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Short Communication

Accumulation of oocysts of *Cryptosporidium parvum* in *Biomphalaria glabrata* (Pulmonata:Planorbidae) under experimental conditions

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Abstract

Introduction: *Cryptosporidium* oocysts are easily transported to various aquatic environments. The objective of this study was to evaluate *B. glabrata* mollusks exposed to food containing *C. parvum* oocysts. **Methods:** Six experimental groups were used with *B. glabrata* either exposed or not to *C. parvum* oocysts. Microscopic and molecular diagnostics were conducted in water samples and tissues of *B. glabrata*. **Results:** By light microscopy, *C. parvum* oocysts were identified in the water of the exposed groups. *C. parvum* DNA was not detected in water but was detected in tissue samples. **Conclusions:** Further studies should be conducted under natural conditions.

Keywords: Protozoa. Mollusca. Molecular biology. Host-parasite relationship.

The Neotropical region has a wide range of snails from *Biomphalaria* genus (Pulmonata: Planorbidae). In Brazil, approximately one-third of these species occur in freshwater ecosystems, which comprise the country's main watersheds[1].

Epidemiological studies have demonstrated that the oocysts, which are the infective form of the protozoa belonging to the genus *Cryptosporidium* and are eliminated into the environment from the feces of infected hosts, can remain infective for several months, depending on the environmental conditions[2].

The infective form of *Cryptosporidium* is easily transported by running water to still water collections[3]. Of the many sources of contamination, the major one is believed to be deposition of feces directly in water bodies or in their environs, either by wild animals or livestock, or by fertilization of nearby pastures and crops with feces[3]. The contamination from adjacent areas occurs by the carriage of oocysts to beds of watercourses by leaching and by infiltration of water with oocytes into the soil[3].

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A major risk is posed by aquatic invertebrates, especially gastropods of commercial interest. These gastropods can accumulate oocysts in their tissue, thus acting as potential carriers of food-borne diseases when eaten raw or undercooked. Among these gastropods are marine bivalves, which are capable of accumulating *Cryptosporidium* spp. oocysts in their tissue. However, these species can also be used for monitoring the water quality of environments[4-9].

Some aquatic organisms that are not of economic interest can internalize oocysts of *Cryptosporidium* and cysts of *Giardia* in their tissues and play an important role in disseminating these microorganisms into the environment[10]. Besides this, oocysts and cysts of protozoa can survive in salt water and fresh water for several months, where aquatic invertebrates can ingest them by filtration and concentrate these infective forms in their tissue[6]. Therefore, aquatic invertebrates can be useful to detect contamination of aquatic habitats instead of analyzing water samples directly, which is more laborious and expensive.

The aim of this study was to experimentally investigate whether individuals of the species *B. glabrata* supplied with food containing *C. parvum* oocysts can accumulate this infective form in their tissue.

The snails of the species *B. glabrata* (BH strain) were obtained from established colonies kept at the Experimental

Schistosomiasis Laboratory of Oswaldo Cruz Institute (LEE/IOC) in Rio Janeiro, Brazil. Before being exposed to *C. parvum* oocysts, the snails remained for one week in aquariums containing 2,000 mL of distilled water, which was changed every two days. The snails were fed with fresh lettuce leaves (*Lactuca sativa* L.), *ad libitum*, previously washed with distilled water and replenished daily. The aquariums were covered with permeable screens, allowing good air circulation while preventing the snails from escaping.

The *C. parvum* oocysts were obtained from the Department of Animal Parasitology, Protozoology Laboratory, Rio de Janeiro Federal Rural University. The sample of *C. parvum* used in the experimental groups was derived from the feces of naturally infected cattle. The species has been characterized genotypically, and the GenBank accession number is JX198273.

Two experimental groups were formed, each with 27 individuals. One group was exposed to *C. parvum* oocysts (n = 81; A = 1, 2, 3). Another group was unexposed, corresponding to control groups (n = 81; B = 1, 2, 3). All experimental groups were formed using triplicates ($n_{Tota\ l}$ = 162).

The snails were then transferred individually to 24-well plates, with a piece of fresh lettuce at the bottom of each well. The same procedure was followed for the snails of the groups that were exposed and not exposed to the *C. parvum* oocysts.

