APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1996, p. 643–647 Vol. 62, No. 2
0099-2240/96/$04.00+0

Simplified Method for Recovery and PCR Detection of *Cryptosporidium* DNA from Bovine Feces†

XIGANG LENG,[1] DEREK A. MOSIER,[1] AND RICHARD D. OBERST[1,2]*

Department of Diagnostic Medicine-Pathobiology,[1] and Food Animal Health and Management Center,[2] College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506-5606

Received 16 October 1995/Accepted 5 December 1995

An assay to identify *Cryptosporidium* DNA in bovine feces has been developed emphasizing standardization of sample preparation and simplification of the DNA recovery process for PCR amplification and DNA hybridization detection. The *Cryptosporidium* DNA recovery-PCR detection procedure (CR-PCR) can recover DNA suitable for PCR amplification without using or generating hazardous chemicals or wastes. In comparisons with a commercial enzyme-linked immunoassay (Color Vue-*Cryptosporidium*; Seradyn, Indianapolis, Ind.), the CR-PCR could detect 10^3 to 10^4 times fewer purified oocysts diluted in solution (water or buffered saline) and 10^2 times fewer oocysts from diarrheic feces and showed earlier detectability from solid, nondiarrheic feces in an experimental infection. This assay may prove useful for detecting *Cryptosporidium* oocysts in feces and in clarifying the role of livestock in waterborne outbreaks of cryptosporidiosis.

The coccidian *Cryptosporidium parvum* infects the intestinal epithelia of humans and many animals, causing mild, self-limiting disease in immunocompetent individuals. However, severe, life-threatening diarrhea can occur in immunocompromised individuals (4, 6–8, 12, 15, 17, 20, 35). Environmentally resistant *Cryptosporidium* oocysts are passed in feces and are acquired by ingestion. Contaminated water is associated increasingly with cryptosporidiosis (7, 28, 37), even when the water meets treatment standards (25). This is due to the resistance of the oocysts to routine water treatments and chemical disinfectants and the low dosage required for infection (13, 29).

Current laboratory protocols for identification of *Cryptosporidium* oocysts in fecal or environmental specimens include microscopic examination of smears stained with Giemsa stain, the modified Ziehl-Nielson technique, the modified Kinyoun acid-fast technique (10, 18, 41), or use of immunofluorescence (16, 38, 42). A reverse passive hemagglutination assay with sensitivity comparable to that of immunofluorescence detection (IFA) has also been described; however, not all oocysts are reactive (14). The IFA is often utilized for detection (3, 19, 21, 34, 38–40, 44, 46); however, the sensitivity of IFA can be influenced by oocyst concentration techniques and extremes in the consistency of the feces (44) or by including a wash process for fecal suspensions prior to staining (46).

Enzyme immunoassays (EIAs) have also been used for coprodiagnosis of human cryptosporidiosis (1, 3, 38, 40, 43). However, these assays have limited sensitivity for detecting low-level asymptomatic *Cryptosporidium* infections or for evaluating dilute environmental samples (3, 31). Differences in sensitivity, specificity, and positive predictive values among different EIAs may be a consequence of the lack of a concentration step for some (1, 38). An EIA was reportedly more sensitive than an IFA at very low concentrations (1 to 10 oocysts per liter of water), and the increased sensitivity was attributed to the ability of the former assay to detect antigens from partial or incomplete oocysts (40).

Difficulties in detecting and enumerating oocysts in fecal specimens are compounded by variation in consistency between individual fecal specimens, the amount of specimen used, and oocyst losses incurred during recovery processes (5, 34, 44). Similarly, oocyst recovery efficiencies from raw water samples are reported to range from 42.0 to 89.9%, and 5- to 50-fold recovery differences occur between laboratories and within a single laboratory by standardized recovery procedures (2) on spiked water samples with known numbers of oocysts (11, 25, 26, 32).

The ability of PCR to amplify suspect DNA thousands of times makes it a logical method for detecting *Cryptosporidium* DNA (19, 23, 24, 45). In this study, we report a technique to standardize the preparation of bovine feces for *Cryptosporidium* DNA recovery, PCR amplification, and DNA hybridization detection.

MATERIALS AND METHODS

Collection and production of secondarily purified oocysts. Neonatal calves were infected orally with ≈10^8 *C. parvum* oocysts. To compare and contrast EIA and CR-PCR detection assays, diarrheic feces were collected from a calf (K-14) on days 4 and 7 postinfection and from another calf (K-17) on a daily basis (days 0, 1, 2, 3, 4, 6, and 8 postinfection). Oocyst shedding was estimated by microscopic examination of unstained formalin-fixed samples from the original specimens. The original specimens were then processed as described below. To produce purified oocysts, diarrheic feces were collected and mixed with equal volumes of 2.5% potassium dichromate solution, and the mixtures were stored at 4°C. Oocysts were concentrated from fecal material by a series of sedimentation, filtration, and centrifugation steps (22, 36). Final cleaning of oocysts was by centrifugation of concentrated material on sucrose density gradients, which was followed by washing and storage in phosphate-buffered saline (PBS) at 4°C. Gradients were constructed by placing 5 ml of primary cleaned oocysts onto a sucrose cushion (10 ml of 1.064 g of sucrose per liter that overlay 10 ml of 1.103 g of sucrose per liter) and were centrifuged (1,500 × *g*, 25 min). Oocysts were collected from the resulting interface. Purified oocyst concentrations were enumerated with a hemocytometer.

