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Review Article

Evaluation of Parasitological and Immunological Techniques in the Diagnosis of *Cryptosporidium* and *Giardia* in Aquatic Mammals

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Abstract

Infections caused by *Cryptosporidium* and *Giardia* are among the main gastro enteric diseases affecting a large number of animals and humans. Oftentimes the disease is asymptomatic, which may render the diagnosis involving aquatic mammals difficult. The aim of this study was to evaluate the use of an immunological technique with parasitological methods in the diagnosis of *Cryptosporidium* and *Giardia* in aquatic mammals. A total of 553 fecal samples and intestinal contents of mustelids, cetaceans and sirenians were submitted to laboratory processing. *Cryptosporidium* oocysts were identified with Kinyoun's technique. *Giardia* cysts were identified using the centrifugation-flotation method. All samples underwent immunological tests through direct immunofluorescent antibody (DFA). The Kappa Index *k* was used to measure the agreement between techniques used for the detection of each parasite addressed in this study. Sensitivity, specificity, real prevalence, estimated prevalence, positive predictive value, negative predictive value, correct classification and incorrect classification were evaluated. *Cryptosporidium* were found in *Pteronurabrasiliensis* [10/24 (41.66%)], *Trichechus inunguis* [22/131 (16.79%), *Lontra longicaudis* [48/314 (15.28%)], *Trichechus manatus* [04/29 (13.79%)] and *Sotalia guianensis* [03/31 (9.67%)]. *Giardia* was identified in *Kogia breviceps* [01/01 (100%)], *Pteronurabrasiliensis* [07/24 (29.16%)], *Kogia sima* [01/04 (25%)], *Trichechus manatus* [04/29 (13.79%)], *Sotalia guianensis* [03/31 (9.67%)], *Lontra longicaudis* [30/314 (9.55%)] and *Trichechusinunguis* [05/131 (3.81%)]. The *k* value for the diagnosis of *Cryptosporidium* was 0.86; for *Giardia* cysts the *k*-value was 0.27. Therefore, the direct immunofluorescent technique demonstrated greater sensitivity both in the diagnosis of *Cryptosporidium* and *Giardia* where the combination of more than one laboratory technique is recommended.

ABBREVIATIONS

DFA: Direct immunofluorescent antibody; AFA: Alcohol; Formaldehyde; Glacial acetic acid

INTRODUCTION

Cryptosporidium and *Giardia* are protozoa that are becoming increasingly important in human and animal health, since in addition to affecting a large number of hosts [1,2] they are also associated with gastrointestinal disorders, especially in immune compromised hosts [3].

These parasites may appear asymptomatic [3,4,5] causing difficulties for an accurate diagnosis. These limitations are further enhanced when involving aquatic mammals [6] in view of the discrete behavior of several species and the environment in which they are encountered.

Therefore, for the diagnosis of these protozoa, it is fundamentally important to use laboratory techniques that allow the visualization of *Cryptosporidium* oocysts and *Giardia* cysts, and/or molecular identification of these etiologic agents [4,7].

Considering the increase in reports of these parasitic agents affecting aquatic mammals [8,5] it is of great relevance to identify laboratory methods that provide good sensitivity, practicality, low cost and be easy to perform [9,10].

Thus, the aim of this study was to evaluate the use of an immunological technique and traditional parasitological methods in the diagnosis of *Cryptosporidium* and *Giardia* in aquatic mammals.

MATERIALS AND METHODS

This biological material was obtained from captive animals or necropsied carcasses, and fresh fecal samples were collected in areas of use of several species (defecation sites, exits of shelters and feeding areas). The activities were carried out between 2011 to 2015 in the northern (Amapá, Amazonas, Pará and Rondônia) and northeastern (Alagoas, Bahia, Ceará, Maranhão, Paraíba and Sergipe) regions of Brazil.

