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MAJOR ARTICLE

Molecular Surveillance Detects High Prevalence of the Neglected Parasite *Mansonella ozzardi* in the Colombian Amazon

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Background: Mansonellosis is an undermapped insect-transmitted disease caused by filarial nematodes that are estimated to infect hundreds of millions of people. Despite their prevalence, there are many outstanding questions regarding the general biology and health impacts of the responsible parasites. Historical reports suggest that the Colombian Amazon is endemic for mansonellosis and may serve as an ideal location to pursue these questions. **Methods:** We deployed molecular and classical approaches to survey *Mansonella* prevalence among adults belonging to indigenous communities along the Amazon River and its tributaries near Leticia, Colombia. **Results:** Loop-mediated isothermal amplification (LAMP) assays on whole-blood samples detected a much higher prevalence of *Mansonella ozzardi* infection (approximately 40%) compared to blood smear microscopy or LAMP performed using plasma, likely reflecting greater sensitivity and the ability to detect low microfilaremias and occult infections. *Mansonella* infection rates increased with age and were higher among men. Genomic analysis confirmed the presence of *M. ozzardi* that clusters closely with strains sequenced in neighboring countries. We successfully cryopreserved *M. ozzardi* microfilariae, advancing the prospects of rearing infective larvae in controlled settings. **Conclusion:** These data suggest an underestimation of true mansonellosis prevalence, and we expect that these methods will help facilitate the study of mansonellosis in endemic and laboratory settings.

Keywords. LAMP; cryopreservation; diagnostics; epidemiology; filariasis; genomics; *Mansonella*; mansonellosis; parasitology.

Mansonellosis is a highly prevalent but undermapped [1] and understudied parasitic disease that infects hundreds of millions of people throughout Africa and Central and South America [2]. Three species of insect-transmitted parasitic nematodes (*Mansonella ozzardi, Mansonella perstans,* and *Mansonella streptocerca*) are responsible for the majority of human cases although other *Mansonella* species have the potential to infect humans, including the recently discovered *Mansonella* sp. "DEUX" which has revitalized demands for allocating resources to study this severely neglected disease [2–5].

There are major gaps in our understanding of the basic biology and clinical or subclinical impacts of mansonellosis in human populations. *Mansonella* infections underlie variable clinical presentations that are likely underrecognized [4] and may affect host immunity, altering vaccine responses and susceptibility to other pathogens [6–8]. Drugs used in mass drug administration campaigns for lymphatic filariasis and onchocerciasis are not as effective against *Mansonella* spp. [9–13], suggesting a unique genetic basis for anthelmintic resistance [14–16]. Additionally, *Mansonella* infections can introduce diagnostic challenges related to species misidentification [17], cross-reactivity of immunochromatographic tests [18, 19], and the inability to easily discern occult or amicrofilaremic infections [20]. Together, these limitations can potentially confound parasite elimination and surveillance programs focused on more prominent filarial parasites in coendemic regions [9–13, 16].

To address these challenges, we carried out epidemiological surveys of *Mansonella* prevalence in adults from villages along the Amazon River around the capital city of Leticia in the Amazonas Department of Colombia. Outdated surveys [21–23] and incidental findings from community health workers in the Amazon basin and neighboring countries [4, 20, 24] suggest a high prevalence of *M. ozzardi* [21–23] and this region is ideally situated to pursue fundamental questions about *Mansonella* biology, clinical presentations, and interactions with other pathogens that are endemic in the region.

The gold standard diagnostic for *M. perstans* and *M. ozzardi* is blood smear microscopy, which requires morphological

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differentiation of the circulating microfilariae stage [4, 17]. Molecular diagnostic approaches including real-time polymerase chain reaction (qPCR) [25–27] are largely restricted to laboratory settings. However, loop-mediated isothermal amplification (LAMP) assays with a simple colorimetric readout are more amenable to field studies [28]. We comparatively profiled LAMP assays with classical microscopy-based techniques to evaluate potential underestimation of parasite prevalence among a cluster of indigenous communities in the Colombian Amazon. Microfilariae were isolated for genomic analyses of *Mansonella* and for the establishment of a cryopreservation protocol to enable laboratory studies. Lastly, we probed associations of *Mansonella* infection status with other clinical and demographic variables. We expect that these data and methods will help facilitate future studies of *Mansonella*.

METHODS

Study Design and Sampling Procedure

This study was conducted between 2021 and 2023 in the Amazonas Department of Colombia. This region of vast biodiversity is largely populated by indigenous communities situated near the Amazon River and the capital city of Leticia and Puerto Nariño municipalities (Figure 1*A*). The study protocol was reviewed and approved by the University of Wisconsin-Madison institutional review board (study No. 2019–1107) and Corporación para Investigaciones Biológicas (No. 17022021). The study population (n = 235) from 13 communities was recruited by community leaders and health promoters who described the study to the community and individuals ≥18 years of age interested in participating were enrolled after providing written informed consent. Peripheral (venous) blood samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes and thin and thick blood smears were examined for the presence of filarial parasites and malaria. Serum/plasma were extracted and aliquoted along with whole blood into 1.5-mL tubes and stored at −80°C (Figure 1*B*). Self-reported sociodemographic and epidemiological data were collected using a structured questionnaire through verbal face-to-face interviews by trained study staff at the enrollment site.

