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ANIMAL SCIENCE

Use of the FLOTAC technique as a new coproparasitological diagnostic method in aquatic mammals and comparison with traditional methods

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Abstract: The inadequate choice of a diagnostic method or the option for techniques that have low sensitivity and specificity may limit the diagnosis of parasitic agents that affect aquatic mammals. The aim of this study was to evaluate the performance of the FLOTAC technique and compare it with three traditional methods (Willis, sedimentation and centrifugation- flotation) used in the diagnosis of gastrointestinal parasites in aquatic mammals. For this, 129 fecal samples from 12 species were collected. Each sample was submitted to laboratory processing using the Willis, Hoffman techniques, Faust method and FLOTAC. Sensitivity, specificity, real prevalence, estimated prevalence, positive predictive value, negative predictive value, correct classification (accuracy) and incorrect classification were evaluated to compare the different diagnostic methods. The highest frequency of positive samples occurred using FLOTAC (46.51%), compared to Hoffman (23.25%), Faust (10.07%) and Willis techniques (6.97%). In the samples analyzed, the occurrence of Strongylidae eggs and Eimeriidae oocysts was frequently observed. The FLOTAC technique proved to be the most appropriate technique and due to its efficacy, is strongly recommended for coproparasitological evaluations in aquatic mammals.

Key words: Diagnosis, FLOTAC, helminths, marine mammals, parasitic diseases, protozoa.

INTRODUCTION

In addition to their economic and health importance, parasites are an integral part of the biosphere (Raga et al. 2009), where due to their diversity and mechanisms of action, infect many free organisms, influencing the host health, size and behavior of populations and the dynamics of the food chain and community structure (Raga et al. 2009).

However, the current knowledge about parasitism in aquatic mammals has is caveats, due to the difficulties in obtaining samples from these animals (Bossart 2001, Borges et al. 2011), the limited understanding of relationships between hosts and the biology of parasites, as well as limitations found in the development of experimental studies (Raga et al. 1997). In addition, the choice of an inadequate diagnostic method or the choice of techniques that have low sensitivity and specificity may limit the evidence of these etiological agents (Appelbee et al. 2010, Rengifo-Herrera et al. 2011, Reboredo-Fernández et al. 2015).

Even recognizing the importance and contribution of traditional methods for the diagnosis of gastrointestinal parasites, such as centrifugation-flotation (Bando et al. 2014),

Willis (Willis 1921) and sedimentation (Bando et al. 2014), the use of FLOTAC has been proposed in recent years, representing a new multivalent technique for the qualitative and quantitative identification of these pathogens (Cringoli et al. 2011, 2013, Maurelli et al. 2014, Capasso et al. 2019).

Researches using FLOTAC have shown that this technique has greater sensitivity when compared to conventional and traditional methods. These studies have been initially focused on domestic animals (Cringoli et al. 2010, Lima et al. 2015) and humans (Becker et al. 2011, Knopp et al. 2014), with no reports of use in aquatic mammals. In all studies developed, the use of FLOTAC showed greater efficiency in the identification of eggs or oocysts (Knopp et al. 2009, Lima et al. 2015).

The aim of this study was to evaluate the performance of the FLOTAC technique and to compare it with three traditional methods (Willis, sedimentation and centrifugation-flotation) used in the diagnosis of gastrointestinal parasites in aquatic mammals.

MATERIALS AND METHODS

A total of 129 fecal samples and intestinal contents from 12 species of aquatic mammals were collected (Table I), both captive and free ranging. The collections of the biological material occurred in eight states of Brazil, covering states from the northern (Amapá and Rondônia) and northeastern (Alagoas, Bahia, Ceará, Maranhão, Paraíba and Sergipe) regions between 2013 and 2014.

After collection, the material was preserved in flasks containing an alcohol-formaldehyde-glacial acetic acid-distilled water (AFA) solution, in proportions suggested by Ueno & Gonçalves (1994), and subsequently sent for laboratory processing.