A total of 553 fecal samples and intestinal contents were collected from 15 species of aquatic mammals from order Carnivora (Neotropical otter - *Lontra longicaudis*, giant river otter - *Pteronurabrasiliensis*), Cetartiodactyla (minke whale - *Balaenoptera acutorostrata*, Risso's dolphin - *Grampus griseus*, pink river dolphin - *Inia geoffrensis*, pigmy sperm whale-*Kogia breviceps*, dwarf sperm whale – *K. sima*, melon-headed whale - *Peponocephala electra*, sperm whale - *Physeter macrocephalus*, Guiana dolphin - *Sotalia guianensis*, pantropical spotted dolphin - *Stenella attenuata*, Clymene dolphin - *Stenella clymene*, Cuvier's beaked whale - *Ziphius cavirostris*) and Sirenia (Amazonian manatee - *Trichechus inunguis*, West Indian manatee – *T. manatus*) (Table 1).

The samples were stored in solution containing alcohol, formaldehyde, glacial acetic acid and distilled water (AFA), in proportions suggested by [11].

[12]. was used for the identification of *Cryptosporidium* oocysts and for *Giardia* cysts were identified using the centrifugation-flotation method in zinc sulphate solution [7,13] Subsequently, all samples were submitted to direct immunofluorescent (DFA) reaction, as recommended by the Cryptosporidium / Giardia Merifluor® Kit, with oocysts and cysts being identified based on their shape, size and fluorescence intensity pattern [14] Samples were considered positive when one of the tests used allowed the identification of *Cryptosporidium* oocysts and *Giardia* cysts [8,4].

To measure the agreement among techniques used to detect each parasite, the Kappa (*k*) index was used, and the values were interpreted according to [15]. In order to compare the different diagnostic methods used, sensitivity, specificity, real prevalence, estimated prevalence, positive predictive value, negative predictive value and correct classification (accuracy) were evaluated [16] and the direct immunofluorescence test was defined as the gold standard in these analyses.

All statistical analyses were performed using the R software [17] and the agreement index among parasite detection techniques was obtained using the IRR software [18].

RESULTS

Among the aquatic mammal species evaluated in this study, the presence of *Cryptosporidium* was found in *Pteronurabrasiliensis* [10/24 (41.66%)], *Trichechus inunguis* [22/131 (16.79%), *Lontra longicaudis* [48/314 (15.28%)], *Trichechus manatus* [04/29 (13.79%)] and *Sotalia guianensis* [03/31 (9.67%)]. *Giardia* was identified in *Kogia breviceps* [01/01 (100%)], *Pteronurabrasiliensis* [07/24 (29.16%)], *Kogia sima* [01/04 (25%)], *Trichechus manatus* [04/29 (13.79%)], *Sotalia guianensis* [03/31 (9.67%)], *Lontra longicaudis* [30/314 (9.55%)] and *Trichechusinunguis* [05/131 (3.81%)]. Simultaneous infections of these protozoa were observed in *P. brasiliensis* [05/24 (20.83%)], *L. longicaudis* [15/314 (04.77%), *S. guianensis* [01/131 (3.22%)] and *T. inunguis* [01/131 (0.76%)].

The *k* value in the diagnosis of *Cryptosporidium* using DFA test and Kinyoun's technique was 0.86. In the identification of *Giardia* cysts through the centrifugation-flotation technique and DFA, *k* value was 0.27.

The sensitivity, specificity, real prevalence, estimated prevalence, positive predictive value, negative predictive value, correct classification (accuracy) and incorrect classification values for each etiological agent are show in Table 2,3.

DISCUSSION

The identification of *Cryptosporidium* oocysts in the aquatic mammal species reported in this study, especially the findings in *Lontra longicaudis, Pteronurabrasiliensis* and *Sotalia guianensis*, as well as the presence of *Giardia* cysts in *L. longicaudis, P. brasiliensis*, *T. inunguis* and *T. manatus*, increase the number of reported hosts affected by these protozoa.

These findings, in addition to the previous descriptions about *Cryptosporidium* and *Giardia* in captive and free-living aquatic mammals in other countries [8,7] as well as in the Brazil [4] may represent an even greater risk to public health [19,20].

The frequency of infection in the aquatic mammal species studied may be related to different factors such as dietary habits, climatic seasonality, sample size, environmental contamination intensity in water resources and sensitivity of diagnostic techniques [21,5].

