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Detection of *Cryptosporidium parvum* in Raw Milk by PCR and Oligonucleotide Probe Hybridization

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***Cryptosporidium* spp. are potential contaminants of food. Suspected cases of food-borne cryptosporidiosis are rarely confirmed because of the limited numbers of oocysts in the samples and the lack of sensitive detection methods adaptable to food. PCR was investigated as a means of overcoming this problem. A PCR assay was designed for the specific amplification of a previously sequenced portion of an oocyst protein gene fragment of *Cryptosporidium parvum* (N. C. Lally, G. D. Baird, S. J. McQuay, F. Wright, and J. J. Oliver, Mol. Biochem. Parasitol. 56:69–78, 1992) and compared with the primer set of Laxer et al. (M. A. Laxer, B. K. Timblin, and R. J. Patel, Am. J. Trop. Med. Hyg. 45:688–694, 1991). The PCR products were hybridized with digoxigenin-labeled internal probes and detected by chemiluminescence to enhance sensitivity. The two sets of primers were compared with regard to their sensitivity and specificity by using a variety of human and animal isolates of *C. parvum* and related parasites. Both assays enabled the detection of 1 to 10 oocysts in 20 ml of artificially contaminated raw milk. The assay based on the PCR set and probe of Laxer et al. detected DNAs from *Eimeria acervulina* and *Giardia intestinalis*. The new assay has good specificity for *C. parvum* bovine isolates and hence has a better potential for monitoring the prevalence of *C. parvum* in raw milk and other environmental samples.**

Cryptosporidium spp. are intracellular parasitic protozoa that are responsible for diarrheal illness in humans and many animals. In immunocompetent individuals, *Cryptosporidium parvum* usually causes short-term gastroenteritis that resolves spontaneously. However, young children with nutritional deficiencies and immunocompromised individuals, especially AIDS patients, may suffer from persistent life-threatening gastroenteritis (13).

The parasite is infective to a wide spectrum of hosts. *C. parvum* is the species mostly responsible for diarrheal diseases in mammals, including humans. The life cycle is completed within one host, and large numbers of infective oocysts are passed in the feces. Evidence of cross-transmission of *C. parvum* among mammals suggests the probability of zoonotic transmission to humans. Oocysts are environmentally resistant and are fully infectious when passed in the feces (37). As few as 10 oocysts can cause illness in infant macaques (29), and as few as 132 oocysts per individual have been reported as the infectious dose for healthy humans (14).

Human infections may be transmitted by livestock. In Canada and the United States, cattle, particularly dairy and beef calves, are commonly infected with cryptosporidia (25, 41, 42, 47). The widespread oocyst contamination on farms and in the environment provides many potential routes of infection. Waterborne outbreaks due to *C. parvum* have been reported. In the spring of 1993, separate outbreaks affected the communities of Kitchener, Ontario, Canada and Milwaukee, Wis. (32).

The reservoirs and transmission routes of *Cryptosporidium* spp. suggest a risk for human infection through contaminated food. Apple cider was the vehicle for transmission in a recent outbreak caused by contamination with oocysts in a farm environment (28). Livestock and their products, such as raw milk and meat products, have been identified as potential sources of infection (11, 16, 39). Indeed, epidemiological evidence has linked the consumption of raw milk and fermented dairy products made from raw milk with cases of cryptosporidiosis (4, 34, 36). However, the incidence of *C. parvum* in raw milk has not been determined, partly because of a lack of detection methods with suitable sensitivity and specificity. Contaminated water is also a risk to the food industry, since it is used for cleaning raw products or included in the products themselves.

The most common methods for the detection of oocysts in feces and in water samples are based on monoclonal antibody procedures. Direct or indirect immunofluorescence assays have been used extensively (6, 15, 24, 35, 38, 45, 48). For the routine examination of water samples, monoclonal antibodies have been used in flow cytometry (43). For increased sensitivity, Campbell et al. (10) developed a method for the enhanced chemiluminescent detection of monoclonal antibodies raised against oocysts, allowing the theoretical detection of 6.25 purified oocysts. These techniques have had limited application to foods because of the need to process large quantities to obtain enough oocysts for detection. Because of this limitation, more sensitive techniques are required to detect low-level contamination of foods by *Cryptosporidium* oocysts.

