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Efficacy of Commercial Cleansing Procedures in Eliminating *Cryptosporidium parvum* Oocysts from Bivalves

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SHELLFISH is one of the most important aquaculture products of Portugal yielding approximately 5,000 tons/yr (Direcção Geral das Pescas, www.dg-pescas.pt, July 2006). Seafood is an excellent natural resource which is common in the Portuguese diet and is in high demand by tourists. Some shellfish are consumed raw or undercooked. Several legal regulatory steps are in place that control processes from production to sale to ensure consumption of uncontaminated live bivalves (Diário da República, Decreto-Lei #293/98, 18 September; Despacho extracto #18 940/2005, 2ª série, 12 July). According to Portuguese law, the location and classification of bivalve production areas are based on several criteria such as indicators of bacterial contamination. However, parasite indicators are not currently evaluated.

Bivalves from production areas classified as A do not need further cleansing. Those collected from B areas can only be eaten if they undergo a cleansing procedure before commercialization. If collected from C areas, intense cleansing or cooking in industrial factories is required before human consumption. The environmental quality of the collection sites vary according to the effectiveness of sewage treatment in rural areas and to discharge centers in urban areas of the country.

Cryptosporidum parvum is an enteric zoonotic parasite that is often discharged into rivers and seas by rain and sewage treatment effluents. Bivalves can concentrate oocysts in their gills and digestive glands during feeding, thus the aim of this study was to evaluate the efficiency of the process for removing bacteria from commercial bivalves in removing *Cryptosporidium* from bivalves consumed in Portugal.

MATERIAL AND METHODS

***Cryptosporidium* detection and identification.** Parasites were visualized using a direct immunoflourescence assay (IFAT) with Kit Crypto Cel (Cellabs, Brookvale, Australia). They were also detected by molecular methods using PCR analysis. Genomic DNA was extracted using a Proteinase K kit (Qiagen, Hilden, Germany, DNA) and then nested PCR was performed (Xiao et al. 1999) The primers used were (a) 18S suRNA (F): 5′-TTCTAGAGCTAATACATGCG-3′; (b) 18S suRNA (R): 5′-CCCTAATCCTTCGAAACAGGA-3′; (c) 18S suRNA (F): 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′; and (d) 18S suRNA (R): 5′-AAGGAGTAAGGAACAACCTCCA-3′. PCR conditions were 94 °C for 5 min followed by 40 cycles of 94 °C for 50 s, 50 °C for 30 s, and 72 °C for 45 s and finally 72 °C for 10 min.

Experimental infections. Controls for the experimental infection of bivalves and subsequent cleansing treatment were the gills and gastrointestinal tissues excised from three to five bivalves collected from A areas. The tissues were pooled, homogenized, and then processed by a modified Ritchie's protocol (Casemore, Armstrong, and Sands 1985) for parasite isolation. The absence of *Cryptosporidium* in these samples were verified microscopically and by PCR as described above.

Twelve to 15 *Cryptosporidium*-free bivalves were grouped and experiments were performed on the following: furrow shell (*Scrobicularia plana*), carpet shell clam (*Venerupis pulastra*), blue mussel (*Mytilus galloprovincialis*), cupped oyster (*Crassostrea angulata*), and cockle (*Cerastoderma edule*) all collected from B areas of the Portuguese coast. *Cryptosporidium* oocysts were obtained from feces of naturally infected Frisian calves. *Cryptosporidium parvum* (previously genotype 2) were quantified in a Neubauer counter. The parasites were identified by PCR-RFLP using the restriction enzymes *Ssp*I and *Vsp*I (Xiao et al. 1999).

Bivalves were placed in glass tanks containing 4.9 L seawater and incubated with *C. parvum* oocysts for 2 h at concentrations ranging from 10^6 to 3.5×10^7 oocysts/ml. Microalgae were added to each tank to stimulate circulation of water by the bivalves thus maximizing filtration and ingestion of oocysts. 100 ml of seawater containing *Tetraselmis* sp. and *Isochyisis galbana* were added to the tanks containing the bivalves; final concentration of each algal species was 4.6×10^4/ml.

After 2 h of incubation, three to six bivalves from each group were analyzed for oocysts by IFAT. The remaining bivalves were subjected to cleansing according to EU/Portuguese procedures. This involved keeping the bivalves in a tank (Fernando Ribeiro Lda., Lisboa, Portugal) with circulating seawater at 18 °C ± 1 °C, pH 7.8, and 33‰ salinity for 24 h during which time they were exposed to UV-irradiation. The water circulating in the cleansing tank is re-circulated and the system was equipped with a filter that eliminates bacteria and oocysts. Cockles incubated with the higher number of oocysts were kept under these cleansing conditions for 42 h. After the experimental trials, water in the tanks and circulation equipment was analyzed by the EPA Method 1623. Bivalves were dissected and analyzed for detection of oocysts.

RESULTS AND DISCUSSION

Furrow shell clams incubated with 1.0×10^6 oocysts/ml were negative for *C. parvum* as determined by IFA and PCR (Table 1), thus 24 h under the cleansing conditions was sufficient to eliminate the oocysts. However, those incubated with 1.76×10^7 oocysts/ml harbored the parasite after treatment for 24 h.

Carpet shell clams infected with oocyst concentrations up to 4.0×10^6/ml did not have detectable oocysts recovered after 24 h of cleansing. Higher oocyst concentrations were not tested in this study.

