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PCR-Restriction Fragment Length Polymorphism Analysis of a Diagnostic 452-Base-Pair DNA Fragment Discriminates between *Cryptosporidium parvum* and *C. meleagridis* and between *C. parvum* Isolates of Human and Animal Origin

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Genomic DNAs from human *Cryptosporidium* isolates previously typed by analysis of the 18S ribosomal DNA locus (*Cryptosporidium parvum* bovine genotype, *C. parvum* human genotype, *Cryptosporidium meleagridis*, and *Cryptosporidium felis*) were used to amplify the diagnostic fragment described by Laxer et al. (M. A. Laxer, B. K. Timblin, and R. J. Patel, Am. J. Trop. Med. Hyg., 45:688–694, 1991). The obtained 452-bp amplified fragments were sequenced and aligned with the homologous *Cryptosporidium wrairi* sequence. Polymorphism was exploited to develop a restriction fragment length polymorphism method able to discriminate *Cryptosporidium* species and *C. parvum* genotypes.

Cryptosporidium spp. are Apicomplexan parasites that infect the gastrointestinal or respiratory tract of humans and animals. In immunocompetent hosts, the infection is typically acute and self limiting, whereas in immunocompromised individuals, such as persons receiving immunosuppressive drugs and AIDS patients, cryptosporidiosis is often a chronic disease. Since drug therapy to control or eliminate these organisms is not yet available, persistent infections in these patients are therefore especially severe and can be life threatening. The potential of *Cryptosporidium* as an opportunistic parasite and the recent reports of major outbreaks of cryptosporidiosis in the United States, United Kingdom, and Australia due to contamination of drinkable water supplies indicate that *Cryptosporidium* should be considered a major public health problem (12, 25).

To date, eight *Cryptosporidium* species have been regarded as valid on the basis of host specificity, pathogenesis, and oocyst morphology (13). These included *Cryptosporidium parvum* in mammals, *Cryptosporidium muris* in rodents and ruminants, *Cryptosporidium felis* in domestic cats, *Cryptosporidium wrairi* in guinea pigs, *Cryptosporidium baileyi* and *Cryptosporidium meleagridis* in birds, *Cryptosporidium serpentis* in reptiles, and *Cryptosporidium nasorum* in fishes. According to this classification, the causative agent of cryptosporidiosis in humans and a range of mammalian species is the species *C. parvum*. Numerous PCR-based assays have been described for detection of *Cryptosporidium* parasites. The primers of these PCR assays are based on either undefined genomic sequences (2, 3, 22, 27, 46) or specific genes (4, 27–29, 35, 40, 41, 43, 45, 48, 49). Most PCR assays have led to the confirmation of *C. parvum* as the major cause of cryptosporidiosis in humans and to the identification of two genotypes within this species: the "human" genotype (genotype 1), which has so far been found exclusively in humans, with the exception of a single nonhuman primate (39) and a dugong (33), and the "bovine" genotype (genotype 2), found in domestic livestock such as cattle, sheep, and goats, etc., which can also infect humans. Additional genotypes have then been distinguished in *C. parvum* (32). However, most of the available genotyping tools were designed to analyze clinical specimens, and their specificities for other *C. parvum* genotypes or other *Cryptosporidium* species were not always established.

Laxer et al. were among the first authors to publish primers for detection of *Cryptosporidium* (22). These primers, which were not known to target a specific gene but to amplify a 452-bp fragment of an unidentified region, as well as the reported 452-bp sequence, have been widely used (1, 6–11, 14, 15, 17, 18, 20, 21, 23, 37, 38). The purpose of the present study was to investigate the extent of sequence heterogeneity for this diagnostic DNA fragment among human isolates of *Cryptosporidium*.

