Veterinary Parasitology 202 (2014) 132–137

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Detection and molecular characterization of *Giardia* and *Cryptosporidium* in common dolphins (*Delphinus delphis*) stranded along the Galician coast (Northwest Spain)

A. Reboredo-Fernández[a], H. Gómez-Couso[a,c,*], J.A. Martínez-Cedeira[b], S.M. Cacciò[c], E. Ares-Mazás[a]

[a] *Laboratory of Parasitology, Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Santiago de Compostela, Campus Vida, 15782 Santiago de Compostela, A Coruña, Spain*
[b] *CEMMA, Camiño do Ceán, 2, 36350 Nigrán, Pontevedra, Spain*
[c] *Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 11 March 2014
Accepted 13 March 2014

Keywords:
Delphinus delphis
Giardia
Cryptosporidium
Molecular characterization
Galician coast (Northwest Spain)

ABSTRACT

The ubiquitous protozoan parasites *Giardia* and *Cryptosporidium* have been detected from many species of captive and free-living wildlife, representing most mammalian orders. Twenty species of marine mammals have been reported to inhabit Galician waters and the region has one of the highest rates of stranding in Europe. Evidence from stranding, reported by-catches and sightings, suggests that the common dolphin (*Delphinus delphis*) is the most abundant cetacean on the Galician coast (Northwest Spain). The objective of this study was to detect and molecularly characterize isolates of *Giardia* and *Cryptosporidium* obtained from common dolphins stranded in this area. Between 2005 and 2012, sections of large intestine from 133 common dolphins stranded along the Galician coast were collected by the personnel of the Galician Stranding Network (*Coordinadora para o Estudo dos Mamíferos Mariños*, *CEMMA*). Using direct immunofluorescence antibody test (IFAT) and PCR amplification and sequencing of the SSU-rDNA, β-giardin genes and the ITS1-5.8S-ITS2 region, *Giardia* and *Cryptosporidium* were detected in 8 (6.0%) and 12 samples (9.0%), respectively. In two samples, co-infection by both parasites was observed. The molecular characterization revealed the presence of *Giardia duodenalis* assemblages A (genotypes A1 and A2) and B and *Cryptosporidium parvum* in these samples. This constitutes the first study in which the presence of *Giardia* and *Cryptosporidium* has been investigated in common dolphins on the European Atlantic coast, and it is also the first report of *C. parvum* in this host. Our findings indicate that these animals could act as reservoir of these waterborne parasites or could be victims of the contamination originated by anthropogenic activities.

1. Introduction

Giardia and *Cryptosporidium*, two ubiquitous protozoan parasites, have been detected from many species of captive and free-living wildlife, representing most mammalian orders. In marine environments, *Giardia* sp. cysts were first reported in intestinal contents of ringed seals

* Corresponding author at: Laboratory of Parasitology, Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Santiago de Compostela, Campus Vida, 15782 Santiago de Compostela, A Coruña, Spain. Tel.: +34 981 563100x14889; fax: +34 981 594912.
E-mail address: hipolito.gomez@usc.es (H. Gómez-Couso).

http://dx.doi.org/10.1016/j.vetpar.2014.03.018
0304-4017/

(*Phoca hispida*) from the western arctic region of Canada using a fluorescent monoclonal antibody test (Olson et al., 1997). Although cysts were morphometrically identical to those of *Giardia* sp., no molecular characterization was undertaken to identify the species found in this host. Likewise, *Cryptosporidium* infection was first described in a dugong (*Dugong dugon*) in a coastal town of Australia by Hill et al. (1997), who observed by transmission electron microscopy numerous forms of *Cryptosporidium* intermediate life stages in small intestine sections, but oocysts were not found. This isolate was subsequently characterized as *Cryptosporidium hominis* (Morgan et al., 2000). Since then, several isolates of *Giardia* and *Cryptosporidium* obtained from different pinniped and cetacean species in a few locations of North America and in the Antarctic continent have been described and sometimes characterized at the molecular level, revealing that marine mammals can act not only as reservoirs for species that infect humans and domestic animals, but also harbour new genotypes of both parasites (Gaydos et al., 2008; Lasek-Nesselquist et al., 2010; Rengifo-Herrera et al., 2011, 2013; Bass et al., 2012).

