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Deep-sequencing reveals occult mansonellosis co-infections in residents from the Brazilian Amazon village of São Gabriel da Cachoeira

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ABSTRACT

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

Key words: *Mansonella perstans*, *Mansonella ozzardi*, Mansonellosis, co-infections, Brazil.

Typically, individuals found to be infected with *Mansonella ozzardi* are treated with ivermectin, whereas individuals infected with *M. perstans* (which cannot effectively be treated with ivermectin) are treated with diethylcarbamazine (DEC) and mebenzadole— neither of which are effective treatments for *M. ozzardi* parasitaemia [1]. How individuals that are co-infected with both parasites should be treated is, thus, not immediately obvious. However, ever since *M. ozzardi* and *M. perstans* were first discovered to occur in the New World more than 100 years ago, the question as to whether the two parasites could co-infect 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* co-infections 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 (rDNA) ITS-1 real-time PCR (rtPCR) 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 rtPCR assay contained *M. ozzardi* (GCAGCAACATATAGTTTTTTGC) and *M. perstans* (CTATTCACTTTTATTTAGCAACATGC) specific and fluorescently labelled probes which allow for the detection of both *M. ozzardi* and *M. perstans* mono and co-infections. Applying this rtPCR to blood samples obtained from residents of São Gabriel da Cachoeira, which microscopy and Multi-Locus Sequence Typing (MLST) classified as being either mono-infected for *M. perstans* (n= 2) or *M. ozzardi* (n= 8), we were able to identify two putative occult co-infections (Table 1).

In order to further clarify the status of these putative co-infected samples, we performed a set of four separate illumina HiSeq shot-gun deep sequencing runs (see table 1). Sequence reads were filtered to remove human DNA sequences and then pooled and used to assemble two mitogenome reference

sequences which have been annotated and deposited in GenBank following an approach described previously [8]. Two large reference ribosomal DNA contigs representing 6,397 nucleotides of the *M. ozzardi* ribosomal locus (MN432519) and 6,976 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 two ribosomal reference sequences we have recovered also share high levels of identity (>99%) with previously published species-specific taxonomic identifier reference sequences [2,8]. As can be seen in Table 1, our deep sequence analysis recovered complete or near-complete *M. ozzardi* and *M. perstans* mitogenome sequences from both of our putative co-infected samples and also managed to detected substantial portions of both parasite´s ribosomal DNA.

We believe these results have two important implications for mansonellosis diagnosis and treatment. Firstly, our results lend weight to the legitimacy of historic, light-microscopy-based, reports that mansonellosis co-infections are very common through-out northern Brazil, Venezuela, Colombia, Guyana, French Guiana and Surinam [4,5,9]. The second related implication is that conventional diagnostic methods, both microscopy and/or standard PCR assays [1], can miss co-infections and this may lead to inappropriate and ineffective treatment selection. In our study both samples that were classified by traditional methods as *M. perstans* mono-infections were found, through rtPCR 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” and “blunt” tailed microfilariae in their blood and proposed these individuals might have *M. ozzardi* and *M. perstans* co-infections [4]. However, Daniels argued that if Manson´s proposal was correct he should be able to find blunt-tailed microfilariae (*M. perstans*) mono-infections 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* mono-infections 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 two different blood stages [5]. Given that we can now be confident that Manson´s co-infection 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 mebenzadole.

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 27.6% (42/152) of individuals found with *Mansonella* parasitaemia were recorded as having co-infections, with 22.4% (35/152) recorded having *M. perstans* mono-infections. This means that >50% of *Mansonella* microfilaraemia in Comisaria del Guainia would not be effectively treated with ivermectin and only between 0 and 24% would be effectively treated with DEC and mebenzadole. Our results, therefore, highlight the potential utility of new diagnostic tools that can detect occult co-infections and in this way minimize the chance of an individual being mis-diagnosed and mis-treated. It is, however, important to note that while the rtPCR we used for this study (and a recently developed field-friendly LAMP assay [10]) have the potential to detect co-infections missed by MLST-based classification, no existing diagnostic method can reliably detect latent infections. There is, thus, still a need for new diagnostic tools and therapies that can detect and treat all kinds of *Mansonella* infection, including mono-infections, co-infections 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* co-infections since *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* microfilaraemia 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 co-infected individuals from São Gabriel da Cachoeira suggesting 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 microfilaraemia-clearing treatments, like ivermectin or DEC and mebenzadole.

Beyond treatment and diagnosis, the fact that our results suggest that *Mansonella* co-infections 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 two parasites appear to be existing 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 two parasites have not yet diverged into more distinct ecological niches or why one of the two 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].

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Table 1 legend

The classification of filarial parasite infections from 11 residents of Amazonas state. The 10 blood samples taken from residents of our study area in Sao Gabriel do Cachoeira have a SGC prefix in their names and were collected and analysed for a project that received ethical clearance from the Instituto Oswaldo Cruz (ref#: 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 (ref#:1504/10). The microscope classification and microfilariae counts show in this table were made using thick blood smears and ~20µL of blood. MLST and real-time PCR classifications were made using DNA extracts prepared with QIAGEN Blood and tissue kits and 200 µL of EDTA-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 four shotgun sequence libraries prepared from samples SGC-136, SGC-190 and, SGC-783 and Tefe-466. Not one sequence read (≥100 nucleotides) recovered from the *M.ozzardi* mono-infected libraries of samples SGC-190 and Tefe-466 matched the *M. perstans* mitogenome reference sequence MN432521 or the variable regions of rDNA refence sequence MN432520 with ≥97% identity and therefor no *M. perstans* data is shown for these samples in this table.

Sample	Microscope classification	Microfilarial load (in microfilariae per 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	*M. ozzardi*	100	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*			
SGC-136	*M. 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 two *Wolbachia* strains were observed in the 16S gene sequence.
SGC-157	*M. ozzardi*	250	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*	*M. ozzardi*			
SGC-190	*M. ozzardi*	9,500	*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.	2,910.9 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	***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	250 kb sequence data mapped to the *Wolbachia* genome NZ_AP013028. SNP patterns consistent with the occurrence of two *Wolbachia* strains were observed in the 16S, coxB, IMP dehydrogenase, insulinase gene sequences.

							MN416134 mitogenome.	ribosomal DNA reference sequence.	
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.