Detection of *Giardia* Cysts and *Cryptosporidium* Oocysts in Edible Shellfish: Choosing a Target

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Abstract

Bivalve mollusks are filter-feeding animals that are often consumed raw or partially cooked. They can harbor a wide variety of microorganisms such as the pathogenic protozoa *Giardia* and *Cryptosporidium*. Both these pathogens are well-known causative agents of diarrhea in humans and have been associated with several water and foodborne outbreaks around the world. Their infective stages, cysts and oocysts, respectively, can remain on the gills and other organs of shellfish, posing a potential threat to human health. There is no standard protocol or valid ISO for the detection of cysts and oocysts from shelled mollusks. The aim of this chapter is to describe the main methods used to detect *Giardia* cysts and *Cryptosporidium* oocysts from shellfish, based on techniques adapted from clinical and environmental parasitology, as well as molecular procedures. The monitoring of these foodborne protozoa in bivalve mollusks is of great relevance to public health, contributing to knowledge of contamination in one of the main food products derived from aquaculture. Indeed, it also reflects the quality of the environmental health surrounding its cultivation, highlighting another important aspect related to global environmental epidemiology.

Key words *Cryptosporidium*, *Giardia*, Protocol, Shellfish

1 Introduction

Shelled mollusks, also known as shellfish, are among the most important animals derived from aquaculture destined for human consumption. The most recent The State of World Fisheries and Aquaculture census revealed that almost 18 million tons of mollusks were produced worldwide, representing 56.3% of the production of marine and coastal aquaculture [1].

Despite its importance as a source of food and income, for decades freshwater and marine bivalve mollusks have been used as "sentinels" of environmental pollution, as they are sedentary filter-feeding species and may therefore be indicators of the sanitary

quality of the surrounding cultivation areas, which are also sometimes used for human recreational purposes [2–5].

Another important public health aspect is infectious outbreaks linked to the consumption of bivalve mollusks, as the tissues of the animals may harbor a wide variety of pathogenic microorganisms, posing a risk to health as they are often consumed raw or with minimal cooking. Moreover, the risk of infection can increase when the animals are sold without the cleaning or purification procedures—especially UV depuration—applied by the mariculture industry [6–10].

The contamination of bivalve mollusks by pathogenic protozoa has only attracted global attention in the last 25 years, with *Giardia* and *Cryptosporidium* being the most commonly detected protozoa in different edible shellfish species, or in those of no commercial interest [11–13].

These parasites are of significant importance to human health as they are recognized agents of diarrheal diseases, and their risks are often neglected [14]. In addition, both are recognized as important foodborne agents in other different food matrices, such as salads, milk, juices, and meat [15–17].

Until now, few giardiasis outbreaks have been related to shellfish consumption, and none have been identified as caused by *Cryptosporidium* [16, 18]. Although there is no apparent relationship between shellfish vehicles and outbreaks of giardiasis or cryptosporidiosis, several factors should be considered: (1) the lack of a system for the reporting of foodborne diseases in many countries, which leads to under-estimation or underreporting of infections; (2) the unavailability of the original food matrix suspected of or responsible for originating the outbreak, for further analysis; (3) the extended incubation period exhibited by both protozoa (1–2 weeks), and the difficulty in performing the retrospective association between the ingestion of bivalves and the appearance of clinical signs or symptoms [13, 16]. Indeed, some biological aspects of both protozoa must be taken into consideration, which reinforces the importance of monitoring these bivalve mollusks. *Giardia* and *Cryptosporidium* are ubiquitous in aquatic environments, and their infective stages (cysts and oocysts, respectively) are immediately released as infectious upon excretion [19]. Also, the infectious dose required to establish an infection is low for both, meaning that along with the high number of (oo)cysts excreted, they can spread easily and pose a great risk to public health [20].

It is also important to highlight that cysts and oocysts exhibit considerable longevity in coastal environments, as they can withstand great variety in temperature and salinity and remain viable outside their hosts in aqueous environments for several months to a year in seawater [21–23]. There is still no correlation between microbiological fecal indicators and pathogenic protozoa in mollusk flesh or in waters where they are cultivated and, unlike other microorganisms, they are not inactivated or quickly removed from the

environment [5, 22, 24]. Finally, both protozoa can remain in the bivalve tissues, even after depuration procedures [4, 6, 8, 10, 24].

