Differentiating Human from Animal Isolates of *Cryptosporidium parvum*

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We analyzed 92 *Cryptosporidium parvum* isolates from humans and animals by a polymerase chain reaction/restriction fragment length polymorphism method based on the thrombospondin-related anonymous protein 2 gene sequence. Used as a molecular marker, this method can differentiate between the two genotypes of *C. parvum* and elucidate the transmission of infection to humans.

Cryptosporidium parasites cause infection in humans and other vertebrates, including mammals, birds, reptiles, and fish. More than 20 species of *Cryptosporidium* have been reported, of which six are considered valid species on the basis of oocyst morphologic features and site of infection (1,2). *Cryptosporidium parvum,* the species that infects humans and most mammals, has a monoxenous life cycle in which all stages of asexual and sexual development occur within one host. The parasite generates large numbers of viable oocysts in feces. Cross-infection studies in various mammalian systems have indicated zoonotic transmission to humans (1,3). *C. parvum* has caused waterborne outbreaks of cryptosporidiosis and (in AIDS patients) life-threatening diarrhea for which no effective treatment exists (4). A waterborne outbreak of cryptosporidiosis in Milwaukee, Wisconsin, in 1993 affected more than 400,000 people (5).

Molecular characterization techniques used to detect intraspecific variations in *C. parvum* include isozyme profiles (6); random amplified polymorphic DNA (RAPD) analyses (7); nucleotide sequence studies of the 18S rRNA (8,9) and DHFR gene (10); and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the undefined repetitive sequence (11), polythreonine motifs, and oocyst wall protein (12,13). Two distinct genotypes of *C. parvum* parasites have been detected in humans.

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In a previous article, we identified several mutations in the gene thrombospondin-related anonymous protein 2 of *C. parvum* (TRAP-C2) that differentiate between anthroponotic and zoonotic infection in humans (14). Our objective in the present study was to develop a simple, rapid protocol that can be used as a diagnostic tool to differentiate between the two genotypes of *C. parvum* and elucidate the transmission of infection in humans. We analyzed 92 *C. parvum* isolates from humans, calves, deer, dogs, and monkeys and found that this new PCR/RFLP method based on the TRAP-C2 gene sequence can be used as a molecular marker to differentiate between the two genotypes of *C. parvum*.

Analytic Approach

Isolates

We summarized data for 92 isolates of *C. parvum,* 50 from human and 42 from animal sources (Tables 1, 2). Twenty-one of the 50 human isolates were from AIDS patients; the rest were primarily from cryptosporidiosis outbreak case-patients. Seven of the human isolates came from a previous TRAP-C2 sequencing study (14), but because of the lack of DNA, other isolates we used in the previous study were not used in this study. Fecal samples were stored at 4° C in 2.5% potassium dichromate before oocysts were isolated. Oocysts were purified from fecal samples by first using the discontinuous density sucrose gradient centrifugation and then the Percoll gradient centrifugation (15,16).

Table 1. *Cryptosporidium parvum* human isolates, restriction pattern, and sequence type

