MOLECULAR IDENTIFICATION OF FOOD-BORNE AND WATER-BORNE PROTOZOA

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Abstract. *Cryptosporidium* and *Giardia* can be transmitted to humans by contaminated food and water, resulting in large outbreaks of diarrheal disease. Sensitive methods for detecting these parasites are needed to control and prevent infection. However, this issue is complicated by the fact that there is still uncertainty about the role played by different species/genotypes with respect to human disease. We are in the process of collecting samples from clinical cases (both sporadic and outbreak-related human infections) and from the environment (tap and waste water samples from different geographic regions), to test the efficacy of methods for detection and genotyping. Concerning *Cryptosporidium parvum*, we have developed new genotyping methods based on highly polymorphic microsatellite markers. The use of microsatellite markers allows the route of transmission to be traced; these methods can also be used not only to distinguish between anthroponotic and zoonotic transmission but also to identify the source(s) of infection. Regarding *Giardia*, which was found very frequently in environmental water samples, we are testing the beta-giardin gene as a marker to discriminate among species/genotypes.

INTRODUCTION

Cryptosporidium parvum and *Giardia duodenalis* are important cosmopolitan protozoa that cause diarrhea in humans and animals. Infected hosts can excrete between 10^9 and 10^{10} oocysts, and both domestic and sylvatic animals may be reservoirs for human infection. Some *C. parvum* and *G. duodenalis* strains show a zoonotic transmission, whereas others show an anthroponotic transmission (Cacciò *et al*, 2000; Thompson *et al*, 2000). The environment can be contaminated through direct deposit of animal and human feces or through the discharge of sewage and wastewater into waters. Therefore, the consumption of contaminated food (vegetables, fruits, etc) and water can cause disease.

Although *G. duodenalis* is the most commonly diagnosed flagellate in the human intestinal tract, the prevalence of *C. parvum* is unknown. In fact, in most laboratories, routine stool examination for ova and parasites does not include procedures for detecting *Cryptosporidium* spp. As a consequence, the true number of outbreaks and of sporadic infections caused by this parasite is probably largely underestimated.

Human infections may result from the ingestion of as few as 5 *Giardia* cysts (De Carneri *et al*, 1977) and 30 *Cryptosporidium* oocysts (DuPont *et al*, 1995). Under favorable conditions of temperature and humidity, oocysts can remain viable for several months (Ortega and Adam, 1997; Tamburrini and Pozio, 1999), increasing the probability of the occurrence of infection.

The objective of the present study was to develop molecular assays for the detection and identification of *Cryptosporidium* and *Giardia* species/genotypes.

MATERIALS AND METHODS

Parasite samples

Samples of human and animal feces and of tap and waste water were collected in Italy. Samples were screened for *Cryptosporidium* sp oocysts and/or *Giardia* cysts by IFA. DNA was extracted from positive samples using the method of da Silva *et al* (1999).

Molecular identification of *Cryptosporidium* oocysts

The N-terminal domain of the COWP gene was amplified by PCR using a previously described primer set (Spano *et al*, 1997). PCR products were purified and sequenced in both strands, and sequences were analyzed and aligned using Sequence Navigator software. Restriction patterns were predicted using the OMIGA software. PCR amplicons were cut with the endonuclease *Rsa* I, and the resulting fragments were separated by agarose gel electrophoresis. To further characterize those isolates typed as *C. parvum*, two microsatellite loci were analyzed. The first locus (G35348) contains GAG repeats (Cacciò *et al*, 2000), and the second locus (SCRP), which corresponds to a new coding sequence, contains AG repeats that are comprised within an intron. PCR products were sequenced and analyzed as described above.

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Molecular identification of *Giardia* cysts

Different regions of the beta-giardin gene were amplified by PCR using primers previously described or newly designed in our laboratory. The amplification, purification and sequencing of PCR products were carried out as described above.

RESULTS

PCR amplifications of DNA extracted from *Cryptosporidium* oocysts belonging to seven species (*C. parvum*, both H and C genotypes; *C. wrairi*; *C. felis*; *C. baileyi*; *C. muris*; *C. serpentis*; *C. andersoni*) generated single amplicons of the expected size (553 bp). Sequencing of the PCR products revealed no deletions or insertions, though a high proportion of positions showed substitutions, most of which were synonymous. Independent sequences from multiple isolates of the same species were found to be identical. Species-specific restriction patterns were obtained for all species and for the two *C. parvum* genotypes, using the endonuclease *Rsa* I. Only in the case of *C. muris* and *C. andersoni* were identical restriction patterns observed: to distinguish these two species, sequencing of the COWP gene fragment would need to be performed.

