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Cryptosporidium spp. and *Giardia* sp. in Neotropical river otters (*Lontra longicaudis*) and giant otters (*Pteronura brasiliensis*) in northern Brazil

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Evaluating the occurrence of diseases in aquatic mustelids is a priority in the conservation strategies for the Neotropical river otter and giant otter. Thus, the objective of this study was to determine the frequency of infection caused by Cryptosporidium *spp. and* Giardia *sp. in both host species in northern Brazil. The collection of biological samples was carried out in the states of Amapá, Amazonas, Pará and Rondônia, totalling 337 faecal samples of these species, which were processed using Kinyoun's technique for the identification of* Cryptosporidium *spp. oocysts, and centrifugal flotation in zinc sulphate solution for visualization of* Giardia *sp. cysts. All samples were also tested by direct immunofluorescence. The frequency of infection by* Cryptosporidium *spp. was higher than* Giardia *sp., in the two otter species. In the analysed samples co-infection by both protozoa was also found in 4.47% (14/313) of Neotropical river otter and 20.83% (5/24) of giant otter samples. Oocysts and cysts of* Cryptosporidium *and* Giardia, *respectively, may remain infectious within specific environmental conditions for long periods of time. The current identification of Neotropical and giant otters as hosts of these protozoa increases the possibility of infection in this species and the transmission of those agents to other aquatic and terrestrial organisms, as well as to human populations. The findings of this study represent the first description of* Cryptosporidium *spp. and* Giardia *sp. affecting* Lontra longicaudis *and* Pteronura brasiliensis.

Keywords: parasites, protozoa, diseases, zoonosis, diagnostics, coccidian, aquatic mammals, Mustelidae, Amazon biome, conservation

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INTRODUCTION

The neotropical river otter (*Lontra longicaudis*) and the giant otter (*Pteronura brasiliensis*) are two mustelid species that occur in the river systems of the Brazilian Amazon (Carter & Rosas, 1997; Coletti *et al.*, 2013.). The main habitats of these species are rivers, lakes, streams and wetlands, with open margins and vegetation (Coletti *et al.*, 2013; Lima *et al.*, 2013; Palmeirim *et al.*, 2014), with low levels of pollution and human occupation, although they do tolerate environments with anthropogenic factors (Palmeirim *et al.*, 2014).

Currently, the alteration of habitats caused by human occupation and the exploitation of natural resources in tropical forests constitute threats to these animals (Carter & Rosas, 1997; Coletti *et al.*, 2013); other dangers include boat traffic, pollution and contamination of water resources (Carter & Rosas, 1997). In addition, these species are also threatened by the drainage of wetlands for agricultural practices, mining and fossil fuel extraction (Palmeirim *et al.*, 2014).

The various anthropogenic factors associated with impacts on water and land resources used by Neotropical river otter and giant otter, as well as the possibility of these species cohabiting with other wild and domestic animals, increase the vulnerability of those mustelids to infections caused by *Cryptosporidium* and *Giardia* (Gaydos *et al.*, 2007).

Due to the spread of *Cryptosporidium* spp. oocysts and of *Giardia* sp. cysts in different water resources (Fayer *et al.*, 2004; Lasek-Nesselquist *et al.*, 2008), occurrence of infections by these aetiological agents has been reported in sirenians (Morgan *et al.*, 2000; Borges *et al.*, 2011), pinnipeds (Rengifo-Herrera *et al.*, 2011), cetaceans (Hughes-Hanks *et al.*, 2005) and mustelid (Gaydos *et al.*, 2007; Méndez-Hermida *et al.*, 2007).

The investigation of diseases in aquatic mustelids is an important priority in the conservation strategies for this taxonomic group (Gaydos *et al.*, 2007), since little is known about the aetiological agents that affect them. Studies of this nature – considering the capability that Neotropical and giant otters have to act as sentinels of environmental quality – enable

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the determination of water resource conditions and of possible risks to other species that use the same habitats (Méndez-Hermida *et al.*, 2007), including human populations (Xiao *et al.*, 2002; Appelbee *et al.*, 2010; Wagnerová *et al.*, 2015).

Therefore, the aim of this study was to determine the frequency of infection caused by *Cryptosporidium* spp. and *Giardia* sp. in Neotropical river otters (*Lontra longicaudis*) and giant otters (*Pteronura brasiliensis*) in northern Brazil.

MATERIALS AND METHODS

Study areas

Samples were collected in the states of Amapá (Jari River), Amazonas (Amanã and Tefé lakes), Pará (Araticum and Saracá streams, Sapucuá lake and Tapajós River) and Rondônia (Madeira River) (Figure 1). These locations are within the Amazon biome, which consists of large tracts of rainforest, with lowland wetlands, flooded forest and upland forests (Ayres *et al.*, 2005).

