Research Note

Temporal Occurrence of Cryptosporidium in the Manila Clam Ruditapes philippinarum in Northern Adriatic Italian Lagoons

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ABSTRACT

In order to evaluate the temporal occurrence of Cryptosporidium oocysts in Ruditapes philippinarum clams bred along the northeastern Italian Adriatic coast and molecularly characterize the isolates, 2,160 specimens (180 clams per month) were collected from three clam farms from January to December 2004. Two farms (sites A and B) were located in Venice (Chioggia, Veneto region) and one (site C) in the Marano Lagoons (Friuli Venezia Giulia region). Clams from 36 pools (i.e., one pool of 60 clams per month per site) were subjected to a high-sensitivity seminested PCR assay specific for a 360-bp diagnostic region internal to the Cryptosporidium spp. outer wall protein gene. Positive amplicons were sequenced and analyzed. Cryptosporidium DNA was found in clams from seven pools (sites A and B) during 1 month of sampling at site A and 6 months of sampling at site B, with Cryptosporidium hominis and Cryptosporidium parvum being detected. The expected infection rate of the clams was 0.36%. Site B showed a significantly higher expected infection rate (1.15%) than did the other sites (A = 0.14% and C = 0%). Given its high sensitivity and specificity, this seminested PCR assay can be considered a reliable tool for detecting and distinguishing species within the Cryptosporidium genus. The seasonal pattern of contamination and the related public health risks are of particular concern.

Shellfish industries in Italy represent a very important economic source for the fishing sector. Italy is the largest producer of clams in the European Union (http://epp.eurostat.ec.europa.eu/cache/ITY_OFFPUB/EN/KS-NN-01-022/EN-KS-NN-01-022-EN.PDF) with 62,000 tons of Ruditapes philippinarum farmed in 1999, 90% of which were produced in the lagoons of northeastern Italy (27). This shellfish species is a Manila clam that was first brought to the northern Italian Adriatic Sea in March 1983 to compensate for the declining market for Tapes decussatus, the native clam, and to boost the local fishing industry (5).

Bivalve mollusks are filter feeders and have the ability to concentrate and retain microorganisms, algae, or inorganic material. Most of these contaminants represent public health hazards, as they may be pathogenic to humans, especially if the shellfish is raw or undercooked at the time of consumption. It is well known that a huge amount of feces produced by livestock, pets, and wild animals harboring zoonotic protozoa is dumped and carried away in runoff and regularly discharged into rivers, estuaries, and coastal waters and is thus filtered and concentrated by shellfish or ends up contaminating beaches (6, 7).

Over the last decade, among the protozoan parasites of animal and human interest, Cryptosporidium has attracted great attention from the scientific community for its public health implications (10, 36). Cryptosporidium is a protozoan parasite that is present worldwide and is responsible for diarrheal disease in various mammalian species, including farm animals and humans. Cryptosporidium can be life threatening in immunocompromised patients and can severely debilitate immunocompetent subjects (11). The major etiological agent of human cryptosporidiosis in Europe, Cryptosporidium hominis, has also been occasionally detected in marine mammals (i.e., dugong), sheep, pigs, and calves (15, 24, 34). The major etiological agent of human cryptosporidiosis in North America, Cryptosporidium parvum (formerly C. parvum genotype II or bovine genotype), is prevalent in preweaned cattle, other young ruminants, and, to a lesser extent, mice and pigs (35). Other species or genotypes can infect humans (such as Cryptosporidium canis, Cryptosporidium felis, Cryptosporidium meleagrisidis, and Cryptosporidium muris), especially immunocompromised subjects (13, 23).

The smallness of the oocysts (3.5 to 6 μm in diameter) and their viability in the environment (up to 1 year in artificial seawater) (9, 28, 31), infectivity for newborn mice after 30 days in shellfish (12), high concentrations reported in the feces (108 oocysts per g) of young calves, low sed-
mentation rate (0.5 μm/s) (11), and resistance to commonly used disinfectants, as well as the low doses required for human infection (≥30 oocysts) (4, 25) and lack of specific treatments, are factors that facilitate their transmission to humans and their accumulation in bivalves (2). The molecular characterization of protozoan isolates is needed to evaluate their role in foodborne illness. Genotyping of isolates is instrumental in determining routes of infection and identifying human food sources that may serve as reservoirs for Cryptosporidium. The presence of internal diagnostic region sequences showing significant differences among Cryptosporidium species and between C. hominis and C. parvum within the gene coding for the Cryptosporidium oocyst wall protein (COWP) makes this gene a useful target for detecting this protozoan and genotyping isolates from different sources (14, 17, 30, 33).

