APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1995, p. 4514–4516 Vol. 61, No. 12
0099-2240/95/$04.00+0

Detection of Viable *Cryptosporidium parvum* Oocysts by PCR

CHRISTIANE WAGNER-WIENING[1] AND PETER KIMMIG[2]*

Landesgesundheitsamt Baden-Württemberg, Stuttgart,[2] and Department of Parasitology, University of Hohenheim, Hohenheim,[1] Germany

Received 21 March 1995/Accepted 26 September 1995

PCR was used to detect and specifically identify a gene fragment from *Cryptosporidium parvum*. An 873-bp region of a 2,359-bp DNA fragment encoding a repetitive oocyst protein of *C. parvum* was shown to be specifically amplified in *C. parvum*. An excystation protocol before DNA extraction allowed the differentiation between live and dead *Cryptosporidium parvum* oocysts.

Cryptosporidium parvum oocysts are present in raw and treated drinking water (6, 12, 14), and they pose a public health risk because this parasite can be transmitted via the fecal-oral route through contaminated water and low numbers of viable oocysts are able to initiate an infection in mammals (10). Several outbreaks of waterborne cryptosporidiosis have been reported (4, 8, 16, 17). The standard method for the detection of *C. parvum* in environmental samples is based on the isolation and identification of the oocysts (7, 11, 13, 19). The oocysts cannot be cultured, so they are stained with a monoclonal antibody coupled with a fluorescent dye and counted by immunofluorescence microscopy. This method of detection does not indicate the viability of oocysts. In vitro excystation, a process in which release of infective sporozoites from oocysts is induced by exposure to bile salts, can demonstrate viability (Fig. 1) (5, 18). The detection of the excystated sporozoites can be used as a viability test for *C. parvum*. Because of the low numbers of cysts usually found in environmental samples, the detection requires a test with high specificity and sensitivity, such as PCR. In this study we developed a method based on PCR for the detection of excysted *C. parvum* sporozoites and therefore allowing discrimination between live and dead *C. parvum* oocysts.

To determine whether PCR amplification of the 873-bp gene fragment of *C. parvum* detects viable sporozoites, feces with oocysts from naturally infected calves were collected and stored in a 2.5% (wt/vol) solution of $K_2Cr_2O_7$ at 4°C for up to 6 months prior to use. Oocysts were purified and concentrated by ether extraction (3 ml of cyst solution and 1.5 ml of ether) and with a percoll step gradient (1, 20). Percoll (Pharmacia) solutions of 1.10, 1.07, and 1.04 g/ml in phosphate-buffered saline (PBS) were prepared and used immediately. The oocysts were recovered from the 1.07-g/ml layer and washed in PBS. Sporozoites were obtained by incubating oocysts in PBS containing 0.25% trypsin and 0.75% (wt/vol) sodium taurocholate for 60 min at 37°C. The trypsin and sodium taurocholate were removed by washing with PBS and centrifugation (800 × *g* for 10 min). The mixture of sporozoites, oocyst walls, and intact oocysts was resuspended in PBS. A subsequent DNase digest destroyed free DNA in the sample. Total genomic DNA of *C. parvum* was extracted from excysted sporozoites by a modified procedure of Ausubel et al. (2). DNA was released by incubation of the sporozoites in digestion buffer with sodium dodecyl sulfate (2%), 15 μl of proteinase K solution (10 mg/ml), 200 μl of 10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl (pH 7.5), and 7 μl of dithiothreitol (1 M) in water. The solution was incubated at 58°C for certain intervals from 1 min to 24 h. DNA was further purified by using phenol-chloroform-isoamyl alcohol (24:24:2) extractions followed by precipitation with 100% isopropanol in the presence of carrier glycogen (200 μg/ml). After centrifugation at 12,000 × *g* for 20 min, the pelleted DNA was washed once with 70% isopropanol and dried. Dried DNA was eluted in a total volume of 10 μl of sterile water (10 min at 55°C).

