A method to detect viable Cryptosporidium parvum oocysts was developed. Polyclonal immunoglobulin G against C. parvum oocyst and sporozoite surface antigens was purified from rabbit immune serum, biotinylated, and bound to streptavidin-coated magnetic particles. C. parvum oocysts were captured by a specific antigen-antibody reaction and magnetic separation. The oocysts were then induced to excyst, and DNA was extracted by heating at 95°C for 10 min. A 452-bp fragment of C. parvum DNA was amplified by using a pair of C. parvum-specific primers in PCR. The method detected as few as 10 oocysts in purified preparations and from 30 to 100 oocysts inoculated in fecal samples. The immunomagnetic capture PCR (IC-PCR) product was identified and characterized by a nested PCR that amplified a 210-bp fragment, followed by restriction endonuclease digestion of the IC-PCR and nested-PCR products at the StyI site and a nonradioactive hybridization using an internal oligonucleotide probe labeled with biotin. PCR specificity was also tested, by using DNAs from other organisms as templates. In the control experiments, inactivated oocysts were undetectable, indicating the ability of this method to differentiate between viable and nonviable oocysts. Thus, this system can be used to specifically detect viable C. parvum oocysts in environmental samples with great sensitivity, providing an efficient way to monitor the environment for C. parvum contamination.

Cryptosporidium parvum is a coccidian parasite that can cause self-limited diarrhea in immunocompetent persons and chronic, life-threatening diarrhea in immunocompromised persons, especially in patients with AIDS (9, 11). Environmental contamination with C. parvum oocysts, shed by infected mammals, can lead to outbreaks of cryptosporidiosis, as evidenced by the 1993 outbreak in Milwaukee, Wis., in which 403,000 people were infected and at least 70 fatalities were reported (20). Since small numbers of C. parvum oocysts are often found in the environment and the number of oocysts required to cause infection is relatively low (5, 12, 21), a method to detect oocysts with great sensitivity is required. The fact that some C. parvum oocysts found in the environment are not viable (22) and do not pose a threat to human health makes it more significant to differentiate between viable and nonviable oocysts.

Conventional methods to detect C. parvum oocysts from stool specimens, including acid-fast-staining (AFS) methods, immunoassays, and immunofluorescence assays (IFA) after stool concentration, are not sensitive enough to detect small numbers of oocysts (17, 25). The method currently recommended for detecting oocysts from drinking water (1) is laborious and time-consuming, as it involves membrane filtration of the water sample, physical manipulation of the filter to dislodge entrapped oocysts, fluorescent-antibody staining, and subsequent microscopic observation of the organisms; the sensitivity is not high enough, either, since many oocysts are lost in the process, resulting in a poor oocyst recovery rate that ranges from 5 to 20% (27). In the past few years, several PCR-based methods have been described (3, 16, 19, 26). The sensitivities of these methods ranged from 1 to 100 oocysts, and specificity can be achieved by choosing C. parvum-specific primers for PCR and confirmed by subsequent restriction enzyme analysis of the amplicon or oligonucleotide hybridization. However, neither these methods nor the conventional methods are able to differentiate viable and nonviable oocysts. Current methods to determine the viability of oocysts include infectivity tests in animal models (13, 18), in vitro excystation procedures (14, 23), and the incorporation of fluorogenic vital dyes (8); all of these methods require a large number of oocysts at high purity to make the test statistically accurate and the results comparable, making them a research tool rather than a detection method. Two PCR-based methods to detect viable oocysts, in which an in vitro excystation procedure was applied to differentiate live from dead oocysts, have been described (15, 24). Although the sensitivity was not accurately determined, the laborious oocyst recovery procedure required by both methods makes them impractical for routine monitoring of the environment.

In this report, we describe the detection of viable C. parvum oocysts directly from environmental samples by an immunomagnetic capture PCR (IC-PCR) technique, in which we combine the specificity and sensitivity of PCR with the ability of in vitro excystation to differentiate viable oocysts from nonviable oocysts.