1.1 Overview of Strategies for the Detection of *Cryptosporidium* Oocysts and Giardia Cysts in Shellfish

No standard validated method for the detection of *Cryptosporidium* and *Giardia* in shellfish is available, making comparison difficult, as each study utilized one or more types of bivalve mollusk, and different analytical methods [13, 25–27]. Another important factor that makes detection complex relates to the transit of the protozoa through the shellfish, which can vary, being concentrated in different animal tissues [28–30]. Thus, prior to detection, it would be reasonable to consider which tissue or other compound will be chosen for further analysis, and also to consider the most edible relevant species destined for human consumption from each specific geographical location [5, 8, 13].

Overall, gills and the digestive tract are frequently employed for this purpose, with tissue homogenates [11, 24, 31, 32] or washings mainly used to concentrate the protozoa [8, 33, 34]. Other strategies have previously been employed to detect the protozoa, with hemolymph extracted from the adductor muscle [30, 35, 36], inner-shell water (intravalvular liquid) [4, 8, 37] or the pooled whole mollusk [26, 38] also used.

Several studies have adopted individual shellfish (whole flesh) as their analytical material [39, 40] or have taken specific parts or organs of animals separately as their samples [12, 34, 41]. However, it should be remembered that the analysis of pooled shellfish or organs, while increasing detection rates, may substantially diminish costs, being considered a more representative sample and facilitating the assessment of foodborne protozoa risk associated with shellfish consumption in low-income food-deficit countries or those with lower financial budgets.

Despite the use of the bivalve (tissue or whole flesh) target, there is a concern that for successful isolation of both protozoa through the shellfish, the protocol applied must obey a minimum of three major steps: *concentration*: successive centrifugations, coarse-sieving, or usage of pepsin digestion solution; *purification*: flotation or immunomagnetic separation (IMS) using magnetic beads coated with anti-*Cryptosporidium* and anti-*Giardia*; *detection method of cysts and oocysts*: microscopy visualization—preferably through the use of direct immunofluorescence, using specific monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) against epitopes of cysts and oocysts—considered the gold standard, and molecular techniques such as PCR [25, 26, 30]. PCR protocols are also used, but present some difficulties, such as the removal of the inhibitors from the matrices. The advantage of this technique is that it allows the source of contamination to be tracked—which may be particularly important in foodborne outbreaks—through the identification of *Cryptosporidium* species and genotypes and *Giardia duodenalis* genetic groups [13, 27].

2 Materials

2.1 Pre-Sampling Harvesting	Prepare all solutions with correct molarity or concentration using ultrapure water at room temperature. After preparation, store solutions at 4 °C (*see* **Note 1**).

2.2 Reagents

1. Elution solution: Prepare 1 L of Tween 80 (0.1%) (*see* **Note 2**).
2. Sterile PBS solution (0.04 M). Adjust pH to 7.2.
3. Diethyl ether (*see* **Note 3**).

2.3 Materials

1. Petri dishes.
2. Sterilized clam knife.
3. Scalpel and scalpel blades.
4. Tissue homogenizer.
5. Centrifuge and micro centrifuge tubes (15 mL and 1 mL, respectively).
6. Sample mixer (RK Dynal®) or similar.
7. Pasteur pipette.
8. Tweezers.

2.4 Sample Collection

1. Samples must be collected using suitable tools. Immediately transport shellfish to laboratory in clean plastic bags and suitable refrigerated containers.
2. Samples must be kept under refrigerated conditions until processing.

3 Methods

3.1 Protocol 1: Detection of Protozoa through Liquid Materials from Mollusks

Open each animal with suitable tools looking for the umbo (the oldest part of the shell; the junction that connects both shells) (Fig. 1a); section the adductor muscles of the bivalve to facilitate opening (Fig. 1b) (*see* **Note 4**).