The Galician coast (Northwest Spain) is an important area for cetaceans, not only within Spain but also at the European level. Up to 20 marine mammal species (16 cetaceans and 4 pinnipeds) have been reported to inhabit this area, which has one of the highest recorded rates of stranding in Europe (Covelo and Martínez, 2001; López et al., 2004; Pierce et al., 2010). Evidence from stranding, reported by-catches and sightings, suggests that the common dolphin (*Delphinus delphis*) is the most abundant cetacean species on the Galician coast, with an estimated population of 7,000–10,000 individuals living in Galician waters (López et al., 2002, 2004; Pierce et al., 2010).

The aim of the present work was to investigate the presence of *Giardia* and *Cryptosporidium* in common dolphins (*D. delphis*) stranded on the Galician coast.

2. Materials and methods

2.1. Sample collection

Between 2005 and 2012, a total number of 133 intestinal samples from stranded common dolphins (*D. delphis*) were collected in the Northwest of the Iberian Peninsula along the Galician coast by experienced personnel of the Galician Stranding Network (*Coordinadora para o Estudo dos Mamíferos Mariños*, *CEMMA*) (Fig. 1). Animals were identified to the level of species, measured and sexed. Among these samples, 54 corresponded to females and 78 to males. Taking into account the age of the animal, 43 samples were from adult individuals (27 females and 16 males); 79 from juvenile specimens (21 females and 58 males); 10 from calves (6 females and 4 males) and in one animal was impossible to determinate the age. After necropsy, sections of large intestine were collected and stored at −20 °C until being processed in the Laboratory of Parasitology of the Department of Microbiology and Parasitology of the University of Santiago de Compostela.

Intestinal contents (2.50 ± 0.48 g) were diluted in 10–20 ml of phosphate buffered saline (PBS) 0.04 M pH 7.2, filtered through a set of two sieves (mesh size 150 and 45 μm), shaken with diethyl ether (2:1, v/v) and centrifuged at 1000 × *g* for 15 min at 4 °C. The resulting two up layers were carefully discarded, the sediment was washed in PBS by centrifugation at 1000 × *g* for 15 min at 4 °C and the pellet was resuspended in 500 μl of PBS 0.04 M pH 7.2.

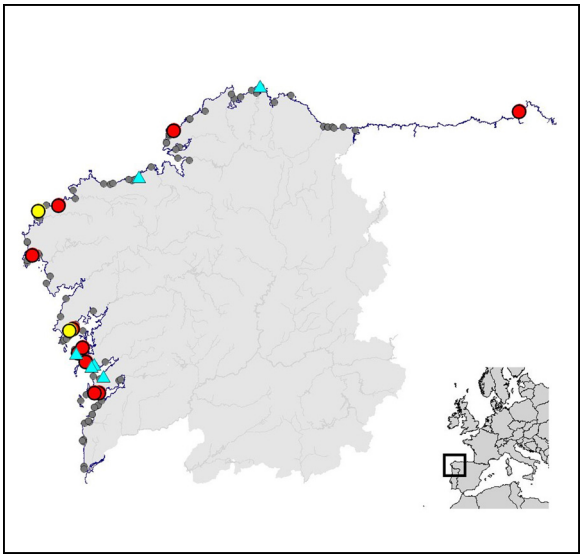

Fig. 1. Locations of the common dolphins (*D. delphis*) stranded along the Galician coast. Grey circles ( ) indicate negative samples, blue triangles ( ) indicate *Giardia*-positive samples, red circles ( ) indicate *Cryptosporidium*-positive samples and yellow circles ( ) indicate co-infection by both parasites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

2.2. Detection of Giardia cysts and Cryptosporidium oocysts by epifluorescence microscopy

Direct immunofluorescence antibody test (IFAT) was performed on 50 μl of the sediments using the AquaGlo™ G/C Direct test (Waterborne, Inc., New Orleans, LA, USA), according to the manufacturer's instructions. The cysts/oocysts were identified by epifluorescence microscopy at 400× magnifications on the basis of their shape, size and the pattern and intensity of immunofluorescence staining.

2.3. Molecular characterization of Giardia spp. and Cryptosporidium spp.

Nucleic acids were extracted from the remaining 450 μl of the sediments obtained previously using the QIAamp® DNA Stool Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions. DNA was stored at −20 °C until use.