**MATERIALS AND METHODS**

**Source of C. parvum oocysts.** Fecal samples from infected calves were collected, and C. parvum oocysts were purified by Sheather’s method of sugar flotation and CsCl gradient centrifugation, following procedures described previously (10). The purified oocysts were stored in 1× Hanks balanced saline solution (HBSS) (Sigma, St. Louis, Mo.) at 4°C, and the viability was determined by fluorogenic vital dye staining (with 4′,6-diamidino-2-phenylindole and propidium iodide [Sigma]) and in vitro excystation as described by Campbell et al. (8).

**Production of immune serum to C. parvum oocysts.** Anti-C. parvum oocyst serum was raised by immunizing a 2.5- to 3.0-kg female New Zealand White rabbit with 1.0 × 109 purified oocysts. After treatment overnight with 10% formalin and extensive washing to remove the formalin, the oocysts were resuspended in 3.0 ml of phosphate-buffered saline (PBS) (pH 7.2). For the first immunization, 1.0 ml of antigen preparation with complete Freund’s adjuvant was injected into the rabbit subcutaneously at multiple sites. Control serum was
obtained before the injection. On days 12 and 25 after the first injection, an additional 1.0 ml of the antigen mixture was injected with incomplete Freund's adjuvant. Starting from day 10 after the final injection, the rabbit was bled at 1-week intervals and the sera were titrated by indirect fluorescent-antibody staining technique as described below. The antiserum was anesthetized and examined after the titers in serum achieved a satisfactory level.

**Antiserum titration.** A small drop of purified oocyst suspension was placed on a fluorescent antibody slide (Becton Dickinson, Franklin Lakes, N.J.) coated with 0.1% poly-l-lysine (Sigma), air-dried, and fixed in acetone for 5 min at room temperature. Twofold dilutions (50 μl) of immunized rabbit serum, in PBS (pH 7.2), were applied to the spots of fixed oocysts and incubated for 30 min at 37°C in a humidified chamber. Before washing with PBS, 50 μl of fluorescein isothiocyanate- or biotin-labeled goat anti-rabbit immunoglobulin G (IgG) (Sigma) was added and the slides were incubated for another 30 min at 37°C in the humidified chamber. The slides were washed again with PBS and examined with a standard fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany). Staining reactions were graded on a scale of brightness from 1 to 4. The preimmunization serum was used as a negative control in the test.

**Specificity test of rabbit anti-*C. parvum***. The indirect fluorescent-antibody staining technique mentioned above was carried out except that purified *C. parvum* oocytes, a mixture of *C. parvum* oocytes and sporozoites (obtained from in vitro cultivation), Cryptosporidium muris (purified from a fecal sample), and *Giardia duodenalis* (ATCC 53098; American Type Culture Collection, Rockville, Md.) were fixed on a fluorescent-antibody slide and stained with rabbit anti-*C. parvum* IgG, followed by fluorescein-labeled goat anti-rabbit IgG. The staining reactions were recorded as positive or negative.

**Coating of anti-*C. parvum* IgG to magnetic particles.** Two commercial kits were used: the MahTrap G II Antibody Purification Kit (Pharmacia Biotech, Alameda, Calif.) for polyclonal IgG purification from immune sera and the MabTrap G II Antibody Purification Kit (Pharmacia Biotech, Alameda, Calif.) for monoclonal IgG purification. *E. coli* (optimal setting). The membrane was washed twice with 5× SSPE (1.0 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7])—0.5% sodium dodecyl sulfate (SDS)—5× Denhardt's solution (0.5 mM EDTA, 0.2% SDS). Hybridization with 5′-biotin-labeled internal probe between bp 681 and 700 (5′-GAA TTA TCC TAT TGG AAC AAC CT-3′; GeneMed) was accomplished by addition of 10 pmol of probe to 25 ml of 5× SSPE-0.5% SDS—5× Denhardt's solution and incubation at 50°C for 1 h. The membrane was washed in 2× SSPE—0.1% SDS at room temperature for 10 min, in 5× SSPE—0.5% SDS at 50°C for 20 min, and in 2× SSPE at room temperature for 10 min. The membrane was then washed and incubated with fluorescein-conjugated rabbit anti-mouse IgG (50 μl; Santa Cruz Biotechnology, Santa Cruz, Calif.) in 2× SSPE at room temperature for 10 min. After two 5-min washes in 100 mM NaCl—1 M urea—0.5% Triton X-100—1% dextran sulfate (Sigma), followed by two 5-min washes in 100 mM sodic citrate (pH 5.0), color was developed by incubating the membrane in 100 mM sodic citrate (pH 5.0) containing 3.3′,5′-tetramethylbenzidine (1 mM [mg/ml]; Sigma) and 0.03% H2O2 (added immediately before use) at room temperature for 10 min. The color reaction was terminated by washing the membrane in distilled water for 2 min.

