Species and Strain-specific Typing of *Cryptosporidium* Parasites in Clinical and Environmental Samples

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Cryptosporidiosis has recently attracted attention as an emerging waterborne and foodborne disease as well as an opportunistic infection in HIV infected individuals. The lack of genetic information, however, has resulted in confusion in the taxonomy of Cryptosporidium *parasites and in the development of molecular tools for the identification and typing of oocysts in environmental samples. Phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene has shown that the genus* Cryptosporidium *comprises several distinct species. Our data show the presence of at least four species:* C. parvum, C. muris, C. baileyi *and* C. serpentis (C. meleagridis, C. nasorum *and* C. felis *were not studied). Within each species, there is some sequence variation. Thus, various genotypes (genotype 1, genotype 2, guinea pig genotype, monkey genotype and koala genotype, etc.) of* C. parvum *differ from each other in six regions of the SSU rRNA gene. Information on polymorphism in* Cryptosporidium *parasites has been used in the development of species and strain-specific diagnostic tools. Use of these tools in the characterization of oocysts in various samples indicates that* C. parvum *genotype 1 is the strain responsible for most human* Cryptosporidium *infections. In contrast, genotype 2 is probably one of the major sources for environmental contamination, and has been found in most oysters examined from Chesapeake Bay that may serve as biologic monitors of estuarine waters.*

Key words: *Cryptosporidium* - phylogeny - genotype - ribosomal RNA

Cryptosporidiosis is a coccidian infection of humans, domestic animals and other vertebrates. In young farm animals, especially preweaned dairy calves, it causes a severe enteritis resulting in significant morbidity, mortality and economic loss. In humans, it results in an acute infection of the digestive system in immunocompetent individuals, and chronic, life-threatening disease in immunocompromised patients. Several transmission routes, including person-to-person, contamination of water or food, and zoonotic infection, are possible. The specific source of *Cryptosporidium* oocysts involved in infection or contamination is frequently unknown, largely due to a lack of detailed epidemiologic investigation and strain-typing tools. The latter results from a current paucity of molecular characterization and lack of acceptance of the taxonomy of *Cryptosporidium* species and genotypes.

CRYPTOSPORIDIUM SPECIES

Since the discovery of *Cryptosporidium muris* and *C. parvum* in rodents, over 20 *Cryptosporidium* species have been described in various animal hosts (O'Donoghue 1995). Species were named based on the historical belief that *Cryptosporidium* spp. are coccidian parasites, and therefore share the strict host specificity demonstarted by many other coccidian parasites. Studies conducted in late 1970s and early 1980s, however, indicated that some isolates of *Cryptosporidium* were infectious for several animal species. Thus, one group of investigators suggested that all *Cryptosoridium* parasites were the same species, *C. muris* (Tzipori et al. 1980). Others demonstrated that host specificity was present among isolates from different classes of vertebrates (O'Donoghue 1995). Based on these observations, Levine (1984, 1986) classified the parasites from mammals, birds, reptiles and fish as *C. muris*, *C. meleagridis*, *C. serpentis*, and *C. nasorum*, respectively. Subsequent studies demonstrated that *C. parvum* from mammals and *C. baileyi* from birds were biologically and morphologically different from *C. muris* and *C. meleagridis*

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(Upton & Current 1985, Current et al. 1986). Thus, *C. parvum*, *C. muris*, *C. baileyi*, *C. meleagridis*, *C. serpentis* and *C. nasorum* were considered valid *Cryptosporidium* species (O'Donoghue 1995). More recently, based on published reports of host specificity, Fayer et al. (1997) added *C. felis* from cats and *C. wrairi* from guinea pigs to the list of valid species, whereas Tzipori and Griffiths (1998)suggested that current evidence does not support the concept that there is more than one species of *Cryptosporidium* parasites.

