Evaluation of *Cryptosporidium parvum* Genotyping Techniques

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We evaluated the specificity and sensitivity of 11 previously described species differentiation and genotyping PCR protocols for detection of *Cryptosporidium* parasites. Genomic DNA from three species of *Cryptosporidium* parasites (genotype 1 and genotype 2 of *C. parvum*, *C. muris*, and *C. serpentis*), two *Eimeria* species (*E. neischulzi* and *E. papillata*), and *Giardia duodenalis* were used to evaluate the specificity of primers. Furthermore, the sensitivity of the genotyping primers was tested by using genomic DNA isolated from known numbers of oocysts obtained from a genotype 2 *C. parvum* isolate. PCR amplification was repeated at least three times with all of the primer pairs. Of the 11 protocols studied, 10 amplified *C. parvum* genotypes 1 and 2, and the expected fragment sizes were obtained. Our results indicate that two species-differentiating protocols are not *Cryptosporidium* specific, as the primers used in these protocols also amplified the DNA of *Eimeria* species. The sensitivity studies revealed that two nested PCR-restriction fragment length polymorphism (RFLP) protocols based on the small-subunit rRNA and dihydrofolate reductase genes are more sensitive than single-round PCR or PCR-RFLP protocols.

Cryptosporidium parasites infect the microvillus border of the gastrointestinal epithelium of a wide range of vertebrate hosts, including humans. To date, at least 21 *Cryptosporidium* species have been named (20). However, only six to eight species (*Cryptosporidium parvum*, *C. muris*, *C. wairi*, *C. felis*, *C. meleagridis*, *C. baileyi*, *C. serpentis*, and *C. nesorum*) are considered valid by most researchers (9, 20). *C. parvum* is the species that infects immunocompetent humans and most mammals. Cross-transmission studies performed with various mammals have suggested the possibility that zoonotic transmission of *C. parvum* to humans occurs (13, 20, 24). However, person-to-person transmission of this parasite has also been demonstrated (2, 8). In recent years, *C. parvum* has been reported to cause waterborne disease outbreaks worldwide (25).

Workers have described many PCR-based protocols for detection of *Cryptosporidium* parasites. The primers of these PCR protocols are based on undefined genomic sequences (14, 18, 29) or specific genes (2, 4, 12, 15). PCR methods have been shown to be more sensitive and specific than traditional microscopic techniques for detecting *Cryptosporidium* spp. in clinical and environmental samples (16, 19). Recently, workers have compared the performance of some PCR techniques commonly used for detection of *Cryptosporidium* parasites (7, 22, 23).

In recent years, researchers have also developed PCR-based techniques for differentiating *C. parvum* of human origin and *C. parvum* of animal origin. These techniques are based on the polymorphic nature of *C. parvum* strains that infect humans and most animals at the β -tubulin, oocyst wall protein (COWP), dihydrofolate reductase (DHFR), thrombospondin-related adhesive protein 1 (TRAP-C1), thrombospondin-related adhesive protein 2 (TRAP-C2), internally transcribed spacer 1 (ITS1), polythreonine repeat (Poly-T), small-subunit (SSU) rRNA, and undefined genomic sequences (2, 3, 5, 6, 11,

15, 17, 21, 26–28, 30–32). These genotyping tools, however, were designed to analyze clinical specimens, and the comparative performance of the protocols has not been evaluated previously. In this study, we compared eight genotyping protocols (3, 5, 6, 11, 17, 21, 26–28), two species-differentiating protocols (2, 15), and one species-differentiating and genotyping protocol (31) for detecting and differentiating *Cryptosporidium* parasites.

MATERIALS AND METHODS

Oocysts and DNA extraction. Fecal samples containing genotype 1 and genotype 2 of *C. parvum* (based on a DNA sequence analysis of the TRAP-C2, β -tubulin, and SSU rRNA genes), *C. muris*, *C. serpentis*, *Eimeria neischulzi*, *E. papillata*, and *Giardia duodenalis* oocysts were obtained from infected humans and animals and were stored at 4°C in 2.5% potassium dichromate solutions. Oocysts and cysts were purified following sucrose and Percoll flotation (1). Genomic DNA was isolated from the purified oocysts as described previously (27, 30) and was stored at –20°C until it was used. The concentrations of DNA samples were determined by UV absorption at 260 nm.

For sensitivity experiments, genomic DNA was extracted from 500,000, 200,000, 5,000, 1,000, 500, 250, 100, and 50 oocysts of a genotype 2 *C. parvum* isolate. The DNA was dissolved in 100 μ l of water. Two microliters of each DNA stock preparation was used in the PCR in order to obtain the equivalent of 10,000, 4,000, 400, 100, 20, 10, 5, 2, and 1 oocysts per PCR mixture.