The aim of the present study was to determine the temporal occurrence of Cryptosporidium spp. in R. philippinarum clams bred on the northeastern Italian Adriatic coast and to molecularly characterize the isolates.

MATERIALS AND METHODS

Sample collection. From January through December 2004, clam rakes were used to collect 2,160 (180/month) clams (R. philippinarum) from three sites: two clam farms in Venice Lagoon (Chioggia, Veneto region) (sites A and B) and one clam farm in the Marano Lagoon (site C) (Friuli Venezia Giulia region) (Fig. 1). The clams were kept at 0 to 5°C until they reached the laboratory within 24 h, where they were measured, weighed (live weight), and pooled (i.e., one pool of 60 clams per month per site) for convenience. A total of 36 pools were produced. Hemolymph was aspirated from each bivalve (approximately 100 μl per clam) and pooled according to the site of collection and to the number of clams per pool, and the pools were concentrated in a 3.5-ml volume of 1 M sucrose solution centrifuged at 4,000 g for 2 min. The resulting pellet was subjected to genomic DNA extraction with the Quantum Prep Aquapure Genomic DNA Kit (Bio-Rad Laboratories, Hercules, Calif.), and all the DNA samples were stored at 4°C. A 360-bp fragment internal to the N-terminal domain of the COWP gene was amplified by seminested PCR. A degenerated primer set, i.e., CRY15D (forward, 5'-GTA GAT AAT GGA AGR GAY TGT G-3') and CRY9D (reverse, 5'-GGA CKG AAA TRC AGG CAT TAT CYT G-3'), was used in a first round of PCR (33) to amplify a 550-bp fragment of the COWP gene, followed by a second round that used the degenerated primer set CRYINT2D (forward, 5'-TTT GTT GAA GAR GGA AAT AGA TGT G-3') and CRY9D (reverse, 5'-GGA CKG AAA TRC AGG CAT TAT CYT G-3'). The first round was performed on a 50-μl PCR mixture containing 10 μl of DNA extract as a template, 100 pm of each primer, 2.5 mM MgCl2, a 200 μM concentration of each deoxynucleoside triphosphate, 1 U of Taq Gold polymerase (Applied Biosystems, Foster City, Calif.), and 1× reaction buffer (100 mM Tris-HCl [pH 8.3] and 500 mM KCl). The conditions of the second round were the same as for the first step, and 10 μl (determined to be optimal by serial dilution and by addition of different undiluted aliquots of the first ampli
con to the second-step mixture) of the first PCR product was used as template.

PCRs of both steps were performed under the following conditions: 94°C for 12 min (Taq Gold activation temperature) and 40 cycles of amplification (50 s at 95°C, 30 s at 52°C, and 50 s at 72°C), with a final 7-min elongation step at 72°C. Samples containing DNA of C. hominis and C. parvum and samples without DNA (i.e., distilled water as a template) were included in all PCRs (to act as positive and negative controls, respectively).

The PCR amplicons were electrophoresed in 2% agarose gel, viewed after ethidium bromide staining, and photographed with the Gel Doc 2000 (Bio-Rad) documentation system. All amplicons were directly sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing Kit in an ABI-PRISM 377 sequencer (Applied Biosystems).

The sequences were aligned with ClustalX (32), further examined manually, and compared with those of Cryptosporidium spp. registered in the GenBank database. All the molecular procedures were performed twice to verify reliability of the results.
FIGURE 2. Agarose gel showing the second-step amplification using primer CRYINT2D-CRY9D of the COWP gene from positive hemolymph pool samples. Lane M, DNA marker (SM0371, GeneRuler; Fermentas); lane 1, positive samples from hemolymph from Chioggia site A; lanes 2 and 3, positive samples from hemolymph from Chioggia site B; and lanes 4 and 5, negative and positive controls, respectively.

