New records of *Mansonella ozzardi*: a parasite that is spreading from the state of Amazonas to previously uninfected areas of the state of Acre in the Purus River region

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Mansonella ozzardi *infections are common in the riverside communities along the Solimões, Negro and Purus Rivers in the state of Amazonas (AM). However, little is known about the presence of this parasite in communities located in regions bordering AM and the state of Acre. The prevalence rate of* M. ozzardi *infections was determined in blood samples from volunteers according to the Knott method. A total of 355 volunteers from six riverine communities were enrolled in the study and 65 (18.3%) were found to be infected with* M. ozzardi. *As expected, most of the infections (25%) occurred in individuals involved in agriculture, cattle rearing and fishing and an age/sex group analysis revealed that the prevalence increased beginning in the 40-50-years-of-age group and reached 33% in both sexes in individuals over 50 years of age. Based on the described symptomatology, articular pain and headache were found to be significantly higher among infected individuals (56 and 65% prevalence, respectively, $p < 0.05$). Sera from volunteers were subjected to ELISA using a cocktail of recombinant proteins from* Onchocerca volvulus *to evaluate the specificity of the test in an endemic* M. ozzardi *region. No cross-reactions between* M. ozzardi*-infected individuals and recombinant* O. volvulus *proteins were detected, thus providing information on the secure use of this particular cocktail in areas where these parasites are sympatric.*

Key words: *Mansonella ozzardi* - prevalence - Purus River - mansonellosis - Amazon

Mansonella ozzardi is a filarial parasite native to the Americas that is widely distributed in the Brazilian state of Amazonas (AM) (Martins et al. 2010), which was the first region in Brazil where it was detected (Deane 1949). Subsequent studies have provided data on the distribution and prevalence of human infection by this filarial species in northern Brazil. Additionally, isolated studies have been conducted along the Purus and Negro Rivers and in other areas of western Amazon (Shelley 1975, Medeiros et al. 2007, 2008, 2009, 2011, Martins et al. 2010, Basano et al. 2011). In these areas, transmission of the parasite is related to the presence of haematophagous blackfly vectors that share the same geographical distribution, including *Simulium amazonicum* Goeldi and *Simulium argentiscutum* (Shelley et al. 1980). Human infection by *M. ozzardi* is still poorly studied and its pathogenicity remains unclear, although symptoms such as fever, articular pain, headache, lymphadenopathy, eosinophilia and pruritic skin eruptions have been reported in parasitised individuals (Batista et al. 1960a, Medeiros et al. 2009). Additionally, some studies have suggested that the ocular impairment observed in individuals from endemic areas could be due to infection with this parasite (Branco et al. 1998, Cohen et al. 2008, Vianna et al. 2012).

Thus, studies conducted to obtain data on epidemiological, clinical and pathological aspects of these infection are necessary, for example, to establish a safe and effective program of treatment and control (Medeiros et al. 2007, 2011, Adami & Maia-Herzog 2008).

Here, we describe the epidemiological, clinical and immunological results of surveys performed in 2006-2008 in riverside communities along the Antimary, Acre and Purus Rivers, in the border regions of the state of Acre (AC) and AM. As there is intense population mobility in this region via rivers, the migration of parasitised individuals may be responsible for the establishment of new active *M. ozzardi* transmission foci. Ten years ago, serum samples collected from volunteers in this area were subjected to ELISA with recombinant proteins from *Onchocerca volvulus* and a significant percentage of the samples reacted positively (Shelley et al. 2001). As this region is not an *O. volvulus*-endemic area, a possible explanation for this finding is that cross-reaction with *M. ozzardi* or another nematode parasite was producing false positives and that the assay needed to be improved. Thus, serum samples were collected from volunteers for two purposes: to reevaluate previous results for this area and to analyse the specificity of an ELISA test employing a cocktail of recombinant proteins from *O. volvulus* in an *M. ozzardi*-endemic region.

