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Detection and Differentiation of *Cryptosporidium* Parasites That Are Pathogenic for Humans by Real-Time PCR

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Cryptosporidiosis is a significant cause of food-borne and waterborne outbreaks of diarrheal diseases. To better understand the route of transmission of *Cryptosporidium* parasites, a number of genotyping techniques have been developed, based on PCR-restriction fragment length polymorphism or sequencing analysis of antigen, structural, and housekeeping genes. In this study, a real-time assay for the detection of *Cryptosporidium* oocysts is described. This technique had a detection limit of five oocysts. By melting curve analysis of PCR products with fluorescence-labeled hybridization probes, this technique was able to differentiate five common *Cryptosporidium* parasites that are pathogenic for humans in a single PCR. We evaluated and validated the test using samples from presently known *Cryptosporidium* parasites that are pathogenic for humans. This technique provides an alternative molecular tool in epidemiologic studies of human cryptosporidiosis.

Cryptosporidium organisms are protozoan parasites that infect various vertebrate hosts. At least 10 species are recognized: *C. parvum*, *C. baileyi*, *C. serpentis*, *C. muris*, *C. andersoni*, *C. wrairi*, *C. meleagridis*, *C. felis*, *C. canis*, and *C. saurophilum.* Within *C. parvum* there are also different genotypes, some of which may be independent species (5, 27). Each *Cryptosporidium* sp. or genotype has a different host specificity, but at least five types of *Cryptosporidium* have been found to infect humans: *C. parvum* human genotype, *C. parvum* bovine genotype, *C. meleagridis*, *C. felis*, and *C. canis* (in the order of prevalence) (26, 27). Thus, the identification of *Cryptosporidium* species and genotypes is important for the assessment of the public health importance of *Cryptosporidium* oocysts of animal or environmental origins and for the tracking of infection or contamination sources.

Presently, the identification of *Cryptosporidium* spp. and genotypes is made mostly by PCR-restriction fragment length polymorphism or sequencing analysis of antigen, structural, and housekeeping genes (1–4, 6–21, 23, 24, 29). These procedures are usually time-consuming. We describe here a LightCycler PCR for the real-time detection and species identification of *Cryptosporidium* parasites. The technique takes advantage of the well-characterized genetic polymorphism in the small-subunit (SSU) rRNA, utilizes fluorescence-labeled probes for real-time detection of *Cryptosporidium*, and incorporates a melting curve analysis of PCR products for the differentiation of *Cryptosporidium* spp. and genotypes that are pathogenic for humans.

MATERIALS AND METHODS

Parasite specimens. The *Cryptosporidium* parasites used in this study included *C. parvum* human, bovine, mouse, ferret, and marsupial genotypes; *C. wrairi*; *C. meleagridis*; *C. felis*; *C. canis*; *C. baileyi*; *C. andersoni*; and *C. serpentis*. All of the isolates used in this study were from naturally infected animals or humans, except for *C. meleagridis*, which was isolated from a turkey and passed through 1- to 2-week-old turkey poults. Identification of *Cryptosporidium* parasites was based on oocyst morphology, the infected host, and other traditional classification guidelines. All of the *Cryptosporidium* isolates used in the study were characterized previously at multiple genetic loci to confirm species and genotypes (22, 29, 30). *Eimeria tenella* and *Eimeria acervulina* from chickens and an *Isospora* sp. from a dog were used as controls. All stool samples containing *Cryptosporidium*, *Isospora*, and *Eimeria* oocysts were stored at 4°C in 2.5% potassium dichromate for less than 12 months before used in DNA extraction.

Oocyst isolation and DNA extraction. For most samples, stools containing oocysts were used in DNA extraction, with the exception of one *C. parvum* bovine genotype isolate (isolate 6), for which oocysts purified by sucrose-Percoll centrifugation were used in DNA extraction. DNA was extracted from oocysts or stool samples by alkaline digestion (100 μl of stool pellets in 33.3 μl of 1 M KOH and 9.3 μl of 1 M dithiothreitol at 65°C for 15 min, followed by neutralization with 4.3 μl of 25% HCl and 80 μl of 2 M Tris-HCl, pH 8.3), phenol-chloroform-isoamyl alcohol extraction (Invitrogen, Carlsbad, Calif.), and DNA purification using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, Calif.).