Food-borne cryptosporidial infections are probably underdetected because of the small numbers of oocysts present, the limited size of the samples, the difficulties encountered in concentrating oocysts, and the lack of in vitro culture techniques for augmentation of the number of oocysts. Recently, molecular techniques, such as PCR and DNA probing, were developed to study the parasite. By combining PCR amplification and DNA hybridization, as little as 30 fg of DNA or 20 oocysts of *C. parvum* can be detected (21, 23, 44, 46). However, the use of these techniques for detection of the organism in food has not previously been described. The detection of *Cryptosporidium* spp. in raw milk, in which the expected number of oocysts is small, is difficult. Therefore, concentration by filtration or centrifugation, combined with more sensitive detection methods, is required for accurate detection. The success of

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TABLE 1. Origin of the *Cryptosporidium* isolates used in this study

Isolate	Species	Host	Location
H-1	*Cryptosporidium* sp.	Human	California
H-2	*Cryptosporidium* sp.	Human	California
H-P1	*Cryptosporidium* sp.	Human	Ontario, Canada
3332	*Cryptosporidium* sp.	Human	Ontario, Canada
H-6	*C. parvum*	Human	France
E-P1	*Cryptosporidium* sp.	Equine	Ontario, Canada
B-4	*Cryptosporidium* sp.	Bovine	Ontario, Canada
B-5	*Cryptosporidium* sp.	Bovine	Ontario, Canada
B-P1	*Cryptosporidium* sp.	Bovine	Ontario, Canada
B-P2	*Cryptosporidium* sp.	Bovine	Ontario, Canada
B-P3	*Cryptosporidium* sp.	Bovine	Ontario, Canada
B-1	*C. parvum*	Bovine	Maryland
B-2	*C. parvum*	Bovine	Georgia
B-3	*C. parvum*	Bovine	Iowa
B-6	*C. parvum*	Bovine	Arizona
B-7	*C. parvum*	Bovine	Arizona
B-8	*C. muris*	Bovine	Idaho

applying PCR to isolates from raw milk depends on the efficiency of the recovery of oocysts and the purification of oocyst DNA.

We describe a new assay for the sensitive detection of *C. parvum* in raw milk, combining procedures for concentration of oocysts from raw milk, DNA purification, and the use of a new PCR primer set and an internal digoxigenin-labeled oligonucleotide probe. Comparisons of the new assay with an assay based on the PCR set and oligonucleotide probe of Laxer et al. (23) were made in terms of the sensitivity for oocyst detection in pure oocyst suspensions and in raw milk and the specificity for isolates of *Cryptosporidium* spp. and other protozoa. The new primer set (21) targets a portion of the gene fragment CpR1, an oocyst protein gene, sequenced by Lally et al. (22). The product is hybridized with a digoxigenin-labeled oligonucleotide and detected with a chemiluminescent substrate.

MATERIALS AND METHODS

Parasites. Isolates of *Cryptosporidium* spp. were obtained from infected animals or humans. In this study, 17 isolates of *Cryptosporidium* spp. were analyzed (Table 1). They were used for the specificity tests, together with *Eimeria acervulina* (University of Guelph strain), *Eimeria bovis* (generously provided by Michael White, Montana State University, Bozeman), *Neospora cauinum* (NC-1 isolate), *Giardia intestinalis* ATCC 50184, *Entamoeba histolytica* ATCC 50007, *Toxoplasma gondii* ATCC 40050, and calf DNA (Sigma, Mississauga, Ontario, Canada). *G. intestinalis* and *E. histolytica* were grown as recommended by the American Type Culture Collection and then subjected to DNA extraction. The DNA of *T. gondii* was extracted directly.

Oocyst purification. Oocysts were concentrated and purified by discontinuous-density sucrose gradient centrifugation (5). The oocysts were counted with a hemacytometer and diluted to standard concentrations.

Specificity analysis. For the specificity analysis, the DNA was extracted from large numbers of oocysts. Quantities of DNA corresponding to 10^4 oocysts of the 17 *Cryptosporidium* isolates were used. DNAs from *Eimeria acervulina*, *Eimeria bovis*, *G. intestinalis*, *E. histolytica*, *T. gondii*, and calves were used at a final concentration of 10 ng in PCR. For *Neospora cauinum*, a DNA concentration corresponding to 10^4 merozoites was used.