Each sample underwent laboratory processing using Willis (Willis 1921) and spontaneous sedimentation - Hoffman (Hoffman et al. 1934), Faust method using zinc sulfate (Cantos et al. 2011) and FLOTAC techniques (Figures 1 and 2) (Cringoli et al. 2010). Eggs and cysts found were identified at family level.

Sensitivity, specificity, real prevalence, estimated prevalence, positive predictive value, negative predictive value, correct classification (accuracy) and incorrect classification were evaluated to compare the different diagnostic methods, and the Willis technique was defined as the gold standard for these analyses (Lima et al. 2015).

The data found of the positivity of four techniques were analyzed using the McNemar's test, with differences considered statistically significant when $p < .0005$. The Partitioning Chi-square test was used to compare the results of families with a significant level of $p \leq .05$. The Cohen's kappa coefficient (k) used to compare the results and to evaluate the agreement between the different techniques (Landis & Koch 1977, Lima et al. 2015). The sensitivity, specificity, positive and negative predictive values, and accuracy of each technique was determined using the InStat software with significance level $p < .05$ (GraphPad Software, Inc., 2000).

All procedures were conducted, under permit number 33.819-1 granted by the Biodiversity Information and Authorization System (SISBIO). In addition, this research was evaluated and approved by the Ethics Research Committee of the Federal Rural University of Pernambuco (010/2014).

Table I. Origin of fecal samples from 12 species of aquatic mammals.

Order	Species	Origin of samples	Location	Total number of samples
Carnivora – Family Mustelidae	*Lontra longicaudis*	Resting places, dens, latrines, rehabilitation enclosure	AP, RO, SE	94
	Pteronura brasiliensis	Resting places, dens, latrines	RO	4
Cetartiodactyla	*Balaenoptera acutorostrata*	Necropsy	MA	2
	Grampus griseus	Necropsy	CE	1
	Kogia breviceps	Necropsy	SE	1
	Kogia sima	Necropsy	CE	2
	Peponocephala electra	Necropsy	AL, CE, SE	7
	Physeter macrocephalus	Necropsy	SE	2
	Sotalia guianensis	Necropsy	BA, PB, SE	11
	Stenella attenuata	Necropsy	SE	1
	Stenella clymene	Necropsy	BA	1
Sirenia	*Trichechus manatus*	Captive animals; Reintroduced animals; necropsy	CE, SE	3

AL (Alagoas); AP (Amapá); RO (Rondônia); SE (Sergipe); MA (Maranhão), CE (Ceará); BA (Bahia); PB (Paraíba).

