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Molecular identification of Wolbachia from the filarial nematode

Mansonella perstans

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Abstract

Wolbachiae are bacterial endosymbionts of insects and many filarial nematodes whose products trigger inflammation in filarial infections. The dependence of the parasites on their endosymbionts has also led to the use of antibiotics directed against the Wolbachiae, therapy that has been demonstrated to have a profound salutary effect on filarial infections. The identification of Wolbachiae in *Mansonella* species has been conclusively shown for *Mansonella ozzardi* (Mo), but not for *Mansonella perstans* (Mp) Using primers known to amplify the 16S ribosomal DNA of other filarial Wolbachiae, an identical 1393 bp band was found in all samples tested. Sequence analysis of these samples demonstrated a single consensus sequence for Mp Wolbachia 16S rDNA that was most similar to Wolbachia sequences from other filarial nematodes. When aligned with the only other *Mansonella* Wolbachia sequence (Mo) there were only 8 nucleotide differences in the 1369 bp overlapping sequence. Phylogenetic dendrograms, showing the relationship of the Mp Wolbachia to other Wolbachia 16S rDNA, tracked almost identically to the 5S rRNA of their parasite host. Wolbachia surface protein (WSP) was also demonstrated in protein extracted from Mp-containing whole blood. In advance of a treatment trial of Mp, a method for the quantitation of Mp Wolbachia was developed and used to demonstrate not only a relationship between microfilarial numbers and Wolbachia copy numbers, but also to demonstrate the effect of antibiotic on ridding Mp of Wolbachia.

Keywords

Wolbachia; endosymbiont; *Mansonella*; ribosomal

1. Introduction

Mansonella perstans is a filarial nematode endemic in tropical portions of Africa and South America. Despite very high prevalences in some regions, very little pathology has been directly attributable to this parasite, although transient angioedema, abdominal pain, and pericarditis each has been associated with infection with *Mansonella perstans* [1-3].

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Wolbachiae are bacterial endosymbionts of insects and many filarial nematodes. It has been postulated that these bacteria and their products trigger inflammatory responses that result in filarial-induced lymphedema and elephantiasis [4,5] and onchocercal ocular disease [6] as well as reactions seen following drug treatment of onchocerciasis [7] and lymphatic filariasis [8, 9]. The dependence of the parasites on their endosymbionts has also led to the use of antibiotics directed against the Wolbachiae, antibiotics that have been demonstrated to have a profound salutary effect on filarial infections [10-12].

The identification of Wolbachiae in *Mansonella* species has been conclusively shown for *Mansonella ozzardi* [13]. For Mp, however, one study using microfilariae of Mp from Gabon was unable to demonstrate Wolbachiae in Mp [14] using PCR. Using microfilariae from microfilaremic patients in Uganda, Fischer et al was also unable to detect Wolbachia in Mp (Peter Fischer personal communication and [15]).

Here we report the molecular identification of a Wolbachia endosymbiont in *Mansonella perstans*, report on its phylogenetic relationship to other Wolbachiae, demonstrate immunologic reactivity with antibodies to the Wolbachia surface protein (WSP) and provide a method for quantitating Mp Wolbachia. Moreover, we have been able to utilize this method to demonstrate the loss of Wolbachiae in Mp microfilaremic patients treated with six weeks of doxycycline.

The presence of bacterial endosymbionts in a largely non-pathogenic filarial species may shed some light on the precise role of Wolbachia in inflammatory conditions associated with other filarial infections. It also suggests that, under the rare circumstances in which Mp is suspected of causing pathology, antibiotics directed against the Wolbachiae (i.e., doxycycline) may be able to modify the disease process and parasite levels.

2. Materials and methods

2.1 *Mansonella perstans*

During a clinical study of post-treatment reactions in *Wuchereria bancrofti* in Mali [16], a number of volunteers were identified who were microfilaremic for *Mansonella perstans* but uninfected with *W. bancrofti* (as determined by circulating filarial antigen testing and midnight blood smears). Individual, frozen, EDTA containing blood samples from 12 of these individuals were the initial source of *Mansonella perstans* DNA. These individuals had microfilarial counts that ranged from 17 to 3,300 Mp mf per ml. Identification of the *Mansonella perstans* was made based on morphologic criteria of diurnal thick smears (unsheathed blood-borne microfilariae, nuclear column extending into the tip of a thick blunt tail).