3.1.1 Bivalve Opening

3.1.2 Sample Processing

Internal Content

1. Each sample represents a pool of one dozen oysters (Fig. 1c), with the gill sets and inner-shell water (intravalvular liquid) removed from each animal.
2. Aspirate all the inner-shell water content of the animals with a Pasteur pipette (Fig. 1d) and place in clean and decontaminated centrifuge tubes (*see* **Notes 5** and **6**).
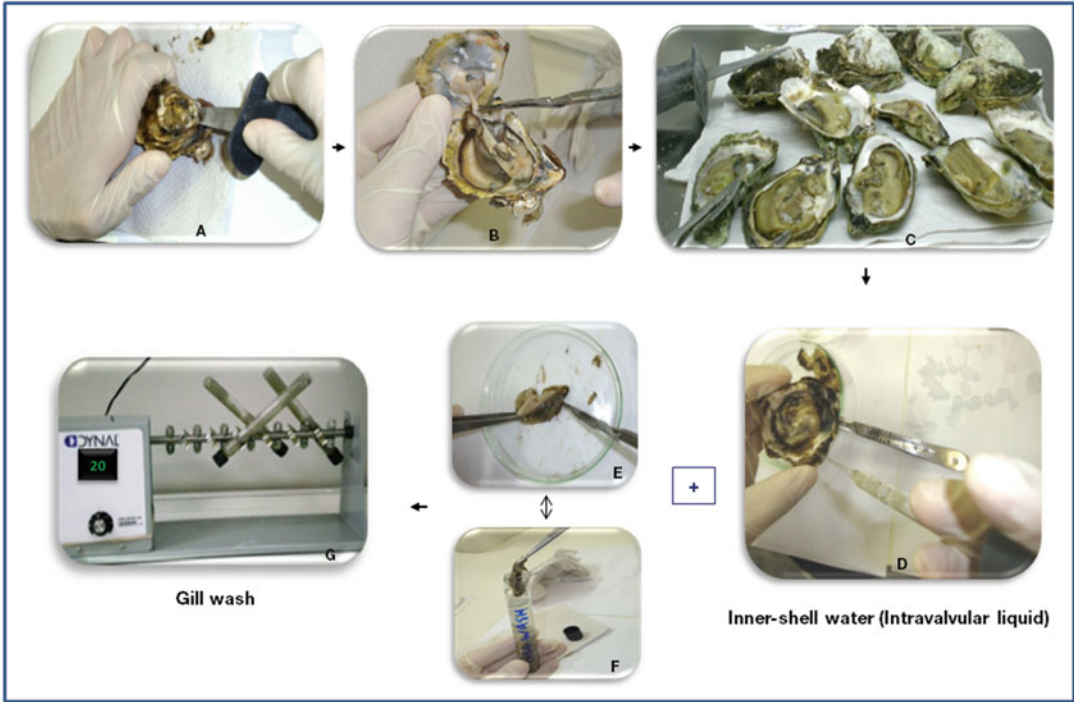
Fig. 1 Shellfish processing: analysis of inner-shell water and gill wash. (**a**) Opening of the umbo (junction that connects both shells); (**b**) Section of the adductor muscles of the bivalve to facilitate opening; (**c**) Each sample represents a pool of one dozen oysters; (**d**) Aspiration of all the inner-shell water content of the animals; (**e**) Extirpation the entire set of gills from each animal; (**f**) Sets of gills on glass tube; and (**g**) Tubes (corresponding to the set of gills from 12 animals) in sample mixer

3. Sieve the liquid content of all the tubes. After this step, centrifuge the liquid ($1050 \times g$ for 10 min) (*see* **Note 7**).

4. Remove all supernatants and maintain a volume of 2 mL of sediment in each tube. Complete the tube with ultrapure water and centrifuge again under the same conditions.

5. Remove the supernatant and transfer the sediment into properly identified micro tubes. Keep all tubes at 4 °C until the purification process using IMS.

Gill Collection and Processing

1. After opening the bivalve (*see* Subheading 3.1.1), excise the entire set of gills from each animal with the aid of a scalpel and tweezers (Fig. 1e) (*see* **Note 8**).

2. Place four sets of gills on each glass tube (Leighton tubes may be used) (Fig. 1f). Next, add about 2 mL of elution solution to the tube and gently shake manually so that the liquid meets the gills.

3. After removing the fourth gill set, complete the tube with Tween 80 (0.1%) elution solution until all gill sets are submerged.