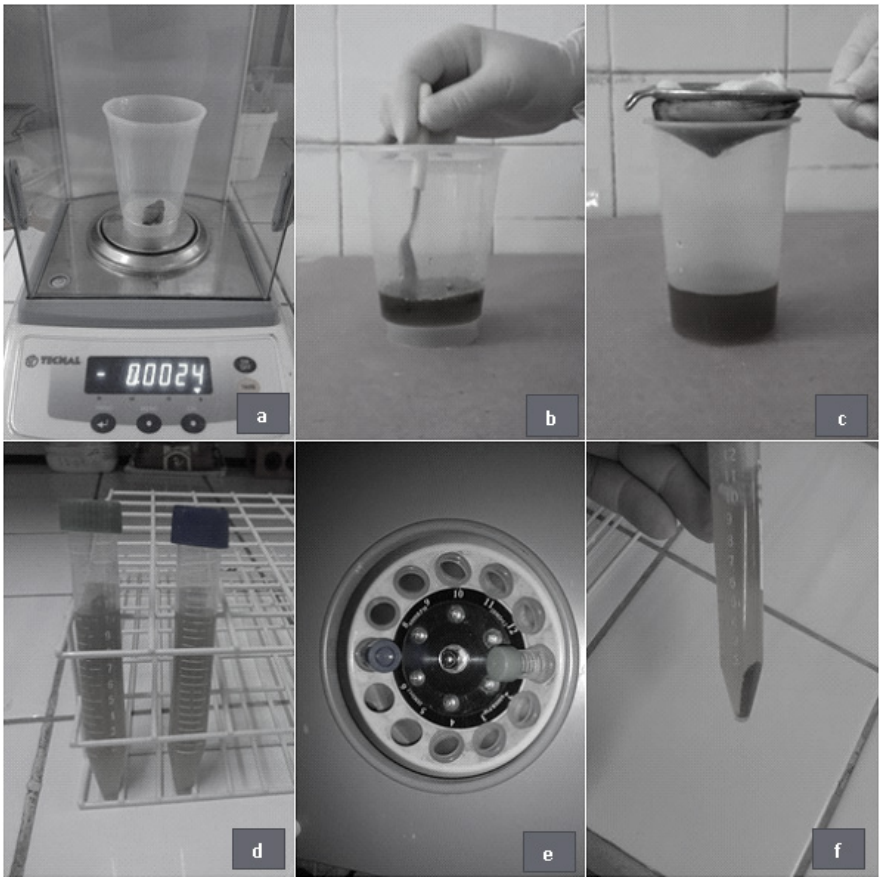

Figure 1. Steps for processing fecal samples using FLOTAC techniques. a) Weigh the sample; b) Homogenization in water; c) Filter; d) Content deposition in Falcon tubes; e) Centrifugation; f) Aspect of the material after centrifugation.

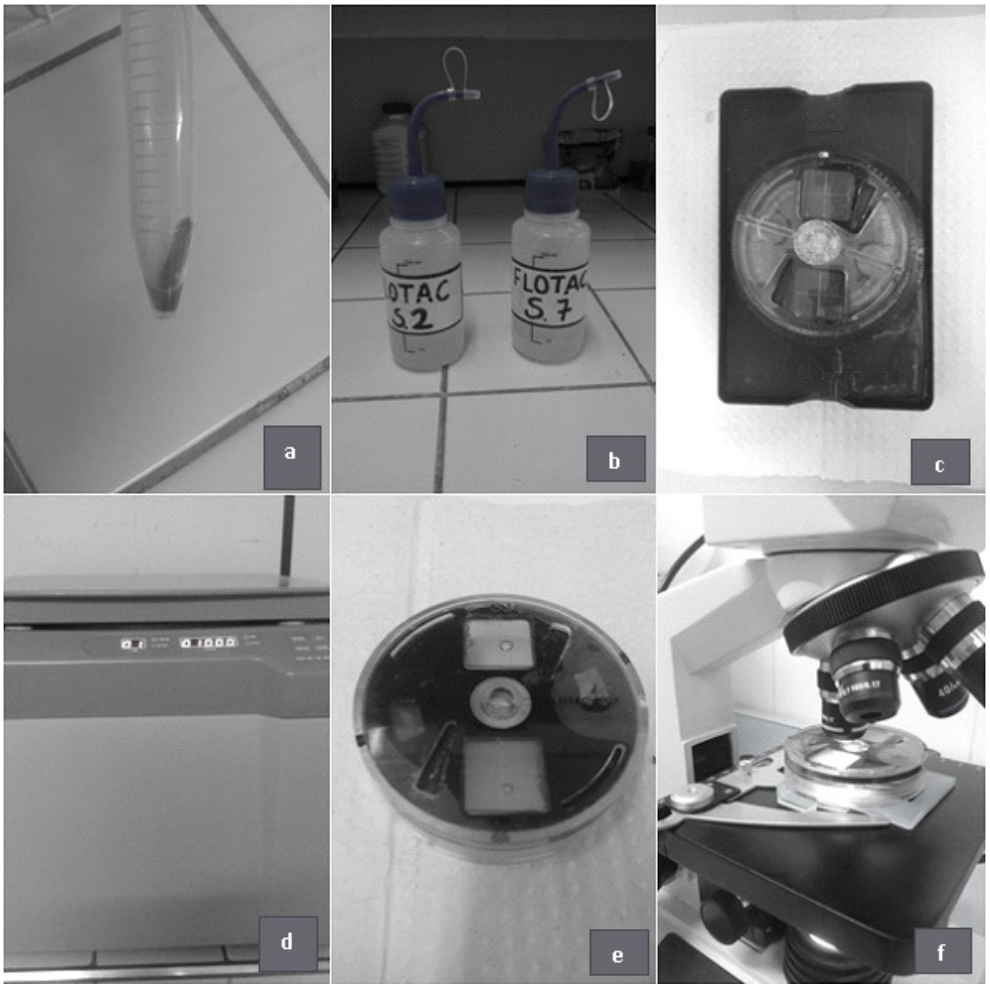

Figure 2. Steps for processing fecal samples using FLOTAC techniques. a) Sediment, after disposal of the supernatant; b) Containers containing the saturated solutions; c) FLOTAC chambers filled; d) Centrifuge used for sample processing; e) After centrifugation, translate the top parts of the flotation chambers; f) Examine under a microscope.