Each snail of the exposed groups was exposed to approximately 600 oocysts, obtained from calf feces utilizing techniques such as centrifugation and floatation in a modified saturated sugar solution[11]. The suspensions of oocytes were then washed with distilled water, counted in a Neubauer chamber, and stored at 4ºC for subsequent addition of aliquots of oocysts through the lettuce leaf fragments the snails were fed. For the control group, equal aliquots of distilled water were placed in the wells with the food. After this procedure, 1 mL of distilled water was added to each well and the plates were kept closed for 24 hours.

Twenty-four hours after exposure to *C. parvum* oocysts, snails from both the exposed and unexposed groups were washed individually in distilled water to remove any oocysts that might be attached to the external surface of their shells. The individual washing water and the water from each recipient that contained the snail and feces and leftover food were filtered through fine plastic disposable sieves with an overlay of gauze and placed in test tubes with a capacity of 15 mL. Then the water samples were processed by centrifuging and floatation in a sugar-saturated solution in order to prepare slides. The slides were examined under a microscope to determine the absence or presence of oocysts. The snails were then kept in aquariums (n = 27) as described previously.

One snail from group A (exposed to *C. parvum* oocysts) was randomly selected (n = 3; A = 1, 2, 3) and one snail of the unexposed group B (n = 3; B=1, 2, 3) was also randomly chosen. The soft tissues of these snails were removed from the shells, macerated, and homogenized in 20 mL of distilled water, then filtered through fine plastic disposable sieves with an overlay of gauze. Post-filtration, 200 µL was used for the extraction of DNA and 15 mL was added to the conical tubes and subjected to centrifugation and floatation in a sugar-saturated solution.

On the first, third, sixth, eighth, twelfth, and fourteenth days of the experiment, water samples containing feces were collected from the aquariums, along with one individual from each group, for processing as described previously.

Total DNA was extracted with a commercial kit (Qiagen QIAamp® Fast DNA Stool Mini Kit), according to the manufacturer's recommendations, with minor modifications, referring to the two periods of incubation of the material. We subjected the samples to a temperature of 95 °C, with a longer incubation period of 10 minutes, utilizing a stirrer with temperature control at a rotation of 800 rpm. At the end of the extraction, the samples were eluted in 100 µL of AE buffer (supplied with the kit).

The polymerase chain reactions (PCRs) were performed in two steps. In the first PCR, the primers 18SF: 5`- TTC TAG AGC TAA TAC ATG CG-3` (forward) and 18SR: 5`- CCC ATT TCC TTC GAA ACA GGA-3` (reverse) were used, obtaining amplicons of approximately 1,325 bp. For the nested PCR, the primers used were 18SNF: 5`- GGA AGG GTT GTA TTT ATT AGA TAA AG-3` (forward) and 18SNR: 5`- AAG GAG TAA GGA ACA ACC TCC A-3` (reverse), obtaining amplicons between 826 and 864 bp. The reaction conditions were those described in the literature[12]. The products obtained were visualized by electrophoresis in 2% agarose gel (100 V for 60 minutes), stained with ethidium bromide (5 µg/mL).

Through centrifugation and floatation in a sugar-saturated solution, it was possible to observe *C. parvum* oocysts in the water containing feces of the exposed snails (**Figure 1**). However, there were very small numbers of oocysts present.

On the first day post-exposure (PE), oocysts were observed in two of the three exposed groups. Thereafter, oocysts were observed in one group on the third, sixth, eighth, and fourteenth days PE, while no oocysts were observed on the twelfth day PE in any of the exposed groups. **Table 1** reports the results obtained from microscopic and molecular diagnosis in function of the exposure time of each experimental group. In the water samples submitted to PCR, no DNA of the parasite was detected, but in the samples of macerated tissue, it was possible to obtain DNA of *C. parvum*. This confirmed that the snails had been in contact with the oocysts and possibly accumulated them in their tissue (**Figure 2**).

In literature, there are no records of the presence of *Cryptosporidium* spp. in *B. glabrata* tissues. However, considering that this snail may live in aquatic environments which directly receive sewage and feces from humans and animals *in natura*, the possibility that they may accumulate oocysts of *C. parvum* is a strong reality.

In this experiment, we observed the presence of *C. parvum* oocysts by microscopic analysis of water samples from the aquariums on the first day PE in two of the three exposed groups, as well as in one group on the third, sixth, eighth, and fourteenth days PE. Few oocysts were observed in the feces, irrespective of the number of days after exposure. This can possibly be explained by the dispersion of oocysts in the aquariums where the snails were kept.

