A New Restriction Fragment Length Polymorphism from Cryptosporidium parvum Identifies Genetically Heterogeneous Parasite Populations and Genotypic Changes Following Transmission from Bovine to Human Hosts

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Length and restriction site polymorphism within a 2.8-kb threonine-rich open reading frame from Cryptosporidium parvum was identified and used to determine the genotypes of isolates from calves and humans. In agreement with observations of other genetic loci, all calf isolates were identical at this locus. In contrast, human isolates showed two profiles, one found exclusively in humans and one a superposition of both profiles, which were indicative of heterogeneous parasite populations. PCR fingerprints were consistent with a change in the genetic profile of C. parvum isolates following transmission from bovine to human hosts.

Cryptosporidium parvum, an enteric protozoan parasite commonly found in neonatal calves, is an opportunistic infection in AIDS patients, frequently causing severe chronic diarrhea (4, 5). To advance our understanding of the epidemiology of C. parvum, genetic polymorphisms suitable for detection by PCR are being identified. Isolation of an open reading frame from C. parvum containing multiple threonine motifs (1) suggested the possibility of length polymorphism which is frequently associated with microsatellites (6). Here, we describe the presence of length and restriction site polymorphism at this locus and the application of these markers to fingerprinting C. parvum isolates from human patients or from calves or transmitted between different host species.

Isolation of a C. parvum threonine-rich open reading frame. A DNA probe was constructed by multimerizing a (ACA/T)4 primer and its complement by PCR and cloning size-selected products into the TA cloning vector (Invitrogen). A genomic clone (pGC91) was identified by screening a λ ZapII C. parvum (GCH1 isolate) (7) phage library with this probe. Sequencing of pGC91 showed the presence of an open reading frame with almost 200-bp difference in the lengths of the amplified fragments. Confirming and extending the observations shown in Fig. 4 (lane 4). In agreement with the detection of a mixed genotype in GCH3 with primers cry40 and cry41 (Fig. 1), RFLP analysis of the cry37-cry44 fragment also revealed a mixed profile (Fig. 3A). The profile recovered from GCH3 (lane 5) was a composite of the bovine profile (lanes 2 and 3) and the human profile seen in GCH2. A comparison of the undigested PCR products showed no length polymorphism in this region (Fig. 3B).

In order to obtain a simple RFLP marker, primer cry37 was replaced with cry39 (Fig. 1), resulting in a PCR fragment of 318 bp spanning the polymorphic RsaI site. Fifteen C. parvum isolates were typed with this marker (Fig. 4). In some samples, restriction with RsaI resulted in a 45-bp fragment and a 273-bp fragment. Confirming and extending the observations shown in Fig. 2, genotypes from nine animal-derived isolates were identical, whereas human isolates fell into two groups. One lacked the RsaI site including GCH2, GCH3, F12, and F16 (Fig. 4; human samples 1, 2, 5, and 6), and two isolates had a mixed profile consisting mostly of Rsal-positive DNA with some uncut PCR product (human samples 3 and 4). Except for GCH3, which was expected based on previous results to generate a mixed RsaI profile, profiles from all samples matched the length polymorphism identified above.

Two ICP samples were included in this analysis, one from oocysts maintained in calves and one from oocysts recovered after a single passage through neonatal BALB/C mice. The mouse-passaged sample had retained its original profile as shown in Fig. 4 (sample 4). Two isolates originating from human patients, 740 and LL, and propagated once through calves had a calf profile (animal samples 8 and 9 [Fig. 4]). The same was found for GCH1, which is of human origin and has been maintained in calves since 1992 (7), and TAMU, which...
was isolated from an accidental human infection and was also propagated in calves.

The human GCH4 and GCH5 isolates from two presumed accidental infections with GCH1 oocysts showed mostly the RsaI-positive profile with some uncut DNA (Fig. 4). The presence of RsaI-negative DNA in these samples suggested a change in the parasite population compared to GCH1. No uncleaved DNA was observed in GCH1 from subsequent passages. Since primers cry44 and cry39 do not amplify DNA from C. parvum-negative human stool (data not shown), we conclude that both the RsaI-positive band and the RsaI-negative band are of C. parvum origin. This view is consistent with sequence analysis of cloned cry44-cry37 PCR products. Clones with point mutations within the RsaI site were identified in GCH2 and GCH3 (data not shown).

To further substantiate the possibility of genotypic changes in human isolates, GCH4 and GCH5 were PCR fingerprinted.
with ribosomal primers within or flanking the ITS1 from bovine isolates. The expected 623-bp band was obtained from GCH1 but not from GCH5 or GCH4 (not shown). However, control amplification of GCH4 and GCH5 samples with conserved SSU ribosomal primers gave the expected products. Together, the RsaI RFLP and the ITS1 fingerprints demonstrate genetic differences between GCH1 and GCH4-GCH5.

In summary, a new polymorphism located within a threonine-rich open reading frame was used to fingerprint 15 *C. parvum* isolates from different host species and geographical origin. In agreement with previous reports (2, 3), the two RFLP genotypes segregated according to host species: a homogeneously RsaI-positive bovine group and a genotypically mixed group from human patients. Analysis of human *C. parvum* isolates detected genetically heterogeneous populations in some individuals. In GCH3, this conclusion is supported by RFLP-PCR analysis with two primer pairs, detection of length polymorphism, and sequence analysis (not shown).

No correlation between the RsaI genotype of the six human isolates and the human immunodeficiency virus (HIV) status of the individuals from which they were isolated was found. GCH2 and GCH3 (the former being an isolate predominantly of human genotype [RsaI negative] and the latter being a mixed genotype) were both from HIV-positive individuals, whereas GCH4, GCH5, F12, and F16 were from HIV-negative individuals and were either of mixed or human genotype.

GCH4 and GCH5 were isolated from two laboratory workers who had recently been hired and who were involved in the purification of GCH1 oocysts and care of calves infected with this isolate. Since none of these patients reported any household members with intestinal symptoms preceding the onset of diarrhea, we assume that these infections resulted from the accidental ingestion of GCH1 oocysts. Based on this assumption, the ribosomal and RsaI fingerprints document a change in genotype between GCH1 and GCH4-GCH5. Similarly, the fingerprints of GCH2 and GCH3, which were two isolates recovered from HIV-positive patients living in the same household, provide circumstantial evidence for changes in *C. parvum* genotype. The individual from whom GCH3 was isolated was diagnosed with cryptosporidiosis before the one carrying GCH2, suggesting that GCH2 originated from GCH3 by direct transmission within the household. Under this assumption, the profiles of GCH3 and GCH2 show a transition from a mixed bovine-human-type parasite population in GCH3 to a human-type profile in GCH2. Together, *C. parvum* fingerprints of human isolates suggest a selective mechanism favoring propagation of parasites with the RsaI-negative genotype in humans.

In conclusion, data consistent with genotypic changes in *C. parvum* populations following transmission between different host species were found. This observation could explain the origin of genotypic differences between *C. parvum* isolates recovered from human and bovine hosts. In order to clarify this aspect of the epidemiology of *C. parvum*, genetic changes following transition from ruminant to nonruminant hosts are currently being investigated with animal models.

**Nucleotide sequence accession no.** The GenBank accession no. of pGC91 is U83169.

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