The lack of genetic information and the presence of erroneous sequences in a few published studies have added to the present state of taxonomic confusion. Cai et al. (1992) compared the small subunit (SSU) ribosomal RNA (rRNA) gene, and showed a greater than 99% identity between one *C. parvum* and one *C. muris* isolate. Alignment of sequences (accession numbers X64430 to 64343) from that study with sequences from us and others indicates that all four sequences from Cai et al. (1992) are the *C. muris* type. Minor sequence errors (one insertion and 12 deletions of nucleotides) were found in the SSU rRNA sequence (L25642) of another published study (Kilani & Wenman 1994). These sequences and five other sequences deposited in the GenBank were used recently by Tzipori and Griffiths (1998) in a phylogenetic analysis of *Cryptosporidium* parasites. Based on this analysis, they concluded that the observed inter-species and intra-species variation did not favor the designation of separate *Cryptosporidium* species, and therefore all *Cryptosporidium* oocysts, including those from lower vertebrates, should be considered hazardous to humans.

We have recently sequenced the SSU rRNA genes from various isolates of *C. parvum*, *C. muris*, *C. baileyi* and *C. serpentis*, and used these sequences in a phylogenetic analysis (Xiao et al. unpub. data). Results of the analysis indicate that *Cryptosporidium* parasites are a multi-species complex containing at least four species: *C. parvum*, *C. baileyi*, *C. muris* and *C. serpentis* (*C. felis*, *C. nasorum* and *C. meleagridis* were not studied). The evolutionary distance between the *Cryptosporidium* guinea pig isolate and *C. parvum* is too small to warrant a separate species designation.

CRYPTOSPORIDIUM PARVUM GENOTYPES

Results of various studies indicate that there is variation within the species *C. parvum*. Two dimensional gel electrophoresis has revealed minor differences between human and bovine *C. parvum* isolates (Mead et al. 1990), which has been confirmed by immunoblot (Nichols et al. 1991, Nina et al. 1992), isozyme (Ogunkolade et al. 1993, Awad-El-Kariem et al. 1995), and restriction fragment length polymorphism (RFLP) analysis (Ortega et al. 1991). More recently, random amplified polymorphic DNA (RAPD) markers have revealed two distinct groups of human *C. parvum* isolates, one containing most human isolates and the other containing some human isolates and all animal isolates (Morgan et al. 1995), indicating the possibility of zoonotic infection. Similar results have been obtained by sequence data or PCR-RFLP analysis of a repetitive sequence (Bonnin et al. 1996), bifunctional dihydrofolate reductase thymidylate synthase (DHFR) (Vasquez et al 1996), rRNA repeats (Carraway et al. 1996), polythreonine motifs (Carraway et al. 1997), oocyst wall protein (COWP) gene (Spano et al. 1997), and thrombospondin anonymous protein-2 (TRAP-C2) gene (Peng et al. 1997 Sulaiman et al. unpub. data). It remains unclear, however, whether the same two genotypes are present in all these polymorphic loci. Results of our multi-locus analysis suggest that indeed the same genotypes are linked across all polymorphic genes (SSU rRNA, TRAP-C1, TRAP-C2, CP15, and ß-tubulin intron) examined (Xiao et al. unpub. data).

Our phylogenetic analyses of the SSU rRNA gene have revealed diversities in *C. parvum* not previously observed (Table I). Human *C. parvum* isolates differ from bovine isolates in four regions of the SSU rRNA gene. Likewise, the *Cryptosporidium* isolate from guinea pigs (*C. wrairi*) also differs from the bovine isolates in four regions, two of which are the same polymorphic regions between the human and bovine genotypes, thus representing a third genotype of *C. parvum*. Partial sequences obtained from a monkey by us and from a koala by Morgan et al.(1997) indicate the presence of two additional genotypes. The difference between the human and bovine genotypes in nucleotides 689-699 has also been observed recently by Morgan et al. (1997). We, however, have observed that some human isolates have the sequence TTTTTT instead of TTTTTTTTTTT. Based on a partial SSU rRNA gene sequence, another group also identified a new *C. parvum* genotype (Carraway et al. 1994, 1996). The new genotype sequence (ICP), however, is identical to the *C. muris* bovine isolate (Xiao et al. unpub. data).