Sensitivity analysis in water and in milk. For the sensitivity test in water, 1-ml dilutions containing 100, 50, 10, 5, or 1 oocyst of isolate B-1 were used. The sensitivity tests in raw milk involved trials composed of five samples of 20 ml of raw milk inoculated with 100, 50, 10, 5, or 1 oocyst of isolate B-1. The artificially contaminated milk (20 ml) was treated with 1 ml of Bacto-Trypsin reconstituted as specified by the manufacturer (Difco, Detroit, Mich.) and 5 ml of Triton X-100 for 30 min at 50°C and then centrifuged at 5,000 × *g* for 10 min. The supernatant was discarded, and the pellet was washed twice with distilled water.

DNA extraction and purification. Concentrated oocysts from milk samples and water suspensions and oocysts from the isolates tested were resuspended in 100 μl of lysis buffer (120 mM NaCl, 10 mM EDTA, 25 mM Tris [pH 7.5], 1% sarcosyl) containing 5 mg of proteinase K per ml. The oocysts were ruptured and the sporozoites were lysed by freeze-thaw cycles as described by Kim et al. (20). The vials were immersed in liquid nitrogen for 5 min an then in water at 65°C for 5 min. The number of cycles was increased from 5 to 10 to release more DNA. After the freeze-thaw breakage, an additional 5 mg of proteinase K per ml was added and the lysate was digested for 60 min at 55°C (20). The DNA was purified with the Isogene kit (Perkin-Elmer Cetus, Norwalk, Conn.) and diluted in water to a final volume of 60 μl.

PCR. A new PCR assay was developed (assay A) and compared with an assay based on the published primer set of Laxer et al. (23) (assay B). The PCR primers of assay A were developed on the basis of the published sequence of gene fragment CpR1 (22) (GenBank accession number M95743); they amplify a 358-base sequence (positions 527 to 884). The sequences of the primers were compared with the GenBank database for sequence homology. Assay A was compared with assay B, in which the primer set of Laxer et al. (23) amplifies a 452-base sequence of a segment of their plasmid pHC1 containing a 2.3-kb insert of *C. parvum* DNA (Table 2).

The PCR mixture for specificity tests (final volume, 50 μl) in reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl) consisted of 0.2 mM deoxynucleoside triphosphate mixture, 0.08 μM each primer, 1% dimethyl sulfoxide, and the DNA sample corresponding to 10^4 oocysts of *Cryptosporidium* spp., 10^4 merozoites of *Neospora cauinum*, or 10 ng of the other organisms tested. The mixture was held at 70°C for 3 to 5 min, 1 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany) was added, and the mixture was overlaid with mineral oil. For the sensitivity test, 60 μl of the DNA sample obtained after extraction and purification was used in a final volume of 100 μl of the PCR mixture.

In an automated DNA thermal cycler (model 480; Perkin-Elmer Cetus), samples with the primer set of assay A were denatured at 94°C for 1 min, annealed at 45°C for 1 min, and extended at 72°C for 1 min. This cycle was repeated 40 times, and the samples underwent a final elongation at 72°C for 2 min. Samples with the primer set of assay B were treated in the same way but with an annealing temperature of 52°C.

The amplified fragments were separated by electrophoresis in a 1.3% agarose gel in Tris-acetate buffer (0.04 M Tris acetate, 0.001 M EDTA [pH 8.0]) for 1 h at 50 V, stained with ethidium bromide (0.2 μg/ml), and photographed on a UV transilluminator.

Confirmation of PCR products by DNA hybridization. After amplification, 80% of the volume of the reaction mixture was denatured for 5 min in boiling water and chilled on ice. An equal amount of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was added. The mixture was transferred to a positively charged nylon membrane (Boehringer) by using a well filtration man-

TABLE 2. Primers and oligonucleotide probes for the detection of *C. parvum*[a]