Oocysts were not recovered from blue mussels infected with 8.8×10^6/ml oocysts after 24 h of cleansing as determined by IFA and PCR. However, after 24 h of cleansing, *C. parvum* oocysts

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Table **1.** Description of 25 experimental infections in five species of bivalves.

Bivalves	No. of biv.	Ooc. conc. (/ml)	2 h PI	After 24 h clean.	After 42 h clean.	Containers water
Furrow shell clam	50	1.0×10^6	nd	−	nd	+
Furrow shell clam	25	1.76×10^7	6+	+	nd	nd
Furrow shell clam	19	1.76×10^7	4+	+	nd	nd
Carpet shell clam	12	2.1×10^5	nd	−	nd	+
Carpet shell clam	25	4.0×10^6	5+	−	nd	+
Carpet shell clam	25	4.0×10^6	5+	−	nd	+
Blue mussel	18	1.76×10^7	4+	+	nd	nd
Blue mussel	18	1.76×10^7	4+	+	nd	nd
Blue mussel	18	8.8×10^6	6+	−	nd	+
Blue mussel	18	8.8×10^6	6+	−	nd	+
Blue mussel	18	8.8×10^6	6+	−	nd	+
Cupped oyster	12	1.76×10^7	3+	−	nd	nd
Cupped oyster	12	1.76×10^7	3+	−	nd	nd
Cupped oyster	15	1.76×10^7	4+	−	nd	nd
Cupped oyster	15	1.76×10^7	4+	−	nd	nd
Cupped oyster	15	3.52×10^7	4+	−	nd	nd
Cupped oyster	15	3.52×10^7	4+	−	nd	nd
Cockle	25	1.76×10^7	5+	+	nd	nd
Cockle	25	1.76×10^7	5+	+	nd	nd
Cockle	25	1.76×10^7	5+	+	nd	nd
Cockle	25	1.76×10^7	5+	+	nd	nd
Cockle	22	1.76×10^7	6+	+	+	nd
Cockle	22	1.76×10^7	6+	+	+	nd
Cockle	22	1.76×10^7	6+	+	+	nd
Cockle	22	1.76×10^7	6+	+	+	nd

No. of biv., number of bivalves infected; Ooc. conc., oocyst concentration; PI, post-infection; clean, cleansing; nd, not done; +, positive; −, negative.

were recovered from mussels incubated with 1.76×10^7 oocysts/ml. Therefore, treatment longer than 24 h was established as the minimum time needed to remove the parasites from blue mussels.

Among the bivalves evaluated, cupped oysters were the most efficient in removing *C. parvum* oocysts. After incubation with 3.52×10^7 oocysts/ml none were recovered after 24 h.

Cryptosporidium parvum was recovered from cockles incubated with 1.76×10^7 oocyts/ml after 24 and 42 h treatment, as shown by IFA and PCR. Thus other treatment protocols or longer periods of this cleansing method is necessary for the removal of oocysts from these bivalves.

In all bivalve samples from which *C. parvum* was recovered, oocysts were also detected in their tank water. The oocyst concentration tested were appropriate since both bivalves and the tank water tested positive for the parasite after 2-h incubation. However, in seven samples in which oocysts were not recovered from the bivalves, they were detected in water taken from the equipment.

Faecal coliform counts are usually used as a water quality indicator in bivalve production areas, but only one indicator microorganism is not sufficient for predicting contamination by all enteric pathogens. *Cryptosporidium parvum* oocysts have been observed in bivalves produced for human consumption but their presence was not correlated to *E. coli* levels (Gómez-Couso, Méndez-Hermida, and Ares-Mazás 2006). The treatment process used in the present study was designed specifically for bacteria, not protozoan parasites. Thus, we intentionally used high concentrations of oocysts because in their natural settings mussels can each contain $>10^3$ (Gómez-Bautista et al. 2000) and clams can harbor up to 1.84×10^6 oocysts/ml of hemolymph (Graczyk et al. 1997).

Several factors might contribute to possible failure of the currently used cleansing protocol. Healthy clams exhibit high filtration rates but parasites can infect hemocytes in bivalve hemolymphs, as shown in *Crassostrea virginica* (Graczyk et al. 1997) and transmission of *Cryptosporidium* oocysts between live clams (*Tapes decussatus*) was experimentally demonstrated (Gómez-Couso et al. 2003). If *Cryptosporidium* oocysts are present in the treatment water, risk of human infection could be avoided. Harsh treatment such as freezing at $-20\,^{\circ}C$ for 72 h or by boiling for 1 min would kill the parasite. On the other hand, oocyst viability could be reduced by milder treatment such as heating at 45 °C or higher for 5–20 min (Havens and Davis 1996). However in Portugal bivalves are usually consumed raw or under-cooked. If they are infected with *Cryptosporidium* species that cause disease in humans they can transmit the parasite to humans (Fayer, Dubey, and Lindsay 2004).

Several factors influence cleansing efficiency. These include the bivalve species involved, water temperature, dissolved O_2, and salinity (Somerset 1995). The cleansing treatment is very stressful to bivalves causing a high degree of mortality, thus increasing treatment time would only increase mortality rates (Cachola and Ruano 2000). According to reports by other workers 72-h treatment or even longer might not be sufficient for removing oocysts from some bivalve species (Freire-Santos et al. 2000). In this preliminary study, we showed that oocysts can persist in bivalves after 24 h of treatment. This represents a serious risk to bivalve consumers with weak immune systems. However, for more precise measurements percent recoveries of oocysts from each bivalve species and the water in tanks and equipment must be determined using this commercial cleansing procedure. We plan to continue these studies to determine the most suitable treatment time needed to effectively remove parasites.

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