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Failure To Differentiate *Cryptosporidium parvum* from *C. meleagridis* Based on PCR Amplification of Eight DNA Sequences

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In order to determine the specificities of PCR-based assays used for detecting *Cryptosporidium parvum* DNA, eight pairs of previously described PCR primers targeting six distinct regions of the *Cryptosporidium* genome were evaluated for the detection of *C. parvum*, the agent of human cryptosporidiosis, and *C. muris*, *C. baileyi*, and *C. meleagridis*, three *Cryptosporidium* species that infect birds or mammals but are not considered to be human pathogens. The four *Cryptosporidium* species were divided into two groups: *C. parvum* and *C. meleagridis*, which gave the same-sized fragments with all the reactions, and *C. muris* and *C. baileyi*, which gave positive results with primer pairs targeting the 18S rRNA gene only. In addition to being genetically similar at each of the eight loci analyzed by DNA amplification, *C. parvum* and *C. meleagridis* couldn't be differentiated even after restriction enzyme digestion of the PCR products obtained from three of the target genes. This study indicates that caution should be exercised in the interpretation of data from water sample analysis performed by these methods, since a positive result does not necessarily reflect a contamination by the human pathogen *C. parvum*.

Organisms of the genus *Cryptosporidium* are widespread coccidian protozoans that develop in epithelial cells lining the digestive and respiratory tracts of vertebrates. On the basis of host specificity, pathogenesis, and oocyst morphology, eight *Cryptosporidium* species are regarded as valid (9): *Cryptosporidium muris* and *C. parvum* in mammals (4, 27), *C. wrairi* in guinea pigs (9), *C. felis* in domestic cats (9), *C. meleagridis* and *C. baileyi* in birds (5, 25), *C. nasorum* in fish, and *C. serpentis* in reptiles (9). According to this classification, *C. parvum* is the agent of clinical cryptosporidiosis in humans and livestock (9). Despite a unique report of *C. baileyi* infection in an immunocompromised patient (6), *C. parvum* is the only *Cryptosporidium* species regarded as a threat to human health.

Human cryptosporidiosis is a worldwide emerging zoonotic disease. Whereas immunocompetent individuals experience short-term gastroenteritis that resolves spontaneously, malnourished children and immunocompromised individuals may suffer from chronic life-threatening diarrhea. Transmission occurs by the fecal-oral route. *C. parvum* oocysts are shed into the environment by infected mammals who contaminate surface waters. The resistance of these oocysts to standard water disinfectants, as well as the low infective dose of viable *C. parvum* oocysts (8), accounts for the risk of waterborne transmission of human cryptosporidiosis and for the serious outbreaks that have been reported (12).

Waterborne cryptosporidiosis thus represents a global public health problem, and reliable detection methods are needed in order to control the presence of the parasite in source and finished waters. PCR amplification of *Cryptosporidium* DNA is a potentially powerful approach in achieving this aim, and several groups have cloned and sequenced *Cryptosporidium* genes as well as proposed PCR-based methods for identifying *C. parvum* DNA. However, environmental waters are likely to be contaminated with *Cryptosporidium* oocysts from diverse vertebrate reservoirs. Therefore, a major requirement regarding the characterization of these techniques should be an accurate evaluation of their specificity with *Cryptosporidium* oocysts of species other than *C. parvum*, in order to ultimately develop a technique capable of unambiguously identifying *C. parvum* oocysts.

In the original studies, PCR-based methods used for the identification and typing of *Cryptosporidium* isolates were evaluated with *C. parvum* (3, 15, 18, 28), with *C. parvum* and *C. muris* (14), or with DNA from *C. parvum*, *C. muris*, and *C. baileyi* (1, 13, 24), and none of these studies included the bird species *C. meleagridis*. The aim of the present study was to thoroughly assess the specificities of the eight PCR assays cited above for *C. parvum*, *C. muris*, *C. baileyi*, and *C. meleagridis.*