PCR. Usually, the PCR mixture contained 50 ng of genomic DNA, each deoxynucleoside triphosphate (Perkin-Elmer, Foster City, Calif.) at a concentration of 200 μ M, 1 $\times$ PCR buffer (GeneAmp 10 $\times$ PCR buffer; 500 mM KCl, 100 mM Tris-Cl [pH 8.3], 15 mM MgCl₂, 0.01% gelatin; Applied Biosystems, Branchburg, N.J.), 5.0 U of *Taq* polymerase (GIBCO BRL, Frederick, Md.), and the required amounts of forward and reverse primers in a total volume of 100 μ l. The Mg²⁺ concentration was adjusted by adding MgCl₂. DNA amplification was carried out with a Perkin-Elmer model PCR 9700 Gene Amp thermocycler for each pair of primers by using the previously described cycling conditions (initial hot start, denaturation, annealing, elongation, final extension, and total number of cycles). A negative control, consisting of a reaction mixture without the DNA template, was included in each experiment. Each PCR product was analyzed by electrophoresis in a 1.5% agarose gel and was visualized after ethidium bromide staining. The same DNA stock preparation was used as the template in all evaluation experiments.

PCR-RFLP analysis. Restriction digestion and PCR-restriction fragment length polymorphism (RFLP) techniques based on Poly-T repeats, COWP, DHFR, TRAP-C1, TRAP-C2, and the SSU rRNA gene were carried out by using previously described protocols (6, 11, 26–28, 31). For the restriction fragment analysis, 20 μ l of amplified products was digested by using 10 U of *Bpu*AI (Boehringer Mannheim, Indianapolis, Ind.), *Bsr*EI (Boehringer), *Hae*III (New England BioLabs, Beverly, Mass.), *Rsa*I (Promega, Madison, Wis.), *Ssp*I (New England BioLabs), or *Vsp*I (GIBCO BRL) and 5 μ l of the appropriate restriction buffer for 1 h under the conditions recommended by the supplier. The digested

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TABLE 1. Sources of the genotyping primers used in this study

Protocol		Target	Product size(s) (bp)	Type of PCR
Authors	Reference			
Awad-El-Kariem et al.	2	SSU rRNA	556	PCR-RFLP
Leng et al.	15	SSU rRNA	1,750	PCR-RFLP
Bonnin et al.	3	Unknown	1,353	PCR-RFLP
Carraway et al.	5	ITS1 of rRNA	513 (human), 519 (bovine)	Genotype-specific PCR
Spano et al.	26	COWP	553	PCR-RFLP
Morgan et al.	17	Unknown	411 (human), 311 (bovine)	Genotype-specific PCR
Gibbons et al.	11	DHFR	408	Nested PCR-RFLP
Carraway et al.	6	Poly-T	318	PCR-RFLP
Spano et al.	27	TRAP-C1	1,200	PCR-RFLP
Sulaiman et al.	28	TRAP-C2	369	PCR-RFLP
Xiao et al.	31	SSU rRNA	~825	Nested PCR-RFLP

products were fractionated by agarose gel electrophoresis and were visualized by ethidium bromide staining.

DNA sequencing and data analysis. When desired, the amplified PCR product was purified by using the Wizard PCR Preps DNA purification system (Promega) and was sequenced with a model ABI 377 automated sequencer by using a dRhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). A multiple alignment of the sequences was prepared by using the Genetics Computer Group program (10).

Nucleotide sequence accession numbers. The COWP sequences determined in this study have been deposited in the GenBank database under accession no. AF161577 to AF161580.

RESULTS

Genotyping techniques evaluated in the present study. In the present study we evaluated 16 primer pairs used for 11 previously described *Cryptosporidium* species differentiation and *C. parvum* genotyping protocols (Table 1). Of these 11 protocols, 4 are based on the rRNA gene (2, 5, 15, 31), 2 are based on unknown sequences (3, 17), and the remainder are based on COWP, DHFR, TRAP-C1, TRAP-C2, and Poly-T repeats (6, 11, 26–28); 10 of the 11 techniques amplified *C. parvum* DNA efficiently, and PCR fragments of the expected sizes were produced. The technique of Bonnin et al. (3) amplified DNA from *C. parvum* genotype 2 isolates. However, we were not able to amplify the DNA of *C. parvum* genotype 1, *C. muris*, and *C. serpentis* isolates under the conditions used in this study. No amplification was observed even after the Mg^{2+} concentration was modified and the amount of template was increased or decreased.