Sample analysis. Fecal samples used in this study were obtained from infected bovine (named B1 isolate) or humans (named H isolates) (Table 1) and were identified as genotype 1 of *C. parvum*, genotype 2 of *C. parvum*, *C. meleagridis*, and *C. felis*, on the basis of 18S ribosomal DNA (rDNA) (16). Genomic DNA samples were prepared as described before (16) and were stored at −20°C until they were used. The 452-bp Laxer fragment was amplified by PCR using a pair of 26-mer primers previously reported (22). The reaction mixtures were prepared in 1× PCR buffer (75 mM Tris [pH 9], 20 mM $[NH_4]_2SO_4$, and 0.01% Tween 20) and contained per 50-μl reaction 2 mM $MgCl_2$, 0.5 μM concentrations of both primers (Eurogentec, Seraing, Belgium), a 200 μM concentration of each deoxynucleoside triphosphate, 1 U of DNA Goldstar Polymerase (Eurogentec), and 10 μl of the purified DNA at the 1/10 dilution. A negative control, consisting of a reaction mixture with water instead of DNA template, was included in

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TABLE 1. Isolates and genotypes of *Cryptosporidium* used in this study

Isolate code[a]	HIV status[c]	Source[b]	Geographical location	Species and genotype by analysis of	
				18S rDNA locus	Laxer locus
C1		INRAT	France	Cattle	Cattle (L1 subgenotype)
H1	Neg.	CHD	France	Cattle	Cattle (L1 subgenotype)
H5	Neg.	CHD	France	Cattle	Cattle (L1 subgenotype)
H4	Neg.	CHRUL	France	Human	Human
H7	Pos.	HSLP	France	Human	Human
H8	Pos.	HSLP	France	Human	Human
H9	Pos.	HSLP	France	Human	Human
H11	Pos.	HSLP	France	*C. meleagridis*	*C. meleagridis*
H14	Pos.	HSLP	France	Cattle	Cattle (L2 subgenotype)
H34	Pos.	HSLP	France	*C. meleagridis*	*C. meleagridis*
H37	Neg.	HSLP	France	Human	Human
H67	Pos.	HSLP	France	*C. felis*	No amplification
H78	Pos.	HSLP	France	*C. felis*	No amplification
H81	Neg.	HSLP	France	*C. felis*	No amplification
H83	Pos.	HSLP	France	*C. felis*	No amplification
H16	Neg.	HHMC	France	Cattle	Cattle (L1 subgenotype)
H15	Pos.	CHRUR	France	Cattle	Cattle (L2 subgenotype)
H17	Neg.	CHUA	France	*C. meleagridis*	*C. meleagridis*
H18	Pos.	CHUN	France	Cattle	Cattle (L2 subgenotype)
H22	Pos.	CHUN	France	*C. felis*	Cattle (L2 subgenotype)
H53	Neg.	CHRUN	France	Cattle	Cattle (L1 subgenotype)
H43	Pos.	GHESKIO	Haiti	*C. felis*	Cattle (L2 subgenotype)

[a] C, cattle isolate; H, human isolate.
[b] INRAT, Institut National de Recherche Agronomique de Tours; CHD, Centre Hospitalier de Dunkerque; CHRUL, Centre Hospitalier Régional Universitaire de Lille; HSLP, Hôpital Saint Louis de Paris; HHMC, Hôpital Henri Mondor de Créteil; CHRUR, Centre Hospitalier Régional Universitaire de Rennes; CHUA, Centre Hospitalier Universitaire d'Angers; CHUN, Centre Hospitalier Universitaire de Nantes; CHRUN, Centre Hospitalier Régional Universitaire de Nice; and GHESKIO, Groupe Haïtien d'Etudes sur le Sarcome de Kaposi et des Infections Opportunistes.
[c] HIV, human immunodeficiency virus; Neg., negative; Pos., positive.

each amplification run. The amplification reactions were initiated by denaturation of the DNA at 94°C for 5 min and were then subjected to 40 cycles of denaturation at 94°C for 30 s, annealing of the primer at 50°C for 30 s, and extension at 72°C for 30 s, with an additional 5-min extension at 72°C (PTC 200 thermocycler; MJ Research, Prolabo, France). The PCR product was analyzed by electrophoresis in a 2% agarose gel and was visualized after ethidium bromide staining. Amplified products were sequenced in both directions on an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.).