MATERIALS AND METHODS

***Cryptosporidium* isolates.** *C. parvum* isolate B-97-11 was provided by G. Harly; it was obtained from the diarrheic feces of a naturally infected newborn calf. Oocysts of *C. muris*, *C. meleagridis*, and *C. baileyi* isolate O.96.2 were provided by M. Naciri. The *C. muris* isolate was obtained from a naturally infected 10-year-old cow with diarrhea. Purified oocysts were ovoid and measured about 7.5 by 5.5 μm. The *C. meleagridis* isolate was obtained from mucosal scrapings of the cecal pouches of a common quail necropsied during an outbreak of diarrhea and was maintained in chickens by oral inoculation and recovery of the cecal contents. Purified oocysts were spherical and measured 4.5 μm in diameter. *C. baileyi* isolate O.96.2 originated from the bursa of Fabricius of a newborn duck and was maintained in ducks or chickens by oral inoculation and recovery of the contents of the bursa of Fabricius and the cloaca. Purified oocysts were ovoid and measured 6.9 by 5.5 μm (19). The second *C. baileyi* isolate utilized in this study, isolate B1, was provided by I. Varga. The oocysts were originally purified from the feces of chickens during an outbreak of avian cryptosporidiosis (7), and the isolate was maintained by serial passage in chickens. Purified oocysts were ovoid and measured 6.2 by 4.2 μm.

Preparation of oocyst lysates as PCR templates. *C. parvum*, *C. muris*, *C. meleagridis*, and *C. baileyi* isolate O.96.2 were extracted from fecal material as

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TABLE 1. Target genes and primers for detection of *Cryptosporidium* DNA

Target	Primary investigator (reference)	Primer pair	Fragment size (bp)
Undefined	Laxer (15)	5′-CCGAGTTTGATCCAAAAAGTTACGAA 5′-TAGCTCCTCATATGCCTTATTGAGTA	452
CpR1	Laberge (14)	5′-GCCCACCTGGATATACACTTTC 5′-TCCCCCTCTCTAGTACCAACAGGA	358
CpR1	Wagner-Wiening (28)	5′-AGTGTCCTCCAGGTACAAACCTGGTA 5′-GCACAGCTGGGACAGAATCAGCTTT	898
Undefined	Morgan (18)	5′-GGTACTGGATAGATAGTGGA 5′-TCGCACGCCCGGATTCTGTA	680
18S rRNA gene	Johnson (13)	5′-AAGCTCGTAGTTGGATTTCTG 5′-TAAGGTGCTGAAGGAGTAAGG	435
18S rRNA gene	Awad-El-Kariem (1)	5′-AGTGCTTAAAGCAGGCAACTG 5′-CGTTAACGGAATTAACCAGAC	556
Hsp70 gene	Rochelle (24)	5′-AAATGGTGAGCAATCCTCTG 5′-CTTGCTGCTCTTACCAGTAC	361
Undefined	Bonnin (3)	5′-TTCATTCTATCATGTC 5′-ATGGTTATATTTGGG	1500

previously described (2). Purified oocysts were resuspended in 10 mM Tris (pH 8.3)–50 mM KCl at 4°C for DNA extraction or stored in 2.5% potassium dichromate at 4°C until analysis. *C. baileyi* oocysts from isolate B1 were purified as described elsewhere (5); oocysts in 2.5% potassium dichromate were washed four times by successive pelleting (10,000 × *g* for 10 min at 4°C) and resuspension in distilled water and were finally suspended in 10 mM Tris (pH 8.3)–50 mM KCl. For DNA extraction, purified oocysts were suspended at a density of 250 oocysts/μl in 100-μl aliquots of 10 mM Tris (pH 8.3)–50 mM KCl containing 0.5% (wt/vol) Tween 20. After freeze-thawing (15 cycles), samples were heated for 15 min at 100°C and then centrifuged for 2 min at 16,000 × *g* to remove particulate matter. Supernatants were recovered and stored at −20°C until used for PCR amplification (3, 10).

PCR primers. The sequences of the primers and the sizes of the expected PCR fragments, with reference to their first description, the last names of the primary authors, and the identification of the target genes when characterized, are given in Table 1.

