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Viability and infectivity of oocysts recovered from clams, *Ruditapes philippinarum*, experimentally contaminated with *Cryptosporidium parvum*

Abstract This study confirms the important role of marine bivalve molluscs, destined for human consumption, as transmitters of cryptosporidiosis, zoonotic diarrhoeal disease caused by *Cryptosporidium parvum*. *C. parvum* oocysts recovered from seawater clams (*Ruditapes philippinarum*) were viable and infective in five of eight infected neonatal CD-1 Swiss mice. Oocysts were observed in clam gill and gastrointestinal tract tissue homogenates as well as in gill histological sections, by an immunofluorescent antibody technique. In vitro viability of recovered oocysts was also determined using fluorogenic vital dyes (75% viability).

Introduction

The coccidian protozoan *Cryptosporidium parvum* causes diarrhoeal disease in humans and other mammals. The infection is usually mild and self-limiting in immunologically healthy individuals, but can be chronic and life threatening in immunocompromised individuals. Moreover, infection causes significant economic losses in farm animals (de Graaf et al. 1999).

Transmission of *C. parvum* oocysts may be direct (person-to-person or animal-to-person contact) or indirect (ingestion of contaminated water and food). In the past 15 years, this parasite has caused numerous massive waterborne epidemics (Fricker and Crabb 1998).

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Contaminated foods (e.g. fresh sausage, unpasteurized milk, apple cider) have been identified as possible sources of oocysts in some outbreaks (Griffiths 1988). *Cryptosporidium* sp. has been described in both natural and experimental infections in filter-feeding molluscs (Fayer et al. 1997; Graczyk et al. 1998a, 1999, 2000; Tamburrini and Pozio 1999; Freire-Santos et al. 2000a; Gómez-Bautista et al. 2000) and detected also in natural waters. Possible sources of water contamination include urban and agricultural slurry, septic tank leakage, recreational baths, agricultural run-off, and erosion of soils exposed to infected faeces (Smith and Rose 1998). Moreover, the infectious stage, i.e. the oocyst (4–6 μm) is long-lived, resistant to standard water disinfection (Graczyk et al. 1997a), and remains viable and infective after 40 days in seawater (salinity 35 ppt) (Freire Santos et al. 1999, 2000b).

The aim of the present study was to investigate the site of location of *C. parvum* oocysts in tissues of experimentally exposed clams, *R. philippinarum*, as well as the potential viability and infectivity of oocysts recovered from the clams.

Materials and methods

Parasite

Cryptosporidium parvum oocysts were collected from a naturally infected neonatal Friesian-Holstein calf. The methods used to concentrate (demineralized water/diethyl ether), purify (discontinuous percoll gradient) and quantify (Neubauer haemocytometer) oocysts have previously been described in detail (Lorenzo-Lorenzo et al. 1993). The technique of inclusion/exclusion of fluorogenic vital dyes [4′, 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI)], as described by Campbell et al. (1992), was used to determine the potential viability of the isolate.

Experimental design

Coccidia-free cultured clams, *R. philippinarum*, were acclimatized in five plastic tanks (eight clams/tank) referred to as tanks 1–5. Each tank contained 2 l of natural seawater (pH 7.2, salinity 35 ppt and

temperature: $15 \pm 3°C$) with aeration. The water in tanks 1–4 was contaminated with 8×10^6 C. parvum oocysts, and tank 5 was used as a control. All clams were sacrificed 48 h after contamination, and gills and gastrointestinal (GI) tissues removed.

Tank 1

Gills and GI tract from clams in this tank were pooled and homogenized in an Ultra-Turrax homogenizer. Lipids were removed by centrifugation at 1,000 g for 5 min with PBS (0.04 M pH 7.2) and diethyl ether (2:1). Two staining procedures were used for the homogenates: negative staining with 0.16% malachite green (malachite green 0.16 g; sodium dodecyl sulfate, 0.1 g; distilled water, 100 ml) and immunofluorescent antibody test (IFAT) (Monofluo Kit *Cryptosporidium*, Sanofi Diagnostics Pasteur).

Tank 2

Gills and GI tract were removed and placed in tissue cassettes, processed routinely, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E).

Tank 3

Gills and GI tract were processed as described for tank 1. The technique involving inclusion/exclusion of fluorogenic vital dyes (DAPI and PI), described by Campbell et al. (1992), was applied to the homogenates. Ten-microlitre aliquots of each sample were viewed under both Nomarski interference optics and epifluorescence. The proportion of ruptured (ghost), PI-positive (dead), DAPI-positive PI-negative (viable at assay), and DAPI-negative PI-negative (viable after further trigger) oocysts were quantified by examining more than 100 oocysts in each sample.