Real-time PCR. The most polymorphic region of the SSU rRNA gene (~820 bp) was amplified from samples by PCR carried out in a LightCycler (Roche Molecular Biochemicals, Indianapolis, Ind.), using *Cryptosporidium*-specific forward primer 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and reverse primer 5′-AAGGAGTAAGGAACAACCTCCA-3′ (29, 30). The PCR mixture contained 2 μl of Perkin-Elmer (Norwalk, Conn.) 10× buffer, 4 mM $MgCl_2$, 100 μM (each) deoxynucleoside triphosphate, 400 nM forward and reverse primers, 200 nM (each) hybridization probes (probe 1, 5′-CCGTCTAAAGCTGATAG GTCAGAAACTTGAATG-flourescein-3′; probe 2, 5′-LCred705-GTCACATT AATTGTGATCCGTAAAG-3′), 0.5 μl of nonacetylated bovine serum albumin (10 mg/ml), 1 U of *Taq* polymerase, and 1 μl of DNA template in a total of 20 μl. Probe 1 was based on the sequence that is conserved among all *Cryptosporidium* parasites, whereas probe 2 was a sequence of the *C. parvum* human genotype and was designed to hybridize to all intestinal *Cryptosporidium* parasites with various extents of mismatches.

In initial assays, the manufacturer-suggested Master Hybridization Probe Kit (Roche) was also used. Each PCR mixture was then subjected to 55 cycles of denaturation at 94°C for 2 s, annealing at 50°C for 10 s, and extension at 72°C for 15 s, with an initial denaturation at 95°C for 3 min. Detection of the fluorescent signal was made after each cycle's annealing phase. For the determination of detection sensitivity, serial dilutions were made from the DNA extracted from a stock concentration of 10,000 *C. parvum* bovine oocysts/μl. Oocysts of the *C. parvum* bovine genotype were used in the sensitivity determination because of the easy acquisition of a large number of purified oocysts, even though one of the probes used in detection had two base mismatches to the *C. parvum* bovine

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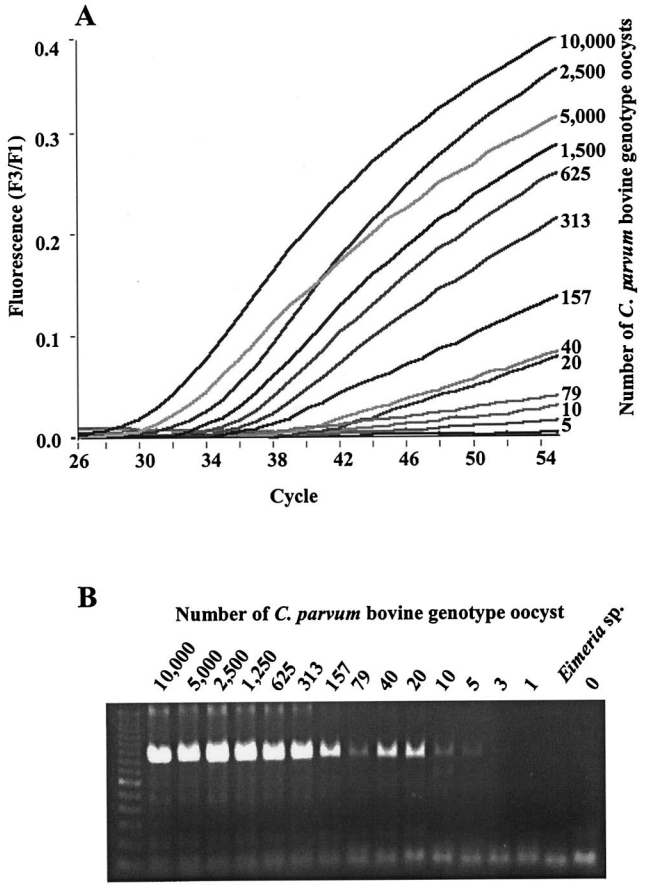