EIAs. The EIAs were performed with unconcentrated fresh fecal specimens according to the instructions of the manufacturer (Color Vue-*Cryptosporidium*; Seradyn, Indianapolis, Ind.). Aliquots from the original fecal specimen were diluted 1:5 (≈1 g of feces to 4 ml of diluted wash concentrate), mixed well, and sedimented (≈15 min). A 50-μl aliquot of the resulting supernatant was recovered from each sample and placed in a test well. The plates were developed as per the manufacturer's instructions and were read spectrophotometrically. A_{450} readings of >0.25 and <0.25 were interpreted as positive and negative reactions, respectively.

* Corresponding author. Mailing address: Food Animal Health & Management Center, College of Veterinary Medicine, Kansas State University, 1800 Denison Ave., VCS Bldg., Manhattan, KS 66506-5606. Phone: (913) 532-4411. Fax: (913) 532-4039. Electronic mail address: oberst@vet.ksu.edu.

† Kansas Agricultural Experiment Station contribution no. 96-135-J.

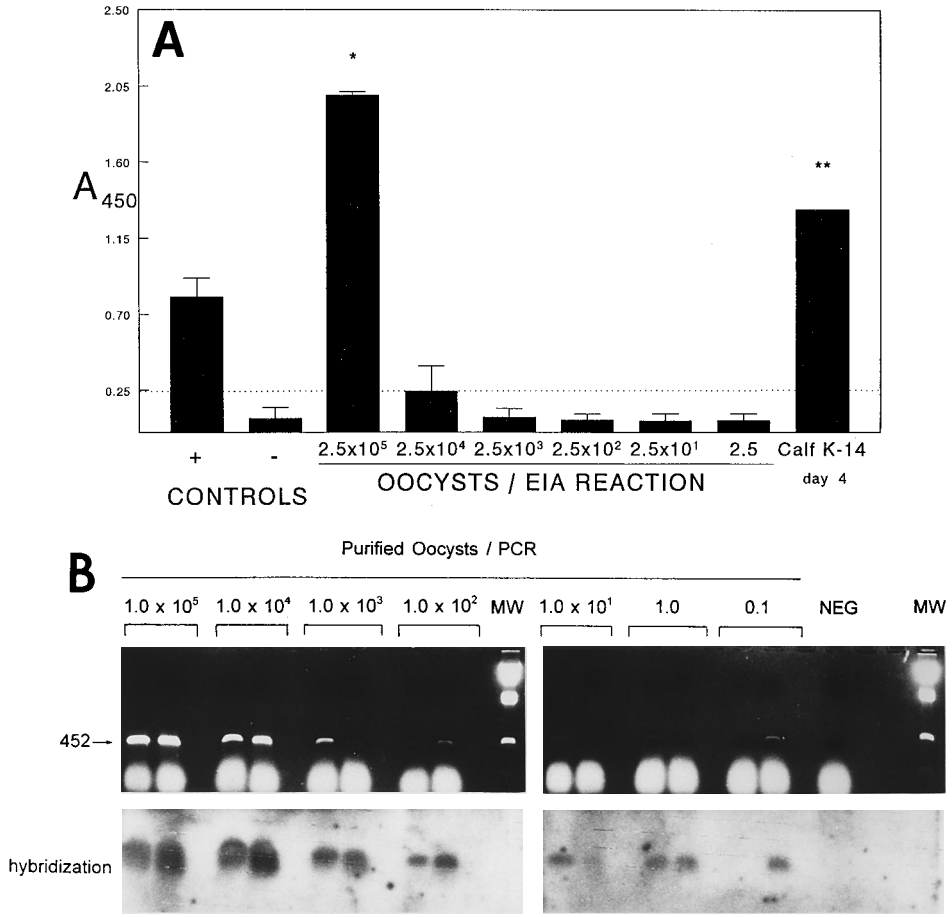


FIG. 1. Comparison of the Color Vue-*Cryptosporidium* EIA and CR-PCR to detect purified *Cryptosporidium* oocysts in solution. (A) Sensitivity of EIA for detection of purified *Cryptosporidium* oocysts. Purified *C. parvum* oocysts (≈5 × 10^7/ml) were serially diluted in solution (water or PBS), processed as a fecal specimen according to the manufacturer's instructions (Color Vue-*Cryptosporidium*), and analyzed at A_{450}. Shown are estimated oocyst concentrations at each serial dilution for each 50-μl EIA well. + and −, positive and negative EIA controls, respectively. Diarrheic feces from calf K-14 (day 4 postinfection) were processed according to EIA instructions. A_{450} values of >0.25 and <0.25 were considered positive and negative, respectively. The data are the means ± standard deviations (error bars) for five replicate samples, except when noted (*, $n = 2$; **, $n = 1$). (B) Sensitivity of CR-PCR for detection of purified *Cryptosporidium* oocysts; estimated oocyst concentrations at each serial dilution for each PCR with the solutions described for panel A. (Top gels) Amplified products for duplicate PCRs at each dilution in 1% agarose and visualized by UV transillumination after ethidium bromide staining. The predicted *Cryptosporidium* PCR product size was 452 bp. MW, *Hin*dIII-digested lambda DNA molecular weight markers. NEG, negative control (no template DNA in the PCR mixture). (Lower gels) Southern blot hybridizations of previous gels with a *Cryptosporidium*-specific internal oligonucleotide probe.

To assess the sensitivity of the EIA, 10-fold serial dilutions of purified oocysts were made with PBS or water, and 50 μl from each dilution was placed in EIA test wells. Similarly, 50 μl of diarrheic feces from calf K-14 on day 4 were diluted serially 10-fold in wash concentrate, and 50 μl from each dilution was placed in an EIA test well. Aliquots from EIA dilutions were also used in assays to determine the sensitivity of the CR-PCR.

