Investigation into *Cryptosporidium* and *Giardia* in bivalve mollusks farmed in Sardinia region and destined for human consumption

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Abstract

Cryptosporidium and *Giardia* are protozoan parasites transmitted by fecal-oral ingestion of (oo)cysts, and are responsible for enteritis in several animal species and humans worldwide. These (oo)cysts can survive for over a year in aquatic environments and can accumulate in bivalve mollusks, which filter large volumes of water. The aim of this study is to evaluate the natural occurrence of *Cryptosporidium* and *Giardia* contamination in different specimens of edible bivalves mollusks from farming sites of the western and north-eastern coasts of Sardinia. From April 2011 to February 2012, 1095 specimens of *Mytilus galloprovincialis* and 240 of *Crassostrea gigas* were sampled from Olbia and Oristano gulf and San Teodoro pond. Hepatopancreas and gills, including the labial palp, were examined for oocysts and cysts after pooling and homogenisation using different techniques: i) staining for light and fluorescence microscopy; ii) direct immunofluorescence (IF) Merifluor® test *Cryptosporidium/Giardia* (Meridian Bioscience Inc., Cincinnati, OH, USA); and iii) molecular procedures. However, in the context under study, all mollusks examined with the three main diagnostic techniques were negative for both parasites pointing out the hypothetically low zoonotic risk related to *Cryptosporidium* and *Giardia* in bivalves, especially *Mytilus galloprovincialis* and *Crassostrea gigas.*

Introduction

The flagellate *Giardia* and the coccidian *Cryptosporidium* are protozoan parasites of vertebrates and well-known causative agents of gastrointestinal diseases. *Giardia* cysts and *Cryptosporidium* oocysts excreted by infected hosts may cause human infection (especially in young and/or immunodeficient subjects) through ingestion of contaminated water and/or food (Rodriguez-Hernandez *et al.*, 1994; Graczyk *et al.*, 1997; Lowery *et al.*, 2001; Glaberman *et al*., 2002). Freshwater contaminated by human and animal waste may be a source of infection in marine environments, as well as of several organisms in estuaries and coastal areas. As protozoan cysts and oocysts are able to survive for a long time in these environments, they can be accumulated in the tissues or organs of some aquatic filtering organisms, such as bivalve mollusks. The presence of *Giardia* and *Cryptosporidium* in bivalve mollusks has been reported in many marine areas in the proximity of contaminated wastewater (Graczyk *et al.*, 1999; Freire-Santos *et al.*, 2000; Gomez-Couso *et al*., 2004, 2005; Guiguet Leal *et al*., 2008; Mladineo *et al.*, 2009). There has been increasing awareness on the presence of these parasites in edible bivalves in Italy, which has led to recent studies along the coastline of the Adriatic sea (Giangaspero *et al*., 2004; Molini *et al.*, 2004; Traversa *et al.*, 2004; Berrilli *et al.*, 2008). As concerns Sardinia, there are currently no relevant data on the presence of these parasites in bivalves.

The aim of the present paper is to evaluate the level of *Giardia* and *Cryptosporidium* contamination of *Mytilus galloprovincialis* and *Crassostrea gigas* collected from Sardinia coast and to assess the validity of different diagnostic techniques for the detection and identification at species level of these protozoan parasites.

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Materials and Methods

Shellfish sampling site

Samples of *Mytilus galloprovincialis* were collected in Olbia and Oristano's gulf, respectively in the northeast and west coast of Sardinia, in the shellfish farms and in purification center; while samples of *Crassostrea gigas* were collected from the breeding pond of San Teodoro (Figure 1; Table 1).

Collection of samples and processing

From April 2011 to February 2012, a total of 1335 specimens of mollusks (1095 *Mytilus galloprovincialis* and 240 *Crassostrea gigas*) were collected. The samples were identified and kept at 0-5°C until they reached the laboratory, where they were weighed (live weight) and pooled (6 pools of 15±2 mussels/month and 5 pools of 4±1 oysters/month). For each pool, gills with labial palps, and digestive gland (hepatopancreas) were collected from each subject. The pooled tissues were homogenised in phosphate-buffered saline (PBS) 0.04 M (pH 7.2-7.4), and the lipids were extracted by addition of diethyl ether. Then, the tube was vigorously hand shaked for 30 sec and centrifuged at 1250 x g for 10 min; the pellet obtained was placed in a 1.5 mL tube.

The light microscopy, immunofluorescence (IF) and polymerase chain reaction (PCR) methods were evaluated using spiked tissues, collected from 20 specimens of *Mytilus galloprovincialis*. For the tissue spiking experiments, from each mussel the digestive gland and gills were excised and inoculated with a 1 mL (oo)cysts suspension obtained from calves and cats feces naturally infected.