FIG. 1. (A) Real-time detection of fluorescent hybridization probes to PCR product formation of a 1:2 serial dilution of a *C. parvum* bovine standard from 10,000 oocysts per reaction down to a single oocyst. The detection limit is five oocysts. (B) Agarose electrophoresis gel stained with ethidium bromide. PCR products of serial dilutions of the *C. parvum* bovine genotype were visualized under UV light after ethidium bromide staining.

genotype sequence. In some experiments, the PCR products were harvested from the LightCycler capillaries by centrifugation of the inverted capillaries after caps were removed. The PCR products were then visualized by staining of 1.2% agarose electrophoresis gels with ethidium bromide to determine whether the amplified product was the proper band size and to compare the sensitivities of gel electrophoresis and fluorescence-tagged detection.

Species differentiation and genotyping by melting curve analysis. For identification of species and genotypes, known *Cryptosporidium* parasites as well as isolates of other apicomplexans were used in real-time PCR under the same conditions as described above. Species differentiation and genotyping were based on differences in melting temperatures of the PCR-probe complexes, which were determined by the extent of complementation of the probes to the target strand of PCR product. For the melting curve analysis, after the completion of the last PCR cycle, a quick denaturation was done at 95°C (0-s holding time), followed by a 30-s annealing step at 45°C with a slow ramp (0.1°C/s) up to 80°C with continuous detection throughout the ramp.

RESULTS

Most of the studies were done with the in-house master mixture described in Materials and Methods. With this master mixture, there was a direct relationship between the starting copies of the DNA template of the *C. parvum* bovine genotype and the cycle of PCR where detection of signal occurred (Fig. 1A). DNAs from the *Eimeria* spp. and the *Isospora* sp. never generated signals greater than background, demonstrating that the primers and probes are specific to *Cryptosporidium* spp. The electrophoresis gel for these samples (Fig. 1B) also showed no amplification for *Eimeria*, further supporting that the primers are *Cryptosporidium* specific. The detection limit for real-time PCR is about five oocysts of the *C. parvum* bovine genotype, which was about the same as that for gel electrophoresis.

Melting curve analysis showed that all PCR products of the *C. parvum* bovine genotype had the same melting curve profile irrespective of the amount of template used. The calculated melting temperatures for PCR products from the DNA of 3 to 10,000 oocysts were all around 59.3°C. Melting curve analysis further confirmed that the PCR product from five oocysts was *Cryptosporidium* specific, because it had the same melting temperature as other PCR products of the *C. parvum* bovine genotype. Melting temperatures calculated from different PCR runs were stable, with interassay variations generally smaller than 0.25°C (data not shown).

The magnesium chloride concentration, however, was found to directly affect the melting temperatures of the probes. As shown in Fig. 2, when the magnesium chloride concentration in the reaction tube was increased from 3 to 5 mM, a difference of greater than 1°C in melting temperature was observed with DNA from the *C. parvum* bovine genotype, no matter whether the commercial (in the Roche Master Hybridization Probe Kit) or the in-house PCR buffers were used. In subsequent studies, we used 4 mM magnesium chloride, because it gave the most consistent PCR amplification for all *Cryptosporidium* parasites (data not shown).

The real-time PCR did not detect the *Isospora* sp., the *Eimeria* spp., *C. andersoni*, or *C. serpentis.* However, DNA samples of all of the intestinal *Cryptosporidium* parasites tested resulted in signals in real-time PCR (Table 1). Agarose gel electrophoresis of the PCR products revealed that DNA samples of *C. andersoni* and *C. serpentis* generated PCR products of the expected size, even though no signals were seen in real-time PCR (Table 1). In contrast, DNA samples of the *Eimeria* spp. and the *Isospora* sp. were not amplified by the