As our sequences, when aligned against the homologous *C. wrairi* sequence, showed polymorphism, a restriction fragment length polymorphism (RFLP)-based assay was developed. Purified PCR product was digested in a 20-μl mixture consisting of 1 U of *Mwo*I (New England Biolabs, Beverly, Mass.), 1 U of *Mlu*I (New England Biolabs), 1 U of *Bpm*I (New England Biolabs), 0.2 μl of 100× bovine serum albumin, and 2 μl of the appropriate 10× restriction buffer (NE buffer 3) under the conditions recommended by the supplier. The digestion mixture was incubated at 37°C for 2 h followed by 60°C for 2 h. The digest products were fractioned by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining.

The DNA diagnostic fragment characterized by Laxer et al. (22) was amplified by PCR from 1 bovine and 21 human isolates of *Cryptosporidium* previously typed by analysis of the 18S rDNA locus (Table 1). The expected size of the amplified product was 452 bp, and all isolates except *C. felis* isolates produced a single amplicon. PCR products were sequenced on both strands, and a multiple alignment was performed with the obtained sequences (Fig. 1). The alignment obtained with our sequences defined four groups of genotypes: one of these matched genotype 1, while another matched *C. meleagridis*. The third and fourth ones matched genotype 2. Two subgenotypes for genotype 2 of *C. parvum* were therefore found at the Laxer locus. They were designated L1 and L2 subgenotypes. Alignment against the original Laxer sequence (22) (accession number M59419) revealed other sequence variations (Fig. 1). Nine common bases of the newly reported sequences were different from the one previously reported. This included three insertions (T at position 54, A at position 341, and A at position 383), three deletions (at positions 152, 206 and 221), a single base mutation (G to C change at position 345) and a 2-base mutation (TA-to-AT change at positions 355 and 356). Most differences between Laxer's sequence and ours did not occur at critical internal diagnostic sites (probe or restriction site). However, two sequences out of the four reported here had a substitution at probe 127 and all had a deletion at probe 325 compared to Laxer's sequence (Fig. 1).

The four sequences presented here were also aligned with the *C. wrairi* sequence reported by Chrisp and LeGendre (6) (Fig. 1). DNA heterogeneity was exploited to identify polymorphic restriction enzyme sites in order to develop an RFLP assay for assessing polymorphisms. Genotyping was made by performing a triple digestion with *Mwo*I, *Mlu*I, and *Bpm*I. The sizes of the restriction fragments are given in Table 2. RFLP profiles (Fig. 2) were confirmed on 28 other human isolates: 12 were identified as genotype 1, while 16 were identified as genotype 2 (10 L1 and 6 L2 subgenotypes) (results not shown). All were in agreement with typing at the 18S rRNA gene locus (16).