For *Giardia*, protocols for the amplification of a ~170-bp fragment of the small subunit ribosomal DNA (SSU-rDNA) gene, of a ~315-bp fragment encompassing the ITS1-5.8S-ITS2 region in the ribosomal unit and of a 511-bp of the β-giardin (bg) gene were used as described previously (Read et al., 2002; Lalle et al., 2005; Cacciò et al., 2010). For *Cryptosporidium*, a two-step nested-PCR technique was

utilized to amplify a 587-bp fragment of the SSU-rDNA gene (Ryan et al., 2003). Positive and negative controls were included in all experiments. The PCR products were subjected to electrophoresis on 2% agarose/ethidium bromide gels.

Positive PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN®) and sequenced in both directions using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Applied Byosystems®, Life Technologies™, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequencing reactions were analysed using the ABI PRISM® 3100 automatic sequencer (Applied Biosystems®) and sequences were assembled using the software SeqMan™ 7.0.0 (DNASTAR®, Madison WI, USA) and BioEdit 7.0.5.3 (©1997–2005 Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). The resulting sequences were compared with those deposited in GenBank® (National Center for Biotechnology Information, Bethesda, MD, USA) using the program BLAST®2.2.28 (http://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology Information). Phylogenetic and molecular evolutionary analyses were made using MEGA 5 (Tamura et al., 2011) on the basis of genetic distances calculated by the Kimura two-parameter model (Kimura, 1980).

2.4. Statistical analysis

Chi-square or Fisher's exact test was used to analyse the data according to sex, age and stranded location along the coast with the GraphPad Instat® 3.05 statistical software (©1992–2009 GraphPad Software, Inc., La Jolla, CA, USA).

2.5. Nucleotide sequence accession numbers

The nucleotide sequences of the isolates analysed in this study have been deposited in the GenBank database under accession numbers KF835548–KF835560.

3. Results

As a result of the application of IFAT and PCR techniques, among the 133 intestinal samples of *D. delphis*, 8 (6.0%) and 12 (9.0%) samples were positive for *Giardia* spp. and *Cryptosporidium* spp., respectively. Co-infection by both pathogens was found in two specimens (Table 1). All samples contained a low number of parasitic forms, between 1 and 5 cysts/oocysts per 50 μl of sediment obtained from intestinal content (10–50 cysts/oocysts per section of large intestine).

Giardia spp. cysts were observed by IFAT in two samples, but the amplification of fragments of the ITS1-5.8S-ITS2 region, β-giardin gene and the SSU-rDNA gene showed that eight samples were positive for *G. duodenalis*, including the two IFAT positive samples. Sequencing analysis of the fragments of the ITS1-5.8S-ITS2 region, showed the presence of *G. duodenalis* assemblage A, genotypes A1 and A2, and assemblage B in 3, 3 and 2 samples, respectively (Fig. 2). At the β-giardin gene, three sequences were identical to those deposited in GenBank, corresponding to *G. duodenalis* assemblage A, genotype A1 (Supplementary Fig. 1). Nevertheless, inconsistent results were found between those and the amplification of fragments of the SSU-rDNA gene, which showed the presence of *G. duodenalis* assemblage A1 in four samples, and *G. duodenalis* assemblage B in another sample (Supplementary Fig. 2). Moreover, some sequences obtained by amplification of a fragment of the ITS1-5.8S-ITS2 region and the SSU-rDNA gene showed single nucleotide polymorphisms (SNPs) with respect to the sequences deposited in GenBank (Supplementary Fig. 3).

Table 1
Prevalence of *Giardia* and *Cryptosporidium* in common dolphins (*D. delphis*) stranded on the Galician coast according to sex and age of the animal.

	Giardia (%)	*Cryptosporidium* (%)
Sex (*n*)		
Female (54)	3 (5.5)	4 (7.4)
Male (78)[a]	5 (6.4)	7 (8.9)
Not determined (1)	–	1 (100)
Age (*n*)		
Adult (43)	1 (2.3)	2 (4.6)
Juvenile (79)[a]	6 (7.6)	8 (10.1)
Calf (10)	1 (10)	1 (10)
Not determined (1)	–	1 (100)
Total	8 (6.0)	12 (9.0)

[a] Co-infection was found in two juvenile males.

Supplementary Figs. 1–3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2014.03.018.

Cryptosporidium spp. oocysts were observed by immunofluorescence microscopy in 12 samples (9.0%), and in three of these samples, from a juvenile female, male calf and adult male, the parasites were identified as *C. parvum* by sequencing of a fragment of the SSU-rDNA gene (Supplementary Fig. 4).