RESULTS

The four techniques used were able to identify helminth eggs and gastrointestinal protozoa oocysts and cysts (Table II). However, the highest frequency of positive samples occurred using FLOTAC (46.51%), compared to the Hoffman (23.25%), Faust (10.07%) and Willis techniques (6.97%) ($p = 0.1250$).

In the samples analyzed, Strongylidae eggs and Eimeriidae oocysts were more frequently observed. Concomitant infections caused by two or three different etiologic agents were found with all techniques used.

The FLOTAC technique also showed greater efficacy in terms of sensitivity, specificity, real prevalence, estimated prevalence, positive predictive value, negative predictive value and correct classification (accuracy) values (Table III). At the *k* analyses, a poor concordance ($k = 0$) was observed among methods.

DISCUSSION

Although the different parasitic agents were diagnosed by means of the four techniques used, the FLOTAC method showed a higher frequency of positive samples, as well as a greater diversity of identified parasites. Similar results have been reported in virtually all studies with parasites of domestic and human species (Cringoli et al. 2010, Becker et al. 2011, Knopp et al. 2014, Lima et al. 2015).

Table II. Simple infection and co-infection by gastrointestinal parasites in aquatic mammals.

Technique	Family	Hosts	Relative Frequency (%)
Willis	Strongylidae	*L. longicaudis*, *P. electra*	3.10 (04/129)
	Eimeriidae	*L. longicaudis*	1.55 (02/129)
	Diphyllobothriidae	*L. longicaudis*	0.77 (01/129)
	Opisthorchiidae	*L. longicaudis*	0.77 (01/129)
	Diphyllobothriidae + Strongylidae	*L. longicaudis*	0.77 (01/129)
Hoffman	Strongylidae	*L. longicaudis*, *P. electra*	5.42 (07/129)
	Eimeriidae	*L. longicaudis*	3.87 (05/129)
	Diphyllobothriidae	*L. longicaudis*	3.10 (04/129)
	Strongylidae + Diphyllobothriidae	*L. longicaudis*	2.32 (03/129)
	Lernaeidae	*L. longicaudis*	1.55 (02/129)
	Lernaeidae + Eimeriidae	*L. longicaudis*	1.55 (02/129)
	Ancylostomatidae	*L. longicaudis*	0.77 (01/129)
	Ancylostomatidae + Strongylidae	*L. longicaudis*	0.77 (01/129)
	Hexamitidae (*Giardia* sp.) + Eimeriidae	*L. longicaudis*	0.77 (01/129)
	Trichinellidae	*L. longicaudis*	0.77 (01/129)
	Diphyllobothriidae + Strongylidae + Kudoidae	*L. longicaudis*	0.77 (01/129)
	Strongylidae + Kudoidae	*L. longicaudis*	0.77 (01/129)
	Diphyllobothriidae + Strongylidae + Eimeriidae	*L. longicaudis*	0.77 (01/129)
Faust	Eimeriidae	*L. longicaudis*, *S. guianensis*, *S. attenuata*	7.75 (10/129)
	Hexamitidae (*Giardia* sp.)	*L. longicaudis*	0.77 (01/129)
	Eimeriidae + Strongylidae	*L. longicaudis*	0.77 (01/129)
	Hexamitidae (*Giardia* sp.) + Lernaeidae	*L. longicaudis*, *P. electra*	0.77 (01/129)
FLOTAC	Eimeriidae	*L. longicaudis*, *P. brasiliensis*, *K. sima*, *P. electra*, *S. guianensis*, *S. clymene*, *T. manatus*	19.37 (25/129)
	Strongylidae	*L. longicaudis*, *S. guianensis*	6.97 (09/129)
	Eimeriidae + Strongylidae	*L. longicaudis*, *P. electra*, *S. guianensis*	3.10 (04/129)
	Ancylostomatidae	*L. longicaudis*, *P. brasiliensis*	1.55 (02/129)
	Ancylostomatidae + Eimeriidae	*L. longicaudis*	2.32 (03/129)
	Eimeriidae + Lernaeidae	*L. longicaudis*, *B. acutorostrata*	1.55 (02/129)
	Ichthyophthiriidae	*L. longicaudis*, *P. brasiliensis*	1.55 (02/129)
	Lernaeidae	*L. longicaudis*	1.55 (02/129)
	Ancylostomatidae + Strongylidae	*L. longicaudis*	0.77 (01/129)
	Ancylostomatidae + Trichinellidae + Strongylidae	*L. longicaudis*	0.77 (01/129)
	Ancylostomatidae + Opisthorchiidae + Lernaeidae	*L. longicaudis*	0.77 (01/129)
	Dioctophymatidae + Eimeriidae	*L. longicaudis*	0.77 (01/129)
	Dioctophymatidae + Gyrodactylidae	*L. longicaudis*	0.77 (1/129)
	Eimeriidae + Myxobolidae	*L. longicaudis*	0.77 (01/129)
	Eimeriidae + Ichthyophthiriidae + Lernaeidae	*L. longicaudis*	0.77 (01/129)
	Kudoidae	*L. longicaudis*	0.77 (01/129)
	Opisthorchiidae	*L. longicaudis*	0.77 (01/129)
	Strongylidae + Lernaeidae	*L. longicaudis*	0.77 (01/129)
	Strongylidae + Gyrodactylidae + Lernaeidae	*L. longicaudis*	0.77 (01/129)