In the present study, six human isolates identified as *C. felis*

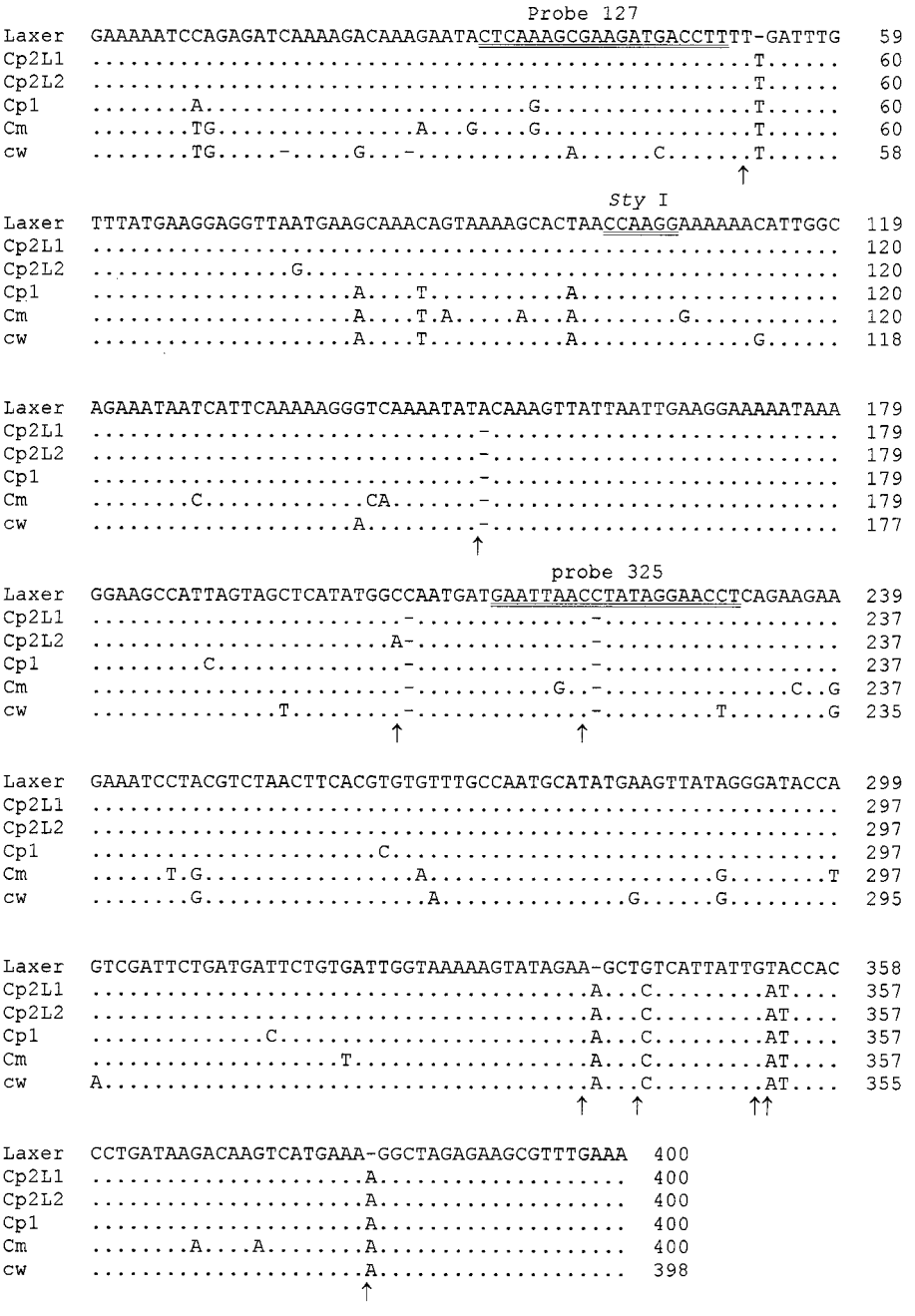

FIG. 1. Sequence alignment of the original sequence obtained by Laxer et al. (22) (Laxer), the *C. wrairi* sequence reported by Chrisp and Legendre (6) (cw), and the *Cryptosporidium* PCR diagnostic fragments obtained in this study for *C. parvum* genotype 2 (L1 subgenotype [Cp2L1] and L2 subgenotype [Cp2L2]), *C. parvum* genotype 1 (Cp1), and *C. meleagridis* (Cm). Dots denote nucleotides identical to those from the Laxer sequence, and dashes indicate deletions. Black arrows indicate mismatched nucleotides or gaps with the original sequence reported by Laxer. Double-underlined nucleotides on the sequence of Laxer et al. correspond to diagnostic probe 127, a *Sty*I restriction site, and diagnostic probe 325. Numbering is arbitrary.

on the basis of 18S rDNA sequences were included. A 452-bp fragment was amplified from two isolates (H22 and H43). For both, sequencing revealed a genotype matching genotype 2 (L2 subgenotype). By using Laxer's primers, we did not succeed in obtaining a positive PCR result from the DNA of the four remaining isolates, in spite of repeated attempts and in the absence of inhibitor.