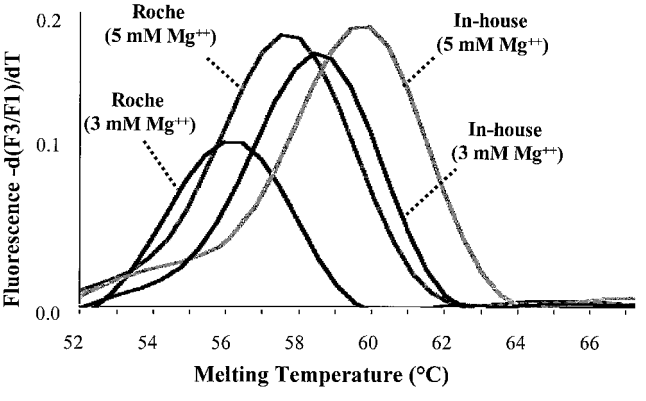

FIG. 2. Effect of magnesium chloride on melting temperature in two PCR buffer systems. Roche, amplification of the *C. parvum* bovine genotype using Roche's Master Hybridization Kit, with 3 or 5 mM $MgCl_2$. In-house, amplification of the *C. parvum* bovine genotype with the PCR master mix described in Materials and Methods, with 3 or 5 mM $MgCl_2$.

TABLE 1. Melting temperatures of the fluorescent hybridization probes for different isolates of *Cryptosporidium* parasites

Species/genotype	Sample	Origin	Result of PCR and gel electrophoresis	Melting temp (°C)
C. parvum/human	120	Human	+	66.50
C. parvum/human	503	Human	+	66.50
C. parvum/bovine	6	Bovine	+	59.30
C. parvum/bovine	11	Bovine	+	59.30
C. parvum/mouse	350	Mouse	+	59.20
C. parvum/marsupial	428	Kangaroo	+	56.60
C. parvum/ferret	351	Ferret	+	55.61
C. wrairi	517	Guinea pig	+	55.55
C. canis	244	Dog	+	58.04
C. canis	715	Dog	+	58.04
C. meleagridis	295	Turkey	+	62.40
C. felis	288	Cat	+	54.23
C. baileyi	764	Chicken	+	58.38
C. andersoni	20	Bovine	+	No detection
C. serpentis	63	Lizard	+	No detection
E. tenella	353	Chicken	−	No detection
E. acervulina	354	Chicken	−	No detection
Isospora sp.	865	Dog	−	No detection

PCR. There was a wide range of melting temperatures (54.23 to 66.50°C) for the probe set for different *Cryptosporidium* parasites. Different *Cryptosporidium* spp. and genotypes generally had different melting curves and melting temperatures (Fig. 3 and Table 1). The following exceptions were noticed: (i) *C. wrairi* and the *C. parvum* ferret genotype had similar melting temperatures, (ii) the *C. parvum* bovine and *C. parvum* mouse genotypes had similar melting temperatures, and (iii) the *Cryptosporidium* dog genotype and *C. baileyi* had a melting temperature difference of less than 0.5°C (Table 1).

DISCUSSION

The results of this study show the potential of real-time PCR in rapid diagnosis of human cryptosporidiosis. In preliminary evaluations, it detected and differentiated all common human *Cryptosporidium* parasites in a single PCR. It had sensitivity compatible to that of traditional PCR but had the advantage of real-time detection of PCR products and did not need restriction digestion or sequence analysis for species differentiation or genotyping. With the use of different fluorescent dyes, multiplex PCR potentially can be developed to allow simultaneous detection of multiple pathogens (*Cryptosporidium* and other enteropathogens). Thus, in clinical laboratories with real-time PCR machines, real-time PCR can be an ideal screening tool for the detection and genotyping of *Cryptosporidium* parasites in stool samples. The only requirement is the use of stools that have not been preserved in formalin, which is one of the commonly used stool preservatives but is problematic for all PCR-based diagnostic tools.

The use of a biprobe format for real-time detection of PCR products in this assay increases the specificity of the detection. The specificity of the detection came from the use of specific primers in the PCR and the use of specific probes in the real-time detection of PCR products. The specificity of primers used in this assay has been evaluated extensively, and they can amplify all *Cryptosporidium* parasites (25, 26, 28–30). The two probes used for the real-time detection added another layer of specificity, because they were chosen to be *Cryptosporidium* specific based on multiple alignment of SSU rRNA sequences of various apicomplexan parasites. The latter was confirmed by the failure to detect PCR products of *C. andersoni* and *C. serpentis* during real-time detection, even though the primers amplified DNAs of *C. andersoni* and *C. serpentis*. This is because one of the two probes, GTCACATTAATTGTGATCC GTAAAG, was designed to hybridize only with PCR products of intestinal *Cryptosporidium* parasites (*C. parvum*, *C. wrairi*, *C. meleagridis*, *C. saurophilum*, *C. felis*, *C. canis*, and *C. baileyi*, etc.). The use of melting curve analysis offers another layer of specificity of detection to the assay, because nonspecific PCR products are likely to have wrong melting temperatures even if they hybridize to the probes.