### Statistical analysis

The following were calculated for each site and for the total data: (i) monthly prevalence (number of positive months/number of months tested); (ii) minimum infection rate (number of positive pools/total number of individual clams tested); and (iii) expected infection rate (EIR), by using the following formula (3):

\[
\text{EIR: } P = 1 - \sqrt{n/N} \cdot 100
\]

where \( n \) is the number of negative pools, \( N \) the number of tested pools, and \( k \) the mean number of clams for each pool (software, Excel). Differences among prevalence and infection rates in the three sites were tested by \( \chi^2 \) test or the exact Fisher’s test (software, Epinfo 6.0 and SPSS for Windows, version 12.01).

### RESULTS

Seven (19.4%) of the 36 pooled samples produced amplicons of 360 bp after the seminested PCR assay (Fig. 2); 2.7% (1 of 36) were from site A, 16.6% (6 of 36) from site B, and none from site C. The positive sample from site A had been collected in August, while the six positive samples from site B had been collected in February, March, June, August, September, and October (Table 1). The sequencing revealed that the amplicons were of 360 bp as expected, and comparison with the *Cryptosporidium* sequences available in the GenBank database revealed that one pooled sample (February) from site B showed 100% identity with the sequence of *C. hominis* (accession number AF248741) and that six pooled samples from sites A and B had 100% identity with the sequence of *C. parvum* (accession number AF266273) (Fig. 3). The clam EIR was an overall 0.36%. Site B showed a significantly higher EIR (1.15%) than did the other sites (A = 0.14% and C = 0%) (Table 2).

### DISCUSSION

Hemolymph has been shown to retain high numbers of *Cryptosporidium* oocysts (21) for as long as 14 days after exposure (22). In the present study the molecular tool applied to this tissue and used to genotype *Cryptosporidium* is a highly sensitive seminested PCR assay amplifying a region internal to the COWP gene. Design of the primer CRYINT2D was based on alignment of the COWP sequences with the most common species of *Cryptosporidium*

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**TABLE 1. Cryptosporidium oocysts in Ruditapes philippinarum from three Italian clam farms (sites A and B, Chioggia, Venice Lagoon, Veneto region and site C, Marano Lagoon, Friuli Venezia Giulia region) detected by the seminested PCR**

<table>
<thead>
<tr>
<th>Mo of sampling in 2004</th>
<th>Chioggia (site A)</th>
<th>Chioggia (site B)</th>
<th>Marano (site C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pools Results</td>
<td>Pools Results</td>
<td>Pools Results</td>
</tr>
<tr>
<td>January</td>
<td>Ch A1 —</td>
<td>Ch B1 —</td>
<td>Ma 1 —</td>
</tr>
<tr>
<td>February</td>
<td>Ch A2 —</td>
<td>Ch B2 + ◇ ◇</td>
<td>Ma 2</td>
</tr>
<tr>
<td>March</td>
<td>Ch A3 —</td>
<td>Ch B3 +□</td>
<td>Ma 3</td>
</tr>
<tr>
<td>April</td>
<td>Ch A4 —</td>
<td>Ch B4 —</td>
<td>Ma 4</td>
</tr>
<tr>
<td>May</td>
<td>Ch A5 —</td>
<td>Ch B5 —</td>
<td>Ma 5</td>
</tr>
<tr>
<td>June</td>
<td>Ch A6 +□</td>
<td>Ch B6 +□</td>
<td>Ma 6</td>
</tr>
<tr>
<td>July</td>
<td>Ch A7 —</td>
<td>Ch B7 —</td>
<td>Ma 7</td>
</tr>
<tr>
<td>August</td>
<td>Ch A8 +□</td>
<td>Ch B8 +□</td>
<td>Ma 8</td>
</tr>
<tr>
<td>September</td>
<td>Ch A9 —</td>
<td>Ch B9 +□</td>
<td>Ma 9</td>
</tr>
<tr>
<td>October</td>
<td>Ch A10 —</td>
<td>Ch B10 +□</td>
<td>Ma 10</td>
</tr>
<tr>
<td>November</td>
<td>Ch A11 —</td>
<td>Ch B11 —</td>
<td>Ma 11</td>
</tr>
<tr>
<td>December</td>
<td>Ch A12 —</td>
<td>Ch B12 —</td>
<td>Ma 12</td>
</tr>
</tbody>
</table>

\( ◇ ◇ = C. hominis\) (GenBank accession number AF248741); \( □ = C. parvum\) (GenBank accession number AF266273).
in order to identify at least 11 different genotypes (36), including the zoonotic C. hominis and C. parvum.