Calf and cat feces were sived through a series of mesh strainers and then purified and concentrate using the method previously described for the mollusks pools.

Oocyst detection and identification

The pellet of each pool was divided in two aliquots: from the first aliquot, one drop of the pellet was streaked onto a glass slide and stained with modified Ziehl-Neelsen (Angus, 1987) and Auramine O for *Cryptosporidium*, and Chlorazol Black and Lugol's iodine (for

Giardia). The slides were observed at light microscopy for modified Ziehl-Neelsen, Chlorazol Black and Lugol's iodine, and fluorescence microscopy for Auramine O.

A direct IF antibody test was used to detect *Cryptosporidium* and *Giardia* (oo)cysts in the pellet of first aliquot. Each pellet was pipetted onto slides. The slides were then processed according to the manufacturer's instructions (Merifluor® test *Cryptosporidium/Giardia*; Meridian Bioscience Inc., Cincinnati, OH, USA).

Molecular procedures

The second aliquot of the pellet was subjected to molecular tests. The pellet was resuspended in a lysis buffer (120 mM NaCl, 10 mM EDTA, 25 mM Tris-HCl, 1% Sarcosyl, pH 8.0), according to the protocol Morgan *et al.* (1997), and subjected to three freeze/thaw cycles (liquid nitrogen 3 min, 37°C 3 min), then incubated with proteinase K and DNA extracted with phenol-chloroform-isoamyl alcohol. DNA was stored at -20°C until used for PCR analysis. *Cryptosporidium* genus-specific primers were used to amplify a fragment of about 300 bp of the 18S rDNA variable region (Morgan *et al.*, 1997). The PCR reactions contained 1 X reaction buffer, 3 mM $MgCl_2$, 0.625 mM dNTP, 25 pmol/µL of each primer, 1U of *Taq* Gold (Roche-Boehringer, Mannheim, Germany) and 1 µL DNA in a 25 µL total reaction volume.

Polymerase chain reactions were performed under the following conditions: 5 min at 96°C, 30 cycles of 40 s at 94°C, 40 s at 60°C, 40 s at 72°C, with a final 10 min elongation step at 72°C. The *Giardia* PCR protocol was designed to amplify a 432 bp segment of the *glutamate dehydrogenase* (*GDH*) gene from multiple genotypes of *Giardia duodenalis* with the degenerated primers described by Miller *et al.* (2005). Polymerase chain reactions contained 1 X reaction buffer, 1.5 mM $MgCl_2$, 200 µm dNTPs, 12.5 pmol/µL of each primer, 0.5 U Taq polymerase, and 2 µL DNA in a 50 µL total reaction volume. Amplification conditions started with one cycle for 3 min at 95°C, 2 cycles of 2 min at 94°C, 1 min at 56°C, 2 min at 72°C, followed by 55 cycles of 30 s at 94°C, 20 s at 56° C and 45 s at 72°C, with a final extension of 7 min at 72°C. Polymerase chain reaction products were separated by gel electrophoresis in 1% agarose gel stained with Sybr® Safe DNA Gel Stain 1X TAE (Invitrogen, Carlsbad, CA, USA).

Results

The mussels tissues spiked using an (oo)cystic suspension were detected by light microscopy, IF and PCR methods. The different diagnostic techniques gave negative results for *Cryptosporidium* and *Giardia* in all pools for both shellfish species.