**Conventional PCR to amplify DNA from other organisms.** The specifics of the primers and PCR methods were tested by amplifying fragment sequences from several other organisms likely to be found in the same environment. Nucleic acids were recovered from the following microorganisms: *C. muris* and *G. duodenalis* (American Type Culture Collection, Rockville, Md.) and *E. coli* (American Type Culture Collection, Rockville, Md.). DNA was extracted by heating ~105 cells (in 25 μl of distilled water) at 95°C for 10 min and cooling to 4°C for 5 min. A modified proteinase K method (2) was used to extract DNA from *C. muris* and *G. duodenalis*. Briefly, 100 μg of DNA was digested with 20 μl of 100 mM EDTA, 10 μg of proteinase K, and 1 M NaOH, followed by neutralization with 2 M HCl. The mixture was centrifuged at 10,000 × g for 20 s, washed, resuspended in 25 μl of distilled water, and DNA from *C. parvum* oocytes was extracted in the same way, as a positive control.

**Differential test of viable oocytes by IC-PCR.** Several inactivation methods, including incubating at 80°C for 1 min, treating overnight with 10% formalin at room temperature, treating with 10% ammonia at room temperature for 1 h, and treating with 90% alcohol at room temperature for 1 h, were investigated. After various treatments and repeated washing, the 105 oocytes were resuspended in 1.0 ml of distilled water and used in IC-PCR. The results were compared with those from a conventional PCR in which DNAs from treated or untreated oocysts were extracted by the proteinase K method. Un-treated oocysts were processed in the same way as positive controls.

**Detection of *C. parvum* from field samples.** To evaluate the system for environmental monitoring and screening, a total of 15 fecal samples were collected from a dairy farm. A 25 ml or 25-g portion of each sample was homogenized, diluted in distilled water containing 0.1% Tween 20, and passed through a series of sieves with the minimum porosity at 100 μm. After centrifugation to remove large volumes, the sample was reduced to 1.0 ml and IC-PCR was performed. The sample was prepared from a cold AFS and the IFA. AFS was performed as described previously (6), using laboratory-prepared reagents. The IFA was done with a Merifluor Cryptosporidium/Giardia Kit (Meridian Diagnostics, Inc., Cincinnati, Ohio) and by following the manufacturer’s instructions. The positive control and a negative control were also included in each IC-PCR. AFS assay, and IFA.
RESULTS

C. parvum oocysts used in the study. Excystation (Fig. 1) was observed after 20 min of incubation at 37°C in excystation solution, while maximum excystation was achieved after 4 h of incubation. The oocysts were found to be fairly stable at refrigeration temperature; there was no significant decrease in excystation efficiency within 8 weeks of storage. Therefore, 88 to 90% of C. parvum oocysts used in the study were viable by this criterion.

Specificity of rabbit anti-C. parvum serum. The antiserum reacted with both C. parvum oocysts and sporozoites, and there was no cross-reaction with C. muris or G. duodenalis (data not shown).

Sensitivity of IC-PCR. The experiments described in Materials and Methods were repeated three times. When a purified oocyst preparation was used, as few as 10 oocysts were detectable, and no PCR product was detected from 3 oocysts. When the oocysts were inoculated into calf feces, the detection sensitivity decreased ~10-fold, in that 100 oocysts were consistently detected, although on one occasion 30 oocysts did give a positive signal; preparations containing 10 oocysts were negative in all experiments (Fig. 2).