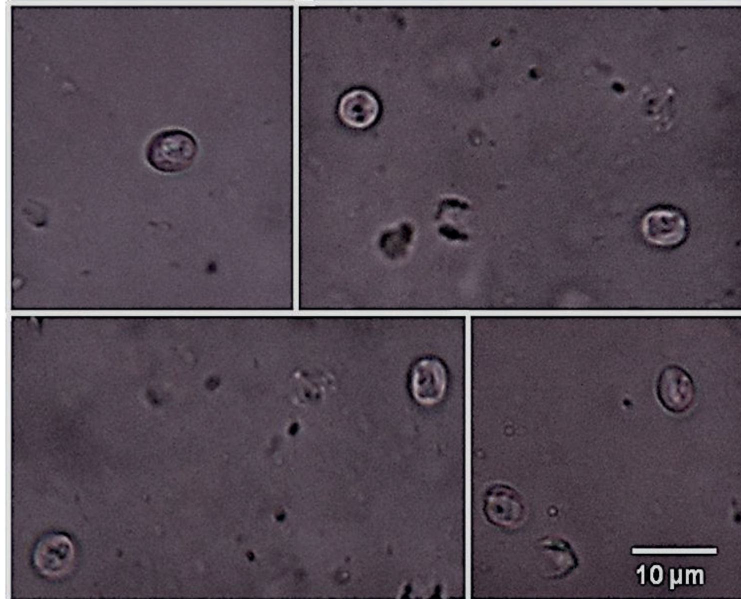

FIGURE 1: *Cryptosporidium parvum* oocysts recovered from the water where snails of the species *Biomphalaria glabrata* were previously exposed during the experiment. Observations made by light microscopy using phase contrast, through the technique of centrifugation and flotation in sugar-saturated solution.

TABLE 1: *Cryptosporidium parvum* oocysts in *Biomphalaria glabrata* snails experimentally exposed to approximately 600 oocysts, in water samples containing feces and tissues detected by centrifugation and floatation technique in a sugar-saturated solution (TCF) and polymerase chain reaction (PCR).

Time after exposure (days)	Groups		Diagnosis		
			TCF	PCR	
			Water	Water	Tissue
1		1	-	-	
	Control	2	-	-	▲
		3	-	-	
		1	+	-	
	Exposed	2	+	-	▲
		3	-	-	
3		1	-	-	
	Control	2	-	-	-
		3	-	-	
		1	+	-	
	Exposed	2	-	-	+
		3	-	-	
6		1	-	-	
	Control	2	-	-	▲
		3	-	-	
		1	+	-	
	Exposed	2	-	-	▲
		3	-	-	
8		1	-	-	
	Control	2	-	-	-
		3	-	-	
		1	+	-	
	Exposed	2	-	-	+
		3	-	-	
12		1	-	-	
	Control	2	-	-	▲
		3	-	-	
		1	-	-	
	Exposed	2	-	-	▲
		3	-	-	
14		1	-	-	
	Control	2	-	-	-
		3	-	-	
		1	-	-	
	Exposed	2	-	-	+
		3	+	-	

- : absence of oocysts/DNA of *Cryptosporidium parvum*; **+ :** presence of oocysts/DNA of *Cryptosporidium parvum*; **▲:** analysis not performed.