Microscopy Smears and Microfilariae Quantification

Routine thin and thick smears were prepared at field sites at the time of blood draws. Slides were allowed to dry at room temperature, Giemsa stained, and examined microscopically. For quantitative microscopy, 40 µL of thawed whole blood was mixed with equal amounts of water and microfilaria were counted using light microscopy. For filtration of thawed whole-blood aliquots, 1 mL of whole blood mixed with 4 mL of phosphate-buffered saline (PBS) was added to a Millipore syringe (Z268429; Sigma-Aldrich) with a 5-µm filter membrane (TMTP02500; Millipore Sigma). Filter membranes were imprinted in a microscope slide, dried before methanol fixation and Giemsa staining for microscopical observation.

Cryopreservation and Recovery of Microfilariae

Blood samples were transported on ice from Puerto Nariño to Leticia, and were mixed with 5% dimethyl sulfoxide at a 1:1 ratio, aliquoted in cryogenic tubes, and stored at −80°C [29]. Processed samples were shipped to Medellin and stored at −80°C. Samples were thawed in a water bath at 37°C for 5 minutes and washed twice with sterile PBS (at 37°C), followed by centrifugation at 2000 rpm for 10 minutes. The microfilariae pellet was gently mixed with 2 mL of RPMI-1640 plus 1× antibiotic-antimycotic (15240096; Gibco) and 1 mL aliquots were added to a 6-well cell culture plate and incubated at 37°C and 5% CO_2. Movement patterns of microfilariae were recorded using an inverted microscope (40× objective). Movement was quantified using an optical flow pipeline [30].

DNA Extraction From Blood and Plasma Samples

DNA was extracted from samples using the Quick-DNA 96-plus kit (D4070/71; Zymo) with minor adjustments to the protocol. For whole-blood samples, the "solid tissue protocol" was followed by adding 50 µL of whole blood in place of water and tissue. After incubation for 2.5 hours, samples were spun at 3500*g* for 2 minutes and supernatant lysate was transferred to the Zymo-spin 96-XL plate. For plasma samples, the "biological fluid and cells" protocol was followed. DNA was eluted with 15 µL of water and concentrations were assessed via NanoDrop.

Loop-Mediated Isothermal Amplification Assays

LAMP reactions were carried out as previously described [28] with slight modifications. Briefly, each LAMP reaction contained 1.6 µM each of primers FIP (5′-CGCAAACAGAAG CCCGAAAC-GCTCGCAATTTCATAGTGG-3′) and BIP (5′-CTTGCGCGTAGCATTAGATCC-TCCGAAATGTATACGA CAGAT-3′), 0.2 µM each of F3 (5′-GCACGAAATGTTTTTG TACG-3′) and B3 (5′-CGTATCACCGTTGATGACG-3′), 0.4 µM each of LF (5′-AAGCCTAAGCCTAAGCCTGA-3′) and LB (5′-GCACATCTTCAATCTCCTCTTGC-3′), 2 µL 10× guanidinium hydrochloride, 10 µL of WarmStart Colorimetric LAMP 2× Master Mix (M1804L; NEB), 4 µL water, and 2 µL of template DNA, or 2 µL water for nontemplate controls, for a total volume of 20 µL per reaction. For a colorimetric readout, reactions were incubated at 63°C for 30 minutes in a SimpliAmp Thermocycler (A24811; Applied Biosystems) and images were acquired using an ImageQuant 800 (Cytiva). A postamplification color of yellow indicated detection of *M. ozzardi*, and pink (or orange) indicated no detection.

For semiquantitative LAMP, simultaneous colorimetric and fluorescent readouts were obtained by adding SYTO 9 green (S34854; Invitrogen) to a final concentration of 1 µM in the colorimetric LAMP reaction. Reactions were performed in a