CRYPTOSPORIDIUM GENOTYPES IN CLINICAL SAMPLES

Results of the molecular characterization have been used by us in the development of molecular diagnostic tools. A PCR-RFLP technique based on the polymorphism in the TRAP-C2 gene was developed and used in the analysis of human clinical samples from various outbreak and non-outbreak cases (Sulaiman et al., unpub. data). Results of our

TABLE I

Differences among genotypes of *Cryptosporidium parvum* in the SSU rRNA gene

	Location of mutations in the SSU rRNA gene[a]					
Genotype	129-135	179-184	262-267	639-656	689-699	795-800
1	TTTTACT	AAACTC	AATTAA	AAAATATTTTGATGAATA	TTTTTTTTTTT or TTTTTT	TTTTTT
2	TTT-ACT	AAACTC	ATTAAA	AAAATATTTTGATGAATA	TATATTTT	TTTCTT
wrairi	TTT-ACT	AGGCCC	ATAAAT	ATAATATTTTGAA-AATA	TATATTTTT	TTTCTT
Monkey	unknown	unknown	AATTAA	AATATATTTTGATGAATA	TTTTTTTTT	TTTTTT
Koala[b]	unknown	unknown	unknown	ATTATACTTTTTAAGGTG	TATTTTTTT	unknown

a: nucleotide positions in the aligned sequences of all *Cryptosporidium* species. Actual positions in individual sequences may vary slightly due to the introduction of gaps in the aligned sequences (1757 bp); *b*: based on the sequence by Morgan et al. (1997).

studies and those by others (Table II) indicate that anthroponotic organisms account for the majority of the cases and person-to-person transmission is likely to be an important transmission route of cryptosporidiosis in non-outbreak cases. This is evident from the large number of genotype 1 parasites in sporadic cases and HIV patients (Sulaiman et al., unpub. data). This is in agreement with some recent observations by others (Table II). Even in outbreak cases, many cryptosporidiosis outbreaks are caused by anthroponotic (genotype 1) parasites (such as the waterborne outbreaks in Milwaukee in 1993, Nevada in 1994, and Florida in 1995; the Atlanta day care outbreak in 1995, and the Washington outbreak in 1997). It is possible that genotype 2 parasites largely cause human infection through contamination of water or food or direct contact with infected animals, especially in rural areas. Examples are the Maine apple cider outbreak in 1993, the British Columbia waterborne outbreak in 1996, and the Pennsylvania multi-family outbreak in 1997. The reason for the high percentage of genotype 2 in AIDS patients (6/13 patients) in France (Bonnin et al. 1996) is not clear. Taken together, there are two distinct populations of *C. parvum* parasites, one cycling only in humans and one cycling predominantly in animals. The latter can cause human infections.

CRYPTOSPORIDIUM PARASITES IN ENVIRONMENTAL SAMPLES

One difficulty facing the investigation of waterborne outbreaks of cryptosporidiosis is the lack of a sensitive, specific diagnostic tool. Most of the current PCR diagnostic and genotyping tools are designed for analysis of clinical samples. Because they cannot differentiate *Cryptosporidium* species and have low sensitivities, they have limitations in the analysis of water samples. Two PCR-RFLP techniques based on the SSU rRNA gene have claimed to differentiate *C. parvum* from other *Cryptosporidium* parasites (Awad-El-Kariem et al. 1994, Leng et al. 1996). One technique (Leng et al. 1996) used conserved sequences for primers and therefore amplify the SSU rRNA gene of all eukaryotic organisms. The other technique (Awad-El-Kariem et al. 1994) used erroneous sequence by Cai et al. (1992) as primers, reducing the efficiency of amplification and making interpretation of the data difficult. Nor have the present genotyping techniques been subjected to cross-species testing, making interpretation of results from environmental samples that could contain non-*parvum Cryptosporidium* virtually impossible.