PCR amplification and gel analysis of PCR products. One-microliter volumes of the oocyst lysates were used as amplification templates in 50-μl reaction mixtures containing 75 mM Tris (pH 9); 20 mM $(NH_4)_2SO_4$; 0.01% (wt/vol) Tween 20; 0.2 mM each dGTP, dATP, dCTP, and dTTP; 2 to 4 mM $MgCl_2$ (Table 2); 50 pM each primer; and 1 to 2 U (Table 2) of GoldStar *Taq* DNA polymerase (Eurogentec). Reaction mixtures were overlaid with 50 μl of sterile mineral oil and were subjected to denaturation, thermal cycling (Minicycler; MJ Research), and then a final elongation at 72°C. The conditions of denaturation, annealing, and elongation varied depending on the primers (Table 2). PCR products were analyzed on horizontal agarose gels in TAE buffer (40 mM Tris acetate, 2 mM $Na_2EDTA \cdot 2H_2O$). Each amplification run included a negative control (PCR water) and a positive control (DNA from *C. parvum*). All negative samples were reamplified in duplicate by adding DNA from 250 *C. parvum* oocysts to the reaction mixtures, to ensure that negative results were not due to the copurification of PCR inhibitors. For restriction fragment analysis of the PCR products, 12-μl aliquots of the amplified DNA were treated with restriction enzymes (Table 2) under conditions recommended by the suppliers, prior to electrophoresis.

RESULTS

The expected PCR fragments were found to be produced upon analysis of *C. parvum* DNA with all the techniques evaluated (Fig. 1A, B, D, E, G, H, J, and K). *C. baileyi* isolates O.96.2 and B1 gave identical results when their DNA was subjected to PCR amplification with the eight techniques utilized (data not shown). No amplification of DNA was detected with the negative-control samples (Fig. 1A, B, D, E, G, H, J, and K). The expected products were obtained by seeding negative samples with *C. parvum* DNA (Fig. 1D, E, G, H, J, and K), suggesting that the negative results were not due to the copurification of PCR inhibitors.

This multilocus analysis showed that isolates of the four *Cryptosporidium* species analyzed were divided into two clearly distinct groups according to the allelic combinations they displayed (Fig. 1; Table 3): in the first group, corresponding to *C. parvum* and *C. meleagridis*, PCRs gave the same-sized fragments with all the primer pairs evaluated (Fig. 1A, B, D, E, G, H, J, and K), while in the second group, which included *C. muris* and *C. baileyi*, only the primer pairs targeting the 18S rRNA gene gave positive results (Fig. 1A and B).

Species in each of the two groups couldn't be differentiated from each other on the basis of DNA amplification at any of the loci examined. In order to identify genetic differences between *C. parvum* and *C. meleagridis*, PCR fragments from 5C12 (Fig. 1I), the 18S rRNA gene (Fig. 1C), and CpR1 (Fig. 1F) were then subjected to restriction enzyme digestion, respec-

TABLE 2. PCRs and thermal-cycling parameters for amplification of *Cryptosporidium* DNA

PCR method (reference)	MgCl2 concn (mM)	Amt of *Taq* (U)	Cycle conditions					No. of cycles	Restriction site(s)
			Initial denaturation	Denaturation	Annealing	Extension	Final extension		
Laxer (15)	2	1	95°C, 10 min	94°C, 1 min	56°C, 90 s	72°C, 90 s	72°C, 7 min	45	
Laberge (14)	2	1	95°C, 10 min	94°C, 1 min	56°C, 90 s	72°C, 90 s	72°C, 7 min	45	
Wagner-Wiening (28)	2	2	94°C, 5 min	94°C, 1 min	50°C, 1 min	72°C, 1 min	72°C, 10 min	35	*Bam*HI
Morgan (18)	2	1	95°C, 10 min	94°C, 1 min	58°C, 90 s	72°C, 90 s	72°C, 10 min	45	
Johnson (13)	4	1	94°C, 5 min	94°C, 30 s	55°C, 30 s	72°C, 1 min	72°C, 10 min	40	
Awad-El-Kariem (1)	2	2	94°C, 5 min	94°C, 90 s	47°C, 90 s	72°C, 3 min	72°C, 10 min	45	*Mae*I
Rochelle (24)	2	1	94°C, 2 min	94°C, 30 s	55°C, 30 s	72°C, 90 s	72°C, 5 min	40	
Bonnin (3)	2.5	1.5	94°C, 20 min	94°C, 90 s	45°C, 90 s	72°C, 90 s	72°C, 10 min	45	*Hin*fI, *Rsa*I