Identification and characterization of IC-PCR product. (i) Nested PCR. In the preliminary experiments, we found that the nested PCR was not highly specific at low primer annealing temperatures. When primer annealing temperatures of 50, 55, and even 60°C were used for PCR, heterologous DNAs were amplified as efficiently as the IC-PCR product (data not shown). Therefore, we increased the annealing temperature to 66°C and included another negative control in which the DNA template was the C. parvum PCR amplification product resulting from another pair of primers. As shown in Fig. 3, the 452-bp IC-PCR product can be efficiently amplified to produce the 210-bp product. When 10 oocysts were used, the 452-bp band signal was weak but the 210-bp band signal became much stronger. The negative control in nested PCR (lane f) was negative, indicating the specificity of nested PCR.

(ii) Restriction endonuclease digestion. Since the SpI site (C ↓ CAAGG [arrow indicates cleavage]) is located between bp 570 and 575, cleavage of the 452-bp IC-PCR product by SpI produces a 127-bp fragment (bp 444 to 570) and a 325-bp fragment (bp 571 to 895), while cleavage of the 210-bp product produces a 64-bp fragment (bp 507 to 570) and a 146-bp fragment (bp 570 to 716), which were confirmed (Fig. 4).

(iii) Nonradioactive oligonucleotide hybridization. Both the 452-bp IC-PCR product and the subsequent nested-PCR product hybridized with the internal probe (Fig. 5). The 452-bp IC-PCR product and the 280-bp nested-PCR product were positioned by both DNA markers on ethidium bromide-stained agarose gel (Fig. 5A); after hybridization and color reaction, two strong bands, as well as the biotinylated DNA marker, appeared (Fig. 5B). By comparing these with the band patterns of biotinylated DNA markers in Fig. 5A, these two bands were determined to be at the same positions as the 452- and the 280-bp bands, respectively, indicating that both the IC-PCR product and the subsequent nested-PCR product hybridized with the internal probe. Although the hybridization signals on the membrane became diffused after 5 min of exposure, this system not only confirmed the specificity of PCR amplification but also was able to enhance the detection sensitivity. In one experiment, we were able to get signals from several half-log dilutions of IC-PCR product on the membrane, but they were invisible on the ethidium bromide-stained gel (data not shown).

Conventional PCR to amplify DNA from other organisms. The PCR amplification was found to be highly specific for the C. parvum template sequence. When heterologous genomic DNA from C. muris, Giardia lamblia, or E. coli was used, no band was detected, while an otherwise identical reaction mixture containing C. parvum template gave the specific product.
lanes 2, the 210-bp nested-PCR product.

Lanes M, DNA size marker; lane 1, the 452-bp IC-PCR product after digestion with StyI; lane 2, same PCR product as that in lane 1, not subjected to StyI digestion; lane 3, the 210-bp nested-PCR product after digestion with StyI; lane 4, same PCR product as that in lane 3, not subjected to StyI digestion.

This demonstrated that all signals and bands seen in PCR could be attributed to primer specificity.

**Differentiation of viable oocysts from nonviable oocysts by IC-PCR.** In conventional PCR, oocysts that had undergone various treatments were consistently detected, and the signals were very strong. In IC-PCR, oocysts treated with 10% formalin or 10% ammonia or by heating were undetectable, whereas oocysts treated with 90% ethanol gave a much weaker signal (Fig. 7). The results were compared with the results from in vitro excystation. No excystation was observed from oocysts heated at 80°C or treated with 10% formalin or 10% ammonia, and the excystation efficiency of oocysts treated with 90% ethanol was decreased to 18.5%, while 89% of the untreated oocysts were completely or partially excysted.