2.2 DNA extraction and PCR

DNA was extracted using a protocol adapted from Williams et al [17]. Briefly, to 200µL of centrifuged whole blood was added a single zinc BB in a microfuge tube. This tube was vortexed in two dimensions over a 20-minute period. DNA was then extracted using the QiaAmp DNAeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The DNA was subsequently stored at 4° until used.

PCR for Wolbachia was performed with primers for the 16S rDNA gene as published previously [18], using the following sequence: 16S forward: GAG AGT TTG ATC CTG GCT CAG; 16S reverse: CTA CGG CTA CCT TGT TAC GA.

Mansonella perstans was confirmed by PCR using primers for the internal transcribed spacer (ITS) in the 5S rRNA gene, according to previously published methods [19] by using primers

with the following sequence: S2: GTT AAG CAA CGT TGG GCC TGG; S16: TTG ACA GAT CGG ACG AGA TG.

Master mix was made for 25 μ L reactions to have 2.5 μ L of 10x master mix (0.75 μ L of 50mM MgCl₂, 0.5 μ L of 2.5mM dNTP's, 0.7 μ L of 10 μ M primers per reaction, 1 μ L of Amplitaq™ [ABI]) with 5 μ L of extracted DNA as the template. Thermal cycling was done with 94° for 1 min followed by 35 cycles at 94° for 30 sec, annealing at 52° for 1min, extension at 72° for 3 min with a final 72° extension for 9 min.

2.3 Cloning and sequencing

PCR products were cloned in the vector pCR®2.1-TOPO (Invitrogen) according to the kit manufacturer's instructions. Purified plasmid was obtained from overnight cultures of transformed Top10 *E. coli* using the QiaPrep Spin MiniPrep Kit (Qiagen) according to manufacturer's instructions. Sequencing of multiple plasmids was done in both directions using standard automated sequencing techniques. The consensus sequence has been deposited in Genbank's non-redundant nucleotide database (accession number AY278355)

2.4 Quantitative PCR

A method for quantitating copy numbers of Mp and the Wolbachia from Mp using the strategy illustrated in Fig. 4A. Because of co-endemicity of *Wuchereria bancrofti* in Mali, speciation of both Wb and Mp and their respective Wolbachia was also developed. Using primers (as illustrated) and FAM-labeled probes, along with standard curves generated by plasmid DNA containing the respective ribosomal genes, quantitation in an ABI 7900HT could be made using standard conditions recommended by the manufacturer.

The cloned 16S plasmid of the Wolbachia endosymbiont was purified, and the plasmid DNA was quantified using a spectrophotometer. This quantified plasmid was aliquoted for use in generating a standard curve for the quantitative PCR reactions. For the reactions, 5 μ L of extracted DNA or serial dilutions of the standards were combined with 20 μ L master mix in 96-well MicroAmp™ optical plates (ABI). Thermal cycling and data analysis were done on the ABI7900HT.

2.5 Immunologic reactivity using anti-WSP antibody

Because only fixed whole blood was available for protein staining, 1 ml of formalin-fixed blood from a patient (*W. bancrofti* negative) with *M. perstans* containing ~2108 microfilariae was pelleted at 14000 rpm. Protein was extracted from the pellet using the Qproteome FFPE kit (Qiagen) exactly as described by the manufacturer. Ninety-six well plates (Immulon4, Dynatech) were coated with this extracted protein at a concentration of 1 μ g/ml overnight at 4° C. The plates were blocked with 5% BSA in PBS-0.05%Tween20 for 1 hour and washed 6 times. High titer, individual rabbit antibodies raised against 2 separate KLH-coupled WSP-specific immunogenic peptides (CAQKDSKTNDLY and CSEEVNKGTSSEDK) were added in multiple dilutions (in PBS/Tween/0.1%BSA) as were rabbit sera obtained prior to immunization (pre-bleeds). The rabbit antisera was incubated for 2 hours at RT and washed. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc-specific) (Jackson ImmunoResearch) was added for 1 hour. The plate was washed and substrate (para nitrophenyl phosphate) was added and read in a multiwell reader (Molecular Dynamics).

2.6 Assessment of Wolbachia levels before and after doxycycline treatment

Blood from a small number of individuals (n = 23) with Mp microfilaremia treated with six weeks of doxycycline was examined for the presence of Mp wolbachia before and at one year following treatment using quantitative real-time PCR.