For PCR, synthetic oligonucleotide primers (DNA Synthesis Lab, University of Hohenheim, Hohenheim, Germany) were designed to amplify an 873-bp region of the *C. parvum* oocyst protein gene, based on the sequence reported by Lally et al. (9). Another two primers, amplifying a 604-bp region, were designed for a nested PCR. Preliminary studies (computer analyses) identified the most suitable primers. Sequence data were analyzed with HUSAR software and compared with data in the GenBank of the Deutschen Krebsforschungszentrum, Heidelberg, Germany, for homology with DNA and amino acid sequences. Paired primers that showed low homology to other sequences in the target fragment and that had closely matched calculated thermal melting points were selected. Primers CP 397 (5′ AGTGTCCTCCAGGTACAAACCTGG TA 3′, corresponding to positions 397 to 423) and CP 1270 (5′ GCACAGCTGGGACAGAATCAGCTTT 3′, corresponding to positions 1270 to 1294), amplifying an 873-bp sequence, were used in the first round (Fig. 2). For nested PCR we used primers NCP 526 (5′ TGCCCACCTGGATATACACT 3′, corresponding to positions 526 to 545) and NCP 1130 (5′ TGCCCATGAGAATGACCATG 3′, corresponding to positions 1130 to 1149), amplifying a 604-bp sequence.

PCR amplification using primers CP 397, CP 1270, NCP 526, and NCP 1130 and a primer annealing temperature of 50°C produced positively amplified DNA bands for *C. parvum* (Fig. 2, lanes 2 and 3). The direct use of PCR to amplify the diagnostic segment of the cryptosporidian gene did not distinguish between live and dead oocysts. Within *C. parvum* cysts, DNA is apparently preserved sufficiently for prolonged periods of time (minimum of 1 week [3]) so that this genetically based detection system cannot be used directly to detect viable oocysts. A way to prove the viability was the detection of the target DNA of active excysted sporozoites after incubation in excystation medium. The excystation followed by a modified DNA extraction method could be used for distinguishing live from dead oocysts. The results of this experiment indicated that a PCR gene probe-positive signal was seen only when viable sporozoites were present. At 5-, 10-, 30-, 60-, and 90-min intervals after digestion buffer addition, only excysted, viable sporozoites gave positive signals. Consequently, a digestion time of 1 h was used

* Corresponding author. Mailing address: Landesgesundheitsamt Baden-Württemberg, Wiederholdstr. 15, D-70174 Stuttgart, Germany.

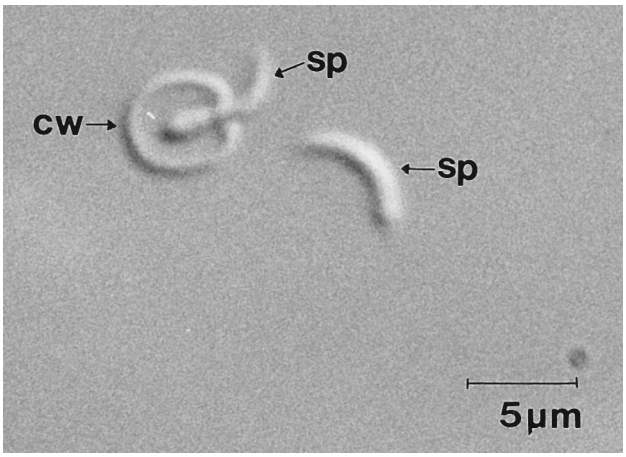

FIG. 1. *C. parvum* oocyst after incubation in excystation medium. sp, sporozoite; cw, cyst wall.

in the following experiments. After 3 h of incubation intact oocysts without preliminary excystation also gave positive signals. After this time the oocyst wall probably became permeable for the digestion buffer. The DNase digestion after excystation avoided false-positive signals caused by free DNA from destroyed sporozoites.

Reaction mixtures for PCR contained 1× PCR buffer (Angewandte Gentechnologie Systeme GmbH [AGS]), 0.2 mmol of each deoxynucleoside triphosphate (AGS) per liter, 50 pmol of each oligonucleotide primer, 2.5 U of *Taq* polymerase (AGS), and 10 μl of template DNA in a final volume of 100 μl. Negative controls without template and a positive control were amplified in parallel. PCR was performed by using a DNA thermal cycler (Pharmacia). Template DNAs were initially denatured at 94°C for 6 min. Then 30 PCR cycles were run under the following conditions: denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and DNA extension at 72°C for 1 min; these cycles were followed by a final incubation at 72°C for 4 min. For nested PCR, 10 μl of the reaction mixture from the first round of PCR was reamplified as described above in a fresh reaction mix with the nested primer pair. PCR products were analyzed by using gel electrophoresis. The amplified DNAs were separated by using a 1.5% horizontal agarose gel. Agarose gels were run in TEB buffer (90 mM Tris-HCl, 2 mM Na-EDTA, 90 mM boric acid). The gels were stained in ethidium bromide solution (1μg/ml), visualized with a UV transilluminator, and photographed.