Specificity of *Cryptosporidium* genotyping tools. The specificity of the primers was examined by using DNA from three *Cryptosporidium* species (genotype 1 and genotype 2 of *C. parvum*, *C. muris*, and *C. serpentis*), two *Eimeria* species (*E. neischiulzi* and *E. papillata*), and one *Giardia* species (*G. duodenalis*). The specificity data are summarized in Table 2. Three SSU rRNA gene-based protocols were used, and the primers of two of these protocols, those developed by Awad-El-Kariem et al. (2) and Leng et al. (15), amplified the DNA of *Eimeria* species in addition to the DNA of *Cryptosporidium* species, generating bands of similar sizes (Fig. 1). In contrast, the *Cryptosporidium*-specific primers developed by us (31) produced an ~1,325-bp band during primary PCR and an ~825-bp band during nested PCR from genomic DNA of all *Cryptosporidium* species but not from genomic DNA of *Eimeria* species or *G. duodenalis*.

The PCR-RFLP protocol of Spano et al. (26) was found not to be *C. parvum* specific in this study, as it also amplified genomic DNA of *C. muris* and *C. serpentis*. The COWP gene-based primers did not amplify the DNA of non-*Cryptosporidium* parasites but did produce a 550-bp band with isolates of the two genotypes of *C. parvum*, *C. muris*, and *C. serpentis*. The Poly-T-based PCR-RFLP protocol of Carraway et al. (6), the TRAP-C1-based protocol of Spano et al. (27), the TRAP-C2-based protocol of Sulaiman et al. (28), and the DHFR-based nested PCR-RFLP protocol of Gibbons et al. (11) were found to be *C. parvum* specific; the primers used in these protocols amplified only the DNA of genotype 1 and genotype 2 isolates

TABLE 2. Specificities of previously described *Cryptosporidium* species differentiation and *C. parvum* genotyping protocols

PCR method		Amplification ^a						
		<i>C. parvum</i>		<i>C. muris</i>	<i>C. serpentis</i>	<i>E. neischiulzi</i>	<i>E. papillata</i>	<i>G. duodenalis</i>
		Human	Bovine					
Authors	Reference							
Awad-El-Kariem et al.	2	+	+	+	+	+	+	–
Leng et al.	15	+	+	+	+	+	+	–
Bonnin et al.	3	–	+	–	–	–	–	–
Carraway et al. ^b	5	+	+	–	–	–	–	–
Spano et al.	26	+	+	+	+	–	–	–
Morgan et al. ^b	17	+	+	–	–	–	–	–
Gibbons et al.	11	+	+	–	–	–	–	–
Carraway et al.	6	+	+	–	–	–	–	–
Spano et al.	27	+	+	–	–	–	–	–
Sulaiman et al.	28	+	+	–	–	–	–	–
Xiao et al.	31	+	+	+	+	–	–	–

^a +, amplification; –, no amplification.

^b Both the genotype 1 and genotype 2 *C. parvum*-specific primers were tested.

FIG. 1. Ethidium bromide-stained gel showing amplification products obtained from various members of the Apicomplexa by using the primers of protocols described by Awad-El-Kariem et al. (2) and Leng et al. (15). Lanes 1 and 9, 100-bp marker; lane 2, *C. parvum*; lane 3, *C. muris*; lane 4, *C. serpentis*; lane 5, *G. duodenalis*; lane 6, *E. neischulzi*; lane 7, *E. papillata*; lane 8, negative control. (A) Primers of Awad-El-Kariem et al. (2), which amplified a ~556-bp product. (B) Primers of Leng et al. (15), which amplified a ~1,750-bp product.

of *C. parvum* and did not amplify the DNA of non-*C. parvum* parasites.

The two ITS1-based genotype-specific primer sets used in the *C. parvum* genotyping protocol developed by Carraway et al. (5) amplified the genomic DNA of genotype 1 and genotype 2 *C. parvum* isolates. As expected, these primers did not amplify *C. muris*, *C. serpentis*, *Eimeria*, and *Giardia* genomic DNA. The *C. parvum*-specific randomly amplified polymorphic DNA diagnostic primers of Morgan et al. (17) produced a 411-bp

band with genotype 1 *C. parvum* DNA when a human-specific primer was used and a 311-bp band with genotype 2 *C. parvum* DNA when bovine-specific primers were used. These primers did not amplify the genomic DNA of non-*C. parvum* parasites in this study.