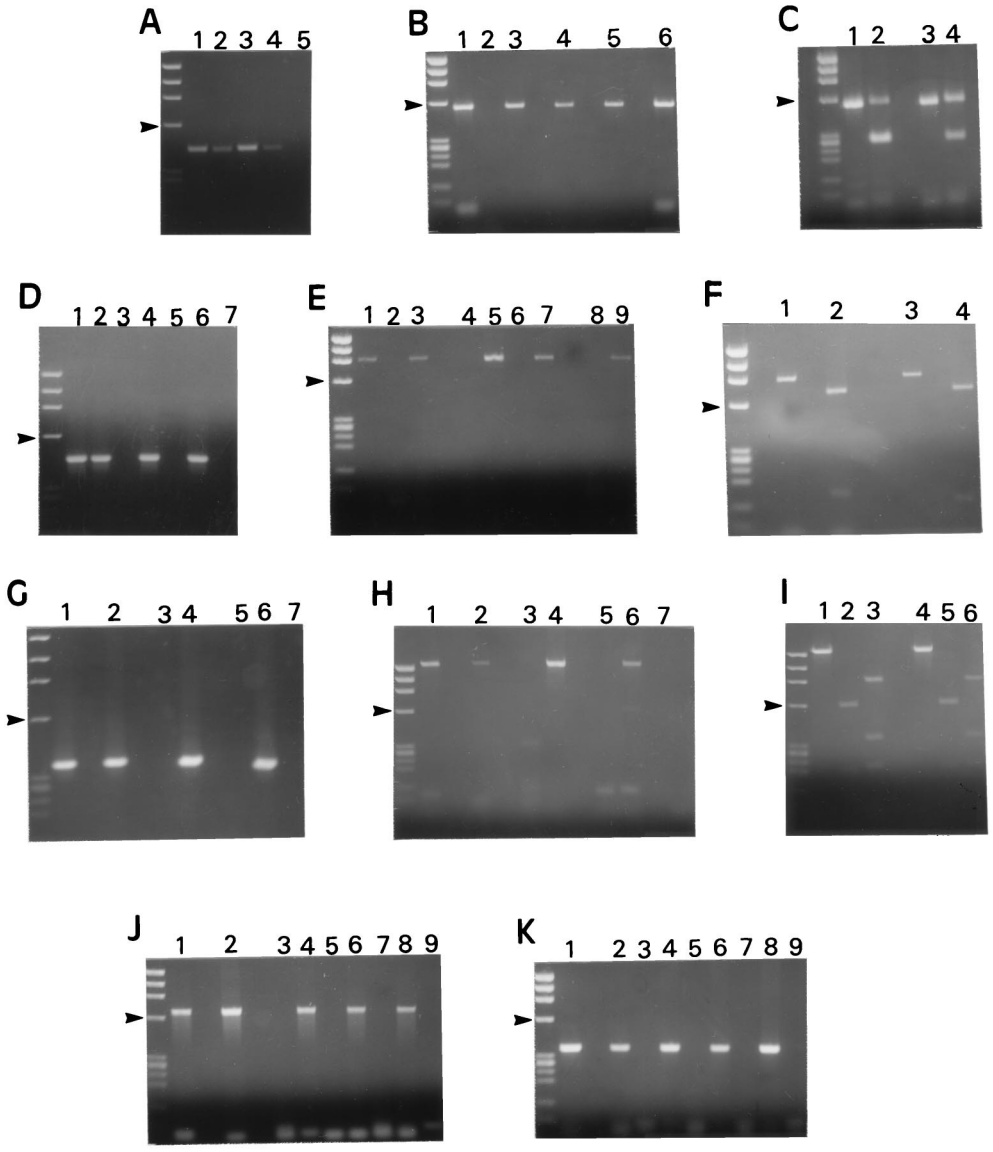

FIG. 1. Ethidium bromide-stained agarose gels of PCR products amplified from *C. parvum*, *C. muris*, *C. baileyi*, and *C. meleagridis* DNA. Size markers are *Hae*III-digested ΦX174; arrowheads in all panels point to the 603-bp fragment. (A) Amplification of a 435-bp product of the 18S rRNA gene (method of Johnson [13]). Lanes: 1, *C. parvum*; 2, *C. meleagridis*; 3, *C. muris*; 4, *C. baileyi* isolate B1; 5, negative control. (B) Amplification of a 556-bp product of the 18S rRNA gene (Awad-El-Kariem [1]). Lanes: 1, *C. parvum*; 2, negative control; 3, *C. meleagridis*; 4, *C. baileyi* isolate B1; 5, *C. baileyi* isolate O.96.2; 6, *C. muris*. (C) Electrophoresis of PCR products obtained in the experiment shown in panel B, prior to and after *Mae*I treatment. As expected (1), incomplete digestion occurred. Lanes: 1, *C. parvum*; 2, same sample as in lane 1 but after restriction with *Mae*I; 3, *C. meleagridis*; 4, same sample as in lane 3 but after restriction with *Mae*I. (D) Amplification of a 452-bp product of an undefined gene (Laxer [15]). Lanes: 1, *C. parvum*; 2, *C. meleagridis*; 3 and 4, *C. muris*; 5 and 6, *C. baileyi* isolate B1; 7, negative control. In lanes 4 and 6, *C. parvum* DNA was added to the reaction mixtures prior to amplification. (E) Amplification of a 898-bp fragment from the CpR1 gene (Wagner-Wiening [28]). Lanes: 1, *C. parvum*; 2, negative control; 3, *C. meleagridis*; 4 and 5, *C. baileyi* isolate B1; 6 and 7, *C. baileyi* isolate O.96.2; 8 and 9, *C. muris*. For lanes 5, 7, and 9, *C. parvum* DNA was added to the reaction mixtures prior to amplification. (F) Electrophoresis of PCR products obtained in the experiment represented in panel E prior to and after treatment with *Bam*HI. Lanes: 