Supplementary Fig. 4 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2014.03.018.

When the sex or age of the infected animals was considered, no statistically significant differences were observed (*Giardia* sp., $P=1$; *Cryptosporidium* sp., $P=1$ and *Giardia* sp., $P=0.43$; *Cryptosporidium* sp., $P=0.56$, respectively) (Table 1). Furthermore, the distribution of individuals that tested positive was proportional along the entire coast and no statistically significant association was found between positive specimens and stranding location (Fig. 1). Thus, the prevalence of *Giardia* was 6.9% and 4.9% on the west and north coast respectively; similarly, the prevalence of *Cryptosporidium* was 9.7% on the west coast and 8.2% on the north coast (*Giardia* sp., $P=0.73$ and *Cryptosporidium* sp., $P=1$).

4. Discussion

This is the first study in which the presence of *Giardia* and *Cryptosporidium* has been investigated in common dolphins (*D. delphis*) from the European Atlantic coast. Using IFAT and PCR analysis, *Giardia* and *Cryptosporidium* were detected in 6.0% and 9.0% of the 133 animals stranded along the Galician coast (NW Spain).

Currently, published data about these protozoan parasites in marine mammals are limited. From an initial

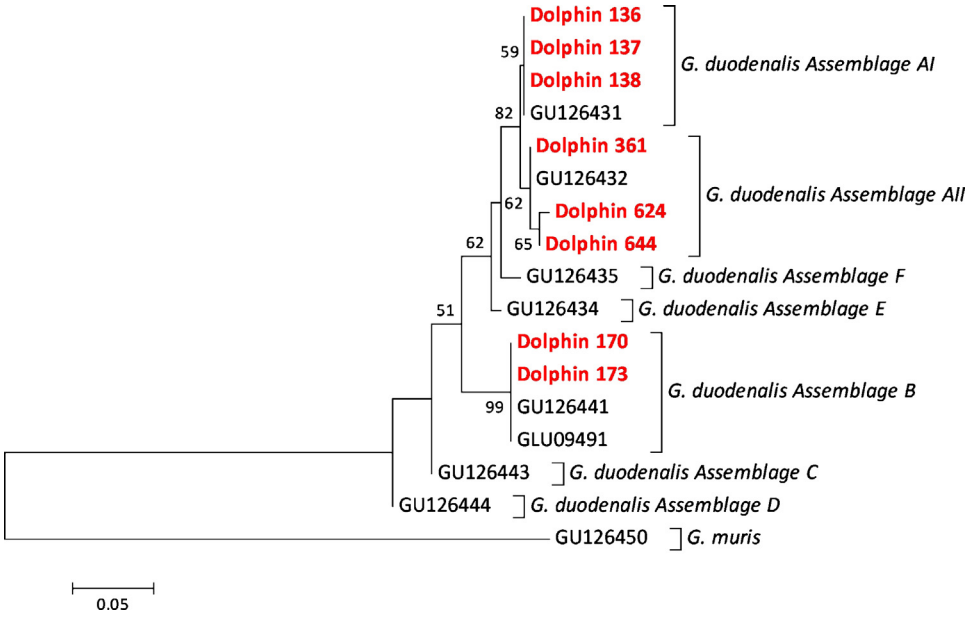

Fig. 2. Neighbor-joining tree analysis of a fragment encompassing the ITS1-5.8S-ITS2 region in the ribosomal unit of *Giardia* showing the phylogenetic relationship between the *G. duodenalis* isolates obtained from common dolphins (*D. delphis*) stranded along the Galician coast and other species/assemblages in the genus. Analysis was based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percentage bootstrap values (>50%) from 1000 replicates. Codes correspond to GenBank accession numbers.

survey of emerging zoonoses in marine mammals and seabirds in the New England area of the Northeast United States, Bogomolni et al. (2006) proved that most *Giardia* and *Cryptosporidium* isolates detected by PCR and fluorescence assay were from a minority of marine mammal species. Altieri et al. (2007) reported the first detection of *Giardia* sp. related with the post-mortem alterations in an estuarine dolphin (*Sotalia guianensis*) stranded in Ceará State, Northeastern Brazil. In a large study that used molecular techniques to characterize *Giardia* from several species of marine mammals, including three dolphin species (*D. delphis*, *Grampus griseus* and *Lagenorhynchus acutus*), a prevalence of 23% was observed. However, *Cryptosporidium* oocysts were not detected in the 19 analysed samples (Bogomolni et al., 2008). At the same time, Lasek-Nesselquist et al. (2008) carried out a molecular characterization of *G. duodenalis* isolated from marine animals and, using a multi-locus sequencing approach, identified *G. duodenalis* assemblages A and B in faecal material of dolphins (assemblages A and B in *D. delphis*; assemblage B in *G. griseus* and *L. acutus*).