Evaluation of PCR-RFLP protocols and nucleotide sequencing. We also evaluated the restriction digestion procedures used in five different PCR-RFLP protocols (6, 11, 26–28, 31). The two species-differentiating protocols of Awad-El-Kariem et al. (2) and Leng et al. (15) were not evaluated further due to nonspecific amplification of DNA from other Apicomplexa species, such as *E. neischulzi* and *E. papillata*. An RFLP analysis of DHFR (11), Poly-T (6), TRAP-C1 (27), and TRAP-C2 (28) PCR products showed that the restriction patterns for genotype 1 and genotype 2 of *C. parvum* were distinct, as reported in the original papers. Restriction digestion of SSU rRNA products could be used to differentiate the *Cryptosporidium* species, as well as the two genotypes of *C. parvum*, as reported previously (31).

The PCR-amplified COWP products (26) of genotype 1 and genotype 2 *C. parvum* and *C. muris* isolates were digested with restriction endonuclease *RsaI*, which resulted in three distinct band patterns for these isolates (data not shown). Since the PCR product of a *C. serpentis* isolate was faint, it was not subjected to restriction digestion. However, all of the COWP PCR products were sequenced. A multiple alignment revealed that the sequences of genotype 1 and genotype 2 *C. parvum*, *C. muris*, and *C. serpentis* isolates were distinct.

Sensitivity of the genotyping primers, as determined by using genomic DNA extracted from known numbers of oocysts from *C. parvum* genotype 2 isolates. Nine PCR protocols were also evaluated to determine their sensitivity for detecting *Cryptosporidium* parasites by using genomic DNA extracted from known numbers of oocysts of genotype 2 *C. parvum* isolates. Genotype 2 *C. parvum* oocysts were used in the sensitivity experiments because of their abundance. Sensitivity tests were not performed for the two protocols (2, 15) that were not specific. Our data on the sensitivity of primers revealed that the nested PCR protocols of Gibbons et al. (11) and Xiao et al. (31) were the most sensitive protocols as the two primer pairs amplified the DNA of even a single oocyst. The results of a comparison of the sensitivities of the genotyping primers are shown in Table 3.

DISCUSSION

Results of recent molecular characterization studies have shown that there are extensive genetic differences among different *Cryptosporidium* species, as well as within *C. parvum*. These inter- and intraspecies differences have been used in the

TABLE 3. Comparison of the sensitivities of various genotyping PCR tools for detection of *C. parvum* oocysts

Protocol		Primers	Target	Detection of the following no. of oocysts								
Authors	Reference			10,000	4,000	400	100	20	10	5	2	1
Bonnin et al.	3	PAP1-PAP2	Unknown	+	+	+	+	–	–	–	–	–
Carraway et al. ^a	5	Cry7-ITS1	ITS1	+	+	+	+	–	–	–	–	–
Carraway et al.	6	Cry39-Cry44	Poly-T	+	+	+	+	+	+	–	–	–
Spano et al.	26	Cry15-Cry9	COWP	+	+	+	+	+	+	–	–	–
Morgan et al. ^a	17	021F-CPCR	Unknown	+	+	+	+	+	–	–	–	–
Gibbons et al.	11	Nested PCR	DHFR	+	+	+	+	+	+	+	+	+
Spano et al.	27	Cp.E-Cp.Z	TRAP-C1	+	+	+	+	+	–	–	–	–
Sulaiman et al.	28	Forward-Reverse	TRAP-C2	+	+	+	+	+	+	+	–	–
Xiao et al.	31	Nested PCR	SSU RNA	+	+	+	+	+	+	+	+	+

^a For these protocols, *C. parvum* genotype 1-specific primers were not tested as the template was from a *C. parvum* genotype 2 isolate.

development of molecular diagnostic tools for *Cryptosporidium* spp. and for genotype differentiation. So far, at least two species-specific (2, 15) and seven genotyping-specific (3, 5, 6, 11, 17, 21, 26–28) protocols have been described. One PCR-RFLP protocol has been developed for both species and genotype differentiation (31). Most of these techniques have not been subjected to evaluation. Recently, several PCR techniques were evaluated to determine whether they detected *C. parvum* in environmental samples (7, 22, 23), but most of the species and genotype differentiation techniques were not included. The nature of environmental samples suggests that multiple *Cryptosporidium* spp. and *C. parvum* genotypes may be present. Therefore, the species differentiation and *C. parvum* genotyping techniques should be evaluated before they are used for analysis of environmental samples.