Table III. Evaluation of Willis, Hoffman, Faust and FLOTAC techniques in the diagnosis of gastrointestinal parasites in aquatic mammals.

Technique	Family	Parameters (%)							
		Sensitivity	Specificity	True Prevalence	Estimated Prevalence	Predictive value (+)	Predictive value (-)	Accuracy	Incorrect Classification
Willis	Strongylidae	15.62	100	24.80	3.87	100	78.22	79.06	20.93
	Eimeriidae	4.4	100	34.88	1.55	100	66.14	66.66	33.33
	Diphyllobothriidae	18.18	100	8.52	1.55	100	92.91	93.02	6.97
	Opisthorchiidae	33.33	100	2.32	0.77	100	98.43	98.44	1.55
Hoffman	Strongylidae	43.75	100	24,80	10.85	100	84.34	86.04	13.95
	Eimeriidae	20	100	34.88	6.97	100	70	72.09	27.90
	Diphyllobothriidae	81.81	100	8.52	6.97	100	98.33	98.44	1.55
	Lernaeidae	33.33	100	9.30	3.10	100	93.6	93.79	6.20
	Ancylostomatidae	22.22	100	6.97	1.55	100	94.48	94.57	5.42
	Hexamitidae	33.33	100	2.32	0.77	100	98.43	98.44	1.55
	Trichinellidae	50	100	1.55	0.77	100	99.21	99.22	0.77
	Kudoidae	100	100	1.55	1.55	100	100	100	0
Faust	Strongylidae	3.12	100	24.80	0.77	100	75.78	75.96	24.03
	Eimeriidae	24.44	100	34.88	8.52	100	71.18	73.64	26.35
	Lernaeidae	8.33	100	9.30	0.77	100	91.40	91.47	8.52
	Hexamitidae	66.66	100	2.32	1.55	100	99.21	99.22	0.77
Flotac	Strongylidae	53.12	100	24.80	13.17	100	86.60	88.37	11.62
	Eimeriidae	82.22	100	34.88	26.68	100	91.30	93.79	6.20
	Opisthorchiidae	66.66	100	2.32	1.55	100	99.21	99.22	0.77
	Lernaeidae	58.33	100	9.30	5.42	100	95.90	96.12	3.87
	Ancylostomatidae	88.88	100	6.97	6.20	100	99.17	99.22	0.77
	Trichinellidae	50	100	1.55	0.77	100	99.21	99.22	0.77
	Kudoidae	50	100	1.55	0.77	100	99.21	99.22	0.77
	Ichthyophthiriidae	100	100	1.55	100	100	100	100	0
	Dioctophymatidae	100	100	1.55	100	100	100	100	0
	Gyrodactylidae	100	100	1.55	100	100	100	100	0
	Myxobolidae	100	100	0.77	0.77	100	100	100	0