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SUBJECTS, MATERIALS AND METHODS

Study area - Blood and serum collections were carried out during four expeditions, three of which were conducted in the rainy season, while one was carried out in the dry season. Six riverside communities along the border of AC and AM were studied: Mapinguari (09º24'08"S 68°08'51.6"W) and Andaraí (09º17'29.8"S 067º45'44.0"W), on the Antimary River, Porto Acre (9º35'18'S 67º31'57''W) and Vila Antimary (09º04'01.0"S 067º23'50.1"W), on the Acre River, and two localities located on opposite edges of the Purus River - Monte Verde (08º68'13''S 67º39'94.8''W) and Praia do Gado (08º74'12''S 67º39'94.4''W), in the municipality of Boca do Acre. The number of inhabitants in the selected communities were as follows: Mapinguari, 142, Andaraí, 10, Porto Acre, 14,309, Vila Antimary, 156, Praia do Gado, 7,550 and Monte Verde, 236 [data provided by the Technology Foundation of Acre, Department of Basic Health Actions/State Department of Health of Acre, National Health Foundation (FUNASA)/Boca do Acre and Brazilian Institute of Geography and Statistics in 2008]. In these areas, with the exception of Porto Acre and Boca do Acre, the inhabitants live in scattered, hidden and very isolated communities. Most of these inhabitants are sustained by subsistence farming and occasional hunting and in some communities they are provided with some basic schooling and primary health care.

Survey procedure - A local radio station advertised the visit of a research team to the area to the inhabitants of the selected communities from the Antimary and Acre Rivers as well as the purposes of the study and the risk factors for simuliid-transmited diseases. Characteristic symptoms such as skin diseases (pruritus, erythema), articular pain, lymphadenopathy and headache were also provided. The communities along the Purus River were contacted through the FUNASA/Boca do Acre and clinical evaluations and blood collections were scheduled in advance.

The nature of our parasitological analysis and the methods involved were explained to the population of each locality and individuals who agreed to be volunteers received and signed a terms of consent form. Personal and clinical data, such as the presence/absence of any of the described symptoms, were recorded during the clinical evaluations. A convenience sampling method was adopted in the area and the sample size was determined by assuming an expected prevalence of 50% with an error of 5% and a confidence interval (CI) of 95%. A total of 355 volunteers from six different communities were enrolled in the study. All enrolled volunteers were 18 years of age or older and, in the case of women, were not pregnant or breast feeding. The study design was approved and registered by the Ethical Committee of Oswaldo Cruz Foundation (Fiocruz) (281/05).

Data on the prevalence of *M. ozzardi* were obtained through venous blood samples collections performed using 10 mL sterile, disposable syringes. One millilitre of venous blood was deposited in a polystyrene tube with 10 mL of a 2% formalin solution (Knott 1939). After a period of 12 h, thin films were prepared with the deposited sediment, then fixed with methanol and stained with Giemsa. To circumvent problems due to the preparation of material in the field, two samples consisting of thin films were prepared from each volunteer.

Prevalence data were evaluated according to the age, sex and occupational activity of the participants. To analyse the disease prevalence in terms of economic activity, the study subjects were split into three different groups based on whether such activities were carried out in primarily indoor, outdoor or mixed environments. Group A included all volunteers who were enrolled in outdoor activities such as agriculture, cattle raising and fishing, whereas Group B consisted of those individuals whose daytime activities consisted of primarily indoor activities, including nurses, policemen, cooks and drivers. Participants involved in activities of a mixed nature, such as housewives, motorboat drivers, teachers, students and salesmen, were placed in Group C. The results of clinical assessments of symptoms were compared between infected and uninfected individuals.

ELISA for *O. volvulus* proteins were performed according to the method described by Bradley et al. (1993) with some modifications. The recombinant proteins included in a cocktail for ELISA were OvMBP/10 (expressed during the L3, L4 and semi-adult stages), OvMBP/11 (expressed in all developmental stages) and OvMBP/16 (expressed only in adult worms) from *O. volvulus*. The recombinant proteins were provided by Dr Mario Rodriguez-Pérez from the Centre of Genomic Biotechnology/National Politechnic Institute, Mexico.