In contrast with this finding, Fayer et al. (2008) did not detect *Giardia* or *Cryptosporidium* in 83 bottlenose dolphins (*Tursiops truncatus*) captured in the estuarine waters of the coasts of South Carolina and Florida (USA). However, Arias León et al. (2008) detected a systemic *Herpesvirus* infection with *Cryptosporidium* spp. in a young female dolphin (*Stenella frontalis*) that was found stranded in the Falcón State, Venezuela. More recently, Borges et al. (2011) reported the occurrence of *Cryptosporidium* spp. in Antillean manatees (*Trichechus manatus*) and Amazonian manatees (*Trichechus inunguis*) from Brazil.

In the present study, *Giardia* cysts were observed by immunofluorescence microscopy only in two samples whereas the amplification of fragments of the ITS1-5.8S-ITS2 region, the SSU-rDNA and β-giardin genes identified eight samples as positive for *G. duodenalis*. This difference in assay sensitivity have been also observed by other authors, and it is supported by a blinded trial which showed that *Giardia* spp. and *Cryptosporidium* spp. were detected 22 times more often by PCR than by conventional microscopic examination of human faecal samples (Amar et al., 2004). In contrast, *Cryptosporidium* spp. oocysts were observed in 12 samples by IFAT but only three samples were positive by PCR and were identified as *C. parvum*. In a previous study on detection of *Cryptosporidium* oocysts in mussels by IFAT and PCR methods, Gómez-Couso et al. (2006a) observed a higher number of positive samples by IFAT than by PCR. The authors proved the existence of empty oocysts in the samples, which were able to bind the monoclonal antibody and were therefore IFAT positive, but that did not contain DNA for PCR amplification. The usefulness of PCR as a diagnostic tool for the detection of *Giardia* and *Cryptosporidium* infections, often characterized by intermittent shedding or by low numbers of cysts or oocysts in faecal samples, has been proven by many studies. In addition, the use of molecular techniques provides data on species/genotypes that can give insights into possible sources of transmission and contamination (Appelbee et al., 2010; Rengifo-Herrera et al., 2013).

In this respect, the present work showed the presence of *G. duodenalis* assemblages A and B and *C. parvum*, the latter being, to the best of our knowledge, the first report of this species in common dolphins. These findings suggest that anthropogenic activities can be a source of contamination for the marine environment and, consequently, highlight the possible role of dolphins as reservoirs of these zoonotic parasites. However, it must be stressed that the finding

of similar genotypes in marine and terrestrial environments is not *per se* a conclusive evidence that zoonotic transmission is occurring between terrestrial and marine hosts. Indeed, only histological examination of the mucosal surface can unequivocally provide evidence of infection, therefore excluding the passive transfer of the parasites in these animals (Appelbee et al., 2010).

Although these primarily fresh-water parasites have been described in marine mammals, *Cryptosporidium* was detected at a lower frequency than *Giardia* (Appelbee et al., 2005, 2010; Dixon et al., 2008; Fayer et al., 2008). Our results on *Cryptosporidium* prevalence in common dolphins are slightly higher than the corresponding values obtained for *Giardia*. Previous studies carried out on environmental samples and mussels, *Mytilus galloprovincialis* (used as bioindicators and collected in harvesting areas of bivalve molluscs along the Galician Coast, where several specimens of *D. delphis* used in this study were stranded), showed the wide contamination by *Giardia* and *Cryptosporidium*, and highlighted a higher concentration of *Cryptosporidium* spp. oocysts in mussels (range 25–250 oocysts *vs* 1–19 cysts per sample of 6–8 mussels). Moreover, molecular characterization studies identified *C. parvum* in all contaminated mussels (Gómez-Couso et al., 2005a,b, 2006b). The authors suggested that cattle, highly parasitized by *C. parvum* in this region, may have been a source of contamination through discharge of runoff into shellfish-farming areas.