4. Place the three tubes (corresponding to the set of gills from 12 animals) in the sample mixer (IMS rotor may be used) and leave to homogenize for 1 h at 20 RPM (Fig. 1g).

5. After this step, remove each tube from the rotor and vortex for 15 s.

6. Open each glass tube separately and remove each set of gills individually, placing each one in a sieve over a beaker. Gently, wash the gills with 3 mL of elution solution while sieving. Aspirate all the sieved liquid and transfer to 15-mL centrifuge tubes.

7. Collect the gill washing liquid from the empty glass tubes and place in 15-mL centrifuge tubes.

8. Add 5 mL of elution solution to the glass tube (empty) and mix by vortexing for 10 s. Aspirate the liquid and add to the centrifuge tubes.

9. Centrifuge all tubes at $1.050 \times g$ for 10 min (*see* **Note 7**).

10. Remove all supernatants and complete with ultrapure water and centrifuge again under the same conditions.

11. Remove supernatant and transfer sediment into properly identified micro tubes. Maintain all tubes at 4 °C until the purification process using IMS.

3.2 Protocol 2: Detection of Protozoa through Homogenized Tissue Materials from Mollusks

Proceed as described in Subheading 3.1.1.

3.2.1 Bivalves Opening

3.2.2 Sample Processing

Gill and Gastrointestinal Tract Removal

1. After opening the bivalve, excise the entire set of gills and gastrointestinal tracts from each animal with the aid of a scalpel and tweezers and transfer them to petri dishes.

2. With the aid of a tweezer, transfer all the sets of both tissues (separately) to tissue homogenizer.

3. Add elution solution containing Tween 80 (0.1%) and distilled water (2:1).

4. Homogenize the tissues until they become a liquid solution.

5. Transfer the solution to glass centrifuge tubes.

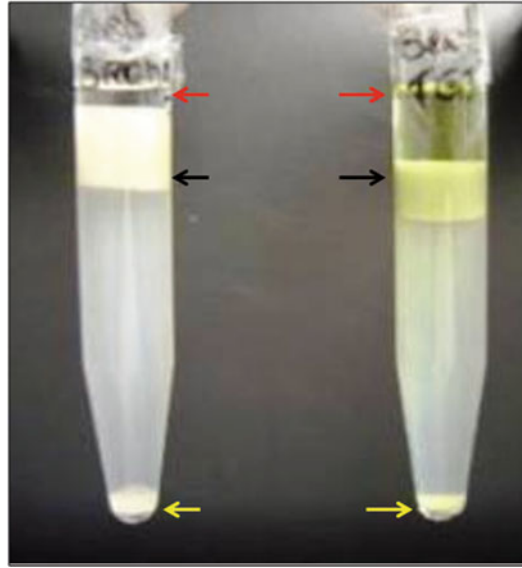6. Add 4 mL of refrigerated diethyl ether (in order to remove lipids) to each tube.

Fig. 2 Analysis of homogenized tissues. Tissues subjected to centrifugation with ether-PBS: yellow arrows: represent the sediments analyzed by IFA, gills (left tube) and gastrointestinal tract (right tube); black arrows: mucus in tissues; red arrows: amount of lipids present in tissues

7. Cover each tube and wrap the edges with cotton.

8. Shake each tube vigorously for 30 s.

9. Complete centrifuge tube with sterile PBS solution (0.04 M; pH 7.2).

10. Centrifuge at $1.250 \times g$ for 5 min. After this, three phases will be produced (Fig. 2).

11. Remove the supernatant and the remaining tissue and lipids with the aid of wooden and cotton toothpicks (Fig. 2).

12. Transfer the sediment to micro tubes.

13. Maintain all tubes at 4 °C until purification using IMS.

3.3 Purification Using Immunomagnetic Separation (IMS)

1. For all pellets, proceed to immunomagnetic separation phase in accordance with reference method 1623.1 [42] or ISO 15553 [43] (*see* **Note 9**).

2. After the IMS procedure, the final volume will be 100 μL.

3. Separate 50 μL per slide (the volume to be used for immunofluorescence assay) and the remaining 50 μL for PCR (polymerase chain reaction). In this case, the total number of oocysts/cysts will be the total number of (oo)cysts visualized on the slide multiplied by 2.