In this sense, considering the possible variations in the sensitivity of the different laboratory techniques, we chose to use two diagnostic methods for each parasite focused in this study. In order to evaluate the agreement between these methods, the Kappa (k) index was used [15,16].

In the relationship established between the direct immunofluorescent antibody test and the Kinyoun technique for the diagnosis of *Cryptosporidium*, *k* = 0.86 was found, being considered an almost perfect or optimal agreement [16]. However in the case of *Giardia* cysts identified using the centrifugation-flotation technique and DFA, the *k* value was 0.27, being in this case considered a reasonable agreement by some authors [15,16].

In the parasitological methods, the dyes used were relatively easy to prepare. The Kinyoun technique showed relevant quality to be used in the laboratory routine, considering its good sensitivity and low cost, although showing limitations inherent in the slowness of procedures, during smear preparation and staining, requiring the performance of microscopy in all fields of the slide, increasing the time to perform procedures [22,23]. The centrifugation-flotation method presented low sensitivity and limited efficiency in the diagnosis of *Giardia* when compared to the DFA technique.

Table 1: Fecal samples from the 15 species of aquatic mammals used in this study.

Order	Specie	Origin of samples	Total number of samples
Cetartiodactyla	*Balaenoptera acutorostrata*	Necropsy	2
	Grampus griseus	Necropsy	1
	Inia geoffrensis	Free-living species under restraint	2
	Kogia breviceps	Necropsy	1
	Kogia sima	Necropsy	4
	Peponocephala electra	Necropsy	8
	Physeter macrocephalus	Necropsy	2
	Sotalia guianensis	Necropsy	31
	Stenella attenuata	Necropsy	1
	Stenella clymene	Necropsy	2
	Ziphius cavirostris	Necropsy	1
Carnivora – Family Mustelidae	*Lontra longicaudis*	Resting places, dens, latrines, rehabilitation enclosure	314
	Pteronurabrasiliensis	Resting places, dens, latrines	24
Sirenia	*Trichechus inunguis*	Floating samples collected in feeding areas; Captive animals	131
	Trichechus manatus	Captive animals; reintroduced animals; necropsy	29

Table 2: Evaluation of the technique ofKinyoun and Centrifugation-Flotation in relation to the DFA technique (gold standard)for the diagnosis of *Cryptosporidium* and *Giardia* in aquatic mammals.

		Parameters (%)							
Etiological Agent	Technique	Sensitivity	Specificity	Real Prevalence	Estimated Prevalence	Predictive Value (+)	Predictive Value (-)	Correct Classification (Accuracy)	Incorrect Classification
Cryptosporidium	Kinyoun	57.47	100	15.73	9.04	100	92.64	93.30	6.69
	DFA	67.81	100	15.73	10.66	100	94.33	94.93	5.06
Giardia	Centrifugation-Flotation	21.56	100	9.22	1.98	100	92.61	92.76	7.23
	DFA	96.07	100	9.22	8.86	100	99.60	99.63	0.36

Abbreviations: DFA: Direct immunofluorescent antibody

Table 3: Infection detected in each technique of two parasites.

Technique	Etiological Agent	Positivity (%)
Kinyoun	*Cryptosporidium*	9.04
DFA		10.66
Centrifugation-Flotation	*Giardia*	1.98
DFA		8.86

Additionally, based on immunological principles, the DFA reaction using anti-*Cryptosporidium* and *Giardia* monoclonal antibodies marked with fluorescein isothiocyanate, provided in many cases, high sensitivity and applicability for processing a larger number of samples in the results found in this study. These findings are similar to those reported by [9] However, the high cost of test kits is a disadvantage, which may represent a limitation for the use of this method in routine evaluations involving aquatic mammals.

CONCLUSION

The DFA technique demonstrated greater sensitivity both in the diagnosis of *Cryptosporidium* and *Giardia*. However, the combination of more than one laboratory technique is recommended when seeking to be more assertive in the detection of these parasites in aquatic mammals.

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