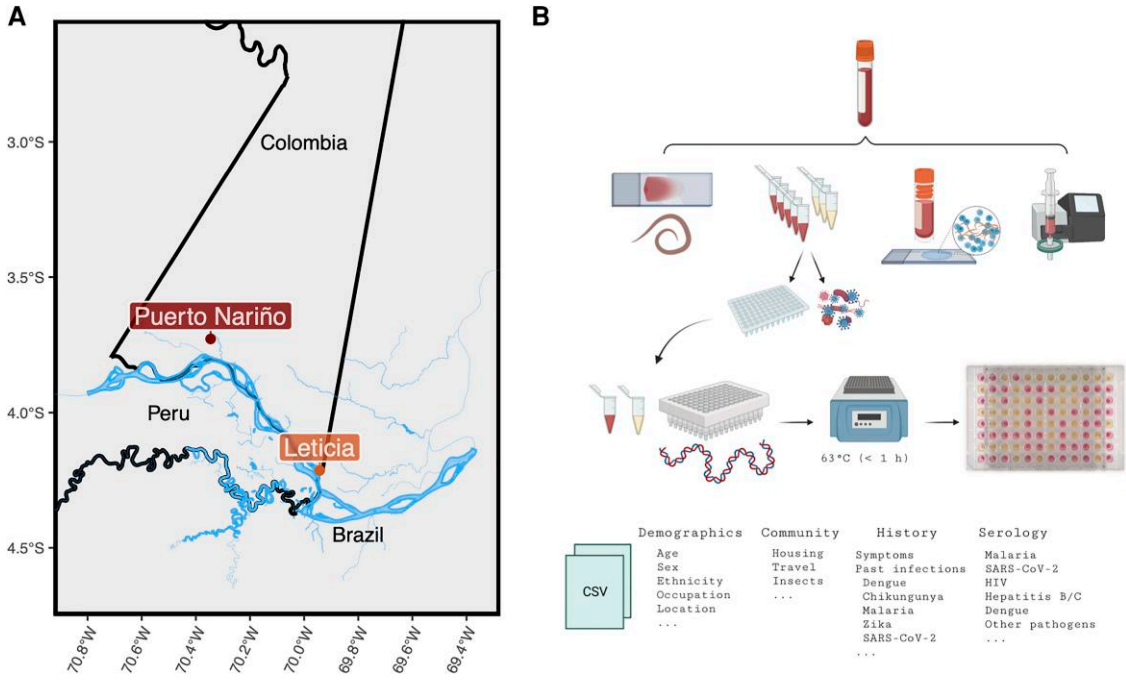

Figure 1. *A*, Map of the study region in the Amazonas Department of Colombia depicting the capital city of Leticia and the Puerto Nariño municipality where samples were collected across 3 surveys. The study region is adjacent to the Amazon River at the borders of Colombia, Brazil, and Peru. *B*, Schematic of sampling efforts and end points for blood samples acquired from adult volunteers. Blood smears and preparations of cryopreserved samples were conducted in the field. Whole blood was obtained in EDTA tubes and transferred on ice to a laboratory in Leticia. Aliquots of whole blood and plasma were prepared from samples and used for downstream serology, molecular diagnostics, and genomic analyses. Questionnaires were filled out at the time of sampling. Recorded data included demographics (age, sex, ethnicity, occupation, place of residence), housing conditions, travel history, medical history (preexisting diseases), and clinical symptoms. Symptoms (fever, headache, muscle pain, joint pain, eye pain, chills, abdominal pain, weakness, skin rash, dizziness, vomit, diarrhea, cough, red eyes, anosmia/ageusia, weight loss, jaundice) were reported as present or absent at the time blood was drawn.

Bio-Rad CFX Opus 96 Real-Time PCR instrument at 65°C with total fluorescence read in the SYBR/FAM channel every 15 seconds for 150 cycles (1 cycle = 21.2 seconds). A cutoff time threshold (Tt) value of 30 minutes was used to differentiate positive/negative reactions, with Tt defined as the time (minutes) to reach the fluorescence detection threshold. Tt ≤ 30 minutes = detection of the target, Tt > 30 minutes or N/A = no detection. Plates were scanned using the Epson Perfection version 600 photo scanner.

DNA Extraction and Illumina Library Construction and Sequencing

DNA was extracted from 200 µL of whole blood using the MagAttract HMW DNA kit (67563; Qiagen) following the manufacturer's instructions. The NEBNext Microbiome DNA enrichment kit (E2612; NEB) was used as directed to enrich *Mansonella* DNA and reduce human DNA prior to library construction. The Illumina libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645; NEB) as described by the manufacturer. The quality and concentration of each library were determined using a 2100 Bioanalyzer with a high-sensitivity DNA chip (Agilent Technologies). Libraries were diluted to 1 nM with 10 mM Tris, 0.1 mM EDTA pH 8. Phi X DNA (5%) was added to balance base pair composition in these A:T rich filarial libraries prior to sequencing on a NovaSeq platform (paired end, 150 bps).

Bioinformatic Analysis

Raw Illumina reads were processed to remove adapters and poor-quality reads using the BBTools package (https://jgi.doe.gov/data-and-tools/bbtools/). For each isolate, the reads were mapped to a combined reference sequence set comprising a *M. ozzardi* reference genome from Brazil [16], a reference mitogenome KX822021.1 [31], and the *Wolbachia w*Moz assembly GCF_020278625.1 [32] using bowtie2 [33]. Reads mapping to mitochondria were extracted from the bam alignment files using SAMtools [34] and assembled into circular mitochondrial genomes using GetOrganelle version 1.7.7.0. The average nucleotide identity scores between pairs of all assembled mitogenomes and published mitogenomes, namely the accessions KX822021.1 and MN416134.1 for *M. ozzardi* [20, 31] and MT361687.1 and MN432521.1 for *M. perstans* [20, 35], were calculated using the OrthoANIu tool [36]. Multiple sequence alignments of all *M. ozzardi* and *M. perstans* mitogenomes were obtained using mafft version 7.149b. The

phylogenetic tree based on this alignment was generated using the iqtree online server [37, 38]. Bootstrap support values were calculated based on ultrafast bootstrap with 1000 replicates. The tree was annotated on the iTOL webserver.

Serological Tests for Viral Infections

Anti-dengue IgG and IgM were detected using SD BIOLINE Dengue Duo rapid test (Abbott), following manufacturer's instructions. Anti-human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV), as well as anti-IgM against severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) were detected by chemiluminescence immunoassay using the Architect I1000 system (Abbott), following manufacturer's instructions.