Giardia and *Cryptosporidium* infections in dolphins are intriguing because they do not drink water and feed on fish, cephalopods and other cold-blooded sea animals (López et al., 2002). These protozoan parasites can be uptake by marine invertebrates such us bivalve molluscs (Fayer et al., 2004; Robertson, 2007; Gómez-Couso and Ares-Mazás, 2012), or even microcrustaceans (Méndez-Hermida et al., 2006), and then enter in the food chain and infect small fish and cephalopods which in turn are the prey of the common dolphins that usually frequent these areas (López et al., 2002). In this respect, several authors have identified zoonotic *Giardia* and *Cryptosporidium* species/genotypes in marine fish, including *G. duodenalis* assemblages A and B and *C. parvum* (Reid et al., 2010; Yang et al., 2010; Koinari et al., 2013).

Several causes of death in marine mammal stranded have been described, including disease cause by viral, bacterial, protozoal and fungal agents, anthropogenic chemical pollution, harmful algal blooms, fatal neurological or renal condition, human interaction (harassment, entanglement and vessel collision), rock and/or sand ingestion, predatory attack, failure to thrive or dependent calf and environmental changes (Bogomolni et al., 2010; Bossart, 2011; Truchon et al., 2013). Specifically, in Galicia, by-catch is recognized as a major threat to small cetaceans, as the main prey species (blue whiting, scad and sardine) are also important local fishery resources so dolphins and fishing may tend to concentrate in the same areas (López et al., 2002). Given the low parasitic load observed in the samples, it is unlikely that *Giardia* and/or *Cryptosporidium* infections cause stranding of these marine mammals. Moreover and because all the studied animals were dead and not studies have been carried out in lived animals, it is impossible to know if *Giardia* and/or *Cryptosporidium* infections are more prevalent in stranding animals or if certain factors that predispose dolphins to stranding could also make them more susceptible to infection with these parasites.

Finally, our results suggest that the population of common dolphins that inhabit Galician waters could spread these protozoan parasites to different locations along the Galician coast. Also, as dolphins are considered as sentinel species for oceans and human health (Bossart, 2011), the results of this study alert us about the contamination of the marine environment by zoonotic pathogens.

Conflict of interest

None of the authors have any commitments, consultancies, or contracts that could be considered as conflicts of interest in this work.

Acknowledgements

This study was funded by the Spanish Ministry of Education and Science (grant no. CGL-2007-60656).

The authors would like to thank the personnel and volunteers of the CEMMA for the sample collection and to Rafael Romero Suances for his assistance.

References

Altieri, B., Viana, D., Meirelles, A., 2007. Isolation of *Giardia* sp. from an estuarine dolphin (*Sotalia guianensis*) in Ceará State, Northeastern Brazil. Latin Am. J. Aquat. Mamm. 6, 113–116.

Amar, C.F., Dear, P.H., McLauchlin, J., 2004. Detection and identification by real time PCR/RFLP analyses of *Cryptosporidium* species from human faeces. Lett. Appl. Microbiol. 38, 217–222.

Appelbee, A.J., Thompson, R.C., Olson, M.E., 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife – current status and future needs. Trends Parasitol. 21, 370–376.

Appelbee, A.J., Thompson, R.C., Measures, L.M., Olson, M.E., 2010. *Giardia* and *Cryptosporidium* in harp and hooded seals from the Gulf of St. Lawrence, Canada. Vet. Parasitol. 173, 19–23.

Arias León, G., Mariani di Lena, M.A., Cornejo Uzcátegui, L., Bermúdez García, V., Ramírez Medina, O.J., 2008. Estudio de infección sistémica por *Herpesvirus* complicada con *Cryptosporidium* spp., en un delfín manchado del Atlántico (*Stenella frontalis*. Cuvier, 1829). Rev. Cient. 18, 243–252.

Bass, A.L., Wallace, C.C., Yund, P.O., Ford, T.E., 2012. Detection of *Cryptosporidium* sp. in two new seal species, *Phoca vitulina* and *Cystophora cristata*, and a novel *Cryptosporidium* genotype in a third seal species, *Pagophilus groenlandicus*, from the Gulf of Maine. J. Parasitol. 98, 316–322.

Bogomolni, A., Ellis, J., Gast, R., Harris, B., Pokras, M., Touhey, K., Moore, M., 2006. Emerging zoonoses in marine mammals and seabirds of the Northeast U.S. In: OCEANS 2006, 18–21 September 2006, pp. 1–5.