1, *C. parvum*; 2, same sample as in lane 1 but after restriction with *Bam*HI; 3, *C. meleagridis*; 4, same sample as in lane 3 but after restriction with *Bam*HI. (G) Amplification of a 358-bp fragment of the CpR1 gene (Laberge [14]). Lanes: 1, *C. parvum*; 2, *C. meleagridis*; 3 and 4, *C. muris*; 5 and 6, *C. baileyi* isolate B1; 7, negative control. For lanes 4 and 6, *C. parvum* DNA was added to the reaction mixtures prior to amplification. (H) Amplification of a 1,500-bp fragment from an undefined DNA region (Bonnin [3]). Lanes: 1, *C. parvum*; 2, *C. meleagridis*; 3 and 4, *C. muris*; 5 and 6, *C. baileyi* isolate B1; 7, negative control. For lanes 4 and 6, *C. parvum* DNA was added to the reaction mixtures prior to amplification. (I) Electrophoresis of PCR products obtained from the 1,500-bp fragment shown in panel H prior to and after treatment with *Hin*fI and *Rsa*I. Lanes: 1, *C. parvum*; 2 and 3, same product as in lane 1 but after digestion with *Hin*fI and *Rsa*I, respectively; 4, *C. meleagridis*; 5 and 6, same product as in lane 4 after digestion with *Hin*fI and *Rsa*I, respectively. (J) Amplification of a 680-bp fragment from an undefined gene (Morgan [18]). Lanes: 1, *C. parvum*; 2, *C. meleagridis*; 3 and 4, *C. muris*; 5 and 6, *C. baileyi* isolate B1; 7 and 8, *C. baileyi* isolate O.96.2; 9, negative control. For lanes 4, 6, and 8, *C. parvum* DNA was added to the reaction mixtures prior to amplification. (K) Amplification of a 361-bp fragment of the Hsp70 gene (Rochelle [24]). Lanes: 1, *C. parvum*; 2, *C. meleagridis*; 3 and 4, *C. muris*; 5 and 6, *C. baileyi* isolate B1; 7 and 8, *C. baileyi* isolate O.96.2; 9, negative control. For lanes 4, 6, and 8, *C. parvum* DNA was added to the reaction mixtures prior to amplification.

