagnostic tools and therapies that can detect and treat all kinds of *Mansonella* infection, including monoinfections, coinfections, and latent infections.

Doxycycline therapies have repeatedly been shown to be effective for the treatment of *M. perstans* infections and could also be effective for *M. ozzardi* and *Mansonella* coinfections as *M. ozzardi* parasites also harbor the *Wolbachia* endosymbionts [1, 11, 12]. Given that *Wolbachia*-targeting treatments are potentially curative, there are clear advantages for the development of *Wolbachia*-targeting treatments for use in the Amazon region. Conventional Amazon treatments only temporarily clear *Mansonella* microfilaremia and, as our work shows, will very likely be ineffective for large numbers of people. Although only doxycycline has been tested for *Mansonella* infections [1, 12], there are a number of other very promising *Wolbachia*-targeting treatments with shorter treatment courses presently being developed for the control and elimination of other filarial diseases such as onchocerciasis and lymphatic filariasis [1, 10]. As can be seen in Table 1, we detected *Wolbachia* in both our coinfected individuals from São Gabriel da Cachoeira, suggesting that anti-*Wolbachia* therapeutics might effectively treat them. There is thus a strong case for developing novel drug treatments by transferring successful *Wolbachia*-targeting treatments from other filarial diseases rather than by developing novel combination therapies based on existing microfilaremia-clearing treatments such as ivermectin or DEC and mebendazole.

Beyond treatment and diagnosis, the fact that our results suggest that *Mansonella* coinfections are common throughout the Amazon region also suggests that applying the ecological theory of competitive exclusion to the distribution of filarial parasites needs to be done with great care, at least in Latin America. Since both *M. perstans* and *M. ozzardi* adults are reportedly found in the peritoneum, the microfilariae lack obvious periodicity and occur commonly in the circulating blood (and occasionally in the skin), and both are transmitted by *Culicoides* biting midges, the 2 parasites appear to exist in similar ecological niches [1, 13]. While the relatively recent (in evolutionary terms) arrival of *M. perstans* in the Americas [2] might explain why these 2 parasites have not yet diverged into more distinct ecological niches or why 1 of the 2 parasites has not yet been competitively excluded, it does also question whether recent mass human migration makes the use of ecological theories to map filarial parasites unsafe [11].

Notes

Financial support. This work was supported by the Fundação de Amparo à Pesquisa do Estado do Amazonas (grant numbers 062.00647/2014, 062.01282/2018, and 002.00200/2019). A. C. P. V. and F. A. C. P. received fellowships from the Brazilian National Council for Scientific and Technological Development (CNPQ).

Potential conflicts of interest. The authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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