Throughout these areas, in particular locations, there are great extensions of preserved lands with low population densities that are rich in animal and plant species (Ayres *et al.*, 2005; Lima, 2009). However, there is also the presence of agricultural and animal husbandry activities, occurrence of domestic animals in the more urbanized areas, damming of rivers for hydroelectric dams (Junk & Mello, 1990) and mineral extraction activities (Monteiro, 2005).

At these sites there are seasonal variations in the water level which influence aquatic ecosystems (Junk *et al.*, 1989) – the periods of flooding and high water generally occur between November and March, and the receding and low water periods occur between the end of June and early November (Ayres, 1993; Lima, 2009).

Collection of biological samples

From 2011 to 2014, 313 faecal samples from Neotropical river otters and 24 from giant otters were collected (Table 1), identified by odour, colour and characteristics of the latrine (Quintela *et al.*, 2008; Cabral *et al.*, 2010). During field trips, the biological samples were found in defecation sites and close to shelters. The activities occurred in both the high water (rainy season) and low water (dry season) periods.

After collection, the material was placed in vials containing AFA solution (absolute alcohol, formaldehyde, glacial acetic acid and distilled water), in proportions suggested by Ueno & Gonçalves (1994). These were then properly identified and referred to further laboratory procedures.

Laboratory processing

For identification of *Cryptosporidium* spp. oocysts, samples were subjected to formol-ether sedimentation with subsequent preparation of smears and staining by Kinyoun's technique (Brasil, 1996). The zinc sulphate centrifugal flotation technique was employed to identify *Giardia* sp. cysts (Gaydos *et al.*, 2007; Bica *et al.*, 2011). Part of the sample was subjected to the direct immunofluorescence test, following instructions by the Kit Merifluor® *Cryptosporidium/Giardia*, and the oocysts and cysts were identified based on their shape, size and pattern of immunofluorescence intensity (Reboredo-Fernández *et al.*, 2015).

Samples were considered positive when one of the tests used allowed the identification of *Cryptosporidium* spp. oocysts and *Giardia* sp. cysts (Borges *et al.*, 2011; Rengifo-Herrera *et al.*, 2011). In order to compare the different diagnostic methods used, sensitivity, specificity, correct classification (accuracy) and incorrect classification were evaluated (Thrusfield, 2004), and the direct immunofluorescence test was defined as the gold standard in these analyses.

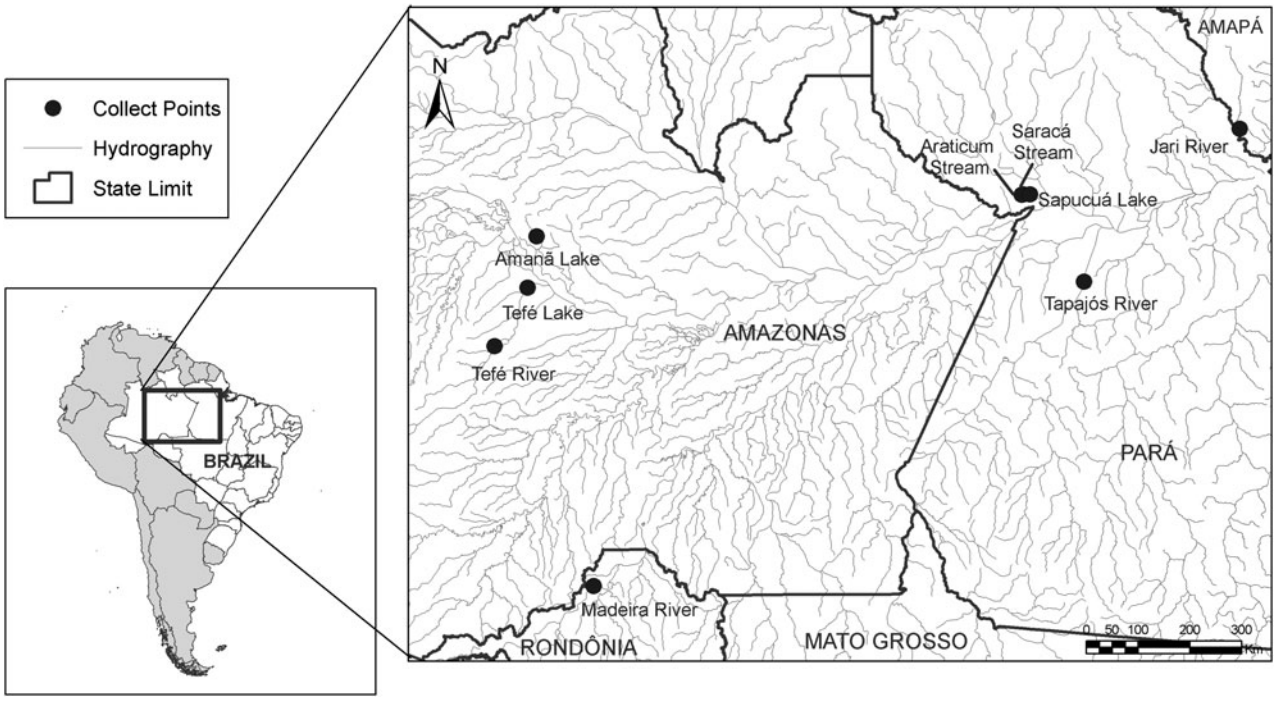

Fig. 1. Map of the states where the samples were collected.