Differences between methodologies used here, particularly the density of solutions, presence of impurities, distortion in the cysts / oocysts / eggs structures and host parasite load are some of the probable factors that may have influenced the different coproparasitological results, as reported in other studies evaluating the laboratory techniques used in the diagnosis of endoparasites (Dubey 1993, Souza-Dantas et al. 2007).

Given the limitations that a particular laboratory technique may present, it is relevant to choose a safe and efficient method. In addition, the coproparasitological diagnosis of gastrointestinal parasites is still the most used laboratory resource because it is easy to perform and has low cost (Souza-Dantas et al. 2007).

Considering these premises for the choice of laboratory techniques used in the diagnosis of gastrointestinal helminths of aquatic mammals and even using direct examination methods such as Willis, Hoffman and coproculture, J.C.G. Borges et al. (unpublished data) did not identify the presence of helminth eggs or larvae. These laboratory techniques have been used in several studies involving parasitological diseases in other species of aquatic mammals (Torres et al. 2004, Uchôa et al. 2004); however, although these traditional methods are part of the laboratory routine, they present significant limitations for adequate diagnosis (Cantos et al. 2011).

As a way of minimizing these limitations found by the use of diagnostic techniques in studies contemplating aquatic mammals, combinations of different coproparasitological diagnostic methods have been performed (Torres et al. 2004, Uchôa et al. 2004).

These strategies have been useful when considering the morphological and biological variability presented by parasites (Mendes et al. 2005) and the specificity that certain techniques present in identifying only eggs or cysts that settled or are on the surface of the solution used (Cantos et al. 2011). However, this leads to additional costs and time to perform laboratory diagnosis.

Using the FLOTAC technique in the current study, it was possible to diagnose helminth eggs and protozoan oocysts and cysts. This technique is highly sensitive and can provide up to 10 times the capacity to identify eggs and cysts of different parasites (Cringoli et al. 2010).

Another relevance observed with the use of FLOTAC was the possibility of working with preserved fresh samples, thus allowing a greater optimization of laboratory activities and safety for the laboratory team through the exposure of these professionals when seeking compliance with protocols that recommend the use of fresh fecal samples (Cringoli et al. 2010).

The diversity of etiological agents identified in this study, among other factors, is directly related to the large number of aquatic mammal species from which samples were obtained, the different habitats, feeding behavior, age and host's immunological condition (McCarthy & Moore 2000, Fahrion et al. 2011). Similarly, the families of these parasites have also been reported in other studies involving mustelids (Hoberg et al. 1997, Torres et al. 2004, Uchôa et al. 2004) and cetaceans (Hughes-Hanks et al. 2005, Altieri et al. 2007, Reboredo-Fernández et al. 2015).

Considering the findings of this study, the FLOTAC technique was more appropriate than the other techniques. Due to its efficiency, it is strongly recommended for coproparasitological evaluations in aquatic mammals without the need to process samples using other sedimentation and flotation methods.

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João Carlos Gomes Borges had a substantial contribution in the concept, study design, data collection, analysis and manuscript preparation; Victor Fernando Santana Lima and Edson Moura da Silva contributed to the processing and analysis of the samples; Danielle dos Santos Lima and Vitor Luz Carvalho contributed to data collection; Miriam Marmontel, Maria Aparecida da Glória Faustino, Giuseppe Cringolli, Laura Rinaldi contribution in the concept, study design, analysis and manuscript preparation; and Leucio Câmara Alves contribution in the concept, study design, analysis, manuscript preparation and supervision.