Plasma samples were obtained from blood collected in heparin-treated tubes, followed by centrifugation for 10 min at 400 *g* to separate the pellets containing packed erythrocytes from the plasma. A total of 355 plasma samples were identified and kept in a container with liquid nitrogen until transportation to Laboratory of Simuliidae and Onchocerciasis/Oswaldo Cruz Institute (LSO/IOC)-Fiocruz in the state of Rio de Janeiro (RJ), where they were stored at -20ºC. Ten plasma samples from onchocerciasis and mansonellosis-naïve donors from RJ were selected and used as negative controls in each experiment. In addition, each ELISA plate contained duplicates of blank and reference positive (n = 3, strongly reacting plasma from onchocerciasis patients) and negative (n = 3, naïve non-endemic donors) controls. The cut-off values for seropositive samples were calculated as the mean optical density (OD) plus three standard deviations obtained from the sera of negative controls from RJ (n = 10; IgG cut-off value = 0.116). To standardise the OD data obtained in different experiments, an OD index was calculated as the ratio of the observed OD/cut-off values. Samples displaying an OD index > 1.0 were considered reactive. The anti-maltose binding protein from *Escherichia coli* (MBP) responses of all analysed sera were determined in parallel. All ELISA OD values were corrected by subtracting the anti-MBP OD values from each serum specimen prior to determining the cut-off values.

Statistical analysis - All data obtained in this study were tested for significance via (2-tailed) Fisher's exact modification of the 2 x 2 chi-squared test with a 95% CI and $p < 0.05$ indicating statistical significance. The Mantel-Haenszel test was applied using Epi Info7™ software and the results are shown in Tables I, II. Fischer's exact test was performed with GraphPad Prism software, version 4.0 for Windows (graphpad.com).

RESULTS

As shown in Table I, a total of 355 blood films prepared according to the Knott (1939) test were examined and 65 (18.3%) were found to be positive for *M. ozzardi* microfilariae. The highest prevalence rates were found among the communities along the Purus River. Monte Verde, which is located on the left bank of this river, exhibited 63% positive samples, whereas on the opposite side of the river, 54.5% of the volunteers in the locality of Praia do Gado presented blood microfilariae of *M. ozzardi* ($p > 0.05$). Only one volunteer from Andaraí, located along the Antimary River, was positive for microfilariae and all of the individuals from Mapinguari presented blood samples that were negative for microfilariae. Vila Antimary and Porto Acre, situated along the Acre River, both presented some infected individuals, with Vila Antimary displaying a higher prevalence rate ($p < 0.05$). When the rates of infection were compared between men and women, a slight difference could be detected only in Porto Acre, whereas in all other communities, the rates of infections were evenly distributed between the two sexes.

The prevalence rates were higher among individuals who primarily performed outdoor tasks (Group A) ($p < 0.05$) compared to those performing mostly indoor activities (Group B) (Table II). No differences were observed between Groups A and C.

All previously described clinical aspects were evaluated and it was found that symptoms such as headache and articular pain were the prevalent symptoms in infected individuals (Table III).

Furthermore, division of the subjects according to age/sex revealed that among volunteers under 40 years of age, the prevalence of infection was higher in males ($p < 0.05$), whereas among those aged 40-50 and over 50 years, the prevalence of infection was distributed evenly between the two sexes ($p > 0.05$). *M. ozzardi* infections were generally more prevalent among males in the study area ($p < 0.05$) (Table IV).

When the results regarding prevalence were compiled, it was possible to note a gradient from the communities where *M. ozzardi* infections are more prevalent in AM to those located in the border regions of AM and AC, where transmission foci may have been recently established (Figure).