Data Analysis

Self-reported data were recorded on printed surveys and entered into Microsoft Excel by double entry. Excel spreadsheets were used for data wrangling and analysis using R Studio software version 4.2.2. To examine sex- and age-based differences of *M. ozzardi* infections, we used a linear model to get the residuals and assess the normality of our data (Shapiro-Wilk test, male age, $W = 0.93224$, P value $= .0002649$; female age, $W = 0.89475$, P value $= 8.05e\text{-}09$). Based on these results, we ran a nonparametric Wilcox rank sum test (P value $< .05$ considered statistically significant). To examine differences between infection prevalence across demographic variables and analyses on reported symptoms and serology results, we used generalized linear models (GLMs) with binomial distributions and log link functions. We conducted model selection analyses using the *aictab* function in the R package AICcmodavg [39]. We built candidate models starting with the full model with all combinations of main effects among relevant biological and methodological factors, while avoiding overfitting. We compared candidate models using Akaike information criterion and ΔAIC and calculated the Akaike weight (ω) (Supplementary Table 1). Using the *Anova* function in the R package car [40], we assessed significance of the effects using Wald χ^2 statistics for the winning model.

Protocol and Data Availability

All pipelines for statistical analysis and data visualization are available at https://github.com/zamanianlab/Mansonella-ms. Raw read data used for assembly of mitogenomes of *M. ozzardi* isolates Moz-Col-195, Moz-Col-204, Moz-Col-220, and Moz-Col-239 are submitted under National Center for Biotechnology Information (NCBI) BioProject PRJNA981507, PRJNA981522, PRJNA981514, and PRJNA981525, respectively. The accession numbers of the assembled mitogenomes are OR271611, OR288092, OR296456, and OR296457, respectively. The mitogenomes of isolates Moz-Brazil-1 and Moz-Venz-1 were assembled from previously reported raw read datasets [16] available from NCBI BioProject PRJNA917722 and PRJNA917766, respectively. The corresponding GenBank accessions for these mitogenomes are OR296458 for Moz-Brazil-1 and OR296459 for Moz-Venz-1.

RESULTS

Parasite Prevalence

To assess *Mansonella* prevalence within our study population, we deployed a species-specific LAMP assay [28] and compared diagnostic results with microscopic examination of thin blood smears. Across the first survey (samples 1–117), whole-blood LAMP results show a higher prevalence of *M. ozzardi* infections (54/115, 46.9%) than single thin-smear microscopy (16/104, 15.3%) and LAMP carried out using DNA from matched plasma (31/115, 26.9%) (Figure 2*A*). Consensus whole-blood LAMP results were derived from experiments involving DNA extractions from independent aliquots of whole blood as well as replicates of LAMP assays carried out in two laboratories using DNA from a single extraction (Supplementary Figure 1*A*).

Treating the whole-blood LAMP as the truth case, thin-smear microscopy showed a 31% sensitivity and plasma LAMP showed a 50% sensitivity. For the 30 samples where whole-blood LAMP was positive and microscopy was negative, lower time (Tt), which is indicative of more parasite material being present in semiquantitative LAMP, was observed (Supplementary Figure 1*B* and 1*C*). We therefore hypothesized that the discrepancy between LAMP results and microscopy is likely associated with lower titers of microfilariae leading to thin-smear false negatives [41] or occult infections [20] that can only be detected using molecular methods. In both cases, we would expect the whole-blood LAMP assay to display better sensitivity.

Subsequent surveys (samples 118–186; samples 187–244) were used to probe potential associations between microfilaremia and *Mansonella* diagnostic results by incorporating quantitative microscopy. Whole-blood LAMP assays showed an *M. ozzardi* prevalence of 33% (40/120). Among the 14 samples that tested positive in microscopy, 13 also tested positive in LAMP. Plasma LAMP (16%) and thin-smear microscopy detected a lower prevalence (12%), reflecting the pattern observed in the first survey (Figure 2*B*). Quantitative microscopy detected microfilariae in 23/122 samples (19%), ranging from 0 to 11 microfilariae/40 µL blood spot (Figure 2*C*). Samples with the highest microfilariae titers were most likely to be positive across all diagnostics, while samples with lower microfilariae titers were more likely to be negative in blood smears and only detected in the whole-blood LAMP assay (Figure 2*D*). This lends support to the hypothesis that low microfilariae titer is a driver of false negatives in both thin smears and plasma LAMP assays.