Tank 4

Gills and GI tract were homogenized, lipids removed (as described for tank 1) and eight neonatal CD-1 Swiss mice were injected intragastrically with 100 μl of this suspension. All mice were killed 7 days post-inoculation and their intestines removed. The intestines from four of the mice were placed in PBS, homogenized in an Ultra-Turrax homogenizer and viewed using an IFAT. Intestines from the remaining mice were processed routinely for histology and then stained with H&E.

Results

Malachite green stain applied to clam tissue homogenates from tank 1 revealed the presence of *Cryptosporidium parvum* oocyst-like bodies (4–5 μm in size, refractile and which did not take up stain). Identification was confirmed with an IFAT according to the criteria proposed by Graczyk et al. (1998b), i.e. bright-green fluorescence, correct size and shape of the fluorescein-stained objects, and clearly visible oocyst wall. The positive reaction confirmed the presence of *C. parvum* oocysts in these clams. Moreover, histological examination of the clams from tank 2 revealed the presence of *C. parvum* oocysts in the gill and GI tract sections.

Use of the inclusion/exclusion of fluorogenic vital dyes technique revealed a total viability of 75% (DAPI-positive PI-negative oocysts: 53% and DAPI-negative PI-negative oocysts: 22%) and the infectivity studies showed that five of the eight mice were infected. In addition, *C. parvum* oocysts were observed by IFAT in three of the four intestinal homogenates, and cryptosporidial stages were also observed in the epithelial border of two of the four intestines processed for histology and stained with H&E.

Discussion

The present study demonstrates that *Cryptosporidium parvum* oocysts taken up from seawater by clams (*R. philippinarum*) can remain viable, and can infect CD-1 Swiss mice, after 48 h. Previous studies have reported that some species of molluscs can take up *Cryptosporidium* sp. oocysts from water (Fayer et al. 1997; Graczyk et al. 1997b; Tamburrini and Pozio 1999). Furthermore, in vitro phagocytosis of *C. parvum* oocysts by Eastern oyster (*Crassostrea virginica*) haemocytes was demonstrated after 15, 30, 60 and 120 min of incubation (Graczyk et al. 1997c). Oocysts contained in Eastern oyster haemocytes were infective in mice (Fayer et al. 1997, 1998a). However, time-related difficulties in the identification of the phagocytosed oocysts under light and phase-contrast microscopy and progressively decreasing intensity of fluorescence of the ingested oocysts suggested that they disintegrated over time (Graczyk et al. 1997c). In our study, non-phagocytosed oocysts were visualized, by IFAT, in gill and GI tract sections after 48 h of exposure. Furthermore, we found most oocysts in the gills, probably because of the short exposure time used (48 h); a longer exposure time may lead to all oocysts appearing in the GI tract and/or haemocytes.

Environmental factors may affect the viability and infectivity of *C. parvum* oocysts (Johnson et al. 1997; Fayer et al. 1998b). Studies carried out in our laboratory (Freire-Santos et al. 1999, 2000b) have shown that both salinity and time affect the viability of *C. parvum* oocysts, but that even small numbers remaining viable are capable of initiating infection in susceptible hosts.

Marine molluscs have been implicated in food-borne outbreaks of gastroenteritis of known and unknown aetiology. Furthermore, these molluscs can serve as biological indicators of human and animal faecal contamination (Graczyk et al. 1997b) and as mechanical vector of *C. parvum* (Fayer et al. 1998a). We have demonstrated that in several molluscan species the depuration process applied to the commercially harvested molluscan shellfish may be ineffective for *Cryptosporidium* sp. oocysts. Moreover, a good correlation between faecal coliform numbers and the presence of *Cryptosporidium* oocysts has been found (Freire-Santos et al. 2000a). Although cryptosporidiosis in humans has not been officially linked to ingestion of raw bivalve molluscs, cases of self-limiting diarrhoea associated with the consumption of raw oysters and clams are often reported in our region (Galicia, NW Spain). The molluscs

consumed are usually harvested from marine estuaries where sewage from dairy farms is known to be discharged. In our opinion, *C. parvum* may be involved in some cases of gastroenteritis of unresolved aetiology.

This study showed that *C. parvum* oocysts recovered from clams remained viable and infective in susceptible hosts; therefore we can conclude that the custom of eating raw filter-feeding bivalve molluscs is a potential health risk. Further studies are needed to determine how long the oocysts remain viable in bivalve molluscs.

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