Preparation of feces for recovering *C. parvum* DNA. The extraction process for oocyst DNA from feces was dependent on the consistency of the specimen. Solid feces (≈1 g or approximately the size of a pea) were diluted 1:5 with *Cryptosporidium* lysis buffer (CLB) that contained 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.05 M NaOH, 1 mg of proteinase K per ml, and 1 mg of RNase per ml (27). The mixture was mixed, and 100 μl was pipetted to a microcentrifuge tube. If the feces were diarrheic, ≈100 μl was collected in a microcentrifuge tube and centrifuged (15,000 × *g*, 5 min); the supernatant was removed by pipetting, and the pellet was resuspended with 100 μl of CLB.

Subsequently, DNA from all fecal specimens (solid or diarrheic) was extracted by four cycles of freeze-thawing (liquid nitrogen for 5 min; 75°C for 5 min) and then by incubation (75°C for 3 h) and centrifugation (15,000 × *g* for 5 min). Fifty microliters of the supernatant resulting from each sample was collected and placed in PCR Select-III spin columns (5′Prime-3′Prime, Inc., Boulder, Colo.) and centrifuged (1,100 × *g* for 2 min) (27). The resulting eluted solutions were collected for use in PCR or were stored (4°C).

To obtain DNA from known numbers of oocysts, ≈5 × 10^7 purified oocysts were diluted serially 10-fold in PBS or distilled water, and 20 μl from each dilution was mixed with 80 μl of CLB. Samples were then subjected to freeze-thawing, centrifugation, spin column purification, and PCR amplification. Similarly, duplicate 100-μl amounts of diarrheic feces from calf K-14 on day 4 postinfection were serially diluted in water prior to beginning the CR-PCR as previously described for diarrheic feces.

PCR amplification. Ten microliters from each spin column was added to a microcentrifuge tube containing the PCR mixture (1× PCR buffer; 2.5 mM $MgCl_2$; 200 mM each the four deoxynucleoside triphosphates dATP, dCTP, dGTP, and dTTP; 2.5 U of *Taq* DNA polymerase [PCR Core Reagents; Perkin-Elmer, Foster City, Calif.]; 1 mM each *Cryptosporidium*-specific primers [primer 1, 5′-CCGAGTTTGATCCAAAAAGTTACGAA; primer 2, 5′-TAGCTCCTCA TATGCCTTATTGAGTA] [24]) to make a final volume of 100 μl. The PCR amplification was completed in 35 cycles (30 s at 94°C; 30 s at 55°C; and 45 s at 72°C), which were followed by 1 extension cycle (10 min at 72°C) on a GeneAmp PCR System 9600 (Perkin-Elmer).

Detection of amplified products. Thirty-microliter aliquots from each PCR amplification were electrophoresed on 1% agarose gels and stained with ethidium bromide. The DNA was transferred onto a nylon membrane (Magna NT; Micron Separation, Inc., Westboro, Mass.) and linked to the membrane by UV cross-linking according to the instructions of the manufacturer (GS Gene Linker UV Chamber; Bio-Rad, Hercules, Calif.). Membranes were washed once with 2× standard saline citrate (SSC)–0.1% SDS at 46°C for 10 min. The membrane was prehybridized (46°C, overnight) in hybridization solution (5× SSC, 10% sodium dextran sulfate, 0.25× PBS, 0.1% SDS, 100 mg of salmon sperm DNA per ml).