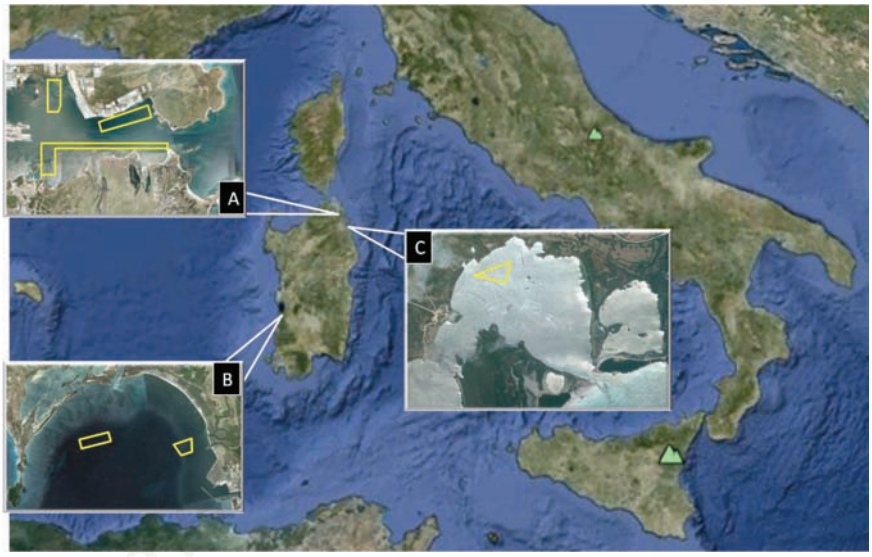

Figure 1. Maps of Sardinia region (Italy) and Oristano's gulf (A), Olbia's gulf (B), San Teodoro pond (C) with shellfish farms and collection site locations (in yellow).

Table 1. Mollusks species and relative sampling sites.

Examined species	Localities	Sample (n)	Shellfish farms	Sampling site	Coordinates	
M. galloprovincialis	Olbia	675	Cocciani cove	1	40°55'50.05"N	9°31'44.60"E
				2	40°55'47.57"N	9°31'44.50"E
				3	40°55'36.18"N	9°31'48.89"E
			Saccaia bay	4	40°55'30.04"N	9°32'28.25"E
				5	40°55'25.61"N	9°32'47.53"E
				6	40°55'25.79"N	9°32'58.63"E
				7	40°55'29.22"N	9°33'22.92"E
			Cavallo island	8	40°55'09.,35"N	9°31'48,23"E
				9	40°55'12.10"N	9°32'09,10"E
				10	40°55'14.10"N	9°32'31,60"E
	Oristano's gulf	420	Oristano's gulf	1	39°52'53.19"E	8°28'25.18"N
				2	39°53'05.52"E	8°29'18.23"N
				3	39°52'45.31"E	8°28'28.94"N
				4	39°52'54.93"E	8°28'50.54"N
				5	39°52'58.15"E	8°29'20.88"N
C. gigas	S. Teodoro	240	S. Teodoro pond	1	9°40'23.80"E	40°48'43.30"N
				2	9°40'27.80"E	40°48'40.90"N

M. galloprovincialis, Mytilus galloprovincialis; C. gigas, Crassostrea gigas.

Discussion

Oocysts of *Cryptosporidium* were recovered in clams (*Chamelea gallina*) collected in Italy at Vomano and Vibrata river mouths on the Adriatic sea (Molini *et al.*, 2004; Traversa *et al.*, 2004; Giangaspero *et al.*, 2005). Other studies have shown the contamination of *Mytilus galloprovincialis* by *Cryptosporidium* oocysts in the lagoon Sacca di Scardovari (northern Adriatic sea), and in Messina area (Berrilli *et al.*, 2008).

The results of this study represent the first data collected in Sardinia about *Cryptosporidium* and *Giardia* (oo)cysts in specimens of *Mytilus galloprovincialis* and *Crassostrea gigas.* In the context studied here, though, unlike those mentioned above, light microscopy, IF and PCR assays showed that the shellfish were negative. Several factors may contribute to this: i) the concentration of (oo)cysts eventually present in breeding water is diluted because of several freshwater streams; ii) the water is often replaced and cleaned by the constant presence of circular currents and tides. Other factors such as chemical (*i.e.* ammonia, pH and salinity) and physical (*i.e.* temperature and solar radiation) parameters were reported to influence (oo)cyst concentration in the environment (Erickson e Ortega, 2006). Also, it should be noted that in summer – and more recently also in spring – the surface water temperature reaches 25°C in the studied area. Considering that shellfish are farmed in water between 50 cm and 2 m depth, direct sunlight may negatively influence the survival of protozoa (Giangaspero *et al.*, 2009). In light of these results, we can reasonably consider the health risk of the farming areas comprised in this investigation to be low. Nevertheless, health risk cannot be considered absent in the areas which have not been examined.

Periodic monitoring is thus recommended, especially after the occurrence of the following conditions: a significant increase in the number of farms or animals in areas along the coastline; an increase in demographic pressure, in turn resulting in an increased wastewater disposal into the sea; and particularly intense and long-lasting rainfall, especially after prolonged periods of drought, in as much as this condition could make higher amounts of (oo)cysts contained in manure residue accumulated in the soil go off into the sea.

Conclusions

In summary, as the source of potential contamination is provided by farms, the reduction of infection risk depends on the strategies for preventing and controlling the spread of intestinal parasites such as *Cryptosporidium* and *Giardia* in Sardinia livestock farms. The lack of such strategies would inevitably result in an increased risk of exposure to (oo)cysts in various food, including edible bivalve mollusks.

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