Genotyping of *Cryptosporidium* Isolates from *Chamelea gallina* Clams in Italy

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***Chamelea gallina* clams collected from the mouths of rivers along the Adriatic Sea (central Italy) were found to harbor *Cryptosporidium parvum* (genotype 2), which is the lineage involved in zoonotic transmission. The clams were collected from the mouths of rivers near whose banks ruminants are brought to graze. This paper reports the environmental spread of *C. parvum* in Italy and highlights the fact that genotyping of seaborne *Cryptosporidium* isolates is a powerful tool with which to investigate the transmission patterns and epidemiology of this microorganism.**

Cryptosporidium spp. are coccidian protozoa that infect the digestive and/or respiratory tracts of animals and humans (40). The major etiological agents of human cryptosporidiosis are *Cryptosporidium hominis* (formerly *Cryptosporidium parvum* genotype 1), which in nature infects only humans but which has been isolated from a primate, a dugong, and experimentally infected pigs and calf (32, 33, 37), and *C. parvum* (formerly *C. parvum* genotype 2), which infects ruminants (cattle, sheep, and goats) (42).

Cryptosporidium infection occurs mainly by fecal-oral, waterborne, and food-associated transmission, although person-to-person and animal-to-person contacts may also represent important sources of infection (12). Among the different kinds of food incriminated in the transmission of cryptosporidiosis, bivalve molluscs collected for human consumption (i.e., mussels, bent mussels, oysters, cockles, and clams) have been shown to be infected by *Cryptosporidium* oocysts in Europe and North America (10, 14, 17, 21, 24, 27–29), possibly due to water contamination by urban or agricultural runoff (31, 35).

Despite the fact that evidence of human anti-*Cryptosporidium* seropositivity has been reported (23), data on the epidemiology of this parasite in Italy are still scant and mainly have been obtained during studies of intestinal infection in children and human immunodeficiency virus-positive patients (5–7, 9). In particular, information on environmental contamination by *Cryptosporidium* spp. in Italy is limited to reports of oocysts in samples from wastewater treatment plants (4, 8), in pooled samples of *Ruditapes philippinarum* clams coming from the lagoon of Venice (21), and in shellfish samples of unknown origin (25). To our knowledge, there are no data on the *Cryptosporidium* species and genotypes present in Italy or on their spread, with the exception of a single report on *C. parvum* in calves (43).

Molecular genotyping of isolates is of paramount importance for identifying the routes of infection and the animals that may act as reservoirs of *Cryptosporidium* spp. and, thus, are potentially hazardous for human health (3, 11, 22, 34). Among the different genes that have been studied, hypervariable regions within the 18S rRNA gene and within the gene encoding the *Cryptosporidium* oocyst wall protein (COWP) have proven to be powerful targets for detection of the protozoan and for genotyping of isolates (14, 27). In fact, the genes reported above present diagnostic sequences that can be PCR amplified with primers relatively conserved across the genus and show significant differences among *Cryptosporidium* species and between *C. hominis* and *C. parvum* (30, 37, 41).

The aim of this investigation was to acquire greater insight into the presence and environmental spread of *Cryptosporidium* spp. in Italy through the genetic identification of isolates from seawater clams collected in that country.

In March 2003, a total of 480 clams (*Chamelea gallina*) were collected from the mouths of the Tronto (site A), Vibrata (site B), Tordino (site C), and Vomano (site D) rivers at about 400 to 500 m from the coast of the Adriatic Sea in the Abruzzo region (central Italy). One hundred twenty clams were obtained at each site and were maintained at 0 to 5°C until they reached the laboratory (within 6 h), where they were identified, measured, weighed, and pooled (30 clams per pool). The hemolymph was aspirated from each clam (approximately 100 µl per clam) and pooled according to the site of collection and to the number of clams per pool. All of the pooled samples ($n = 16$) were concentrated by sucrose gradient centrifugation ($400 \times g$, 15 min). The gradient interface was aspirated, resuspended in 4 ml of physiological saline (0.9% NaCl), and then subjected to centrifugation at $600 \times g$ for 10 min. The supernatant was aspirated off, leaving about 1 ml of the concentrated sample volume, including the pellet.

Genomic DNA was extracted from 200 µl of each concentrated sample. Briefly, samples were subjected to three freeze-thaw cycles (each consisting of 3 min in liquid nitrogen followed by 3 min at 80°C) and then centrifuged at $6,500 \times g$ for 2 min. After each sample was washed with phosphate-buffered

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saline, the resulting pellet was digested overnight in 200 μ l of a lysis buffer containing proteinase K, as previously described (27). Finally, the samples were purified in spin columns (DNeasy kit; QIAGEN GmbH, Hilden, Germany) and stored at 4°C until molecular analysis was performed.

All DNA extracts were subjected to PCR amplification with a degenerate primer set, i.e., CRY15D (5'-GTAGATAATGG AAGRGAYTGTG3') and CRY9D (5'-GGACKGAAATRC AGGCATTATCYTG3'), to amplify a ~550-bp fragment of the N-terminal domain of the gene encoding *Cryptosporidium* spp. COWP (37, 41). The reaction mixtures consisted of 50 μ l containing 10 μ l of DNA extract as a template, a 2 mM concentration of each primer, 2.5 mM MgCl₂, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1 U of *Taq* Gold polymerase (Applied Biosystems), and 1 $\times$ reaction buffer (10 mM Tris-HCl [pH 8.3]–50 mM KCl). Amplification runs were performed in an Applied Biosystems model 2700 thermal cycler with the following protocol: 12 min at 94°C (*Taq* Gold activation temperature); 40 cycles of 50 s at 95°C, 30 s at 52°C, and 50 s at 72°C; and a final 7-min elongation step at 72°C. Samples containing DNA of *C. hominis* or *C. parvum* (provided by the Public Health Laboratory Service's Central Public Health Laboratory, London, United Kingdom) and samples without DNA were used as positive and negative controls, respectively, in all PCR runs.