Source	Isolate	Host	Restriction pattern	Sequence type
1993 Milwaukee	HM3	Hum[a]	Hum	Hum[b]
1993 Milwaukee	HM5	Hum	Hum	Hum
1993 Milwaukee	HM7	Hum	Hum	ND[c]
1995 Florida	HFL1	Hum	Hum	Hum[b]
1995 Florida	HFL5	Hum	Hum	Hum[b]
1995 Florida	HFL6	Hum	Hum	ND
1995 Atlanta	HGA1	Hum	Hum	Hum[b]
1995 Atlanta	HGA4	Hum	Hum	Hum
1995 Atlanta	HGA5	Hum	Hum	ND
1995 Atlanta	HGA6	Hum	Hum	ND
1996 Canada	HCAN9	Hum	Bov[d]	Bov[b]
1995 Canada	NC30	Hum	Hum	ND
1994 Nevada	HCNV2	Hum	Hum	ND
1994 Nevada	HCNV4	Hum	Hum	ND
1997 Pennsylvania	PA41	Hum	Bov	Bov[b]
1997 Pennsylvania	PA46	Hum	Bov	Bov[b]
1997 HIV-Guatemala	HGMO7	Hum	Hum	Hum
1997 HIV-Guatemala	HGMO8	Hum	Hum	ND
1997 HIV-Guatemala	HGMO9	Hum	Hum	Hum
1997 HIV-Guatemala	HGMO10	Hum	Hum	Hum
1997 Minnesota	HMOB1	Hum	Bov	Hum
1997 Minnesota	HMOB3	Hum	Bov	Bov
1997 Minnesota	HMOB4	Hum	Bov	Bov
1997 Minnesota	HMOB5	Hum	Bov	Bov
1997 HIV-New Orleans	HNO2	Hum	Hum	Hum
1997 HIV-New Orleans	HNO3	Hum	Hum	Hum
1997 HIV-New Orleans	HNO4	Hum	Hum	ND
1997 HIV-New Orleans	HNO5	Hum	Bov	Bov
1997 HIV-New Orleans	HNO6	Hum	Hum	Hum
1997 HIV-New Orleans	HNO7	Hum	Hum	Hum
1997 HIV-New Orleans	HNO8	Hum	Hum	Hum
1997 HIV-New Orleans	HNO10	Hum	Hum	Hum
1997 HIV-New Orleans	HNO11	Hum	Bov	ND
1997 HIV-New Orleans	HNO12	Hum	Hum	Hum
1997 HIV-New Orleans	HNO13	Hum	Hum	Hum
1997 HIV-New Orleans	HNO14	Hum	Hum	Hum
1997 HIV-New Orleans	HNO15	Hum	Hum	Hum
1997 HIV-New Orleans	HNO16	Hum	Hum	Hum
1997 HIV-New Orleans	HNO17	Hum	Hum	Hum
1997 HIV-New Orleans	HNO18	Hum	Hum	Hum
1997 HIV-New Orleans	HNO19	Hum	Hum	ND
1997 India	HIND4	Hum	Hum	Hum
1997 India	HIND5	Hum	Hum	ND
1998 Washington State	HWA1	Hum	Hum	Hum
1998 Washington State	HWA2	Hum	Hum	ND
1998 Washington State	HWA3	Hum	Hum	ND
1998 Washington State	HWA4	Hum	Hum	ND
1998 Washington State	HWA5	Hum	Hum	Hum
1998 Washington State	HWA6	Hum	Hum	Hum
1998 Washington State	HWA7	Hum	Hum	Hum

[a]Hum=human
[b]Sequencing data reported earlier (14)
[c]ND= Not done.
[d]Bov=bovine.

Table 2. *Cryptosporidium parvum* bovine isolates, restriction pattern, and sequence type

Source	Isolate	Host	Restriction pattern	Sequence type
1996 Alabama	AAL35	Calf	Bov[a]	Bov
1996 Georgia	AGA43	Calf	Bov	Bov
1996 Georgia	AGA44	Mon[b]	Bov	Bov
1997 Georgia	AGA75	Calf	Bov	Bov
1996 Idaho	AID21	Calf	Bov	Bov
1996 Kansas	AKA19	Calf	Bov	Bov
1996 Maryland	AMD36	Calf	Bov	Bov
1996 Maryland	AMD38	Deer	Bov	Bov
1996 Massachusetts	AMA61-GCH1	Calf	Bov	Bov
1997 Iowa	AIO62	Calf	Bov	Bov
1996 Ohio	AOH6	Calf	Bov	Bov
1996 Ohio	AOH7	Calf	Bov	Bov
1996 Ohio	AOH8	Calf	Bov	Bov
1996 Ohio	AOH9	Calf	Bov	Bov
1996 Ohio	AOH10	Calf	Bov	Bov
1996 Ohio	AOH11	Calf	Bov	Bov
1996 Ohio	AOH12	Calf	Bov	Bov
1996 Ohio	AOH13	Calf	Bov	Bov
1996 Ohio	AOH14	Calf	Bov	Bov
1996 Ohio	AOH15	Calf	Bov	Bov
1996 Ohio	AOH16	Calf	Bov	Bov
1996 Ohio	AOH17	Calf	Bov	Bov
1997 Ohio	AOH45	Calf	Bov	Bov
1997 Ohio	AOH47	Calf	Bov	Bov
1997 Ohio	AOH48	Calf	Bov	Bov
1997 Ohio	AOH49	Calf	Bov	Bov
1997 Ohio	AOH50	Calf	Bov	Bov
1997 Ohio	AOH52	Calf	Bov	Bov
1997 Ohio	AOH53	Calf	Bov	Bov
1997 Ohio	AOH54	Calf	Bov	Bov
1997 Ohio	AOH55	Calf	Bov	Bov
1997 Ohio	AOH56	Calf	Bov	Bov
1997 Ohio	AOH57	Calf	Bov	Bov
1997 Ohio	AOH58	Calf	Bov	Bov
1997 Ohio	AOH59	Calf	Bov	Bov
1997 Ohio	AOH107	Dog	Bov	Bov
1996 Oklahoma	AOK3	Beef cattle	Bov	Bov
1996 Oklahoma	AOK29	Calf	Bov	Bov
1997 Pennsylvania	APE89	Calf	Bov	Bov
1996 Utah	AUT37	Calf	Bov	Bov
1996 Washington	AWA5	Beef cattle	Bov	Bov
1997 West Virginia	AWV65	Calf	Bov	Bov

[a]Bov=bovine.
[b]Mon=monkey.