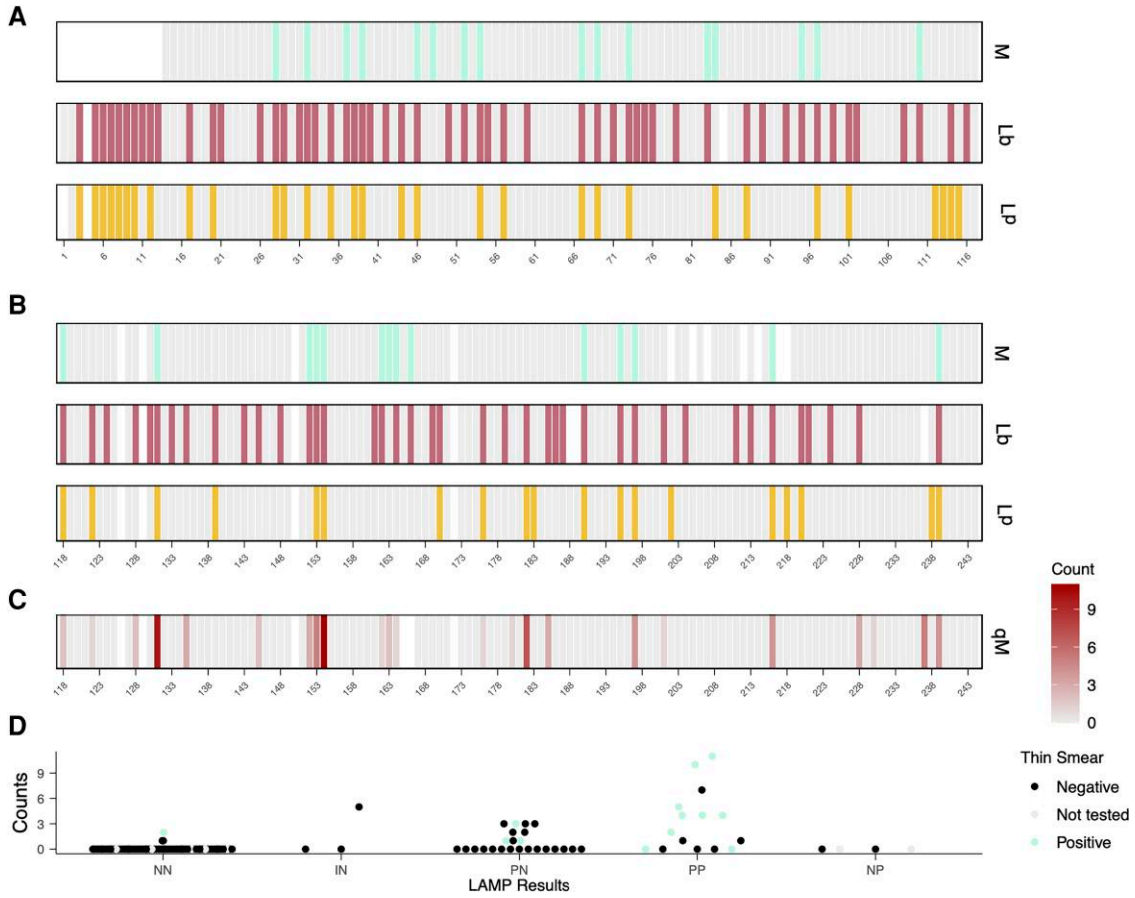

Figure 2. *A*, Diagnostic results derived from survey 1 blood samples (1–117) for M, Lb, and Lp. *B*, Diagnostic results derived from surveys 2 and 3 (samples 118–244) for M, Lb, and Lp. *C*, qM derived from surveys 2 and 3 blood samples (118–244). *D*, qM of microfilariae stratified by Lb and Lp results and colored by microscopy smear results. *A–C*, LAMP assay data are specific to Mansonella *ozzardi*. Grey = negative for *M. ozzardi*; white = no result. Abbreviations: IN, inconclusive Lb and negative Lp; LAMP, loop-mediated isothermal amplification; Lb, LAMP using DNA extracted from whole blood; Lp, LAMP using matched DNA extracted from plasma; M, in-field microscopy smear; NN, negative Lb and negative Lp; NP, negative Lb and positive Lp; PN, positive Lb and negative Lp; PP, positive Lb and positive Lp; qM, quantitative microscopy counts.

Table 1. Summary and Comparison of *Mansonella ozzardi* Diagnostic Results for the Entire Study Population

	Microscopy Smear Positive / LAMP-Plasma Positive	Microscopy Smear Negative / LAMP-Plasma Negative	No Result	Total
LAMP-blood positive	27 / 42	57 / 52	10 / 0	94
LAMP-blood negative	3 / 8	129 / 132	9 / 1	141
Total	30 / 50	186 / 184	19 / 1	235

Abbreviation: LAMP, loop-mediated isothermal amplification. Microscopy and LAMP-plasma diagnostic results stratified by LAMP-blood test results.

To explore whether occult infections could also be a factor contributing to the positive whole-blood LAMP samples that had negative microscopy results, we resampled blood from 6 individuals that fit this pattern. A larger volume of blood (1 mL) was filtered to identify microfilariae that could be circulating at titers not routinely detectable via thick- and thin-smear microscopy. All 6 samples were still microscopy negative, supporting the likelihood of occult infections. It is possible that the freeze-thaw process preceding filtration of these samples reduced the sensitivity of this approach. Whole-blood LAMP was the most sensitive diagnostic test and identified a prevalence of 40% for *M. ozzardi* across all 3 surveys (Table 1).

Population Demographics

The 3 surveys consisted of individuals who reside in 13 different communities situated between −02°50′14.9496″ north latitude and −03°52′39.1980″ south latitude; and −069°44′10.7880″ and −070°35′52.2240″ west longitude (Figure 3*A*). The majority of our sampled population were centered in Puerto Nariño (n = 136) (Table 2), where the prevalence of *M. ozzardi* as determined by whole-blood LAMP was 44%.

