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A histological study of the transit of *Cryptosporidium parvum* oocysts through clams (*Tapes decussatus*)

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Abstract

A histological study was carried out to investigate the transit of *Cryptosporidium parvum* oocysts through the clam *Tapes decussatus*. Spat of approximately 5–7 mm shell length were maintained in a tank of natural sea water contaminated with purified *C. parvum* oocysts. The experiment lasted 240 h and, every 24 h, five specimens were killed, placed in Bouin's fixative, and processed routinely for histological examination. Sections (3 μm) cut from the all body tissues were stained with modified Gomori's trichrome for their accurate identification; the oocysts were detected by a direct immunofluorescence procedure. Oocysts were detected in siphons, gills, stomach, digestive diverticula, and intestine. The oocysts present in the intestine were free or mixed with the intestinal contents; therefore release of these oocysts with the feces should favour dissemination of contamination. Oocysts were found in branchial mucus and within the interfilamentary spaces, which suggests the occurrence of repeated filtrations and the possibility that the retained oocysts maintain their infective capacity.

Keywords: *Cryptosporidium*; Protozoa; Shellfish; Histology; Detection

1. Introduction

Bivalve molluscs have the capacity to filter large volumes of water and therefore can retain infective stages of *Cryptosporidium* present in aquatic environments. The presence of *Cryptosporidium parvum* oocysts has been demonstrated in different species following experimental contamination (Fayer et al., 1997; Graczyk et al., 1998; Tamburrini and Pozio, 1999; Freire-Santos et al., 2001). Natural contamination of oysters, mussels, clams, and cockles from different coastal regions has also been confirmed (Chalmers et al., 1997; Freire-Santos et al., 2000; Gómez-Bautista et al., 2000; Gómez-Couso et al., 2003a, 2004). The contamination is usually closely associated with the presence of waste water discharge sites and, for example, cattle or sheep farms, from

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which high levels of fecal contamination are expected, although it can also be associated with the presence of certain wild animals, such as seabirds that frequent the coastal areas where these molluscs are cultivated (Slifko et al., 2000).

Although the taxonomic classification of *Cryptosporidium* into species remains controversial, several *Cryptosporidium* species have been described in mammals, birds, reptiles, and fish (Xiao et al., 2004). The main causal agents of cryptosporidiosis in humans are *C. parvum* genotype I—proposed as *Cryptosporidium hominis* (Morgan-Ryan et al., 2002), associated almost exclusively with anthroponotic transmission, and *C. parvum* genotype II, associated with zoonotic transmission. However, in recent years, species of *Cryptosporidium* usually associated with birds (*Cryptosporidium meleagridis*) or with other mammals (*Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium muris*, and *Cryptosporidium andersoni*) have been also implicated in human infection (Tzipori and Ward, 2002).

Furthermore, previous studies have demonstrated the interaction in vitro between hemocytes of the mollusc species *Corbicula fluminea* and *Crassostrea virginica*, and oocysts of *C. parvum* (Graczyk et al., 1997a,b). Oocysts have also been found in the hemolymph, gills, gastrointestinal tract, and feces of different bivalve molluscs following experimental infection. In some of these studies, the infective capacity of the oocysts retained in the different tissues was confirmed using a suckling mouse model (Fayer et al., 1997; Graczyk et al., 1998; Tamburrini and Pozio, 1999; Freire-Santos et al., 2001).

In a previous study of experimentally contaminated oysters (*Ostrea edulis*) and clams (*Tapes decussatus*), we found that—despite detecting a sharp decrease in oocyst viability during the first 4 days, with 15–25% viable oocysts remaining thereafter—the oocysts retained by both species remained infective to suckling mice at 31 days post-contamination (Freire-Santos et al., 2002). Furthermore, our research group has also shown that infective stages can be transmitted between coexisting species (Gómez-Couso et al., 2003b).

The present histological study was undertaken to detect the location of *C. parvum* oocysts in the tissues of clam (*T. decussatus*) spat and thereby determine the sites of retention, the existence of which substantiates the maintenance of the infective capacity of the oocysts over time, as well as their dispersal in the environment.

2. Materials and methods

A total of 60 clam (*T. decussatus*) spat, between 5 and 7 mm shell length (i.e., approximately 8 months old) and free of *Cryptosporidium* spp. oocysts, were used in the study. The spat were acclimatized for 240 h in a tank (20 l) containing natural sea water at a constant temperature of 15 ± 3 °C and with controlled aeration. The molluscs were fed ad libitum with a mixture of equal proportions of five species of microalgae usually used in bivalve culture (*Tetraselmis suecica*, *Skeletonema costatum*, *Phaeodactilum tricornutum*, *Chaetoceros calcitrans*, and *Monochrysis lutheri*). The food mixture was added to the tank twice during the assay, at 0 h and 120 h, in order to stimulate filtration and induce the ingestion of infective stages of the parasite.

The tank was then contaminated with 20×10^6 purified *C. parvum* oocysts (approximately 3.3×10^5 oocysts/specimen). The oocysts were purified as described previously (Freire-Santos et al., 2002). At 24 h post-contamination, 10 specimens were sacrificed, the complete body was homogenized, and the lipids were extracted with PBS/diethyl ether, and the sediments obtained were analyzed for the presence of *C. parvum* oocysts by direct immunofluorescence antibody technique (IFAT), as described previously (Freire-Santos et al., 2002).

Every day, from 24 h to 240 h, five specimens were killed by forcing open their shell and the whole body was placed in Bouin's fixative for 4–5 h (750 ml of saturated solution of picric acid in artificial sea water (pH 7–7.2 and 930 mOsm/kg); 250 ml formaldehyde (40%) and 50 ml glacial acetic acid) as described by Hernández-Córdova (2001). The sample was then subjected to the following dehydration procedure: three steps of 1 h each in 70% EtOH (saturated with lithium carbonate for modified Gomori trichrome staining); 96% EtOH for 1 h; two steps each of 1 h in 100% EtOH; the samples were then washed twice (×30 min) with xylene and then embedded in paraffin (Paraplast Plus; Sherwood Medical, St. Louis, MO, USA) for 2 h with two changes and, finally, were