The results obtained via ELISA using the recombinant proteins Ov10, Ov11 and Ov16 coupled to MBP revealed that IgG responsiveness to the cocktail was negative in 354 of the 355 samples tested, with the one exception coming from Porto Acre.

TABLE II

Distribution of *Mansonella ozzardi* infected individuals according to economic activities in six riverside communities of Acre and Purus Rivers bordering region of the states of Acre and Amazonas, Brazil (2006-2008)

Occupation groups	*M. ozzardi* prevalence np/n (%)
A	37/152 (24.3)
B	4/54 (7.4)
C	24/149 (16.1)
Total	65/355 (18.3)

Group A: individuals enrolled in outdoor activities such as agriculture, fishing, cattle raising; Group B: individuals enrolled in indoor activities such as nurses, policemen, cookers, drivers; Group C: individuals enrolled in activities of mixed nature such as students, teachers, housewives, motor boat drivers; n: number of subjects examined; np: number of positive subjects.

TABLE I

Prevalence of *Mansonella ozzardi* infections in six riverside communities of Antimary, Acre and Purus Rivers bordering region of the states of Acre and Amazonas, Brazil (2006-2008)

	M. ozzardi prevalence						
Rivers	Acre		Antimary		Purus		
Community	Porto Acre np/n (%)	Vila Antimary np/n (%)	Andaraí np/n (%)	Mapinguari np/n (%)	Monte Verde np/n (%)	Praia do Gado np/n (%)	Total np/n (%)
Sex							
Males	4/39 (10.3)	17/55 (31)	1/6 (16.7)	0/39 (0)	14/19 (73.7)	7/12 (58.3)	43/170 (25.7)
Females	1/90 (1.1)	6/33 (18.2)	0/4 (0)	0/29 (0)	10/19 (52.6)	5/10 (50)	22/185 (11.7)
Total							
Community	5/129 (3.9)	23/88 (26.1)	1/10 (10)	0/68 (0)	24/38 (63.2)	12/22 (54.5)	65/355 (18.3)
River	28/217 (13)		1/78 (1.3)		36/60 (60)		-

n: number of subjects examined; np: number of positive subjects.

DISCUSSION

The surveyed localities were chosen based on previous field studies revealing the presence of *Simulium* species such as *S. amazonicum* and *Simulium oyapockense* in this region, which are potential vectors of filarial parasites (Nascimento et al. 2009) and could serve as risk factors for simuliid-transmitted diseases in the population. *M. ozzardi* infections are well known along the Purus River and its tributaries (Medeiros et al. 2008, 2009, 2011), but no epidemiological studies aimed at determining the presence and dispersion of this species through strategic points on the border of AM and AC have been performed previously. The results of the present survey demonstrated varying rates of human infection at the studied sites. The thin films obtained from all volunteers from Mapinguari, which is situated along the Antimary River, were negative, suggesting that transmission was likely not occurring at this point along the river, although breeding sites of simuliids had been detected (Nascimento et al. 2009). In Porto Acre, a city bordering the Acre River, low but detectable rates of *M. ozzardi* infection were observed. Indeed, as Porto Acre is moderately developed compared to other riverine communities, most of the volunteers from this site were involved in indoor activities such as nursing, teaching and domestic care, reducing their exposure to simuliid bites. The geographical proximity of riverine communities to this particular city makes it very attractive in terms of economic activities such as trade, fishing and agriculture. Porto Acre is still very important to individuals searching for medical assistance, resulting in frequent human migration to the city. The results obtained in Monte Verde and Praia do Gado support the contention that *M. ozzardi* infections are scattered along the Purus River and that the infected individuals in these areas exhibit high parasitic loads on average (data not shown). Indeed, the infection rates observed in Monte Verde were much higher than have previously been described (Medeiros et al. 2009), possibly due to differences in methodology, such as the use of the Knott (1939) method, which improves sensitivity.