2.7 Phylogenetic analyses

Using the 16S rDNA sequence obtained from *M. perstans* as the query sequence we performed a BLAST search against the nonredundant nucleotide databases to identify homologues in the main Wolbachia lineages [20]. These 16S rDNA sequences were then aligned using the global alignment program ClustalW [21] with the default parameters. The 5S rDNA spacer sequences for the corresponding filarial nematodes [13,19] were aligned using the local alignment program, Muscle [22]. All multiple sequence alignments were visually inspected and manually improved before further analysis.

Phylogenetic analysis was performed with PAUP* 4.0b10 [23] using maximum likelihood distances with Neighbor-joining clustering and 2000 bootstrap replicates. Phylogenetic trees were drawn using Interactive Tree of Life (iTOL [24]).

2.8 Statistical analyses

Spearman rank correlation was used to determine correlations among variables. The analyses were performed using GraphPad (v4.0).

3. Results

Identification of the species as *Mansonella perstans* was made on morphologic criteria from thick smears of peripheral blood (Fig. 1). The absence of concomitant *Wuchereria bancrofti* was confirmed by midnight blood filtration and negative tests (ICT™ and TropBio™) for circulating filarial antigen.

Molecular speciation of the *M. perstans* was confirmed by sequencing the internal spacer region of the 5S rRNA gene (100% identity with published sequence, accession number U31640.1; GI:975833).

When primers known to amplify the 16S ribosomal DNA of other Wolbachia were used to amplify DNA obtained from blood of multiple, individual, whole blood samples of known Mp-infected individuals, an identical 1393 bp band was found in all samples tested. These products were cloned and sequenced in both directions. Results of sequence similarity searches using BLAST demonstrated that the 16S rDNA gave the best scores with the Wolbachia sequences from other filarial nematodes and indicated that the endosymbiont of *M. perstans* belonged to the genus Wolbachia. When aligned with the only other *Mansonella* wolbachia sequence (that from *Mansonella ozzardi* (AJ2790434) there were only eight nucleotide differences over the 1369 bp overlapping sequence (data not shown).

A fine-scale phylogenetic analysis of 16S rDNA sequences was performed on an alignment including most of the sequences available for filarial Wolbachia, some of the available sequences for the arthropods capable of transmitting filarial parasites (i.e. *Culex* spp) and representatives of most of the known Wolbachiae supergroups [20,25,26]. Phylogenetic dendrograms showing the relationship of the Mp Wolbachia 16S rDNA in relation to multiple other Wolbachiae (labeled according to host species) is shown in Fig. 2A. The similar, though not identical, dendrogram based on parasite 5S internal transcribed spacer sequences is shown in Fig. 2B. As can be seen, both the *M. perstans* 5S rRNA sequence and the Mp Wolbachia 16S rRNA sequences track with the only other *Mansonella* species for which both sequences were available. Moreover, based on the known phylogenetic relationships among the Wolbachiae, the Wolbachia from Mp groups with the F supergroup, the only supergroup that contains Wolbachia from both arthropods (i.e., termites) and filarial nematodes (i.e. *Mansonella ozzardi*). These analyses help to corroborate the notion that an independent horizontal transfer between the Wolbachia bacteria and the Mp parasites occurred ~ 100 million years ago [20].

To determine if Wolbachia protein could be detected in Mp, protein was extracted from formalin-fixed whole blood containing 2108 Mp mf/ml obtained from an Mp-infected individual. Using Wolbachia surface protein (WSP)-specific polyclonal antisera, it can be seen (Fig 3) that WSP could be specifically recognized in protein extracts of the mf-containing blood.

In advance of a treatment trial of *Mansonella perstans*, a method was developed for the quantitation of Mp Wolbachia and to differentiate it from the Wolbachia from *Wuchereria bancrofti*, a filarial species often found co-infecting patients with Mp, (Fig. 4). As can be seen, one can easily quantitate the Mp Wolbachia, specifically. Moreover, there was a correlation between the numbers of microfilariae in a given human blood sample and the relative copy number of the 16S Mp Wolbachia sequence ($p < 0.001$).

Finally, this real-time PCR method was used to demonstrate that antibiotics directed against the endosymbiont in Mp was useful in clearing the Wolbachia (Fig. 5). As seen, in 23 individuals followed longitudinally before and one year after a six-week course of doxycycline, all but one was able to completely clear their Wolbachia endosymbiont.

4. Discussion

We present molecular evidence that *Mansonella perstans* contains a bacterial endosymbiont with high sequence homology to Wolbachiae of other filarial nematodes, most notably *Mansonella ozzardi*.