Based on sequence information on the SSU rRNA gene, we have developed a PCR-RFLP technique for both species identification and genotyping of *Cryptosporidium* parasites. Because the technique employs nested PCR and targets the multi-copied rRNA gene, it has sufficient sensitivity for use in environmental samples. We have used this technique in the analysis of *Cryptosporidium* oocysts recovered from the gill washings and hemolymph of oysters (*Crassostrea virginica*) collected from the Chesapeake Bay. We are interested in oysters because they are filter feeders that concentrate and accumulate *Cryptosporidium* oocysts they have removed from surface waters. The use of oysters enables investigators to avoid the poor recovery rate often associated with filtering hundreds of liters of water to determine the presence or absence of *Cryptosporidium* oocysts. Before applying our technique *Cryptosporidium* oocysts were morphologically identified in oysters, but the species of most of the oocysts was unconfirmed (Fayer et al. 1998).

TABLE II

Prevalence of genotype 2 in human clinical samples reported in various studies

Location	Sample source	# of samples	genotypes 1/2	Technique used	Reference
England & Guinea Bissau	Sporadic cases	11	10/1	Isozyme	Awad-El-Kariem et al. 1995
Western & South Australia	Sporadic cases	14	12/2	RAPD	Morgan et al. 1995
USA	Sporadic cases	3	2/1	ITS1 and SSU rRNA repeat	Carraway et al. 1996
Northeast France	HIV+ patients	13	6/7	PCR-RFLP of repetitive DNA	Bonnin et al. 1996
UK	Sporadic cases	7	5/2	PCR-RFLP of oocyst wall protein	Spano et al. 1997
Western Australia	Sporadic cases	32	28/4	PCR of RAPD fragment	Morgan et al., 1997
USA & Canada	Outbreaks & sporadic cases	16	13/3	TRAP-C2 sequencing	Peng et al. 1997
USA, Canada, India & Guatemala	Outbreaks & sporadic cases	50	42/8	PCR-RFLP of TRAP-C2	Sulaiman et al. unpub. data

Preliminary analysis of 65 pooled oyster samples using the SSU rRNA-based PCR-RFLP technique has shown the presence of *Cryptosporidium* oocysts in 26 samples. Twenty four of these positive samples were *C. parvum*, and each of the others was *C. baileyi* and *C. serpentis*. The majority of *Cryptosporidium* oocysts were of genotype 2 (22 samples), indicating animals maybe the most likely the source of most *Cryptosporidium* oocyst contamination in the Chesapeake Bay. Even though this is a highly populated area, only two samples had genotype 1 sequences . These results demonstrate that oysters can serve as a biologic monitor for *Cryptosporidium* oocyst contamination in waters. Because raw oysters are often consumed by humans, *Cryptosporidium* oocysts in oysters also pose a potential health concern. Other filter-feeders such as freshwater clams and marine mussels have also been shown to accumulate *Cryptosporidium* oocysts (Graczyk et al. 1998, Chalmers et al. 1997). They may serve as similar biologic monitors for *Cryptosporidium* oocyst contamination.

CONCLUSIONS

Although the traditional classification of species based on the vertebrate classes of their hosts is largely accurate, it has greatly underestimated the diversity various *Cryptosporidium* isolates. This has presented problems in the identification of parasites in environmental samples. Molecular techniques are now available to identify species of *Cryptosporidium* and to differentiate known genotypes of *C. parvum*, and should be very useful in the investigation of clinical outbreaks of cryptosporidiosis. The performance of these techniques in the analysis of environmental samples, however, has yet to be thoroughly demonstrated. Because of the nature of environmental samples, *Cryptosporidium* isolates from various hosts must be more extensively characterized before enough data have been acquired and interpreted to instill full confidence in the method.

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