TABLE 3. Specificity of PCRs for detection of *C. parvum*, *C. muris*, *C. baileyi*, and *C. meleagridis*

PCR method (reference)	Result[a] for:			
	C. parvum	*C. muris*	*C. meleagridis*	*C. baileyi*
Laxer (15)	+	−	+	−
Laberge (14)	+	−	+	−
Wagner-Wiening (28)	+	−	+	−
Morgan (18)	+	−	+	−
Johnson (13)	+	+	+	+
Awad-El-Kariem (1)	+	+	+	+
Rochelle (24)	+	−	+	−
Bonnin (3)	+	−	+	−

[a] +, positive PCR; −, negative PCR.

tively, with *Rsa*I and *Hin*fI, *Mae*I, and *Bam*HI. No restriction fragment length polymorphisms (RFLPs) were detected in the PCR products (Fig. 1C, F, and I).

DISCUSSION

In order to determine the specificities of PCR-based diagnosis assays used for detecting *C. parvum* DNA, we conducted a thorough assessment of eight PCR techniques with isolates of four *Cryptosporidium* species infecting birds and mammals.

The specificities of the primer pairs targeting the 18S rRNA gene (1, 13) and the Hsp70 gene (24) were determined with *C. parvum*, *C. muris*, and *C. baileyi* in the studies on which the original descriptions of the primers were based, and the results of the present study are similar to those previously reported, confirming that the 18S rRNA regions, but not the 361-bp sequence of the Hsp70 gene, are amplified from *C. muris* and *C. baileyi*. Our data also confirm a study by Rochelle et al. (23) showing that the primer pair originally described by Laxer et al. (15) produced no PCR fragment with *C. muris* or *C. baileyi* DNA. Similarly, and in agreement with its first description (14), the 358-bp fragment of the *C. parvum* CpR1 gene was not amplified from *C. muris*. These results, based on isolates from France and Hungary, strengthen previous reports based on studies done with isolates essentially obtained in North America or the United Kingdom. The other three primer pairs evaluated herein, which target a larger fragment of the CpR1 gene of *C. parvum* (28) and two undefined sequences of the *C. parvum* genome (3, 18), were tested with *C. parvum* DNA only prior to this study. Our data show that the corresponding regions are amplified from *C. parvum* DNA, but not from *C. muris* or *C. baileyi.*

In none of the previous studies was *C. meleagridis* DNA included to assess PCRs. The isolate used in this study originated from the cecal pouches of a common quail with diarrhea and was maintained by oral inoculation of chickens and recovery of the cecal contents. Thus, host specificity, together with the site of infection and the small size of the oocysts, supports the identification of this isolate as *C. meleagridis* (5, 17, 25). The hypothesis that PCR fragments obtained with *C. meleagridis* resulted from nonspecific amplification of DNA from cells or microorganisms copurified with *C. meleagridis* oocysts is unlikely since all the PCR fragments had the predicted sizes. Amplification of the expected PCR fragments from *C. meleagridis* DNA with the eight PCRs evaluated is an important finding with respect to the monitoring of water contamination, since a positive PCR result with the primer pairs evaluated in the present paper would not necessarily imply the presence of *C. parvum* oocysts. We therefore sought for RFLP of the PCR products to differentiate between *C. parvum* and *C. meleagridis*. Two of the DNA sequences utilized in the present work contain restriction sites that were previously shown to be polymorphic: *Mae*I digestion of the 18S rRNA gene distinguished between *C. parvum* and a group of *C. muris-C. baileyi* isolates (1), and 5C12 contained *Rsa*I and *Hin*fI sites that distinguished two subpopulations among *C. parvum* isolates (3). Digestion with the appropriate enzymes showed that the *C. meleagridis* amplicons had an internal organization identical to that of *C. parvum* at these loci, ruling out the possibility of discriminating between *C. parvum* and *C. meleagridis* based on these RFLPs. Similarly, the *Bam*HI site in the *C. parvum* CpR1 PCR fragment (28) was present in the corresponding *C. meleagridis* amplicon. Sequencing of PCR products from both species, or cloning of further regions of the *Cryptosporidium* genome, may thus be necessary to identify genetic differences between *C. meleagridis* and *C. parvum* and to develop appropriate typing assays.