The internal oligonucleotide probe (5-GAATTAACCTATAGGAACCT [24])

was labeled with digoxigenin (DIG) with minor modifications (Oligonucleotide 3′-End Labeling kit, Genesis 5; Boehringer Mannheim, Indianapolis, Ind.). Briefly, 100 ng of probe DNA was mixed with 4 μl of 5× reaction buffer, 4 μl of $CoCl_2$ solution, 1 μl of DIG-dUTP, and 1 μl of terminal transferase in a total volume of 20 μl, and the mixture was incubated (37°C, 20 min). One microliter of glycogen solution and 1 μl of 0.2 M EDTA solution were added to the mixture, which was boiled (5 min). The DIG-labeled probe was then added to the hybridization solution, and the solution was incubated with the membrane (46°C, 20 h). Following hybridization, the membranes were washed six times (2× SSC, 0.1% SDS at 42°C for 10 min each time) and blocked (0.2% I-BLOCK; TROPIX, Bedford, Mass.) in PBS (37°C, 1 h). The anti-DIG antibody (α-DIG-AP-Fab fragments; Boehringer Mannheim) was added (1:7,500) and incubated with the membranes (37°C, 1 h). The membranes were washed four times with PBS-Tween 20 and twice with substrate assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$), each for 5 min at 37°C. One milliliter of substrate solution (1:200 AMPPD; TROPIX) was spread evenly over the membranes, which were placed in a plastic bag and exposed to X-ray film.

RESULTS

Sensitivity of CR-PCR and EIA to detect purified oocysts in solution. The Color Vue-*Cryptosporidium* EIA demonstrated positive reactions, indicating the presence of *Cryptosporidium* antigens, for $>5.0 \times 10^3$ oocysts per μl (Fig. 1A). At dilutions containing an estimated 2.5×10^4 purified oocysts per EIA reaction mixture, the average A_{450} readings were <0.25 ($n = 5$; mean, 0.2445; standard deviation, ± 0.1495) (Fig. 1A). The CR-PCR detected the presence of purified *Cryptosporidium* oocyst DNA when ≥50 oocysts per ml (equivalent to >0.1 oocysts per PCR) were present (Fig. 1B); however, more consistent positive results were evident when $>5.0 \times 10^2$ to 5.0×10^3 oocysts per ml (equivalent to 1 to 10 oocysts per PCR) were present (Fig. 1B). The CR-PCR used 10 μl or ≈1/5 of the total eluted sample volume from each spin column. In direct comparisons, we estimate that the CR-PCR detection procedure demonstrated a sensitivity for detecting purified oocysts in fluids such as water or PBS that was 10^3 to 10^4 times greater than that of the Color Vue-*Cryptosporidium* EIA.

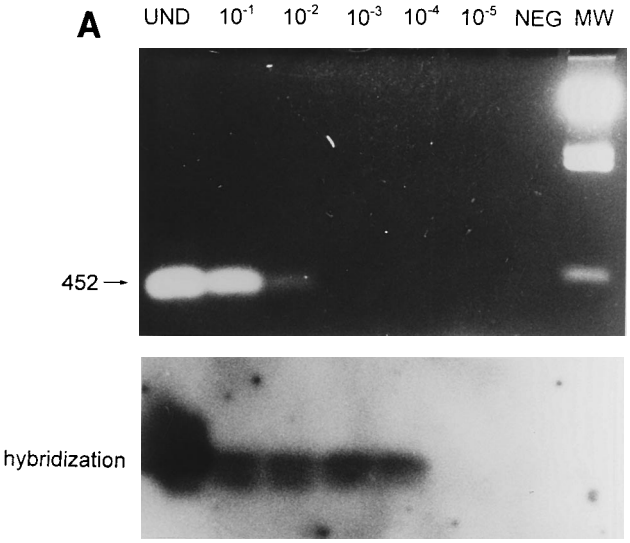
Sensitivity of EIA and CR-PCR to detect *Cryptosporidium* oocysts in serially diluted diarrheic calf feces. Diarrheic feces collected from calf K-14 on days 4 and 7 postinfection were positive when assayed by EIA (Fig. 1A and 2B). Day 7 feces from calf K-14 contained $>10^6$ oocysts per ml of feces as determined by microscopic examination of diluted formalin-fixed feces. Tenfold serial dilutions of these fecal specimens resulted in positive EIA readings for all dilutions ≤1:100 (Fig. 2B). The same dilutions assayed by CR-PCR had positive hybridization signals at dilutions of ≤1:10,000 (Fig. 2A), indicating at least a 100-fold difference in detection sensitivity between the two assays.