Assay	Oligonucleotide	Positions	Sequence[b]
A	Forward primer	527–548	5′ GCC CAC CTG GAT ATA CAC TTT C 3′
	Reverse primer	861–884	5′ TCC CCC TCT CTA GTA CCA ACA GGA 3′
	Internal probe	645–669	5′ GAT CGA TGC TAT CTG CCC AGA TGG A 3′-DIG
B	Forward primer	444–469	5′ CCG AGT TTG ATC CAA AAA GTT ACG AA 3′
	Reverse primer	870–895	5′ TAG CTC CTC ATA TGC CTT ATT GAG TA 3′
	Internal probe (probe 127)	501–520	5′ CTC AAA GCG AAG ATG ACC TT 3′-DIG

[a] The primers and the oligonucleotide probe of assay A were designed to amplify a 358-base sequence of the gene fragment CpR1 reported by Lally et al. (22) (GenBank accession number M95743) and to hybridize to an internal portion of the amplified sequence. Assay B is based on the primers and probe 127 reported by Laxer et al. (23) (GenBank accession number S74588). The primers amplify a 452-base sequence, and the oligonucleotide probe (probe 127) hybridizes to an internal portion.

[b] DIG, digoxigenin.

TABLE 3. Specificity of assay A and assay B for *Cryptosporidium* spp. and other organisms by PCR amplification and hybridization with oligonucleotide probes[a]

Isolate	Assay A[b]		Assay B[b]	
	PCR product	Hybridization signal	PCR product	Hybridization signal
Human				
Cryptosporidium sp. (H-1)	++	+	++	−
Cryptosporidium sp. (H-2)	+	+++	+	+
Cryptosporidium sp. (H-P1)	−	−	ND	ND
Cryptosporidium sp. (3332)	++	+	ND	ND
C. parvum (H-6)	++	+++	++	++
Cryptosporidium sp. (E-P1)	+	+++	ND	ND
Cryptosporidium sp. (B-4)	++	+++	++	+++
Cryptosporidium sp. (B-5)	++	+++	++	++
Cryptosporidium sp. (B-P1)	++	+++	ND	ND
Bovine				
C. parvum (B-1)	++	+++	+	++
C. parvum (B-2)	++	+++	++	++
C. parvum (B-3)	++	+++	++	++
C. parvum (B-6)	++	+++	ND	+++
C. parvum (B-7)	++	++	ND	+++
C. muris (B-8)	−	−	ND	−
Giardia intestinalis	−	−	−	+
Entamoeba histolytica	−	−	−	−
Toxoplasma gondii	−	−	−	−
Eimeria acervulina	−	−	++	++
Eimeria bovis	−	−	ND	ND
Neospora sp.	−	−	ND	ND
Calf DNA	−	−	ND	ND

[a] Amplified fragments were separated by agarose gel electrophoresis. Agarose gels (0.8% agarose) were stained with ethidium bromide and viewed under UV light. The hybridizations were detected by a chemiluminescence assay.

[b] +++, very strong hybridization signal; ++, strong hybridization signal; +, weak hybridization signal; −, hybridization signal not visible; ND, not determined.

ifold (Hybri Dot; GIBCO-BRL, Gaithersburg, Md.) with a transfer time of 30 min. DNA was cross-linked on the membrane by exposure to UV light for 5 min.

The PCR products of the primer set of assay A were hybridized with a 25-base oligonucleotide probe homologous to an internal portion of the amplified fragment. The sequence of the designed probe was compared for homology with the GenBank database. The PCR products from the primers of assay B were hybridized with the 20-base oligonucleotide internal probe described by Laxer et al. (23) (probe 127) (Table 2). The probes were labeled at the 3′ end with digoxigenin-11-dUTP, using the digoxigenin oligonucleotide-tailing kit (Boehringer).

The probe of assay A was hybridized as follows. The membrane was prehybridized for 30 min at 50°C in 30 ml of prehybridization solution (5× SSC, 2% [wt/vol] blocking reagent [no. 1096 176; Boehringer], 0.02% [wt/vol] sodium dodecyl sulfate, 0.1% [wt/vol] lauryl sarcosine) and hybridized for 4 h at 50°C in 2 ml of hybridization solution containing 50 pmol of probe. Probe 127 (assay B) was hybridized in the same way, using a prehybridization step of 1 h at 42°C and a hybridization step of 4 h at 42°C, which was determined experimentally to produce a clearer signal with *C. parvum* B-1 DNA.

The hybridization signals were developed as described by Boehringer Mannheim Biochemica (8) with AMPPD and chemiluminescence was detected with a BIQ Bioview biomedical image quantifier (Cambridge Imaging Ltd., Cambridge, United Kingdom).