Detection of *Cryptosporidium* spp. by Laxer's PCR-based assay. Numerous PCR methods for the detection of *Cryptosporidium* have been reported (12, 31, 47). The method of Laxer et al., which uses primers specific for an unidentified region, was among the first to be published (22). In their study, the authors selected a clone containing a 2.3-kb insert of *C. parvum* DNA according to its specificity for *Cryptosporidium* spp. upon screening the *Escherichia coli* transformants with DNA of *C. parvum*, *Giardia lamblia*, *Plasmodium falciparum*,

TABLE 2. RFLP (in base pairs) in the 452-bp PCR fragment of various *Cryptosporidium* organisms

Species	Sizes of restriction fragments			
	Single digestion			Triple digestion
	*Mwo*I	*Mlu*I	*Bpm*I	
C. parvum cattle L1 subgenotype	226, 188, 38			226, 188, 38
C. parvum cattle L2 subgenotype	414, 38			414, 38
C. parvum human genotype	226, 188, 38	284, 168		226, 130, 58, 38
C. meleagridis	226, 188, 38		397, 55	188, 171, 55, 38
C. wrairi	412, 38		395, 55	357, 55, 38

Toxoplasma gondii, and *Trichomonas vaginalis* by colony hybridization. After sequencing, they designed a primer set allowing the amplification of a 452-bp segment of the sequence containing a unique *Sty*I site. Two internal oligonucleotide probes, designed as probes 127 and 325, were also defined for confirming specificity by hybridization (Fig. 1). Following this work, other authors have used Laxer's primer set or the reported 452-bp sequence in order to develop other PCR-based diagnostic tests for *C. parvum* (1, 6–11, 14, 15, 17, 18, 20, 21, 23, 37, 38). These assays proved useful on a routine basis for detecting *C. parvum* parasites in livestock and humans. However, the utility of such methods seems now limited, since *C. meleagridis*, *C. felis*, and *C. muris* have been recognized in human infections (16, 19, 24, 26, 34, 36, 50).

In the present study, distinct sequences were obtained at Laxer's locus for *C. parvum* (for both genotypes 1 and 2) and *C. meleagridis*. Alignment of these sequences against the original one reported by Laxer et al. revealed nine differences between the newly reported sequences and Laxer's (Fig. 1). The likeliest explanation is that Laxer's sequence contains some errors. This hypothesis is supported by three previous studies in which the same differences were reported (6, 7, 11). In the first one, which targeted species differentiation, Chrisp and LeGendre attempted to adapt the PCR assay of Laxer et al. (22) for the specific detection of *C. wrairi* DNA and demonstrated variations between the two species (6). The internal probe used in the previously assay reported by Laxer et al. (probe 127) could not detect the amplified *C. wrairi* DNA. A new probe based on a sequence homologous to *C. parvum* and *C. wrairi* was successful in detecting both species, but their attempt to specifically recognize *C. wrairi* DNA using a specific oligonucleotide probe based on sequence differences was not successful. In the second study, das Graças C. Pereira et al. sequenced the Laxer locus for 11 animal-derived *Cryptosporidium* isolates and one environmental sample (7). As obtained sequences were very similar, the authors concluded that the Laxer marker did not appear to be sufficiently polymorphic to allow a reliable discrimination between *C. parvum* isolates. Alternatively, they proposed that all their isolates could belong to the same strain of *C. parvum*. The results presented in our study support the last hypothesis. In the third study, Deng et al. reported the existence of *C. parvum* in California sea lions (11).

In the present study, restriction analysis revealed different electrophoresis band patterns for genotype 1, genotype 2, and *C. meleagridis*. The sequence also predicts that *C. wrairi* generates a different band pattern. Results from human isolate genotyping for this locus were in agreement with those for the 18S rDNA locus (Table 1). Moreover, intragenotype variation was detected in the genotype 2 isolates. Two subgenotypes of *C. parvum* of cattle origin were identified. Both subgenotypes were retrieved in France, whereas only the L2 subgenotype was retrieved among 25 Haitian human isolates identified as genotype 2 of *C. parvum* at the 18S rDNA locus (data not shown).