Amplicons were resolved by electrophoresis in a 1.8% agarose gel, visualized after ethidium bromide staining, and photographed with a documentation system (Gel Doc 2000; Bio-Rad). Then amplicons were purified over minicolumns (Ultrafree-DA; Millipore) and sequenced using a *Taq* Dye-DeoxyTerminator cycle sequencing kit (Applied Biosystems) and an ABI-PRISM model 377 sequencer. Sequence accuracy was ensured by two-directional sequencing, and all sequence electropherograms were manually checked and edited as deemed necessary. The sequences were aligned with each other by using the ClustalX application (39) and compared with those of *Cryptosporidium* spp. registered in the GenBank database by using the nucleotide-nucleotide BLAST tool (1) available online at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

All of the molecular procedures were performed twice to verify the reliability of the results. Of the 16 pooled samples of hemolymph subjected to PCR, 2 produced amplicons of about 550 bp that were detectable on the agarose gel (Fig. 1); these positive samples came from two of the four river mouths included in the study, namely, sites B and D. Comparison with the *Cryptosporidium* sequences available in the GenBank database showed 100% identity of the sequences obtained from the PCR-positive clam samples with the bovine *C. parvum* COWP sequence already available in the GenBank database (accession number AF266273).

Detection of *Cryptosporidium* spp. oocysts by light microscopy and their morphological identification to the species level have proven to be unreliable in most cases, since all of the techniques used are highly time-consuming and labor-intensive and require highly skilled personnel. Oocysts may be difficult to retrieve because of their small size, and they can be difficult to identify to the species level because of the frequent similarity of the morphological features (i.e., size, shape, and internal structures) of the oocysts of the various species. For example,

FIG. 1. Example of an agarose gel showing PCR amplification (using primer pair CRY15D-CRY9D) of the *Cryptosporidium* spp. COWP gene from positive hemolymph pool samples. Lanes: 1, size marker; 2 and 3, positive samples of hemolymph from the Vibrata (site B) and Vomano (site D) rivers (pools 3 and 2, respectively); 4 and 5, negative samples of hemolymph from the Tronto (site A) and Tordino (site C) rivers (pools 1 and 2, respectively); 6, positive control; 7, negative control.

C. hominis and *C. parvum* oocysts cannot be easily distinguished from those of *Cryptosporidium bailey* or *Cryptosporidium meleagridis* and are indistinguishable from *Cryptosporidium canis* oocysts (13, 24, 42).

The conventional acid-fast stain used in microscopy has minimal applicability to mollusc screening because it may yield false-positive results due to the presence of microorganisms with morphological features similar to those of *Cryptosporidium* spp. or of other acid-fast stain-positive pathogens (e.g., *Haplosporidium* spp.) (20, 26). The other staining methods (e.g., dimethyl sulfoxide-carbol fuchsin or safranin-methylene blue) are laborious and have low sensitivity and specificity (18). Another difficulty in attaining reliable microscopic detection of *Cryptosporidium* spp. oocysts is the need to distinguish them from other small particles that can be present in the samples (e.g., debris, yeasts, and algae) (19).

Finally, to date, no extensive data on the morphology of putative marine *Cryptosporidium* spp. have been available, since they have been studied only recently (2, 36).

Examination of mollusc hemolymph or tissues for the presence of *Cryptosporidium* spp. may be successfully performed by immunofluorescence assays, but this technique is often limited by the occurrence of cross-reactions with other microorganisms, the lack of monoclonal antibodies for all *Cryptosporidium* species, or its low sensitivity and high cost (3, 16, 18, 19, 24, 26). Furthermore, when antibody-based methods are used for shellfish samples, some identification problems arise due to the fact

that microorganisms and particulate materials of different sizes and shapes may appear as brightly fluorescent as *Cryptosporidium* oocysts (14, 19). Hence, molecular characterization of the isolates remains the most reliable technique with which to detect and distinguish species within the genus *Cryptosporidium*.

In this study, evidence of the presence of *C. parvum* in clams collected at the mouths of the Vomano and Vibrata Rivers led us to hypothesize that ruminants grazing near the rivers might be sources of contamination. Similarly, *Cryptosporidium* oocysts were found in oysters harvested from sites near livestock farms (17), and *C. parvum* was also found in mussels and cockles harvested in northwestern Spain at the mouths of rivers with a high density of grazing ruminants along their banks (24).

The presence of *C. parvum* in marine *C. gallina* clams is noteworthy since these clams are often eaten raw in Italy. It has been demonstrated that *C. parvum* oocysts may survive in seawater for 4 weeks to 1 year (17, 38) and remain infectious for mice (24), and it has also been shown that a minimum *Cryptosporidium* burden of 30 oocysts is needed to infect an immunocompetent human being (12).

This paper provides new insights into environmental contamination by *C. parvum* in Italy and, as recently demonstrated (15), highlights the power of molecular genotyping of isolates for the specific identification of this protozoan, as this could be instrumental in defining the origin of contamination and studying the epidemiology and transmission patterns of cryptosporidiosis. Since no effective treatment or vaccine for *C. parvum* infection is available, understanding its routes of transmission and the unequivocal identification of isolate genotypes and their sources of infection are of paramount importance for the prevention of outbreaks and the control of this disease.

Nucleotide sequence accession number. Nucleotide sequence data obtained in this work are in the GenBank database under accession number AY497420.

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