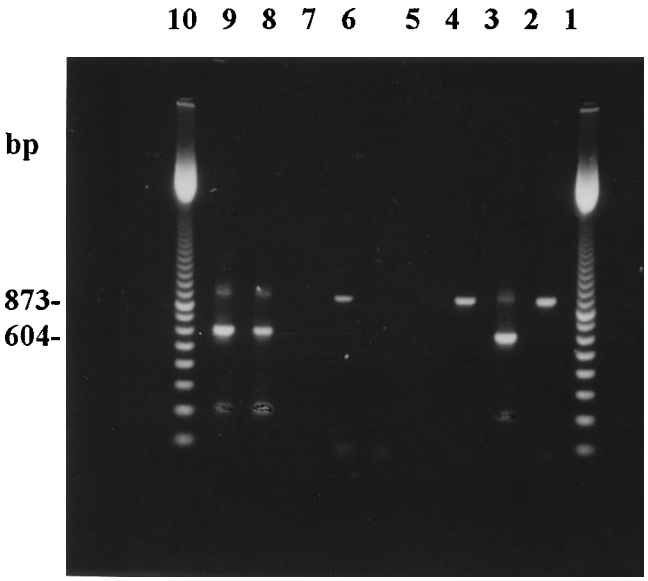

FIG. 2. Agarose gel electrophoresis of PCR products stained with ethidium bromide. Lanes 1 and 10, 100-bp ladder (Pharmacia); lane 2, the 873-bp target sequence of the DNA of 1,000 *C. parvum* sporozoites after the first amplification round; lane 3, the 604-bp target sequence from the second amplification round with 10 μl of the reaction mixture from the first amplification round; lane 4, positive control; lane 5, negative control; lanes 6 and 7, amplification products from the first round with 100 and 10 sporozoites, respectively; lanes 8 and 9, amplification products from the second round with 100 and 10 sporozoites, respectively.

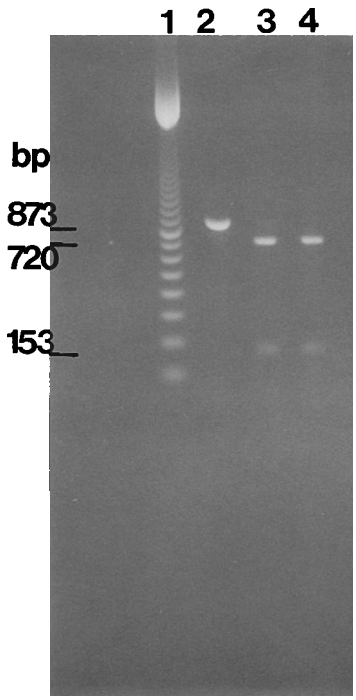

FIG. 3. Agarose gel electrophoresis of PCR products stained with ethidium bromide. Lane 1, 100-bp ladder (Pharmacia); lane 2, the 873-bp target sequence of the DNA from 1,000 *C. parvum* sporozoites; lanes 3 and 4, amplification product after restriction with *Bam*HI restriction enzyme.

To determine the specificity of the developed PCR method for *C. parvum*, the restriction enzyme *Bam*HI with one restriction site for the target sequence was used as a marker to test the amplification product. The restriction endonuclease gave two fragments of 153 and 720 bp (Fig. 3). The specificity of the designed primers was demonstrated by digestion with the restriction endonuclease *Bam*HI. The 873-bp region contains a unique restriction endonuclease site that serves as a useful marker for specificity. The restriction endonuclease *Bam*HI gives two fragments of 153 and 720 bp (Fig. 3). PCR-amplified DNAs were precipitated with isopropanol in the presence of glycogen (200 μg/ml). After centrifugation at 12,000 × *g* for 20 min, the pelleted DNA was dried. Reaction mixtures for restriction contained 1× restriction buffer (AGS) and 8 U of *Bam*HI in a final volume of 10 μl. After incubation at 37°C for 1 h, the products were analyzed by gel electrophoresis.