Although Wolbachiae have been implicated as a source of pro-inflammatory products among those filarial infections of humans, the presence of a similar endosymbiont in a largely non-pathogenic filarial species suggests that the mere presence of bacterial endosymbionts is not sufficient to trigger pathology. This same conclusion could be made based on the fact that most filarial infections, even those known to induce pathology (*W. bancrofti*, *B. malayi*, *B. timori*, *O. volvulus*), cause generally asymptomatic (or subclinical) infection.

Other investigators, using methods similar to ours, have concluded that *Mansonella perstans* does not possess an endosymbiotic Wolbachia [14,15]. This may simply reflect technical or methodological differences in that we used a bead-based DNA extraction method shown to break open filarial parasites more efficiently than other methods [17].

The possibility that some geographically distinct strains of *Mansonella perstans* possess an endosymbiont while others do not remains a possibility. However, the notion that these Wolbachia and *Mansonella* species share a very long and co-dependent relationship [13,26], as suggested by the similarity in phylogenetic relationships (Fig. 2) makes this a little less likely. Nevertheless, there is clearly data to suggest that some very closely related filarial species (e.g. *Onchocerca volvulus* and *Onchocerca flexuosa*) have evolved in such a manner that one has and the other does not have the Wolbachia endosymbiont [27]. Moreover, the very recent data that lateral transfer can occur of an entire Wolbachia genome to its host, suggests that there is the possibility at least, that this process might have begun to occur in some geographic isolates of *Mansonella perstans* [28].

To date, there has been no effective therapy for clearing infection with *Mansonella perstans* [29,30]. With the demonstration that *M. perstans* possesses a Wolbachia endosymbiont, studies using doxycycline therapies are being investigated for their ability to clear Mp microfilariae. As a proof of concept, we have been able to demonstrate the loss of measurable Wolbachia DNA up to one year following a six-week course of doxycycline (see Fig. 5). These data suggest that Wolbachia can be targeted to rid humans of Mp when necessary, and also suggests there

may be effective therapeutic approaches to those rare patients whose symptoms or persistent eosinophilia have been attributed to this filarial species.

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Abbreviations

Mp	<i>Mansonella perstans</i>
Mo	<i>Mansonella ozzardi</i>
Mf	Microfilaria

WSP

Wolbachia surface protein

Fig. 1.
Individual microfilaria of *Mansonella perstans* (both panels) used for amplification of Wolbachial DNA. Note the lack of sheath, blunt tail with nuclei extending to the tail.

Fig. 2.

Phylogenetic relationships of *Mansonella perstans* and its Wolbachia sp. **Panel A:**

Phylogenetic relationship of the endosymbiont of *Mansonella perstans* among the Wolbachia of nematodes and arthropods. The tree, based on the 16S rDNA sequences, was obtained using a distance algorithm in PAUP. Bootstrap confidence values after 2000 replicates are shown.

Panel B: Phylogeny of filarial nematodes based on the 5S internal spacer. A distance algorithm-based tree was obtained. Bootstrap confidence values after 2000 replicates are shown.

Mansonella spp are in red.

Fig. 3. Rabbit anti-WSP recognizes Mp protein extracted from fixed whole blood. Optical densities (ODs) shown for two separate WSP-specific rabbit polyclonal antisera (solid lines) and the corresponding ODs using control (pre-bleed) rabbit sera (dashed lines).

A.

B.

C.

Fig. 4.

Quantitative and specific amplification of the endosymbiont of *Mansonella perstans*. **Panel A:** Sequence based strategy to detect the Wolbachia endosymbiont *Mansonella perstans* (Mp) and *Wuchereria bancrofti* (Wb). **Panel B:** Standard curve used to quantitate the Mp Wolbachia using dilutions of known quantities of Mp Wolbachia DNA plotted in terms of CT. Note: the higher the CT value, the lower the amount of input DNA. **Panel C:** Relationship between microfilarial numbers per ml of whole blood and the relative copy number of the Wolbachia of Mp.

Fig. 5.

Effect of doxycycline on *Mansonella perstans* Wolbachia. **Left panel:** shows relative copy number of Mp Wolbachia DNA before (2005) and one year following (2006) the administration of 6 weeks of doxycycline. Each dot represents an individual patient. **Right panel:** The percentage of individuals with demonstrable Mp wolbachia DNA before (2005) and 1 year (2006) following the administration of six weeks of doxycycline.