# Differentiating Human from Animal Isolates of *Cryptosporidium parvum*

**Irshad M. Sulaiman, Lihua Xiao, Chunfu Yang, Lilian Escalante, Anne Moore, Charles B. Beard, Michael J. Arrowood, and Altaf A. Lal**
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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.

Address for correspondence: Altaf A. Lal, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Mail Stop F-12, 4770 Buford Highway, Atlanta, GA 30341-3717, USA; fax: 770-488-4454; e-mail: aal1@cdc.gov.

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), *Bset*EI (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

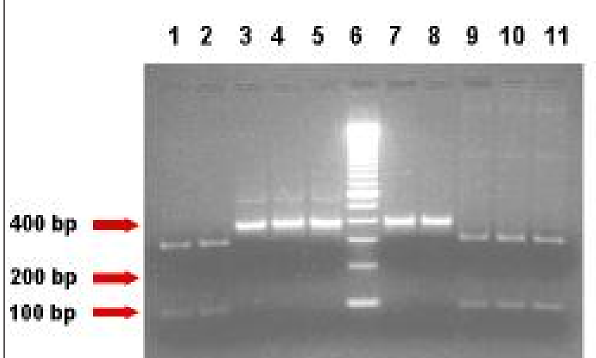


Figure. Human- and bovine-specific restriction enzymes showed distinct banding pattern for genotypes of *Cryptosporidium parvum* isolates. The different lanes represent the TRAP-C2 PCR-amplified products belonging to AGA43, AMD36, AOH6, HM3, and HM5 isolates of *C. parvum*, respectively, after digestion 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.

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).

### 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.

## Acknowledgments

We thank Barbara Herwaldt, Corinne S. L. Ong, P. Vijyalakshmi, Bruce Anderson, William Shulaw, A. Morse, and J. Inungu for providing *Cryptosporidium* isolates.

This work was supported in part by funding from Environmental Protection Agency (DW 75937984-01-1).

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.

## References

1. O'Donoghue PJ. *Cryptosporidium* and cryptosporidiosis in man and animals. Int J Parasitol 1995;25:139-95.
2. Dubey JP, Speer CA, Fayer R. General biology of *Cryptosporidium*. Dubey JP, Speer CA, Fayer R, editors. Cryptosporidiosis of man and animals. Boca Raton (FL): CRC Press; 1990. p. 1-29.
3. Smith HV. Environmental aspects of *Cryptosporidium* species: a review. J R Soc Med 1990;83:629-31.
4. Colford JM, Tager IB, Hirozawa AM, Lemp GF, Aragon T, Petersen C. Cryptosporidiosis among patients infected with human immunodeficiency virus. Am J Epidemiol 1996;144:807-16.
5. Mac Kenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, et al. A massive outbreak in Milwaukee of *Cryptosporidium* transmitted through the public water supply. N Engl J Med 1994;331:161-7.
6. Awad-El-Kariem FA, Robinson HA, Dyson DA, Evans D, Wright S, Fox MT, et. al. Differentiation between human and animal strains of *Cryptosporidium parvum* using isoenzyme typing. Parasitology 1995;110:129-32.
7. Morgan UM, Constantine CC, O'Donoghue P, Meloni BP, O'Brien PA, Thompson RCA. Molecular characterization of *Cryptosporidium* isolates from humans and other animals using random amplified polymorphic DNA analysis. Am J Trop Med Hyg 1996;52:559-64.
8. Carraway M, Tzipori S, Widmer G. Identification of genetic heterogeneity in the *Cryptosporidium parvum* ribosomal repeat. Appl Environ Microbiol 1996;62:712-6.
9. Morgan UM, Constantine CC, Forbes DA, Thompson RCA. Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. J Parasitol 1997;83:825-30.
10. Vasquez JR, Gooze L, Kim K, Gut J, Petersen C, Nelson RG. Potential antifolate resistance determinants and genotypic variation in the bifunctional dihydrofolate reductase-thymidylate synthase gene from human and bovine isolates of *Cryptosporidium parvum*. Mol Biochem Parasitol 1996;79:153-65.
11. Bonnin A, Fourmaux MN, Dubremetz JF, Nelson RG, Gobet P, Harly G, et al. Genotyping human and bovine isolates of *Cryptosporidium parvum* by polymerase chain reaction-restriction fragment length polymorphism analysis of a repetitive DNA sequence. FEMS Microbiol Lett 1996;137:207-11.
12. Carraway M, Tzipori S, Widmer G. A new restriction fragment length polymorphism from *Cryptosporidium parvum* identifies genetically heterogeneous parasite populations and genotypic changes following transmission from bovine to human hosts. Infect Immun 1997;65:3958-60.
13. Spano F, Putignani L, McLauchlin J, Casemore DP, Crisanti A. PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. FEMS Microbiol Lett 1997;150:209-17.
14. Peng MP, Xiao L, Freeman AR, Arrowood MJ, Escalante A, Weltman AC, et al. Genetic polymorphism among *Cryptosporidium parvum* isolates supporting two distinct transmission cycles. Emerg Infect Dis 1997;3:1-9.
15. Arrowood MJ, Sterling CR. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic Percoll gradients. J Parasitol 1987;73:314-9.
16. Arrowood MJ, Donaldson K. Improved purification methods for calf-derived *Cryptosporidium parvum* oocysts using discontinuous sucrose and cesium chloride gradients. J Eukaryot Microbiol 1996;43:89.
17. Kim K, Gooze L, Petersen C, Gut J, Nelson RG. Isolation, sequence and molecular karyotype analysis of the actin gene of *Cryptosporidium parvum*. Mol Biochem Parasitol 1992;50:105-14.
18. Wisconsin Package Version 9.0, Madison (WI): Genetics Computer Group; 1996.