It is of note that the rates of *M. ozzardi* infection in this area (the Purus River) have been the subject of various studies and appear to be increasing with time. In 1975, Shelley studied riverine communities along the Purus River and reported an index of 4.4%. Thirty-three years later, Medeiros et al. (2009, 2011) found prevalence rate of 20% to almost 40% depending of the locality. In general, *M. ozzardi* infections are observed at prevalence rates of approximately 20-47% in endemic areas such as those in Bolivia, Venezuela and Colombia (Kozek et al. 1982, Formica & Botto 1990, Bartoloni et al. 1999). The present work was conducted in localities bordering the Antimary, Acre and Purus Rivers, which have never been surveyed previously. These areas were presumed to display prevalence rates of approximately 50%, which is the value employed when there is an absence of conclusive data. Instead, the actual global prevalence rate recorded was 18.3% and by examining each community, the highest rates were shown to occur along the Purus River. In studies conducted in communities along the Pauini and

TABLE IV

Prevalence of *Mansonella ozzardi* infected individuals according to age and gender in six riverside communities of Acre and Purus Rivers bordering region of the states of Acre and Amazonas, Brazil (2006-2008)

Age group	Males np/n (%)	Females np/n (%)	Total np/n (%)
< 40	20/95 (21)	05/109 (4.6)	25/204 (12)
40-50	05/25 (20)	07/41 (17)	12/66 (18)
> 50	18/47 (38)	10/38 (26)	28/85 (33)
Total	43/167 (26)	22/188 (12)	65/355 (18)

n: number of subjects examined; np: number of positive subjects.

TABLE III

Comparative analysis of clinical aspects of *Mansonella ozzardi* infections between infected and uninfected individuals from six riverside communities of Acre and Purus River bordering region of the states of Acre and Amazonas, Brazil (2006-2008)

	M. ozzardi infections				
Subjects	Positive (n = 65) np (%)	Negative (n = 290) np (%)	p	OR	CI
Symptomatic individuals					
Coldness in legs	19 (29)	80 (28)	0.7623	1.084	0.5990-1.963
Headache	43 (66)	96 (33)	< 0.0001	3.950	2.236-6.978
Articular pain	37 (57)	94 (32)	0.0003	2.755	1.591-4.772
Lymphadenopathy	4 (6)	52 (18)	0.023	0.3001	0.1045-0.8623
Asymptomatic individuals	21 (32)	182 (63)	< 0.0001	0.2832	0.1599-0.5018

CI: confidence interval; n: total examined; np: number of positive individuals; OR: odds ratio.

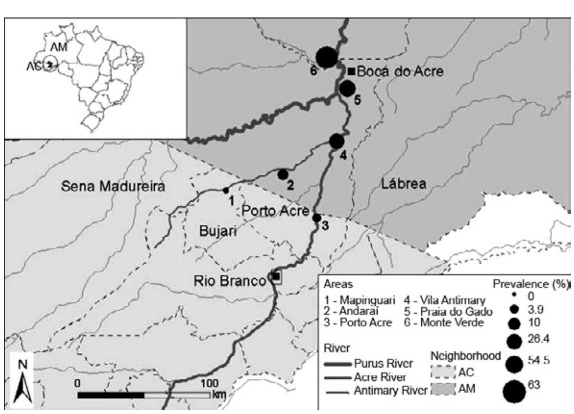
Purus Rivers as well as in Lábrea, Medeiros et al. (2009, 2011) found prevalence rates close to 21%. As some localities along the Purus River are very important economically, they appear to be exporting infected individuals to previously uninfected areas, such as areas of AC.

When the prevalence of *M. ozzardi* infections was compared between males and females, the frequency of infection was observed to be higher in males, in agreement with previously reported data (Tavares 1981, Medeiros et al. 2011). Indeed, our data showed that for individuals involved in outdoor and mixed indoor-outdoor activities, there was an enhanced probability of exposure to infective bites. Farmers, fishermen, housewives and teachers are examples of occupations that are frequently reported to exhibit high prevalence rates (Batista et al. 1960b, Medeiros et al. 2009, 2011). Additionally, the infection rate increased with age and became more evident from 40 years of age onward, with very similar rates being detected between men and women, as reported for other areas (Moraes et al. 1978, Medeiros et al. 2008).