Monitoring *Cryptosporidium* infections by EIA and CR-PCR. The experimental infection of calf K-17 resulted in clinical signs of diarrhea by day 3 postinfection and continued through the last test date (day 8). The Color Vue-*Cryptosporidium* EIA detected *Cryptosporidium* antigens on day 2 postinfection (Fig. 3A). The highest A_{450} readings coincided with the development of diarrhea on day 3 and continued through the remainder of the experiment (Fig. 3A). Multiple CR-PCR experiments of the same fecal samples indicated that *Cryptosporidium* DNA was present in the feces prior to experimental infection with purified oocysts (Fig. 3B). A faint positive hybridization signal was also evident on day 1 postinfection and was followed by increasingly intense hybridization signals on subsequent sampling dates. The increased intensity of the signals coincided with the development of diarrhea (Fig. 3B).

DISCUSSION

Specific primers have been used to detect *Cryptosporidium* DNA from purified oocysts and paraffin-embedded tissues (23, 24). However, extensive DNA extraction procedures that utilize hazardous reagents and problems with false-negative results have limited *Cryptosporidium* PCR applications for diagnostic or environmental samples (19). The present report describes a simplified DNA extraction procedure that allows recovery from bovine feces of *Cryptosporidium* DNA that is suitable for PCR amplification and DNA probe detection. The addition of spin columns to segregate total recovered DNA, including *Cryptosporidium* genomic DNA, from PCR inhibitory substances eliminates the need for phenol or chloroform and greatly simplifies the extraction process. The procedure is also efficient, because one technical person can process ≈40 fecal samples for PCR amplification in 4 to 5 h.

Important observations resulting from this study include the following. (i) The CR-PCR detected 10^3 to 10^4 times fewer oocysts than the Color Vue-*Cryptosporidium* EIA in serial dilutions of purified oocysts in water or PBS. (ii) The CR-PCR detected 10^2 times fewer oocysts than the Color Vue-*Cryptosporidium* EIA in serial dilutions of diarrheic bovine feces. (iii) The CR-PCR detected *Cryptosporidium* oocysts in solid feces earlier in an experimental infection than the Color Vue-*Cryptosporidium* EIA. (iv) The CR-PCR recovered DNA suitable



B

	UND	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	NEG	POS
Color Vue	+	+	+	-	-	-	-	+
Results	1.043	0.775	0.351	0.147	0.113	0.120	0.080	0.720

FIG. 2. Comparison of the Color Vue-*Cryptosporidium* EIA and CR-PCR for detection of *Cryptosporidium* oocysts in diarrheic bovine feces. (A) Sensitivity of CR-PCR for detection of *Cryptosporidium* DNA in diarrheic feces from calf K-14 on day 7 postinfection. Feces were serially diluted 10-fold in water and processed by CR-PCR. (Top gel) UND, undiluted. Amplified products from duplicate PCRs from each dilution in 1% agarose and visualized by UV transillumination after ethidium bromide staining. Predicted *Cryptosporidium* PCR product size was 452 bp. MW, *Hin*dIII-digested lambda DNA molecular weight markers. NEG, negative control (no template DNA in the PCR mixture). (Lower gel) Southern blot hybridization from previous gel with internal probe. (B) Sensitivity of Color Vue-*Cryptosporidium* EIA for detection of *Cryptosporidium* antigens in diarrheic feces from calf K-14 on day 7 postinfection. Feces were serially diluted 10-fold in Color Vue-*Cryptosporidium* wash concentrate, processed according to the manufacturer's instructions, and analyzed at A_{450}. Positive (POS) and negative (NEG) EIA controls were provided in the kit. Absorbance readings of >0.25 and <0.25 were considered positive (+) and negative (−), respectively. Data are the EIA interpretation (positive or negative result/A_{450} reading) at each dilution.