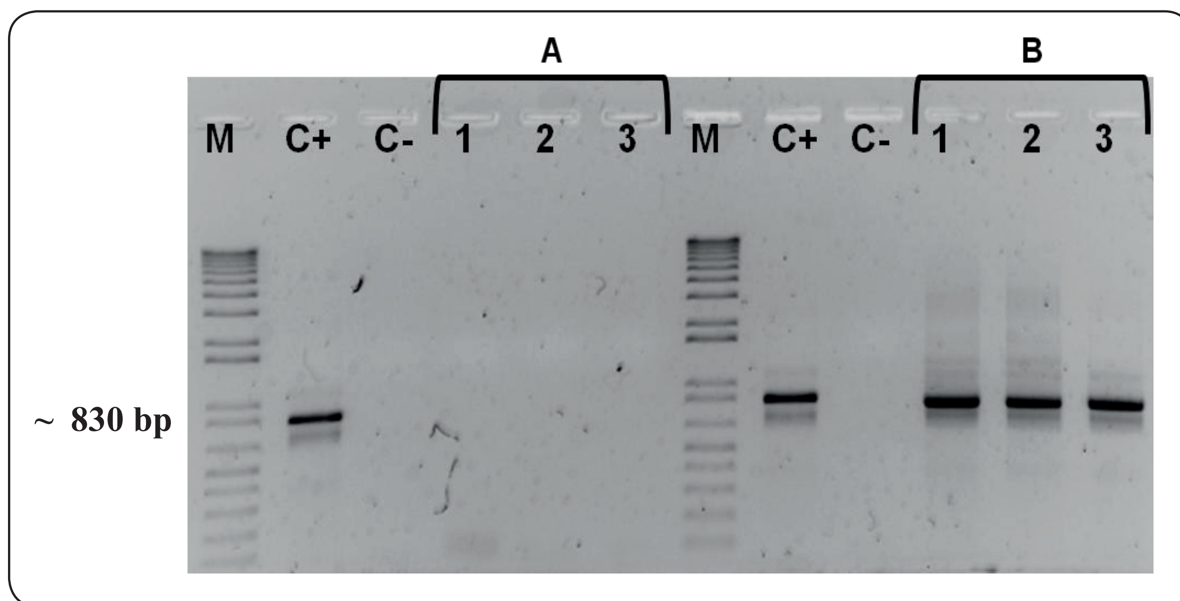

FIGURE 2: *Cryptosporidium parvum* DNA detected in tissues of *Biomphalaria glabrata* previously exposed to this protozoan. PCR-nested reactions using the 18S gene visualized on 2% agarose gel using ethidium bromide. **M:** molecular weight marker; **C +:** positive control; **C-:** negative control; **(A)** (1, 2, 3) snails not exposed to oocysts of *C. parvum*; **(B)** (1, 2, 3) snails exposed to oocysts of *C. parvum*.

For diagnosis of *Cryptosporidium* in mollusks, various methods and target sites are used. To detect the presence of *Cryptosporidium* oocysts in mollusks, techniques generally used involve the analysis of their gills and the intestinal tract[13]. Polymerase chain reaction (PCR) has been widely used in scientific research to confirm the presence and classify species of *Cryptosporidium* in the tissue of shellfish[5]. In the present study, it was possible to detect DNA of *C. parvum* in macerated tissue of *B. glabrata*, indicating that this accumulation was possibly due to the ingestion of oocysts. Therefore, genetic testing is important to provide information on the potential of reservoir hosts.

In a study to diagnose the level of contamination of oysters (*Crassostrea gigas*) in an estuary in southern Brazil[14], the researchers found the presence of *Cryptosporidium* spp. oocysts in the gills, suggesting continuous contamination of water, mainly in regions near rivers that receive wastewater discharges and have low salinity. Based on this, we can surmise that contamination of rivers in Brazil associated with the wide distribution of *B. glabrata* enables dissemination of pathogens by this snail. Similarly, *Cryptosporidium* oocysts and *Giardia* cysts were detected in rivers in Malaysia[15] along reaches flowing through ranchlands, due to runoff containing cattle stools. The presence of these oocysts and cysts in the river water is a probable source of human and animal infection, as well as of the aquatic mollusks that live in this habitat. Based on this, we can surmise that contamination of rivers in Brazil associated with the wide distribution of *B. glabrata* enables dissemination of pathogens by this snail. In experimental conditions, it was possible to recover *C. parvum* oocysts in the water in which the *B. glabrata* specimens were kept, in a tissue pool diagnosed by DNA of *C. parvum*. We observed oocysts up to the eighth day after exposure. We did not detect any oocysts on the twelfth day but did observe them on the fourteenth day. Although the number of oocysts observed in the water can be classified as low, this still holds relevance for environmental quality.

Further studies should be conducted to diagnose *C. parvum* in *B. glabrata*. These snails act as intermediate hosts of various parasites with medical and veterinary importance. Because these parasites do not have narrow specificity, it is possible that *B. glabrata* shelters various microorganisms in its tissue, possibly serving as a transport host, disseminating etiological agents in its habitat and increasing the environmental contamination, thus posing a risk of infection in various vertebrate hosts.

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