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Table 1. Origin of faecal samples from Neotropical and giant river otters.

Species	States/number of samples				
	Pará	Amapá	Rondônia	Amazonas	Total
Neotropical otter	33	230	38	12	313
Giant otter	6	8	9	1	24
Total	39	238	47	13	337

Data analysis

Chi-square tests were performed to evaluate the association between the species investigated in this study and the variables – origin (place of origin of the samples), infection by *Cryptosporidium* spp. and by *Giardia* sp., and seasonality (rainy and dry seasons). When the expected frequencies were less than five in more than 20% of the respective data set, the Fisher test was used (Quinn & Keough, 2002; Crawley, 2013). Analyses were performed using the software R (R Core Team, 2014).

RESULTS

Out of all samples analysed, 27% (94/337) scored positive for at least one pathogen studied. The frequency of infection by *Cryptosporidium* spp. was higher than that caused by *Giardia* sp., for both Neotropical river otters and giant otters (Table 2). In the samples analysed, some cases of co-infection by both protozoa were detected.

Subsequently, the association between the presence of *Cryptosporidium* spp. and of *Giardia* sp. was tested. In Neotropical river otters, an association between these two variables was found ($\chi^2 = 30.708$, gl = 1; $P < 0.00001$), but not in giant otters ($P = 0.08501$).

In Neotropical river otters, infection by *Cryptosporidium* spp. was higher during the rainy season (22.8%) compared with the dry season (12.21%), thus this protozoan was associated with the rainy season ($\chi^2 = 5.6309$; gl = 1; $P = 0.01765$). In this same mustelid, despite the fact that the occurrence of *Giardia* sp. was also higher during the rainy season (13.04%) compared with the dry season (8.16%), there was no significant difference ($\chi^2 = 1.79877$; gl = 1; $P = 0.179861$) in relation to seasonality.

In giant otters, the presence of infection by *Cryptosporidium* spp. ($P = 0.2391$) and *Giardia* sp. ($P = 0.5296$) occurred independently from season.

The results did not show any association between the studied areas and infection by the aetiological agents studied.

The sensitivity, specificity, correct classification (accuracy) and incorrect classification values for each aetiological agent are shown in Table 3.

DISCUSSION

The findings of this study highlight the concern for infections caused by *Cryptosporidium* spp. and *Giardia* sp. in Amazonian aquatic mustelids, particularly in giant otters. The frequency of these pathogens in *Pteronura* samples was higher than that found in studies with the European otter *Lutra lutra*, in Spain (Méndez-Hermida *et al.*, 2007) and the Canadian otter *Lontra canadensis*, in the USA and Canada (Gaydos *et al.*, 2007).

The clinical significance of these pathogens for mustelids is unknown (Gaydos *et al.*, 2007). However, in Antillean manatees *Trichechus manatus* (Borges *et al.*, 2011), some animals exhibited diarrhoea, abdominal discomfort, and increased respiratory intervals.

In studies with other species of aquatic mammals, prevalence of *Cryptosporidium* spp. was found in 5.1% of bowhead whales, *Balaena mysticetus*; in 24.5% of North Atlantic right whales, *Eubalaena glacialis* (Hughes-Hanks *et al.*, 2005); 21.4% of bottlenose dolphins, *Tursiops truncatus* and 22.2% of striped dolphins, *Stenella coeruleoalba* (Reboredo-Fernández *et al.*, 2015); 4.3% of Amazonian manatees, *Trichechus inunguis*; and 25% of Antillean manatees, *Trichechus manatus* (Borges *et al.*, 2011). Research studies on *Giardia* sp. found the occurrence of this parasite in 64.5% of ringed seals, *Phoca hispida* (Hughes-Hanks *et al.*, 2005); 50% of Greenland seals, *Phoca groenlandica* (Measures & Olson, 1999); and 33.3% of bowhead whale (Hughes-Hanks *et al.*, 2005).