**Detection of oocysts from field samples.** Among 15 field samples, only 2 were negative by all three methods. Thirteen samples (87%) were IC-PCR positive, while 11 samples (73%) were IFA positive and 8 samples (53%) were AFS positive. All the AFS-positive samples were both IFA and IC-PCR positive; no sample was positive by the AFS method but negative by IFA or IC-PCR. All the IFA-positive samples were also IC-PCR positive; no sample was IFA positive but IC-PCR negative.

**DISCUSSION**

The sensitivity of the IC-PCR is a function of the efficiencies of oocyst separation and of DNA extraction from sporozoites and partially excysted oocysts after excystation is induced, as well as the ability of PCR to increase the DNA copy number exponentially. In our study, the IC-PCR was able to detect as few as 100 oocysts from stool samples. Since the detection limit of AFS and IFA was 10,000 to 500,000 oocysts (25), the sensitivity of the IC-PCR was at least 100 times those of AFS and IFA.

In our preliminary experiments, the sensitivity of IC-PCR was similar to that of a conventional PCR (data not shown), but unlike the conventional PCR, the IC-PCR does not involve laborious and time-consuming DNA extraction. Another advantage the IC-PCR has over the conventional PCR is that it combines the specificity of IC of the oocysts and specific DNA amplification in PCR. Only *C. parvum* gave positive reactions in the PCR, and no PCR products were observed when several other non-*C. parvum* organisms were tested. The IC-PCR produced the expected 452-bp amplification product, and its nucleotide sequence was confirmed by nested PCR, restriction endonuclease digestion, and an oligonucleotide probe hybridization.

This technique has the great potential to be used for monitoring environmental samples for *C. parvum* presence. Current viability assays require a large number of oocysts (8, 13, 14), which limits the usefulness of infectivity tests for environmental samples; *C. parvum* detection methods based on oocyst antigens are often not sensitive enough for testing environmental samples; and conventional PCR might be sensitive enough but cannot differentiate viable oocysts from nonviable oocysts. The IC-PCR described in this paper is certainly a promising technique in these respects.

Among the 15 field samples we tested, the two samples negative by IC-PCR were also negative when tested by AFS or IFA. Two samples were positive by IC-PCR but negative as determined by IFA; five samples were positive by IC-PCR but negative by AFS. Since we took precautions to avoid carryover contamination and included negative controls, we think that our positive IC-PCR results were not false-positive results and that IC-PCR has greater sensitivity than AFS and IFA. Environmental samples have often been found to contain substances that inhibit PCR; but in our experiments, the IC-PCR procedure seemed to be efficient in eliminating those inhibitors.

A positive reaction in conventional PCR, AFS, and IFA demonstrates the presence of *C. parvum* oocysts, but it does not indicate whether the oocysts in the sample are probably infectious. In our procedure, noninfectious oocysts can be captured if their surface antigens are not destroyed, but the oocysts will not excyst under induction; thus, a positive signal demonstrates the presence of probably infectious oocysts. The IC-PCR can thus be used in place of other cumbersome and
time-consuming procedures to screen environmental samples. Furthermore, the IC-PCR can be used as a molecular approach to study the epidemiology of *C. parvum* and effects of different inactivation or disinfection methods on oocyst infectivity.

The nested PCR procedure and the simple nonradioactive hybridization techniques described in this paper further enhance the sensitivity of IC-PCR. Although the nested PCR might be nonspecific in conventional PCR (4), it causes no problems following the IC-PCR. The specific immunoaffinity separation ensures that only *C. parvum* oocysts are collected from a sample and that free nucleic acids from other organisms or nonviable oocysts in the sample are removed during the magnetic separation steps. In other experiments, we found that heating at 95°C for 10 min was insufficient to extract DNA from unexcysted oocysts (data not shown); thus, all the PCR product from first-round PCR will be from viable *C. parvum* oocysts. Although we think the IC-PCR is sensitive enough for routine environmental monitoring purposes, the nested PCR or the nonradioactive hybridization can be added to the procedure if higher sensitivity is desired.

In summary, a basic method for detection of viable *C. parvum* oocysts from environmental samples was developed, experimental conditions were established, and the efficiency of the procedure if higher sensitivity is desired. 

**REFERENCES**