DNA from *C. parvum* B-1 was used as a positive control in the PCR and hybridization assays.

RESULTS

Specificity tests. A small-scale study was conducted to compare assay A with assay B. Both assays amplified specific fragments of the expected sizes (358 and 452 bp, respectively). The primer sets and internal probes of both assays could detect variability in the intensity of the hybridization signal among human *Cryptosporidium* isolates; however, both assays consistently detected the bovine *Cryptosporidium* isolates. Among the other protozoa tested in the comparative study, *Eimeria acervulina* was amplified by the primer set of assay B, and the internal probe could detect the amplified DNA of *Eimeria acervulina* and the DNA of *Giardia intestinalis*. Assay A did not detect DNA from other protozoa tested (Table 3).

More extensive investigations were performed with assay A. The primer set of assay A amplified all *Cryptosporidium* isolates except the human isolate H-P1 and *C. muris* B-8, in which no amplification was observed. The H-P1 isolate was not determined to the species level and may not have been a *C. parvum* strain. Unfortunately, there was insufficient sample to determine if this isolate was detectable by assay B. The intensity of amplification was variable among human isolates, and a lower amplification resulted from the equine isolate (E-P1). No amplification was observed with DNA from sources other than *Cryptosporidium* spp. The internal probe of assay A hybridized with all samples in which specific fragments of DNA were amplified. Variable intensities of hybridization were observed among the human isolates, but the hybridization signals were of comparable intensities among bovine isolates. Hybridization with the equine isolate generated a strong signal. The internal probe did not hybridize with any of the other protozoa studied (Table 3). The variability in the intensity of the hybridization signal observed with the human isolates may have been due to many factors including variations in DNA concentrations in the assays and differences in the stringency of the hybridization reaction.

Sensitivity tests. Both PCR sets allowed sensitive detection of *C. parvum* oocysts (isolate B-1) in pure suspension and in milk. In pure solution, the amplified fragment from only one oocyst was observed in few cases. In both assays, however, the concentration of the PCR products was often insufficient for their detection on the ethidium bromide-stained agarose gel. Hybridization with the oligonucleotide internal probes im-

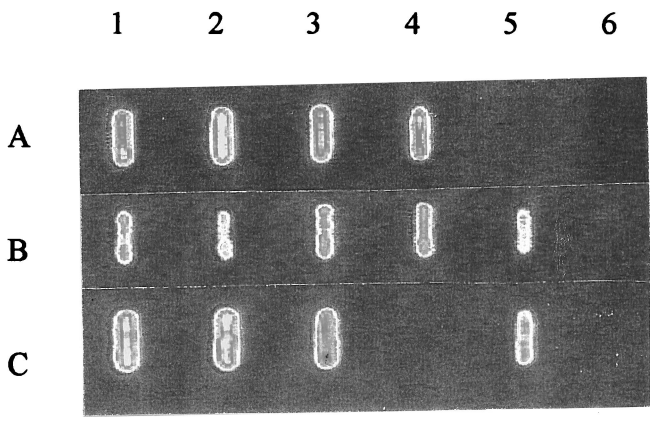

FIG. 1. Chemiluminescent detection of *C. parvum* for the sensitivity test by PCR amplification and slot blot hybridization by assay A. (A) Sensitivity in water suspension; (B and C) sensitivity in 20 ml of raw milk (two trials). Slots 1 through 5 correspond to 100, 50, 10, 5, and 1 oocyst of *C. parvum* B-1, respectively; slot 6 contains negative controls.

proved the sensitivity of the assay and enabled the detection of amplified fragments that were invisible on the gel (Fig. 1).

PCR detection of oocyst DNA in raw milk rarely resulted in consistent amplified products visible on an agarose gel. The hybridization with oligonucleotide probes increased the sensitivity of the assay and generated unambiguous signals. The assays could detect consistently 10 oocysts per 20 ml of raw milk and, in some cases, as few as 5 and 1 oocysts per 20 ml of raw milk (Fig. 1).