Bogomolni, A.L., Gast, R.J., Ellis, J.C., Dennett, M., Pugliares, K.R., Lentell, B.J., Moore, M.J., 2008. Victims or vectors: a survey of marine vertebrate zoonoses from coastal waters of the Northwest Atlantic. Dis. Aquat. Organ. 81, 13–38.

Bogomolni, A.L., Pugliares, K.R., Sharp, S.M., Patchett, K., Harry, C.T., LaRocque, J.M., Touhey, K.M., Moore, M., 2010. Mortality trends of stranded marine mammals on Cape Cod and southeastern Massachusetts, USA, 2000 to 2006. Dis. Aquat. Organ. 88, 143–155.

Borges, J.C.G., Alves, L.C., da Gloria Faustino, M.A., Marmontel, M., 2011. Occurrence of *Cryptosporidium* spp. in Antillean manatees (*Trichechus manatus*) and Amazonian manatees (*Trichechus inunguis*) from Brazil. J. Zoo Wildl. Med. 42, 593–596.

Bossart, G.D., 2011. Marine mammals as sentinel species for oceans and human health. Vet. Pathol. 48, 676–690.

Cacciò, S.M., Beck, R., Almeida, A., Bajer, A., Pozio, E., 2010. Identification of *Giardia* species and *Giardia duodenalis* assemblages by sequence analysis of the 5.8S rDNA gene and internal transcribed spacers. Parasitology 137, 919–925.

Covelo, P., Martínez, J., 2001. Strandings of marine mammals in the coasts of Spain and Portugal between 1999 and 1998: ATLANCETUS. Galemys 13, 93–106.

Dixon, B.R., Parrington, L.J., Parenteau, M., Leclair, D., Santin, M., Fayer, R., 2008. *Giardia duodenalis* and *Cryptosporidium* spp. in the intestinal contents of ringed seals (*Phoca hispida*) and bearded seals (*Erignathus barbatus*) in Nunavik, Quebec, Canada. J. Parasitol. 94, 1161–1163.

Fayer, R., Dubey, J.P., Lindsay, D.S., 2004. Zoonotic protozoa: from land to sea. Trends Parasitol. 20, 531–536.

Fayer, R., Fair, P.A., Bossart, G.D., Santin, M., 2008. Examination of naturally exposed bottlenose dolphins (*Tursiops truncatus*) for Microsporidia, *Cryptosporidium*, and *Giardia*. J. Parasitol. 94, 143–147.

Gaydos, J.K., Miller, W.A., Johnson, C., Zornetzer, H., Melli, A., Packham, A., Jeffries, S.J., Lance, M.M., Conrad, P.A., 2008. Novel and canine genotypes of *Giardia duodenalis* in harbor seals (*Phoca vitulina richardsi*). J. Parasitol. 94, 1264–1268.

Gómez-Couso, H., Ares-Mazás, E., 2012. Chapter IV. *Giardia duodenalis*. Part 2: Contamination of bivalve molluscs. In: Robertson, L.J., Smith, H.W. (Eds.), Foodborne Protozoan Parasites. Nova Science Publishers, Inc., New York, pp. 133–150.

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005a. *Giardia* in shellfish-farming areas: detection in mussels, river water and waste waters. Vet. Parasitol. 133, 13–18.

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005b. Occurrence of *Giardia* cysts in mussels (*Mytilus galloprovincialis*) destined for human consumption. J. Food Prot. 68, 1702–1705.

Gómez-Couso, H., Méndez-Hermida, F., Ares-Mazás, E., 2006a. Levels of detection of *Cryptosporidium* oocysts in mussels (*Mytilus galloprovincialis*) by IFA and PCR methods. Vet. Parasitol. 141, 60–65.

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2006b. *Cryptosporidium* contamination in harvesting areas of bivalve molluscs. J. Food Prot. 69, 185–190.

Hill, B.D., Fraser, I.R., Prior, H.C., 1997. *Cryptosporidium* infection in a dugong (*Dugong dugon*). Aust. Vet. J. 75, 670–671.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Koinari, M., Karl, S., Ng-Hublin, J., Lymbery, A.J., Ryan, U.M., 2013. Identification of novel and zoonotic *Cryptosporidium* species in fish from Papua New Guinea. Vet. Parasitol. 198, 1–9.