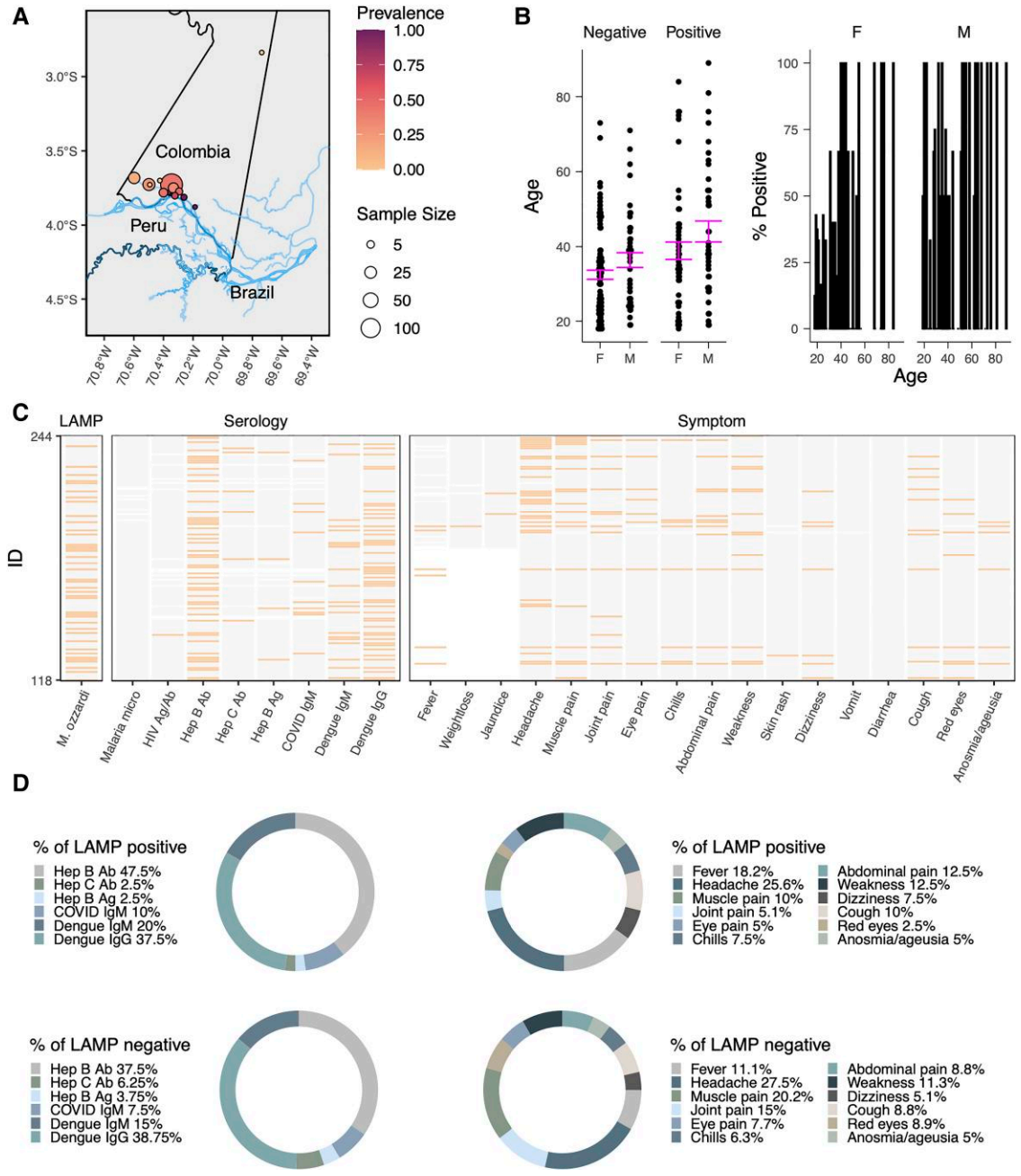

Figure 3. *A*, A map of the study region in the Amazonas Department of Colombia depicting the community where individual participants reside. Circle size reflects the number of samples collected and color represents the prevalence of *Mansonella ozzardi* infection for each community. *B*, *M. ozzardi* prevalence as determined by whole-blood LAMP assay stratified by sex and age (95% confidence interval) (left) and histogram of *M. ozzardi* prevalence distribution by age (right). *C*, *M. ozzardi* infection status and reported symptoms and serological test results for survey 2 and 3 individuals (n = 120); orange, positive, present; gray, negative, not present; white, missing data. *D*, Donut plots reflecting relative fractions of *M. ozzardi* Lb-positive and Lb-negative individuals who self-reported symptoms and laboratory-confirmed serological test results. Abbreviations: Ab, antibody; Ag, antigen; COVID, coronavirus disease; F, female; Hep, hepatitis; HIV, human immunodeficiency virus; Ig, immunoglobulin; LAMP, loop-mediated isothermal amplification; Lb, LAMP using DNA extracted from whole blood; Lp, LAMP using matched DNA extracted from plasma; M, male.

There was a higher prevalence of *M. ozzardi* microfilariae identified via blood LAMP in men (49%; n = 84; mean age = 40.1 years) compared to women (32%; n = 148; mean age = 34.7 years). The mean age of *M. ozzardi*-positive individuals was higher than the mean age of negative individuals in women (Wilcox, $P = .026$) but not men (Wilcox, $P = .07$) (Figure 3*B*). To further examine differences between infection prevalence across demographic variables, we assessed significance of the fixed effects using Wald χ^2 statistics and identified education and place of residence as drivers of prevalence (education

Table 2. Summary of Demographic and Diagnostic Data

Characteristic	Number
Age Group	
Young adult 18–32 y	101
Adult 33–54 y	104
Mature adult 55–89 y	29
Total	234
Sex (female/male)	
Young adult 18–32 y	69/32
Adult 33–54 y	68/36
Mature adult 55–89 y	12/17
Total	149/85
Mansonella ozzardi (Lb/Lp/microscopy)	
Young adult 18–32 y	31/16/9
Adult 33–54 y	42/24/16
Mature adult 55–89 y	20/10/5
Total	93/50/30
Education	
Primary	59
High school	81
Technical	52
University	16
Other	2
Not reported	24
Ethnicity	
Bora	1
Cocama	5
Senu	1
Ticuna	151
Tokami	1
Multiethnic	3
Not reported	72
Location	
Lomas Lindas	11
12 De Octubre	25
San Francisco	9
Villa Andrea	15
Puerto Narino	136
San Pedro De Tipisca	21
Other	14
Not reported	3

Data include a subset of the total study population where all summarized demographic information was provided on the questionnaire.