DISCUSSION

An ideal assay for the detection of oocysts of *C. parvum* in milk requires a relatively high specificity to detect only the organism of interest and a high sensitivity to allow the detection of few oocysts in large volumes, and it should include a sample process easily adaptable to milk, taking into account the large numbers of substances that can potentially interfere. Assay A, combining PCR amplification and DNA probe hybridization, was designed to address these specifications. The assay was investigated for its potential use in raw milk and was compared with assay B based on the published work of Laxer et al. (23) in terms of the specificity and sensitivity of the two assays.

The specificity tests revealed differences between the two sets of primers under comparison. The assay based on that of Laxer et al. (23) (assay B) was significantly less specific than assay A, since it amplified DNA from *Eimeria acervulina*, and detected the DNA of *Giardia intestinalis*. The genus *Eimeria* belongs to the same subclass as the genus *Cryptosporidium*, i.e., Coccidiasina, and must share DNA homology in the region amplified by the primer set of assay B. The DNA of *G. intestinalis* did not generate a visible band after PCR amplification. However, probe 127 hybridized with the PCR products or the template DNA. In their specificity testing, Laxer et al. (23) reported *G. lamblia* J-156, the former name for *G. intestinalis*, as being unamplified by their primer set and undetected by Southern blotting with probe 127. This different result could be due to strain variation or could suggest the presence of the analogous sequence in an unamplified region. *G. intestinalis* has been reported as a potential food-borne pathogen transmitted through raw foods (11, 39). Assay B based on that of Laxer et al. (23) therefore might not be suitable for the specific detection of *Cryptosporidium* spp. in food.

Ranucci et al. (33) cloned a DNA fragment encoding an oocyst protein (COWP-190) of *C. parvum*. The first 2,359 bp of their clone was identical to the DNA sequence reported by Lally et al. (22). They developed a PCR assay targeting the COWP-190 gene, which partially overlaps the sequence detected in our assay. Ranucci et al. (33) speculated that the COWP-190 gene was conserved among bovine and human isolates. However, this suggestion was based on the analysis of only three *C. parvum* isolates. DNA from *G. lamblia*, *P. falciparum*, or *Sarcocystis* spp. was not amplified by the PCR assay of Ranucci et al. (33).

Wagner-Wiening and Kimmig (44) described a PCR assay for *C. parvum* that was also based on amplification of a fragment of the oocyst protein gene sequenced by Lally et al. (22). However, they amplified an 873-bp sequence corresponding to positions 397 to 1294, but the nested primers they used targeted the same region (positions 526 to 1149) as that used in the present study. Good specificity was reported, but no other species of *Cryptosporidium* and no species of *Eimeria* were tested by Wagner-Wiening and Kimmig (44). Also, the *C. parvum* oocysts that they analyzed were from naturally infected calves, and no human isolates were examined. Use of the nested PCR allowed detection of 10 sporozoites, but this proved unreproducible.

All *C. parvum* isolates from our collection, including the *C. parvum* human isolate (H-5) and all bovine *Cryptosporidium* isolates, were detected by the primer set of assay A. However, variability in the intensity of reactions among human isolates was observed, and this included one human isolate (H-P1) that was not amplified by the PCR primer set. In addition, the equine isolate (E-P1) was poorly amplified by the PCR set. The human isolates under study were obtained from various regions in the United States, Canada, and France. Four of those isolates, including the H-P1 strain that could not be detected by assay A, were not identified at the species level. The DNA variation among human isolates is probably caused by strain variations within *C. parvum*, the species known to commonly infect humans. Such variations based on host and geographical location have been reported previously. In a recent study, Morgan et al. (30) analyzed the genetic diversity of *C. parvum* isolates by the randomly amplified polymorphic DNA method. They found that *Cryptosporidium* isolates could be divided into two main groups, one comprising only isolates of human origin and the other including all the bovine and three human isolates. A restriction fragment length polymorphism analysis conducted by Ortega et al. (31) revealed differences between isolates from human and bovine sources originating from different geographical areas, indicating the possible presence of a subspecies of *C. parvum*. Geographical variations within the 18S rRNA gene sequence were also reported for *C. parvum* isolates. Kilani and Wenman (19) compared the 18S rRNA gene of their *C. parvum* isolate with the two fully and partially sequenced 18S rRNA genes of previously reported *C. parvum* isolates (9, 17) originating from different geographical areas and revealed 3 and 8.4% sequence variations. In addition, variations in sporozoite protein patterns were distinguished by two-dimensional gel electrophoresis among *C. parvum* isolates from samples of human, horse, and calf origin (27).