Although the presence of Cryptosporidium spp. is often determined by an immunofluorescence assay, this technique can be limited by the occurrence of cross-reactions between Cryptosporidium and other microorganisms living in marine environments (18, 20–22), lack of monoclonal antibodies for all Cryptosporidium species, low sensitivity, the technique’s elevated costs, and finally the ability of some particulate materials to fluoresce as brightly as Cryptosporidium oocysts do (8, 16, 20). Hence, highly sensitive molecular tools such as a seminested PCR assay provide the most reliable means to detect and distinguish species within the Cryptosporidium genus. This fact is of importance, since the unequivocal identification of isolate genotypes and their sources of infection is fundamental to studying both the epidemiology of cryptosporidiosis and the presence of protozoa in food destined for human consumption. Despite an overall low EIR, this study revealed the presence of Cryptosporidium in R. philippinarum in Italy. This one-year survey—the longest of any thus far reported in Europe—indicates that fecal contamination from human and animal sources is protracted over time, at least in Chioggia B, where clams collected during 6 of 12 months were infected with Cryptosporidium.

In this 12-month survey, Cryptosporidium was detected in clam farms during the summer at Chioggia A and throughout the year at Chioggia B, which showed a significantly higher infection rate ($P < 0.05$). Detection of the C. parvum bovine genotype in six out of seven pools may be related to the presence of numerous breeding farms in this area, and the bovine genotype has been reported to be infectious for humans in the literature (35).

C. hominis is generally specific to humans and primates, although it has been isolated from a marine mammal as well. The presence of C. hominis was reported by Gomez-Couso et al. (17) in samples of shellfish of unknown origin, and C. parvum was recently detected by both immunofluorescence assay and molecular tools in Chamelea gallina in central Italy (14). The Italian literature provides a wealth of information on cryptosporidiosis in humans. Despite a decrease in the overall prevalence of cryptosporidiosis subsequent to highly active antiretroviral therapy in human immunodeficiency virus patients, cases of Cryptosporidium infection in children (2%) and human immunodeficiency virus patients (21%) and, more important, several undiagnosed single cases or outbreaks of human cryptosporidiosis have been reported in Italy (1, 26).

Rain runoff is discharged into rivers, estuaries, and coastal waters; sewage treatment does not presently remove pathogens from the effluent and contributes to beach, sea, and, consequently, shellfish contamination. The presence of Cryptosporidium in farmed clams may also be due to the particular geographic features of the land, given that rivers flow into the lagoons, where water stagnation is likely. The Lagoon of Venice is a transitional environment suffering from industrial, urban, and agricultural impacts (29).
though the exact waste sources for the area surveyed are unknown, illegal disposal of wastewater has been denounced by environmental associations (http://www.salve.it/uk/banchetedati/f/documenti.htm). Bivalves are subject to depuration; however, the treatment process does not totally remove Cryptosporidium (19). Some oocysts remain unaltered and viable and may be retained by the gills since the pores and oocysts are of similar size (12). As bioindicators, clams are regularly tested by the Italian national or regional control institutions for fecal pathogens such as coliforms and Escherichia coli, Salmonella spp., and metals (Legislative Decree no. 530, 1992, Gazzetta Ufficiale, no. 107, 9 May 1996). The European Union legislation does not protect consumers from the risk of Cryptosporidium infection. The shellfish examined in this investigation came from areas classified as category B (i.e., areas where shellfish must be marketed after depuration), but Gomez-Couso et al. (19) found Cryptosporidium spp. in mussels, from areas graded as category A, that can be marketed directly without undergoing depuration. In this report, the presence of both human (C. hominis) and zoonotic (C. parvum) genotypes indicates a high level of pollution in the lagoons surveyed that may create considerable health risks.

Considering that popular swimming resorts have been examined and large numbers of R. philippinarum clams are mainly eaten raw each year in Italy, the data presented herein are of social and economic importance. In our opinion, public health institutions must be alerted so as to include Cryptosporidium spp. among the pathogens used as indicators of marine environmental contamination.

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