Co-infection by *Cryptosporidium* spp. and *Giardia* sp. was diagnosed in both Neotropical river otters and giant otters. This may be due, among other factors, to inherent conditions of the affected animals, such as immunity and age (Xiao *et al.*, 2002; Carey *et al.*, 2004; Lasek-Nesselquist *et al.*, 2008), as well as to characteristics of these agents, which present high resistance, fast dissemination through water courses (LeChevallier *et al.*, 1991; Fayer *et al.*, 2004) and high morbidity (Cacciò *et al.*, 2005). The simultaneous occurrence of these protozoa was also found in the European otter (Méndez-Hermida *et al.*, 2007), minke whale, *Balaenoptera edeni*, and striped dolphin (Reboredo-Fernández *et al.*, 2015), California sea lions, *Zalophus californianus* (Deng *et al.*, 2000), North Atlantic right whale, bowhead whale and the ringed seal (Hughes-Hanks *et al.*, 2005).

Although no association was found between the areas studied and infection by the protozoa evaluated, the occurrence of *Cryptosporidium* spp. in Amazonian rivers was previously evidenced by this coccidian affecting Amazonian manatees (Borges *et al.*, 2007). At the time, among the probable factors related to the spread of these aetiological agents, the limited sanitary conditions of several riverine communities studied in the region were highlighted, as well as livestock waste products and those released by vessels (Borges *et al.*, 2007). Similar results were observed in infections caused by *Cryptosporidium* spp. and *Giardia* sp. in Canadian otters

Table 2. Absolute (AF) and relative (RF) frequency of infection by *Cryptosporidium* spp., *Giardia* spp. and co-infection in Neotropical and giant otters, using the Kinyoun technique, centrifugal flotation and direct immunofluorescence.

Species	No. of sample	*Cryptosporidium* spp.		*Giardia* sp.		Co-infection	
		AF	RF (%)	AF	RF (%)	AF	RF (%)
Neotropical otter	313	48	15.33	29	9.26	14	4.47
Giant otter	24	10	41.66	7	29.16	5	20.83

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Table 3. Evaluation of laboratory techniques used for the diagnosis of *Cryptosporidium* spp. and *Giardia* sp. in Neotropical river otter and giant otter.

Parameters (%)					
Aetiological agent	**Technique**	**Sensitivity**	**Specificity**	**Correct classification (Accuracy)**	**Incorrect classification**
Cryptosporidium spp.	Kinyoun	55.17	100	92.28	7.71
	Direct immunofluorescence	67.24	100	94.36	5.63
Giardia sp.	Centrifugal flotation	29.72	100	92.28	7.71
	Direct immunofluorescence	91.89	100	99.10	0.89

and other species of aquatic mammals (Deng *et al.*, 2000; Hughes-Hanks *et al.*, 2005; Gaydos *et al.*, 2007).

Although most areas evaluated in this study presented good environmental conservation status, in many locations – in addition to the reported anthropogenic impacts – there were large deforested areas, river damming for hydroelectric dams and mineral extraction. These factors can alter the ecological balance and contribute to the contamination of wild hosts by pathogens (Patz *et al.*, 2000; Lallo *et al.*, 2009; Palmeirim *et al.*, 2014).

Since the frequency of infection by *Cryptosporidium* spp. and *Giardia* sp. in both Neotropical and giant otters was higher in the rainy season than in the dry season, it is important to point out that throughout the studied areas in northern Brazil there is a seasonal variation of up to 10 metres in the river water levels. This phenomenon is caused by the melting on the Andes associated with the rainy season (Lima, 2009) and influences the various associated aquatic ecosystems (Junk *et al.*, 1989). For example, most pastures used for animal husbandry activities are flooded during the rainy season. These sites may be used by Neotropical and giant otters, rendering them more vulnerable, especially considering the great potential for environmental contamination presented by cattle, where one single infected calf can eliminate from one to 10 billion oocysts in its faeces per week (Fayer *et al.*, 2004).

During this rainy season, the areas occupied by Neotropical and giant otters undergo flooding, directly affecting resource use. During the dry season these species leave the flooded areas and occupy the main water body channels, where there is greater availability of space, food (Lima, 2009) and refuges (Duplaix, 1980).

Having identified Neotropical river otters and giant otters as hosts of *Cryptosporidium* spp. and *Giardia* sp., it becomes evident that the ability of the oocysts and cysts of these protozoa, respectively, to remain infectious within specific environmental conditions for long periods of time (LeChevallier *et al.*, 1991) increases the possibility of transmission of these agents to other aquatic and terrestrial organisms, as well as to human populations.

The findings of this research study highlight the importance of carrying out the genetic characterization of the studied protozoa, as a way to elucidate the impact of human activities as potential sources of infection of Neotropical river otters and giant otters by these coccidia. The findings of this study provide the first description of infection by *Cryptosporidium* spp. and *Giardia* sp. affecting *Lontra longicaudis* and *Pteronura brasiliensis*.

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