As shown in this study, most *Cryptosporidium* species and *C. parvum* genotypes could be differentiated from each other by melting curve analysis, judged by the melting temperatures calculated. The *C. parvum* human genotype had a melting temperature different from that of the *C. parvum* bovine genotype. Likewise, *C. meleagridis* and *C. felis* also had melting temperatures different from those of each other and other *Cryptosporidium* parasites. Thus, all five *Cryptosporidium* parasites that are pathogenic for humans (the *C. parvum* human and bovine genotypes, *C. meleagridis*, *C. felis*, and *C. canis*) could be differentiated from each other by melting curve analysis. A few other *Cryptosporidium* parasites had melting temperatures similar to those of the *Cryptosporidium* parasites that are pathogenic for humans. For example the *C. parvum* mouse genotype had a melting temperature similar to that of the *C. parvum* bovine genotype, and *C. baileyi* had melting temperature close to that of *C. canis*. These *Cryptosporidium* parasites have not been detected in humans, and thus this should not present a problem in real-time PCR analysis of clinical samples.

The melting curves of the PCR products from various *Cryptosporidium* parasites are directly related to the sequence diversity in the region of the SSU rRNA gene covered by the LCred probe. Because the probe sequence was based on the *C. parvum* human genotype, isolates of the *C. parvum* human genotype had the highest melting temperature (66.5°C) during

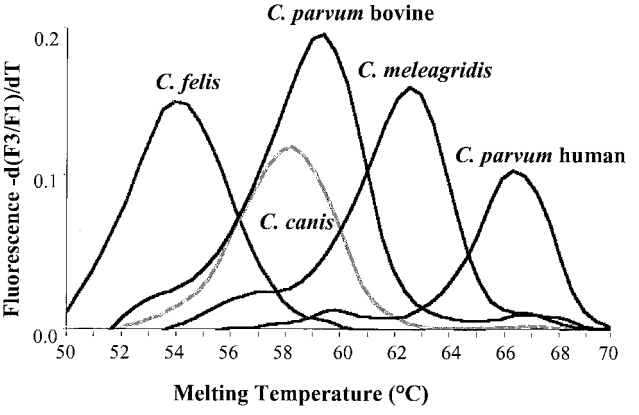

FIG. 3. Melting curve profiles of the fluorescent probes for the five *Cryptosporidium* parasites that are pathogenic for humans (*C. parvum* human and bovine genotypes, *C. meleagridis, C. felis*, and *C. canis*). The differences in the melting temperatures are due to DNA sequence polymorphism in the probing region of the SSU rRNA gene.

melting curve analysis. *C. meleagridis* had one base mismatch and thus had a somewhat lower melting temperature (62.4°C). The bovine and mouse genotypes of *C. parvum* had two mismatches and thus had even lower melting temperatures (59.2 to 59.3°C). *C. felis* had the most sequence divergence and thus had the lowest melting temperature (54.2°C). The position of mismatches also affected the melting temperature; *C. canis* had a melting temperature lower than those of the *C. parvum* bovine and mouse genotypes (58.0 versus 59.2 to 59.3°C), even though they all had two base mismatches with the probe sequence.

In summary, the real-time PCR developed in this study can detect and differentiate all five common *Cryptosporidium* parasites that are pathogenic for humans. The sensitivity was similar to that of conventional PCR, but the specificity was increased because of the use of two hybridization probes during the detection phase and melting curve analysis during the differentiation phase. It is possible to increase the sensitivity of detection by further standardization and reducing the size of amplicon. Real-time PCR has a more rapid turnaround time for reporting results and with further development can be quantitative, both of which will be important in investigations of waterborne outbreaks of cryptosporidiosis.

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