Since 1960, when Batista et al. described the most frequent symptoms presented by infected individuals with a high parasitic load, mansonellosis, as a disease, has been a subject of controversy. Briefly, Restrepo and Ochoa (1986) carried out clinical trials and observed symptomatology similar to that reported previously in Brazil. In Argentina, Jorg (1983) described a unique case of mansonellosis in an immunocompromised patient. However, in studies carried out in Bolivia, no correlation was found between infections and clinical signs (Bartoloni et al. 1999). Additionally, the suspected involvement of *M. ozzardi* in ocular impairment was studied in volunteers from a endemic area along the Solimões River in AM (Cohen et al. 2008) and, more recently, an association with corneal lesions was found (Vianna et al. 2012). In the present study, the clinical analysis indicated a significant relationship between infection and two of the traditional symptoms described in the literature: headache and articular pain. The physiological reasons for these symptoms have not been well studied, but circulating immune complexes associated with *M. ozzardi* infections have previously been described and could serve as an explanation for the articular pain observed in infected individuals (Godoy 1998). Furthermore, asymptomatic individuals were present in significant numbers, some of whom exhibited high parasitaemia levels (data not shown), which may be a result of a well-balanced host/parasite relationship.

Finally, 40% of the serum samples collected previously from subjects in Vila Antimary and tested at LSO/IOC-Fiocruz were found to be positive when subjected to ELISA against a cocktail of recombinant proteins from *O. volvulus* (Shelley et al. 2001), which is considered suggestive of exposure to this parasite (Bradley & Unnasch 1996). The use of non-invasive methods such as serological tests has increased, but although such methods are more sensitive, extensive cross-reaction between different nematodes has been observed, compromising their specificity (Cabrera et al. 1989). Controversial findings and variations in sensitivity and specificity depending on the geographical area and the parasite proteins employed have been reported (Bradley & Unnasch 1996). The antigens used in the present work were selected due to showing promising results in other studies and despite one inconclusive positive result from an uninfected volunteer from Porto Acre, the ELISA applied here using the Ov10, Ov11 and Ov16 recombinant proteins showed high specificity (99.7%), with no cross-reactivity with *M. ozzardi*-infected individuals being detectable. These results contrast with those from previous studies (Shelley et al. 2001) in which cross-reaction with serum samples from *M. ozzardi*-infected individuals was detected. However, these previous assays employed Ov29 rather than Ov16. Furthermore, blood microfilarias similar to those of *O. volvulus* in the cephalic space, but with a posterior region morphology similar to *M. ozzardi* have been found in the study area (Adami et al. 2008). It is possible that the presence of these parasites in blood samples could be responsible for the previously observed ELISA cross-reactivity. However, even in infected volunteers with atypical microfilarias, the serological profile has not changed. Atypical microfilarias have also been found in Peru and a molecular analysis performed on these samples clustered the atypical microfilarias into the *Mansonella* group, which is indistinguishable from *M. ozzardi* (Arróspide et al. 2012, Marcos et al. 2012). Other factors, such as the preparation of materials and variations in protocols, may have had some effect on the obtained results, as a different combination of recombinant proteins was employed in our cocktail.

In conclusion, epidemiological surveillance should be sustained through border regions of AM and AC to prevent the critical dispersion of *M. ozzardi* infections to non-endemic areas where simuliid species are present and can act as competent vectors. As long as mansonellosis remains a poorly understood filarial disease and scattered infections continue in human populations situated along the Purus River and some of its tributaries, further studies will be needed to increase our understanding of its pathology and to develop safe treatment strategies.



Map showing the location of the six communities in the border region of the states of Amazonas (AM) and Acre (AC) (western Brazilian Amazon) and the prevalence of *Mansonella ozzardi* infections found in each one.

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