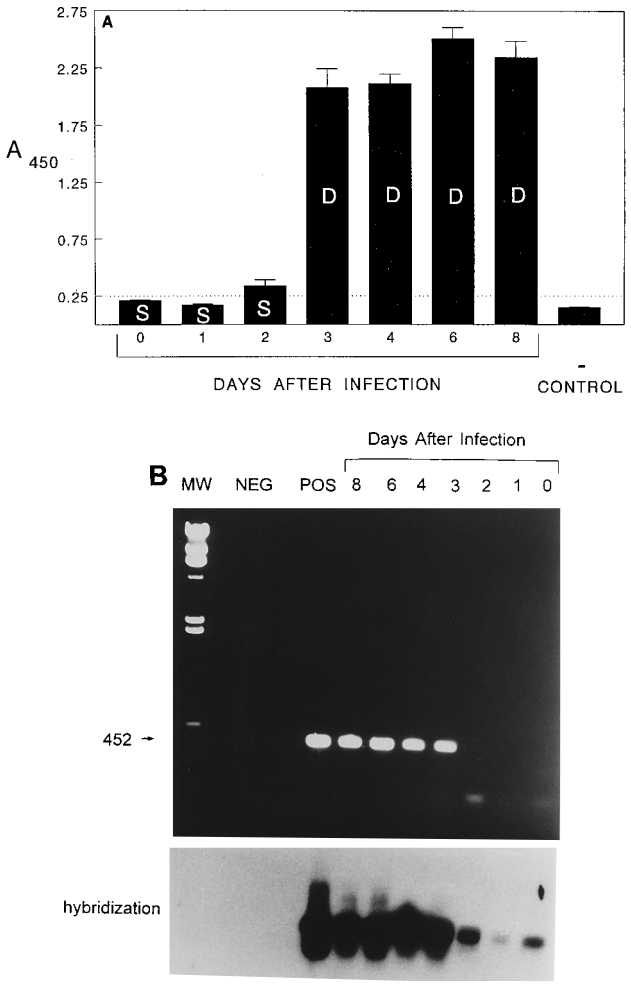


FIG. 3. Comparison of the kinetics of *C. parvum* fecal shedding by the Color Vue-*Cryptosporidium* EIA and CR-PCR. Calf K-17 was experimentally infected with $\approx 10^8$ *C. parvum* oocysts on day 0, and feces were collected daily through day 8 postinfection. (A) Kinetics of infection assayed with Color Vue-*Cryptosporidium* EIA. Fecal specimens were processed according to EIA kit instructions and were analyzed at A_{450}. −, negative EIA control. The data are the means ± standard deviations (error bars) for three replicate samples. Absorbance readings of >0.25 and <0.25 were considered positive and negative, respectively. Consistency of feces was scored as solid (S) or diarrheic (D). (B) Kinetics of infection assayed by CR-PCR. (Top gel) PCR products in 1% agarose and visualized by UV transillumination after ethidium bromide staining. Predicted *Cryptosporidium* PCR product size was 452 bp. MW, *Hin*dIII-digested lambda DNA molecular weight markers. NEG, negative control (no template DNA in the PCR mixture); POS, positive control (452-bp *Cryptosporidium* PCR product contained in the PCR mixture). (Lower gel) Southern blot hybridization from previous gel with internal probe.

for PCR amplification from feces without using or generating hazardous components.

The recovery and detection of *Cryptosporidium* oocysts from naturally infected cattle are not generally difficult because of the large numbers of oocysts shed in diarrheic feces ($\approx 10^8$ to 10^{10} oocysts per day over a period of 1 to 2 weeks) (9, 30). However, more-sensitive and specific methods are needed to rapidly and accurately detect small numbers of oocysts in asymptomatic infections and in dilute environmental samples, such as water and soil.

The CR-PCR detected *Cryptosporidium* DNA in solid, nondiarrheic feces similar to those expected with asymptomatic infections. The procedure detected *Cryptosporidium* DNA in the feces of a calf both prior and subsequent to experimental infection, whereas the EIA was first positive on day 2 postinfection (1 day prior to the onset of diarrhea). We have subsequently observed similar positive PCR results from feces of asymptomatic neonatal calves that were negative by direct microscopic examination of formalin-fixed feces (33). Preexposure results that are positive may be due to low-level contamination of the environment. Additional research will be required to clarify these results and to explore the use of PCR detection for asymptomatic bovine cryptosporidiosis.

Direct comparisons of CR-PCR and IFA were not conducted. Previous studies comparing and contrasting the sensitivities of various methods detecting *Cryptosporidium* oocysts in feces generally agree that IFA is more sensitive than other staining protocols for conventional microscopic evaluations (16, 17, 31, 44) and that EIA is comparable to IFA for evaluating diarrheic feces (21, 31, 38). Our sensitivity results with the Color Vue-*Cryptosporidium* EIA are consistent with previous studies that demonstrated its reliability for coprodiagnosis of cryptosporidiosis when the feces were diarrheic and contained $>10^3$ to 10^4 oocysts per ml (1).

The ability to detect *Cryptosporidium* DNA in feces or water will be increasingly important for preventing waterborne outbreaks of cryptosporidiosis and in defining the effect livestock production and management practices have on water quality. Although no DNA recovery process has yet been demonstrated to be applicable for all environmental samples, CR-PCR has the potential for use in detecting *Cryptosporidium* DNA from a wide variety of samples.

ACKNOWLEDGMENTS

This work was supported by USDA-CSRS Special Water Quality grant no. 93-34214-9159 and the College of Veterinary Medicine, Kansas State University.