Lalle, M., Jiménez-Cardosa, E., Cacciò, S.M., Pozio, E., 2005. Genotyping of *Giardia duodenalis* from humans and dogs from Mexico using a beta-giardin nested polymerase chain reaction assay. J. Parasitol. 91, 203–205.

Lasek-Nesselquist, E., Bogomolni, A.L., Gast, R.J., Welch, D.M., Ellis, J.C., Sogin, M.L., Moore, M.J., 2008. Molecular characterization of *Giardia intestinalis* haplotypes in marine animals: variation and zoonotic potential. Dis. Aquat. Organ. 81, 39–51.

Lasek-Nesselquist, E., Welch, D.M., Sogin, M.L., 2010. The identification of a new *Giardia duodenalis* assemblage in marine vertebrates and a preliminary analysis of *G. duodenalis* population biology in marine systems. Int. J. Parasitol. 40, 1063–1074.

López, A., Santos, M.B., Pierce, G.J., González, A.F., Valeiras, X., Guerra, A., 2002. Trends in strandings and by-catch of marine mammals in north-west Spain during the 1990. J. Mar. Biol. Assoc. U.K. 82, 513–521.

López, A., Pierce, G.J., Valeiras, X., Santos, M.B., Guerra, A., 2004. Distribution patterns of small cetaceans in Galician waters. J. Mar. Biol. Assoc. U.K. 84, 283–294.

Méndez-Hermida, F., Gómez-Couso, H., Ares-Mazás, E., 2006. *Artemia* is capable of spreading oocysts of *Cryptosporidium* and the cysts of *Giardia*. J. Eukaryot. Microbiol. 53, 432–434.

Morgan, U.M., Xiao, L., Hill, B.D., O'Donoghue, P., Limor, J., Lal, A., Thompson, R.C., 2000. Detection of the *Cryptosporidium parvum* "human" genotype in a dugong (*Dugong dugon*). J. Parasitol. 86, 1352–1354.

Olson, M.E., Roach, P.D., Stabler, M., Chan, W., 1997. Giardiasis in ringed seals from the western arctic. J. Wildl. Dis. 33, 646–648.

Pierce, G.J., Caldas, M., Cedeira, J., Santos, M.B., Llavona, A., Covelo, P., Martínez, G., Torres, J., Sacau, M., López, A., 2010. Trends in cetacean sightings along the Galician coast, north-west Spain, 2003–2007, and inferences about cetacean habitat preferences. J. Mar. Biol. Assoc. U.K. 90, 1547–1560.

Read, C., Walters, J., Robertson, I.D., Thompson, R.C., 2002. Correlation between genotype of *Giardia duodenalis* and diarrhoea. Int. J. Parasitol. 32, 229–231.

Reid, A., Lymbery, A., Ng, J., Tweedle, S., Ryan, U., 2010. Identification of novel and zoonotic *Cryptosporidium* species in marine fish. Vet. Parasitol. 168, 190–195.

Rengifo-Herrera, C., Ortega-Mora, L.M., Gómez-Bautista, M., García-Moreno, F.T., García-Parraga, D., Castro-Urda, J., Pedraza-Díaz, S., 2011. Detection and characterization of a *Cryptosporidium* isolate from a southern elephant seal (*Mirounga leonina*) from the Antarctic peninsula. Appl. Environ. Microbiol. 77, 1524–1527.

Rengifo-Herrera, C., Ortega-Mora, L.M., Gómez-Bautista, M., García-Pena, F.J., García-Parraga, D., Pedraza-Díaz, S., 2013. Detection of a novel genotype of *Cryptosporidium* in Antarctic pinnipeds. Vet. Parasitol. 191, 112–118.

Robertson, L.J., 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: a review. Int. J. Food Microbiol. 120, 201–216.

Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69, 4302–4307.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Truchon, M.H., Measures, L., L'Herault, V., Brethes, J.C., Galbraith, P.S., Harvey, M., Lessard, S., Starr, M., Lecomte, N., 2013. Marine mammal strandings and environmental changes: a 15-year study in the St. Lawrence ecosystem. PLoS ONE 8, e59311.

Yang, R., Reid, A., Lymbery, A., Ryan, U., 2010. Identification of zoonotic *Giardia* genotypes in fish. Int. J. Parasitol. 40, 779–785.