Extraction of Genomic DNA and PCR Amplification

We followed the protocol of Kim et al. in isolating the total genomic DNA from the purified oocyst (17). A 369 base pair (bp) fragment of the TRAP-C2 gene of *C. parvum* was amplified by using a forward (cua cua cua cua CAT ATT CCC TGT CCC TTG AG) and a reverse (cau cau cau cau TGG ACA ACC CAA ATG CAG

AC) primer (lower case represents nucleotide used for cloning); these primers correspond to positions 848-867 (positive strand) and 1,180-1,199 (negative strand) of the GenBank sequence X77586, respectively. The PCR reaction consisted of 50 ng genomic DNA, 200 µM of each dNTP (Perkin Elmer, Foster City, CA), 40 ng of primer, 1X PCR buffer, and 0.5 units of Taq polymerase (GIBCO BRL, Frederick, MD) in a total volume of 100 µl. DNA amplification was carried out for 35 cycles, each consisting of denaturing (94° C, 45 sec), annealing (48° C, 45 sec), and elongating (72° C, 60 sec), with an initial hot start at 94° C for 5 min in a Perkin Elmer Gene Amp PCR 9600 thermocycler. An additional cycle of 7 min at 72° C was done for final extension. Each experiment used three negative controls (reaction mixtures without Taq polymerase, primers, or template DNA) and a positive control.

DNA Sequencing and Analysis

PCR products were purified by the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and cloned by the CLONEAMP pAMP1 System for Rapid Cloning of Amplification Products (GIBCO BRL, Frederick, MD) according to the manufacturer's protocol. DNA sequencing of recombinant clones that had the correct size insert was carried out on an ABI 377 Automated Sequencer by the dRhodomine Terminator Cycle Sequencing Kit (Perkin Elmer-Applied Biosystems).

RFLP

To develop an RFLP technique for differentiating between the two genotypes of *C. parvum*, the TRAP-C2 sequences were aligned and mapped for restriction enzyme sites by the Genetics Computer Group program (18). Enzymes with predicted exclusive cutting in each genotype were used in RFLP development and analysis. For RFLP analysis, 10 µl of amplification products was digested in a 30-µl reaction mix consisting of 10 units of *Bfa*I (New England BioLabs, Beverly, MA), *Bse*tEI (Boehringer Mannheim, Indianapolis, IN), *Eco*571 (MBI Fermentas, Gariciuno, Vilnius, Lithuania), *Hae*III (New England BioLabs), *Hph*I (New England BioLabs), *Mae*III (Boehringer Mannheim, Germany), *Nru*I (New England BioLabs), *Pac*I (New England BioLabs), or *Tsp*45I (New England BioLabs), and 3 µl of respective restriction buffer for 1 hr, under conditions recommended by the supplier. The digested products were fractionated on 2.0% agarose gel and visualized by ethidium bromide staining.

Findings

Sequence Analysis of Human and Bovine Isolates

Two genotypes of *C. parvum* exist in humans, as shown by the primary sequence of the TRAP-C2 gene (14). Nucleotide sequences differed at five positions between most human and bovine isolates. To confirm and extend this observation, we sequenced additional human and bovine isolates, as well as isolates from dogs, deer, and monkeys. We obtained 42 additional sequences of the TRAP-C2 gene from animal sources and 27 additional sequences of the TRAP-C2 gene from human sources; results of DNA sequencing confirmed that *C. parvum* is highly conserved at the TRAP-C2 locus. All animal isolates, including those from nonbovine animals, showed bovine genotype characteristics (Table 2). Differences between the two genotypes are shown in Table 3. Of the additional 23 human isolates showing human genotype pattern, four isolates (HGMO7, HGMO9, HGMO10, and HNO18) showed "C" at the fifth place, whereas the rest showed "T".

Table 3. Human and bovine *Cryptosporidium parvum* isolates based on multiple alignment[a]

Position (nt)	Human genotype	Bovine genotype
51	G	A
78	C	T
100	T	G
147	C	T
280	T or C	C

[a]Representative sequences have been deposited in the GenBank, with accession numbers AF082521 to AF082524.

PCR-RFLP Method To Discriminate between Human and Bovine Genotype Isolates

To avoid expensive and lengthy DNA sequencing when determining the genotype of *C. parvum* isolates, we developed a simpler, quicker method—PCR amplification of the TRAP-C2 gene followed by RFLP. Restriction

enzyme mapping on the aligned sequences of both genotypes showed five human-genotype–specific (*Hae*I, *Hae*III, *Nru*I, *Pac*I, and *Tha*I) and six bovine-genotype–specific (*Bfa*I, *Bse*tEI, *Eco*571, *Hph*I, *Mae*III, and *Tsp*45I) restriction enzymes. All human-genotype– and bovine-genotype–specific restriction enzymes except *Hae*I and *Tha*I were tested for the TRAPC-2 PCR-amplified products of genomic DNA of *C. parvum*. After restriction and gel electrophoresis, the resulting bands were the size predicted by the mapping analysis (Figure). Digestion of PCR products with these enzymes resulted in a distinct band pattern for the human genotype and bovine genotype isolates. In all cases, the DNA sequencing and PCR-RFLP mapping data matched.