Abbreviations: Lb, loop-mediated isothermal amplification using DNA from whole blood; Lp, loop-mediated isothermal amplification using DNA from plasma.

binomial GLM, $\chi^2 = 16.2647$, $P = .01240$; case origin GLM, $\chi^2 = 13.6850$, $P = .03336$). However, given the limitation of the data we were unable to confirm significant differences within these groups.

To explore potential associations between clinical symptoms and infections with other pathogens, clinical histories were collected and additional serological tests were carried out for survey 2 and 3 samples (n = 117) (Figure 3*C*) and compared across *M. ozzardi* whole-blood LAMP results (Figure 3*D*). Symptoms and serology results that were differentially reported between whole-blood LAMP-positive and -negative individuals were examined further to determine if fixed effects or *M. ozzardi* infections were more likely to explain these data. Using Wald χ^2 statistics we determined that *M. ozzardi* infection status did not significantly correlate to reported symptoms or serology results. Fixed effects including sex and age were identified as potential drivers of some of the reported symptoms and serology results but unequal and small sample sizes amongst subsets of data limited our post hoc analysis.

Genomic Analysis

Whole-genome sequencing was performed on four samples (ID numbers 195, 204, 220, and 239) which tested positive in the *M. ozzardi*-specific whole-blood LAMP assay and where larger volumes of whole blood were available for DNA extractions. Only sample 239 had detectable microfilariae in the quantitative microscopy assay. While a mapping analysis of the sequencing data showed that over 94% of the reads were from the human host, 1% to 6% of reads mapped to the reference *M. ozzardi* genome assembly and to the *Wolbachia w*Moz genome assembly (Supplementary Figure 2).

Sufficient read coverage was obtained for successful assembly of complete, circular mitogenomes for each isolate. The assembled mitogenomes displayed more than 99.6% sequence identity to each other and to previously reported *M. ozzardi* mitogenomes from Brazil [20, 31], as well as to the newly assembled mitogenomes from isolates Moz-Brazil-1 and Moz-Venz-1 obtained from Brazil and Venezuela, respectively [16]. A phylogenetic analysis of all *M. ozzardi* mitogenomes in conjunction with *M. perstans* mitogenomes from Brazil [20] and Cameroon [35] showed a distinct *M. ozzardi* clade with very short branch lengths between various isolates and a clear separation from the *M. perstans* clade (Figure 4). Together, these results confirm the presence of *M. ozzardi* infection in these individuals.

Cryopreservation of Blood-Dwelling Parasite Stage

As a first step towards establishing the lifecycle of *M. ozzardi* in a laboratory environment, we sought to functionally cryopreserve microfilariae and observe viable and active worms after thawing samples. We collected and cryopreserved peripheral blood from individuals who were positive for *M. ozzardi* via blood LAMP assay and thin-smear microscopy in our initial survey. We observed revitalization of microfilariae upon thawing at 37°C, whereby motility increased over the first 24-hour incubation period before steadily declining (Figure 5*A*). Videos showed viable and healthy microfilariae (Figure 5*B*), which may allow for membrane feeding of suitable insects and the production of infective-stage larvae (L3) in a laboratory setting.

DISCUSSION

We conducted three seroprevalence surveys to better map the distribution of *Mansonella* infections among indigenous

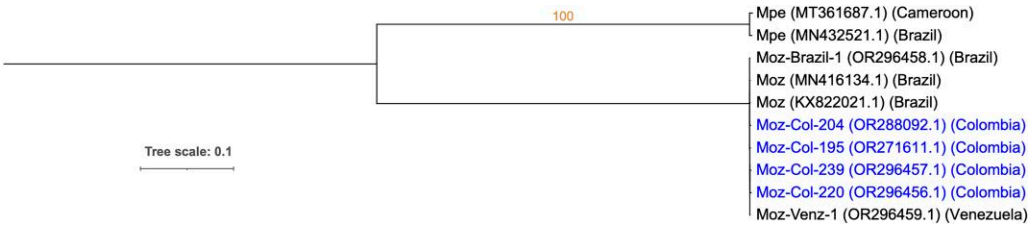

Figure 4. Maximum-likelihood tree based on whole-mitogenome alignments of various *Mansonella ozzardi* and *Mansonella perstans* isolates. The GenBank accessions are indicated in parenthesis next to isolate names, followed by the country of origin in the second parenthesis. Blue colors mark the isolates sequenced in this study. The DNA substitution model HKY + F + I was found to be the best fit according to Bayesian information criteria in ModelFinder. Values of ultrafast bootstrap support calculated with 1000 replicates is shown for branches with value higher than 80. Abbreviations: Moz, *M. ozzardi*; Mpe, *M. perstans*.