3.4 Detection of Protozoa by Direct Immunofluorescence-Assay

1. Immunofluorescence assay (IFA) must be processed according to the manufacturer's instructions. The only change is in the volume placed in the slide well (50 μL), with the rest utilized in PCR (*see* **Notes 10** and **11**).

2. Keep slides incubated in a humid chamber. After drying, fixing, and staining, the entire smear in each well must be examined at 400× or 600× magnification using an epifluorescence microscope. DAPI and DIC should be applied as per USEPA 1623.1 protocol [42].

3.5 Detection of Protozoa by Molecular Methods

1. Use the 50 μL remaining from the IMS procedure to extract the DNA (*see* **Note 12**).

2. After DNA extraction, amplify the DNA by nested-PCR protocols (*see* **Note 13**).

If the molecular analyses of the samples are positive, the use of two or three genes is encouraged to determine the genotype present.

4   Notes

1. Shellfish farming producers recommend the consumption of animals within 5 days of harvest or purchase. However, from our personal experience, the animals should be processed within 48 h, as even in areas of high microbiological quality, specimens spoil quickly, generating a pungent smell (bad odor), and bacterial proliferation.

2. Add 100 μL of Anti Foam A to the elution solution. Use the magnetic stirrer to homogenize the solution until the reagents are completely dissolved.

3. Must be stored at 4 °C prior to use.

4. Use individual protection equipment before starting: coat, gloves, and safety goggles, as oysters may be harvested from areas impacted by sewage.

5. Rinse all centrifuge tubes and Pasteur pipettes with Tween 80 (0.1%) prior to the experiments to reduce the likelihood of parasite attachment. The use of glass materials is preferable as adhesion of cysts and oocysts is greater with plastic, reducing the possible loss of protozoa.

6. Take care not to suck grease or fragments from the shell into the animals, as this may interfere with visualization and the IMS process.

7. All tubes containing inner-shell water and gill wash liquid may be also centrifuged following the recommendations of the last version of the USEPA method (1623.1) for liquid materials ($1500 \times g$ for 15 min) [42].

8. Avoid moving material from the gastrointestinal tract (hepato-pancreas) to the glass tube, as well as the mantle (the layer on top of all the other organs).

9. Consider using thermic rather than acid dissociation [44]. It is important to perform this step twice (80 °C for 10 min) as shellfish are rich in mucous tissue and lipids.

10. Use only IFA commercial kits recommended by validated methods, as per the standard procedures established for water samples:

    (a) MeriFluor® *Cryptosporidium/Giardia*, Meridian Diagnostics Cincinnati, OH.

    (b) Aqua-Glo™ G/C Direct FL, Waterborne, Inc. New Orleans, LA.

    (c) Crypt-a-Glo™ and Giardi-a-Glo™, Waterborne, Inc. New Orleans, LA.

    (d) EasyStain™C&G, BTF Pty Limited, Sydney, Australia.

11. Gastrointestinal tract homogenate analysis by IFA may be more difficult than gill homogenate examinations, due to the presence of thick layers on the slides. Therefore, (oo)cysts may not be detected due to masking [32, 34].

12. For DNA extraction, use commercial kits. Freezing–thawing cycles may also be used for *Cryptosporidium*. The number of cycles employed in the extraction process is critical, with a greater number of cycles potentially leading to DNA degradation [45].

13. For nested PCR protocols, consider the genes described in Table 1 for each pathogenic protozoan. In the event that nested PCR second reactions are positive, proceed to sample purification and then to gene sequencing.

Table 1
Most commonly used locus for the amplification of *Giardia* and *Cryptosporidium* genes in environmental samples

Protozoan	Locus	Reference
Giardia	18S rRNA	[46, 47]
	ß-giardin	[48, 49]
	Tpi	[50]
	Gdh	[51]
Cryptosporidium	18S SSU rRNA[a]	[52, 53]

[a]It is important to proceed to the nested PCR protocol for locus *gp60* to confirm the sample contamination of *Cryptosporidium parvum* or *Cryptosporidium hominis* genotypes

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