Specificity of Laxer's protocol. The primer pair originally described by Laxer et al. produced no PCR fragment with *C. muris* (5, 7, 10, 37) or *C. baileyi* (5, 37) DNA. Champliaud et al. (5) reported that this primer pair and other seven primer pairs previously proposed to detect *C. parvum* cross-reacted with *C. meleagridis*. These authors found that *C. parvum* and *C. meleagridis* could not be differentiated even after RFLP of the 452-bp PCR product. However, Morgan and Thompson (30) suggested that the bird isolate used in the referenced study could in fact be an isolate of *C. parvum*. This hypothesis was probably right because (i) the study of Champliaud et al. (5) was the first attempt at molecular characterization of *C. meleagridis*; for this reason, the authors did not have any molecular data as reference, and (ii) further studies have confirmed the existence of a genotype specific to *C. meleagridis* at the 18S rDNA locus (42, 52), at the *Cryptosporidium* oocyst wall protein locus (34, 51), at the locus of thrombospondin-related adhesive protein of *Cryptosporidium* 1(34), and at the heat shock protein 70 locus (44). In none of these studies was *C. felis* DNA included to assess PCR.

The *C. felis* case. We succeeded in detecting *Cryptosporidium* DNA only in two isolates out of six found positive by PCR at the 18S rDNA locus. Curiously, genotyping revealed the identification of genotype 2 (L2 subgenotype). Our conclusion was that Laxer's primers were not specific of *C. felis* and that the amplification of genotype 2 DNA could be explained by coinfection. Actually, since the primer pair of Laxer was unable to

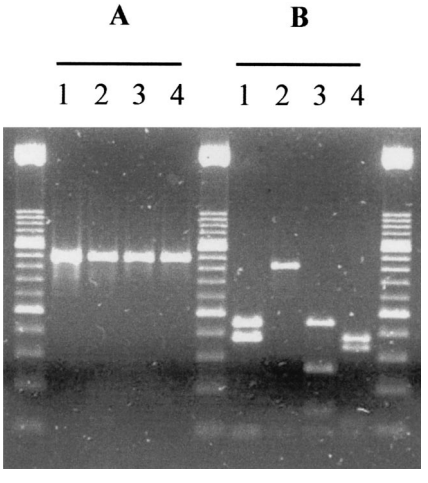

FIG. 2. Ethidium bromide-stained agarose gel of the 452-bp PCR product amplified from *C. parvum* genotype 2/L1 subgenotype (lane 1), *C. parvum* genotype 2/L2 subgenotype (lane 2), *C. parvum* genotype 1 (lane 3), and *C. meleagridis* (lane 4) before (A) and after (B) triple digestion with *Mwo*I, *Mlu*I, and *Bpm*I. Size markers are DNA molecular weight marker XIII (Boehringer Mannheim).

recognize the hybridization site on the *C. felis* DNA, only *C. parvum* DNA was amplified. According to the hypothesis of *C. parvum* and *C. felis* cocarriage, *C. parvum* should be present at a low level compared to *C. felis*. In fact, the proportion between the two species could be such that only *C. felis* DNA was amplified by PCR at the 18S rDNA locus. A competitive phenomenon with generic primers can explain this result. In consequence, direct sequencing of the 18S rDNA *C. felis* fragment produced a beautiful electrophoregram without any reading ambiguity. Contamination risks can practically be excluded as we routinely perform PCR experiments in our laboratory and have therefore developed strict procedures to avoid cross-contamination. Procedures included physical separation of the rooms set aside for DNA extraction, PCR medium preparation, and electrophoresis. In addition, individual samples are treated in independent experiments.

Conclusion. Findings of this study have practical implications on the use of Laxer's tool for detecting *Cryptosporidium* parasites. A positive PCR result with Laxer's primer pair does not necessarily imply the presence of *C. parvum* in the analyzed sample, as *C. meleagridis* DNA is also amplified at this locus. RFLP of PCR products should be performed to differentiate *C. parvum* and *C. meleagridis* (and *C. wrairi*) species as well as genotypes 1 and 2 of *C. parvum*. Furthermore, the specificity of Laxer's primers should be tested with the other genotypes of *C. parvum* as well as with the species *C. serpentis* and *C. nasorum*. As the used primers are not generic, the utility of this tool in the analysis of environmental or even clinical samples is therefore severely limited because of the narrow spectrum of *Cryptosporidium* species or genotypes that it can effectively detect.

Nucleotide sequence accession number. The sequences determined in this study have been published in the GenBank database under accession numbers AF400130 to AF400133.

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