REFERENCES

1. **Aarnaes, S. L., J. Blanding, S. Speier, D. Forthal, L. M. de la Maza, and E. M. Peterson.** 1994. Comparison of the ProSpecT and Color Vue enzyme-linked immunoassays for the detection of *Cryptosporidium* in stool specimens. Diagn. Microbiol. Infect. Dis. **19:**221–225.
2. **American Society for Testing and Materials.** 1991. Proposed test method for *Giardia* cysts and *Cryptosporidium* oocysts in low-turbidity water by a fluorescent antibody procedure, p. 925–935. *In* Annual book of ASTM standards, vol. 11.01. American Society for Testing and Materials, Philadelphia.
3. **Anusz, K. Z., P. H. Mason, M. W. Riggs, and L. E. Perryman.** 1990. Detection of *Cryptosporidium parvum* in bovine feces by monoclonal antibody capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. **28:**2770–2774.
4. **Arnaud-Battandier, F., M. Naceri, and C. Maurage.** 1985. Cryptosporidiosis in immunocompetent patients. N. Engl. J. Med. **313:**1019.
5. **Arrowood, M. J., M. R. Hurd, and J. R. Mead.** 1995. A new method for evaluating experimental cryptosporidium parasite loads using immunofluorescent flow cytometry. J. Parasitol. **81:**404–409.
6. **Baxby, D., and C. A. Hart.** 1986. The incidence of cryptosporidiosis: a two-year prospective survey in a children's hospital. J. Hyg. **96:**107–111.
7. **Berkelman, R. L.** 1994. Emerging infectious diseases in the United States, 1993. J. Infect. Dis. **170:**272–277.
8. **Black, R. E., G. Lopez de Romana, K. H. Borown, N. Bravo, O. G. Bazalar, and H. C. Kanashiro.** 1989. Incidence and etiology of infantile diarrhea and major routes of transmission in Huascar, Peru. Am. J. Epidemiol. **129:**785–799.
9. **Blewett, D. A.** 1989. Quantitative techniques in *Cryptosporidium* research, p. 85–89. *In* K. W. Angus and D. A. Blewett (ed.), Cryptosporidiosis. Proceedings of the First International Workshop. Animal Diseases Research Association, Edinburgh.
10. **Casemore, D. P., M. Armstrong, and R. L. Sands.** 1985. Laboratory diagnosis of cryptosporidiosis. J. Clin. Pathol. **38:**1337–1341.
11. **Centers for Disease Control and Prevention.** 1995. Assessing the public health threat associated with waterborne cryptosporidiosis: report of a workshop. Morbid. Mortal. Weekly Rep. **44:**1–14.
12. **Current, W. L., N. C. Reese, J. V. Ernst, W. S. Bailey, M. B. Heyman, and M. D. Weinstein.** 1983. Human cryptosporidiosis in immunocompetent and immunodeficient persons: studies of an outbreak and experimental transmission. N. Engl. J. Med. **308:**1252–1257.