Organization of the four *Cryptosporidium* species analyzed herein in two groups with respect to results of PCR analysis of eight loci located in six distinct genes is a striking result of the present work. Little is known about the comparative structures of *Cryptosporidium* species at the molecular level. ^{125}I labeling of outer oocyst wall proteins of *C. parvum*, *C. muris*, and *C. baileyi* revealed common as well as species-specific molecules (26). In a study based on Western blotting, *C. parvum* and *C. baileyi* were easily differentiated, while *C. muris* produced weak bands that were difficult to interpret (20). Isoenzyme analysis showed that *C. parvum*, *C. muris*, and *C. baileyi* had distinct phosphoglucomutase electrophoresis patterns (22). Similarily, double digestion of a large fragment of the 18S rRNA gene produced distinct profiles for *C. parvum*, *C. muris*, and *C. baileyi* (16). Reports based either on the analysis of an undefined DNA sequence of the *Cryptosporidium* genome (29) or on antigenic reactivity of anti-*Cryptosporidium* antibodies (21) suggested that *C. parvum* and *C. baileyi* are more closely related to one another than to *C. muris*. On the other hand, PCR-RFLP analysis of the 556-bp region of the 18S rRNA gene showed that *C. muris* and *C. baileyi* have a common pattern that differs from the pattern displayed by *C. parvum* (1). Moreover, in an enzyme immunoassay, *C. parvum* and *C. meleagridis* oocysts gave positive reactions while *C. muris* and *C. baileyi* gave negative reactions (11), a profile of antigenic reactivity identical to the profiles for the two groups described herein which were separated on the basis of genomic-DNA organization. Whether these molecular profiles have any biological significance, especially with respect to species phylogeny, host adaptation, or differences in virulence will require further investigations.

Differentiation between *C. baileyi* and *C. muris* would not be as critical for the quality control of water supplies as a mix-up involving *C. parvum*, since neither of the first two species is considered a human pathogen. Anyway, two readily available PCR-based techniques may potentially differentiate between *C. muris* and *C. baileyi* provided the procedures are optimized for diagnosis samples: a double digestion of cloned PCR products from the 18S rRNA gene produced patterns specific for *C. muris* and *C. baileyi* (16), and an undefined DNA region described by Webster et al. was amplified from *C. parvum* and *C. baileyi* but not from *C. muris* (29). However, Rochelle et al. reported low PCR efficiency with the latter sequence (23).

Results of the present study, together with the observation that *C. parvum* and *C. meleagridis* share epitopes that are cross-reactive in an enzyme immunoassay (11), indicate that these two species are closely related at the molecular level and emphasize the complexity of the molecular mechanisms involved in host-parasite adaptation. Studies of additional *C.*

meleagridis isolates are needed in order to determine the extent of this biochemical homology and characterize markers that differentiate between *C. parvum* and *C. meleagridis*. Furthermore, the specificity of PCR methods available should also be examined with isolates of *C. serpentis* and *C. nasorum*, as well as isolates of *C. wrairi* and *C. felis*. Insofar as cryptosporidiosis increasingly appears as an environmental threat, our understanding of the epidemiology of cryptosporidiosis and our ability to define appropriate measures to prevent transmission will rely on accurate characterization of the techniques used for testing environmental samples.

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