Using PCR-RFLP in Outbreak Investigations

We validated the PCR-RFLP technique by using isolates from outbreaks and sporadic cases of human cryptosporidiosis. Human genotype characteristics were evident in all samples from HIV-infected patients from Guatemala and most patients with sporadic clinical cases, as well as samples from the following outbreaks: Milwaukee (1993), Florida (1995), Atlanta (1995), Canada (1995), Nevada (1994), and Washington (1998). Of the 17 samples from HIV-infected patients in New Orleans, two demonstrated bovine-genotype pattern, while the rest were similar to human genotype. However, bovine-genotype characteristics were evident in the human isolates from outbreaks in British Columbia, Canada (1996), Minnesota (1997), and Pennsylvania (1997).

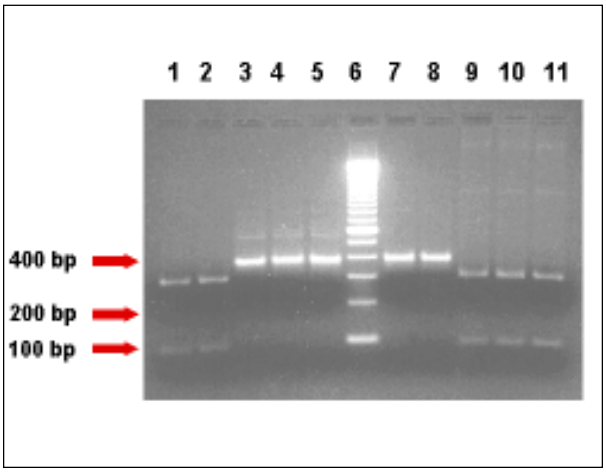

Figure. Human- and bovine-specific restriction enzymes showed distinct banding pattern for genotypes of *Cryptosporidium parvum* isolates. The different lanes represent the TRAP-C2 PCR-amplified products belonging to AGA43, AMD36, AOH6, HM3, and HM5 isolates of *C. parvum*, respectively, after digestion with *Hae*III (Lanes 1-5, human-specific marker) and *Bst*E II (Lanes 7-11, bovine-specific marker) restriction enzymes and agarose gel electrophoresis. Lane 6 is the 100 bp marker. Samples AGA43, AMD36 and AOH6 are bovine (bovine genotype) and samples HM3 and HM5 are human (human genotype). Human- or bovine-specific restriction enzyme markers can cut only the TRAP-C2 amplified product of the respective genotype of *C. parvum* isolates.

Conclusions

We examined a large number of *C. parvum* isolates (92) from human and animal sources from patients in outbreak and nonoutbreak settings to determine the two transmission routes of the parasite in humans. Molecular markers were generated by restriction digestion of PCR-amplified TRAP-C2 products with one of the 12 enzymes to differentiate the two genotypes of *C. parvum*. The results based on TRAP-C2 gene PCR-RFLP showed that this method could also be used in future cryptosporidiosis outbreak investigations.

Results of our characterization of outbreak and nonoutbreak cases of human cryptosporidiosis indicate that anthroponotic organisms account for most cases. We find a large number of human genotype parasites in sporadic cases and in HIV-infected patients. Most cryptosporidiosis outbreaks examined are caused by anthroponotic (human genotype) parasites. Our results suggest similar epidemiologic features of cryptosporidiosis in HIV-infected persons from New Orleans and Guatemala because both were infected with human genotype parasites.

The results of this study confirm the polymorphic nature of *C. parvum*. As we showed in a previous study, two alleles of the TRAP-C2 gene exist, each representing a distinct genotype of *C. parvum* with different transmission cycles in humans. The simple PCR-RFLP technique we developed can effectively differentiate between these two genotypes and transmission cycles and can be used as a tool in outbreak investigations of cryptosporidiosis. Information generated from these investigations will be useful not only in

identifying the sources of contamination but also in controlling the disease.

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Dr. Sulaiman is a postdoctoral research associate in the Molecular Vaccine Section, Division of Parasitic Diseases, National Center for Infectious Diseases, CDC. For the last 7 years, he has focused on the genetic polymorphism of various organisms. He is now conducting molecular typing of *Cryptosporidium* to understand the transmission routes of the parasite.

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