The specificity of the primers was further tested by attempting to amplify target sequences from a number of other organisms likely to be found in the same environment. Nucleic acids

were recovered from the following microorganisms: *Toxoplasma gondii* (K. Naser, Landesgesundheitsamt Baden-Württemberg, Stuttgart, Germany); a *Sarcocystis* sp. (University of Hohenheim, Stuttgart, Germany); *Giardia lamblia* (P. Karanis, University of Bonn); *Trichomonas vaginalis*, *Entamoeba coli*, *Entamoeba histolytica*, *Trichuris trichiura*, a *Taenia* sp., *Ascaris lumbricoides*, and *Hymenolepis nana* (Landesgesundheitsamt Baden-Württemberg); a *Cyclops* sp., a *Rhodospirillum* sp., *Sphaerotilus natans*, *Saccharomyces cerevisiae*, a *Micromonospora* sp., a *Dictyostelium* sp., *Daphnia pulex*, *Gammarus roeseli*, a *Gerris* sp., an *Isoperla* sp., a *Potamophylax* sp., and *Ephemera vulgata* (University of Hohenheim); and *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, a *Lactobacillus* sp., *Listeria monocytogenes*, a *Micrococcus* sp., a *Pneumococcus* sp., a *Pseudomonas* sp., *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella flexneri*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Yersinia enterocolitica*, *Aspergillus niger*, *Candida albicans*, a *Nocardia* sp., a *Penicillium* sp., *Mus musculus*, and *Homo sapiens* (Landesgesundheitsamt Baden-Württemberg). DNA was recovered and purified by the procedure described for *C. parvum*. With the stringent reaction conditions described above, no amplified products were detected from any of the strains examined. It was not possible to test other species of *Cryptosporidium*, for example, *C. meleagridis* and *C. nosarum*. These species should be examined in further studies.

As well as having the required specificity, a PCR-based detection system must be highly sensitive. In this case, sensitivity will be a function of the number of excysted sporozoites present as well as the efficiency of isolation of semipurified DNA suitable for amplification. To determine the practical levels of sensitivity, the number of excysted *C. parvum* sporozoites was determined by direct counting. DNA prepared as described above from serially diluted sporozoites was then used in the PCR assay. The isolation of amplifiable DNA from low numbers of sporozoites was easily practicable by addition of glycogen as an adsorber before precipitation of the DNA. The specific 873-bp fragment was faintly visible after a single round of 30 amplification cycles when as few as 100 sporozoites were used as the starting material (Fig. 2, lane 6). To see whether the sensitivity could be improved, an aliquot of the amplified material was removed and reamplified for a further 30 cycles after addition of fresh reagents with another primer pair amplifying a 604-bp fragment inside the 873-bp fragment from the first round. This second round of amplification increased the sensitivity such that 10 sporozoites could sometimes be detected (Fig. 2, lane 8), but this was not yet reliable or routinely predictable. Therefore, the practical level of sensitivity with this two-phase amplification scheme has to be determined in further studies. Furthermore, it is important to run positive and negative controls in parallel, to avoid false-positive signals caused by contamination of the probes and false-negative results caused by inhibitors in the probes.

In summary, we have developed a specific and sensitive test using PCR technology for identification and detection of *C. parvum* DNA and for distinguishing live from dead organisms. The specificity and sensitivity of the primers selected for the present study appear to be sufficient for use in detection of low numbers of viable organism, for example, the routine monitoring of drinking water and other environmental samples. The nested PCR offers enhanced sensitivity derived from a second round of amplification, which should allow the detection of viable *C. parvum* in small amounts; the use of a different, nested primer pair for the second round maintains specificity.

We gratefully thank R. Müller, Staatliches tierärztliches Untersuchungsamt Stuttgart, for providing positive feces samples used in this study.

This work was financially supported by the Grimminger Stiftung für Zoonosenforschung, Stuttgart, Germany.