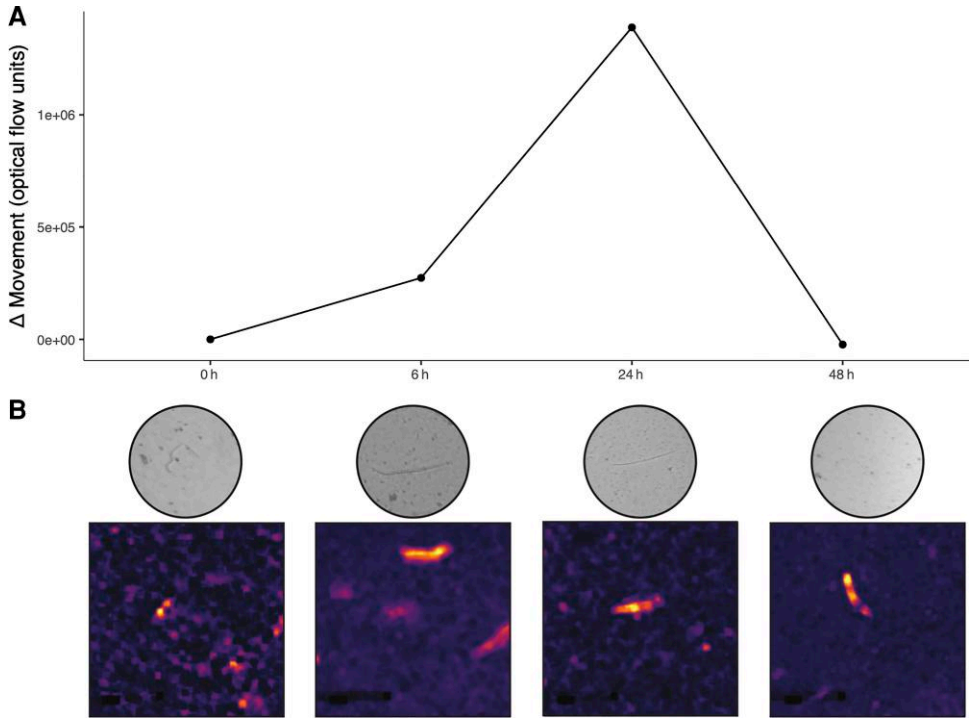

Figure 5. *A,* Motility of cryopreserved *Mansonella ozzardi* microfilariae over 48 h as measured by an optical flow algorithm. *B,* Brightfield images and optical flowmaps of videos used for motility analysis of thawed cryopreserved *Mansonella* samples aligned with the time points in (*A*). Brighter regions of the flowmaps reflect areas where more parasite movement was detected.

communities in the Amazon basin of Colombia. Blood smear microscopy identified microfilariae in 12.7% of sampled individuals, recapitulating historical studies in the region [22, 23, 42]. Whole-blood LAMP assays detected *M. ozzardi* DNA in 40% of sampled individuals, agreeing with recent PCR-based surveys in the Amazon region of neighboring countries [24, 26, 43]. Our survey data adds to the growing body of evidence that microscopy-based approaches can drastically underestimate the true prevalence of global mansonellosis [5, 14, 24–28, 43, 44].

Molecular diagnostic assays can capture a broader range of infection states, including low-microfilaremias or occult infections [20], where adults are the likely source of blood-detectable DNA. The recovery of mitogenomes confirms the presence of *M. ozzardi* DNA in blood samples where parasites were not detectable via microscopy, providing further confidence in LAMP diagnostic results. LAMP assays provide a sensitive, specific, and potentially cost-effective approach for the surveillance of filarial nematode infections in human and vector populations [28, 45, 46]. The development of direct LAMP assays not reliant on DNA extraction will add to its utility in endemic settings.

The establishment of a cryopreservation protocol can potentially facilitate the rearing of *M. ozzardi* in laboratory settings, including through the controlled blood-feeding of reanimated microfilariae to susceptible colonies of biting midges or via

intrathoracic infection of mosquitoes [47, 48]. In vitro phenotyping of *Mansonella* drug responses [49] can help resolve the genetic and molecular basis for observed differences in antifilarial drug susceptibility across species [9, 10, 12, 13, 15] and allow for the screening and discovery of new therapeutic leads.

Mansonella is neglected even among neglected pathogens due to a lack of investment in studying its biology and potential health impacts [4, 8]. Nonspecific clinical effects and the potentially skewed baseline health of our study population may have hampered our ability to resolve associations with infection status and more epidemiological data is required to assess pathogenicity and risk factors. Adult worms disperse somewhat randomly into various tissues and body cavities [50], resulting in varied consequences, which have historically been summarized as relatively nonpathogenic. Growing clinical case reports suggest appreciable pathogenicity [4], but difficulties arise establishing causal associations with subtle health impacts. The current threshold for addressing human filariasis is a causal association with significant morbidity. Investigation of subclinical or secondary impacts of human infection would shift this threshold of concern in mansonellosis-endemic regions to the same threshold that often triggers action for subclinical nematode infections of livestock and companion animals. We expect that improved diagnostic tools, growing genomic resources, and methods to study *Mansonella* in laboratory settings will facilitate this goal.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. C. K. S. C., Z. L., A. S., and S. R. are employed by New England Biolabs, a manufacturer and vendor of molecular biology reagents. All other authors report no potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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