Neither of the two assays used in this study could detect *C. muris*. This species has been found in beef and dairy cattle and causes abomasal cryptosporidiosis (1–3). This may indicate that the assays are specific for *C. parvum*. Chrisp and LeGendre (12) reported additional studies, using the assay described by Laxer et al. (23), on the detection of *C. wrairi*. The primer set generated a fragment of the correct size, but probe 127 could not detect the amplified DNA. Other DNA varia-

tions among species have been reported. In a karyotype study conducted by Mead et al. (26), *C. parvum* was differentiated from *C. baileyi* by a distinct banding pattern of chromosome-sized DNA. No differences were noted among *C. parvum* isolates. Furthermore, the sequence homology calculations show that over 99% of the 18S rRNA gene of *C. parvum* is identical to that of *C. muris*. The sequence variations, however, were similar to those exhibited by four clones of *C. parvum* (9). Awad-El-Kariem et al. (7) exploited the small variations within the 18S rRNA gene to differentiate *C. parvum* from *C. muris* and *C. baileyi* by PCR combined with digestion of the products by restriction endonucleases.

The specificity analysis indicates that assay A is a more appropriate detection tool than assay B for the evaluation of the prevalence of *C. parvum* in raw milk. All the bovine isolates, the more likely type to be retrieved in raw milk, were detected, and no cross-reaction was observed with *C. muris* and the other protozoa under study.

Both assays allowed sensitive detection of *C. parvum* oocysts in pure suspension. A clarification procedure was necessary for good recovery from raw milk. Some of our preliminary work for the investigation of centrifugation as a means of oocyst recovery indicated an interaction of oocysts with milk fat. Observation by epifluorescence microscopy, using the immunofluorescence assay Merifluor kit (Meridian Diagnostics Inc., Cincinnati, Ohio), indicated that even after centrifugation, large numbers of oocysts remained in the fat (results not shown). Treatment with Bacto-Trypsin and Triton X-100 not only resulted in a more manageable pellet, but also prevented the oocysts from interacting with the fat layer, thus increasing the efficiency of the recovery.

When dealing with raw milk, the limiting factor to achieving a sensitive assay was the lack of reproducibility when few oocysts were present in the sample. The numerous steps prior to PCR, involving washing and resuspension of the pellets, increased the risk of material loss. Theoretically, only one copy of DNA, including the sequence to be amplified, is necessary for detection by PCR. Oocysts of *Cryptosporidium* spp. contain four genomic copies, one in each sporozoite. The use of a sequence that is present in multiple copies in the genomic DNA provides greater sensitivity. The use of sequences from the rRNA-encoding genes may give a similar advantage. Taghi-Kilani et al. (40) identified, cloned, and characterized three tandemly repeated 5S rRNA-encoding genes. Awad-El-Kariem et al. (7) developed a PCR assay on the basis of 18S rRNA-encoding gene sequences. They did not calculate the number of gene copies available or test the sensitivity of their assay. Johnson et al. (18) designed another PCR assay targeting 18S rDNA of *C. parvum*. Specificity was evaluated with a broad spectrum of bacterial and protozoal DNAs, and no foreign DNA was amplified by the assay. However, specificity within the genus *Cryptosporidium* was not evaluated. Sensitivity tests were performed with purified oocysts only. When preparations less than 2 months old were tested, one oocyst could be detected. The use of rRNA-encoding genes may have a greater potential for sensitive detection by PCR in food and environmental samples. Many regions of those genes, however, are strongly conserved among related organisms and hence render the choice of the stretch to be amplified very critical for the specific detection of *C. parvum.*

Assay A, combining PCR amplification and oligonucleotide probe hybridization, offers good potential for evaluation of the prevalence of *C. parvum* in raw milk. The assay detected all bovine *Cryptosporidium* isolates, excluding the isolate of *C. muris*, and did not detect the other protozoa under study. The sensitivity reached was as low as 1 to 10 oocysts in 20 ml of raw milk. Given that the infectious dose of *C. parvum* may be as low as 10 oocysts (14), this level of sensitivity would allow the detection of a potentially harmful number of oocysts in a single serving (approximately 250 ml) of milk.

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