REFERENCES

1. **Arrowood, M. J., and C. R. Sterling.** 1987. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous sucrose and isopycnic percoll gradients. J. Parasitol. **73:**314–319.
2. **Ausubel, M., R. Brendr, R. F. Kingsdon, D. D. Moore, J. K. Seidmann, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Green and Wiley, New York.
3. **Dinkel, A.** 1994. *Cryptosporidium parvum* und Trinkwasser. Eignung der PCR zum Sporozoitennachweis nach Chlorung und Ozonung. Diplomarbeit. Fakultät für Parasitologie der Universität Hohenheim, Hohenheim, Germany.
4. **Edwards, D. D.** 1993. Troubled waters in Milwaukee. ASM News **59:**342–345.
5. **Fayer, R., and R. G. Leek.** 1984. The effects of reducing conditions, medium, pH, temperature, and time on in vitro excystation of *Cryptosporidium*. J. Protozool. **31:**567–569.
6. **Gornik, V., and M. Exner.** 1991. Cryptosporidium sp.—Vorkommen und Bedeutung eines neuen Erregers im Wasser. Forum Stadte-Hyg. **42:**106–108.
7. **Greenberg, A. E., R. R. Trussell, and L. S. Clesceri (ed.).** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
8. **Hayes, E. B., T. D. Matte, T. R. O'Brien, T. W. McKinley, T. G. Logsdon, J. B. Rose, L. Ungar, D. M. Word, P. F. Pinsky, M. Cummings, M. A. Wilson, G. Long, E. S. Hurwitz, and D. D. Juranek.** 1989. Large community outbreak of cryptosporidiosis due to contamination of a filtered public water supply. N. Engl. J. Med. **320:**1372–1376.
9. **Lally, N. C., G. D. Baird, S. J. McQuay, F. Wright, and J. J. Oliver.** 1992. A 2359-base pair DNA fragment from *Cryptosporidium parvum* encoding a repetitive oocyst protein. Mol. Biochem. Parasitol. **56:**69–78.
10. **Miller, R. A., M. A. Bronsdon, and W. R. Morton.** 1986. Determination of the infectious dose of *Cryptosporidium* and the influence of inoculum size on disease severity in a primate model, abstr. 8-148, p. 49. *In* Abstracts of the Annual Meeting of the American Society for Microbiology 1986. American Society for Microbiology, Washington, D.C.
11. **Olson, B. H.** 1989. The safety of our drinking water. N. Engl. J. Med. **320:**1413–1414.
12. **Ongerth, J. E., and H. H. Stibbs.** 1987. Identification of *Cryptosporidium* oocysts in river water. Appl. Environ. Microbiol. **53:**672–676.
13. **Rose, J. B., and K. Botzenhardt.** 1990. *Cryptosporidium* und *Giardia* im Wasser, Nachweisverfahren, Häufigkeit und Bedeutung als Krankheitserreger. GWF Wasser-Abwasser **131:**563–572.
14. **Rose, J. B., A. Cifrino, M. S. Madore, C. P. Gerba, C. R. Sterling, and M. J. Arrowood.** 1986. Detection of *Cryptosporidium* from waste-water and freshwater environments. Water Sci. Technol. **18:**233–239.
15. **Rose, J. B., H. Darbin, and C. P. Gerba.** 1988. Correlations of the protozoa, *Cryptosporidium* and *Giardia* with water quality variables in a watershed. Water Sci. Technol. **20:**271–276.
16. **Rush, B. A., P. A. Chapman, and R. W. Ineson.** 1990. A probable waterborne outbreak of cryptosporidiosis in the Sheffield area. J. Med. Microbiol. **32:** 239–242.
17. **Smith, H. V., W. J. Patterson, R. Hardie, L. A. Greene, C. Benton, W. Tulloch, R. A. Gilmour, R. W. Girdwood, J. C. M. Sharp, and G. Forbes.** 1989. An outbreak of waterborne cryptosporidiosis caused by posttreatment contamination. Epidemiol. Infect. **103:**703–715.
18. **Wagner, C., and P. Kimmig.** 1992. Vitalitätstest für *Cryptosporidium parvum*. Methode und praktische Anwendung. Ber. Hohenh. Semin. Dtsch. Veterinarmed. Ges. Giessen **4:**189–203.
19. **Wagner, C., and P. Kimmig.** 1992. *Cryptosporidium parvum* und *Giardia lamblia*—Vorkommen in Oberflächen- und Trinkwasser. Gesundheitwes. Desinfekt. **54:**662–665.
20. **Waldman, E., S. Tzipori, and J. R. L. Forsyth.** 1986. Separation of *Cryptosporidium* species oocysts from feces by using a Percoll discontinuous density gradient. J. Clin. Microbiol. **23:**199–200.