13. **DuPont, H. L., C. L. Chappell, C. R. Sterling, P. C. Okhuysen, J. B. Rose, and W. Jakubowski.** 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. N. Engl. J. Med. **332:**855–859.
14. **Farrington, M., S. Winters, C. Walker, R. Miller, and D. Rubenstein.** 1994. *Cryptosporidium* antigen detection in human feces by reverse passive hemagglutination assay. J. Clin. Microbiol. **32:**2755–2759.
15. **Garber, L. P., M. D. Salman, H. S. Hurd, T. Keefe, and J. L. Schlater.** 1994. Potential risk factors for *Cryptosporidium* infection in dairy calves. J. Am. Vet. Med. Assoc. **205:**86–91.
16. **Garcia, L. S., T. C. Brewer, and D. A. Bruckner.** 1987. Fluorescence detection of *Cryptosporidium* oocysts in human fecal specimens by using monoclonal antibodies. J. Clin. Microbiol. **25:**119–121.
17. **Garcia, L. S., D. A. Bruckner, and T. C. Brewer.** 1988. Cryptosporidiosis in patients with AIDS. Am. Clin. Product Rev. **7:**38–41.
18. **Garcia, L. S., D. A. Bruckner, T. C. Brewer, and R. Y. Shimizu.** 1983. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. J. Clin. Microbiol. **18:**185–190.
19. **Johnson, D. W., N. J. Pieniazek, and J. B. Rose.** 1993. DNA probe hybridization and PCR detection of *Cryptosporidium* compared to immunofluorescence assay. Water Sci. Technol. **27:**77–84.
20. **Juranek, D.** 1987. Shedding of oocysts in immunocompetent individuals infected with *Cryptosporidium*. Am. J. Trop. Med. Hyg. **36:**338–342.
21. **Kehl, K. S. C., H. Cicirello, and P. L. Havens.** 1995. Comparison of four different methods for detection of *Cryptosporidium* species. J. Clin. Microbiol. **33:**416–418.
22. **Kuhls, T. L., R. A. Greenfield, D. A. Mosier, D. L. Crawford, and W. A. Joyce.** 1992. Cryptosporidiosis in adult and neonatal mice with severe combined immunodeficiency. J. Comp. Pathol. **106:**399–410.
23. **Laxer, M. A., M. E. D'Nicuola, and R. J. Patel.** 1992. Detection of *Cryptosporidium parvum* DNA in fixed, paraffin-embedded tissue by the polymerase chain reaction. Am. J. Trop. Med. Hyg. **47:**450–455.
24. **Laxer, M. A., B. K. Timblin, and R. J. Patel.** 1991. DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. Am. J. Trop. Med. Hyg. **45:**688–694.
25. **LeChevallier, M. W., W. D. Norton, and R. G. Lee.** 1991. *Giardia* and *Cryptosporidium* spp. in filtered drinking water supplies. Appl. Environ. Microbiol. **57:**2617–2621.
26. **LeChevallier, M. W., W. D. Norton, J. E. Siegel, and M. Abbaszadegan.** 1995. Evaluation of the immunofluorescence procedure for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl. Environ. Microbiol. **61:** 690–697.
27. **Leng, X. G., D. A. Mosier, and R. D. Oberst.** Differentiation of *Cryptosporidium parvum*, *C. muris*, and *C. baileyi* by PCR-RFLP analysis of the 18S rRNA gene. Vet. Parasitol., in press.
28. **Ma, P., and R. Soave.** 1983. Three-step examination for cryptosporidiosis in 10 homosexual men with protracted watery diarrhea. J. Infect. Dis. **147:**824–827.
29. **Miller, R. A., M. A. Bronsdon, and W. R. Morton.** 1990. Experimental cryptosporidiosis in a primate model. J. Infect. Dis. **161:**312–315.
30. **Müller, H. M., L. Ranucci, E. Pozio, and A. Crisanti.** 1993. A method for collecting large quantities of *Cryptosporidium* parasites. Parasitol. Today **9:**261–263.
31. **Newman, R. D., K. L. Jaeger, T. Wuhib, A. A. M. Lima, R. L. Guerrant, and C. L. Sears.** 1993. Evaluation of an antigen capture enzyme-linked immunosorbent assay for detection of *Cryptosporidium* oocysts. J. Clin. Microbiol. **31:**2080–2084.
32. **Nieminski, E. C., F. W. Schaefer, and J. E. Ongerth.** 1995. Comparison of two methods for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl. Environ. Microbiol. **61:**1714–1719.
33. **Oberst, R. D.** Unpublished data.
34. **Parisi, M. T., and P. M. Tierno.** 1995. Evaluation of new rapid commercial enzyme immunoassay for detection of *Cryptosporidium* oocysts in untreated stool specimens. J. Clin. Microbiol. **33:**1963–1965.
35. **Petersen, C.** 1992. Cryptosporidiosis in patients infected with the human immunodeficiency virus. Clin. Infect. Dis. **15:**903–909.
36. **Riggs, M. W., and L. E. Perryman.** 1987. Infectivity and neutralization of *Cryptosporidium parvum* sporozoites. Infect. Immun. **55:**2081–2087.
37. **Rose, J. B.** 1988. Occurrence and significance of *Cryptosporidium* in water. J. Am. Water Works Assoc. **80:**53–58.
38. **Rosenblatt, J. E., and L. M. Sloan.** 1993. Evaluation of an enzyme-linked immunosorbent assay for detection of *Cryptosporidium* spp. in stool specimens. J. Clin. Microbiol. **31:**1468–1471.
39. **Rusnak, J., T. D. Hadfield, M. M. Rhodes, and J. K. Gaines.** 1989. Detection of *Cryptosporidium* oocysts in human feces specimens by an indirect immunofluorescence assay with monoclonal antibodies. J. Clin. Microbiol. **27:** 1135–1136.
40. **Siddons, C. A., P. A. Chapman, and B. A. Rush.** 1992. Evaluation of an enzyme immunoassay kit for detecting *Cryptosporidium* in faeces and environmental samples. J. Clin. Pathol. **45:**479–482.
41. **Smith, H. V., W. J. Patterson, R. Hardie, L. A. Greene, C. Benton, W. Tulloch, R. A. Gilmour, R. W. A. Girdwood, J. C. M. Sharp, and G. I. Forbes.** 1989. An outbreak of waterborne cryptosporidiosis caused by post-treatment contamination. Epidemiol. Infect. **103:**703–715.
42. **Sterling, C. R., and M. J. Arrowood.** 1986. Detection of *Cryptosporidium* sp. infections using a direct immunofluorescent assay. Pediatr. Infect. Dis. **5:**S139–S142.
43. **Ungar, B. L. P.** 1990. Enzyme-linked immunoassay for detection of *Cryptosporidium* antigens in fecal specimens. J. Clin. Microbiol. **28:**2491–2495.
44. **Weber, R., R. T. Bryan, H. S. Bishop, S. P. Wahlquist, J. J. Sullivan, and D. D. Juranek.** 1991. Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence of low sensitivity of current diagnostic methods. J. Clin. Microbiol. **29:**1323–1327.
45. **Webster, K. A., J. D. E. Pow, M. Giles, J. Catchpole, and M. J. Woodward.** 1993. Detection of *Cryptosporidium parvum* using a specific polymerase chain reaction. Vet. Parasitol. **50:**35–44.
46. **Xiao, L., and R. P. Herd.** 1993. Quantitation of *Giardia* cysts and *Cryptosporidium* oocysts in fecal samples by direct immunofluorescence assay. J. Clin. Microbiol. **31:**2944–2946.