DNA sequencing and multiple alignment of *C. parvum* isolates at the G35348 locus revealed the presence of 6 different alleles (or subgenotypes): two in the human (H) genotype and four in the animal (C) genotype. The alleles were observed to have differed by expansion/contraction of the microsatellite repeat units, which are likely to be produced by slippage during the DNA replication.

DNA sequencing and multiple alignment of *C. parvum* isolates at the SCRP locus revealed the presence of 8 different alleles (or subgenotypes) among animal and human isolates collected in Italy.

PCR amplification of DNA extracted from *Giardia* cysts was performed with primers targeting the beta-giardin gene. The isolates represent different genotypes of *G. duodenalis*, which were typed as Assemblage A or B. In each case, single amplicons of the expected size (about 800 bp) were obtained. The sequences of the amplicons were determined and compared to those available in the GenBank database. Mutations specifically associated with isolates belonging to Assemblage A or B were identified, which will rapidly lead to the development of a diagnostic PCR-RFLP assay.

DISCUSSION

We have developed new molecular assays that are useful for the detection and molecular discrimination of different species/genotypes of the cosmopolitan parasites *Cryptosporidium* and *Giardia*. The final goal of our research is to apply these methods to detect species/genotypes pathogenic to humans that may be present in water and food samples.

However, whereas these techniques can routinely detect approximately 10-100 oocysts from experimentally spiked water or food samples, their use on "real" samples poses several problems related to the nature of the samples. Specifically, inhibitory substances were frequently found in water samples. Although these inhibitors may be eliminated by purification of oocysts using immunomagnetic separation, the recovery rate of the method varied considerably from sample to sample, a fact that probably reflects the diverse chemical composition of the samples. Furthermore, the number of oocysts present in these samples is frequently below the detection level of current methods, or recovered organisms simply cannot be typed by established methods (McIntyre *et al*, 2000). Finally, the identification of *Cryptosporidium* oocysts and *Giardia* cysts that are pathogenic to humans is difficult, because the zoonotic potential of some of the species/genotypes of these organisms is still unknown. However, we expect that in the very near future rapid improvements in molecular and immunological assays will allow these technical problems to be overcome.

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REFERENCES

Cacciò S, Homan W, Camilli R, Traldi G, Kortbeek T, Pozio E. A microsatellite marker reveals population heterogeneity within human and animal genotypes of *Cryptosporidium parvum*. *Parasitol* 2000; 120:237-44.

da Silva AJ, Bornay-Llinares FJ, Moura IN, Slemenda SB, Tuttle JL, Pieniazek NJ. Fast and reliable extraction of protozoan parasite DNA from fecal specimens. *Mol Diagn* 1999;4:57-64.

De Carneri I, Trane F, Mandelli V. *Giardia muris*: oral infection with one trophozoite and generation time in mice. *Trans R Soc Trop Med Hyg* 1977;71:438.

DuPont HL, Chappell CL, Sterling CR, Okhuysen PC, Rose JB, Jakubowski W. The infectivity of *Cryptosporidium parvum* in healthy volunteers. *N*

Engl J Med 1995;332: 855-9.

McIntyre L, Hoang L, Ong CS, Lee P, Isaac-Renton JL. Evaluation of molecular techniques to biotype *Giardia duodenalis* collected during an outbreak. *J Parasitol* 2000;86:172-7.

Ortega YR, Adam RD. *Giardia*: overview and update. *Clin Infect Dis* 1997;25:545-9.

Spano F, Puri C, Ranucci L, Putignani L, Crisanti A. Cloning of the entire COWP gene of *Cryptosporidium parvum* and ultrastructural localization of the protein during sexual parasite development. *Parasitol* 1997;114:427-37.

Tamburrini A , Pozio E. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). *Int J Parasitol* 1999;29:711-5.

Thompson RCA, Hopkins RM, Homan WL. Nomenclature and genetic groupings of *Giardia* infecting mammals. *Parasitol Today* 2000;16:210-13.