Two of the three *Cryptosporidium* species differentiation techniques based on the SSU rRNA gene were found not to be *Cryptosporidium* specific. The primers used in these two protocols (2, 15) amplified DNA from two *Eimeria* species (*E. neischulzi* and *E. papillata*). As RFLP was used for species differentiation and the taxon Apicomplexa contains thousands of species, the nonspecificity of the two techniques makes diagnosis of *Cryptosporidium* species unreliable. The major reason for the nonspecificity is the fact that conserved 18S rRNA sequence data were used when the primers were designed. The primers used by Leng et al. (15) are the universal primers used by most researchers to clone the SSU rRNA gene of eukaryotic organisms. However, another SSU rRNA-based PCR-RFLP protocol, the protocol of Xiao et al. (31), was shown to be *Cryptosporidium* specific. The primers used in this protocol did not amplify the DNA of non-*Cryptosporidium* species. This protocol differentiated three *Cryptosporidium* species and two genotypes of *C. parvum*.

Six *C. parvum* genotyping protocols, which were based on DHFR, TRAP-C1, TRAP-C2, ITS1, Poly-T repeats, and an unknown genomic sequence (5, 6, 11, 17, 27, 28), were found to be *C. parvum* specific. The primers used in these protocols amplified the DNA of genotype 1 and genotype 2 isolates of *C. parvum* but not the DNA of non-*C. parvum* species, *Eimeria* spp., or *G. duodenalis*. In the techniques of Carraway et al. (5) and Morgan et al. (17) genotype 1- and genotype 2-specific primers are used. The genotype 1-specific primers amplify DNA of *C. parvum* genotype 1 and not DNA of genotype 2 isolates, whereas the genotype 2-specific primers amplify DNA of *C. parvum* genotype 2 and not DNA of genotype 1 isolates. Our studies revealed that these four genotype-specific primers are indeed *C. parvum* genotype 1 or genotype 2 specific, as they amplified and distinguished the two genotypes of *C. parvum*, as claimed previously. The DHFR-, TRAP-C1-, TRAP-C2-, and Poly-T-based PCR-RFLP techniques (6, 11, 27, 28) were found to be *C. parvum* specific, and following restriction digestion of single-round PCR and nested PCR products two distinct band patterns were obtained for the genotype 1 and genotype 2 *C. parvum* isolates. However, a PCR protocol based on an unknown genomic sequence (3) was shown to be less sensitive, as it amplified only *C. parvum* genotype 2 isolates even after the Mg^{2+} concentration and the amount of DNA template were modified.

We found that the PCR-RFLP protocol based on the COWP gene (26) was not *C. parvum* specific, as the primers also amplified genomic DNA from other *Cryptosporidium* spp. However, the primers did not amplify the DNA of non-*Cryptosporidium* parasites. The COWP gene-based PCR-RFLP protocol has been reported to differentiate *C. wrairi* (some workers consider this organism *C. parvum*) from the two genotypes of *C. parvum* (26). We evaluated this technique by

using *C. muris* and *C. serpentis* and identified distinct band patterns for these parasites. Furthermore, nucleotide sequencing of the PCR products also revealed similar distinct sequences for different species and genotypes. Therefore, the set of primers may be utilized for species- and genotype-specific diagnosis of *Cryptosporidium* parasites after the primer sequences are modified.

In summary, the PCR-RFLP protocols of Awad-El-Kariem et al. (2) and Leng et al. (15) for *Cryptosporidium* species differentiation described previously are not *Cryptosporidium* specific. We found that the set of primers developed by Bonnin et al. (3) has poor diagnostic sensitivity for genotype 1 *C. parvum* isolates. The PCR-RFLP method of Spano et al. (26) has the potential to distinguish different *Cryptosporidium* species (*C. muris* and *C. serpentis*), as well as the two genotypes of *C. parvum*. We found that the genotype 1-specific and genotype 2-specific primers of Carraway et al. (5) and Morgan et al. (17) are *C. parvum* specific, as these primers detected *C. parvum* genotype 1 and genotype 2 isolates. Similar results were obtained for the DHFR-, TRAP-C1-, TRAP-C2-, and Poly-T-based PCR-RFLP techniques of Gibbons et al. (11), Spano et al. (27), Sulaiman et al. (28), and Carraway et al. (6). The nested PCR-RFLP protocols developed by Gibbons et al. (11) and Xiao et al. (31) are the most sensitive protocols evaluated in this study. Additional evaluations with other apicomplexan parasites and *Cryptosporidium* species may be needed before these techniques can be used for analysis of environmental samples.

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