Evaluation of Two DNA Template Preparation Methods for Post-Immunomagnetic Separation Detection of *Cryptosporidium parvum* in Foods and Beverages by PCR[▽]

Christian D. Frazar and Palmer A. Orlandi*

Division of Virulence Assessment, Center for Food Safety and Applied Nutrition,
Food and Drug Administration, Laurel, Maryland 20708

Received 19 July 2007/Accepted 10 September 2007

***Cryptosporidium parvum* oocysts were recovered by immunomagnetic separation from six artificially contaminated foods. Two DNA isolation methods were subsequently evaluated by PCR. The FTA Concentrator-PS filter provided rapid and reproducible detection, although variability increased at lower inoculum levels (88% and 15% detection in high- and low-inoculum-level samples, respectively). Total DNA extraction generated consistent results at all oocyst levels but resulted in longer analysis time (100% and 59% detection in high- and low-inoculum-level samples, respectively). Also reflected in this study was that the matrix played an important role in the ability to recover oocysts, as sample turbidity, pH, and PCR inhibitors all influenced detection.**

Cryptosporidiosis, a severe diarrheal disease in humans, is caused by several *Cryptosporidium* species, including *Cryptosporidium hominis* and *Cryptosporidium parvum*. As a source for many domestic outbreaks, the prevalence of *C. parvum* in cattle presents a continual threat to domestic water sources, raw beverages, and fresh produce (7, 9). Epidemiological studies have implicated contaminated raw milk and apple cider in many outbreaks of this gastrointestinal disease (2). *C. parvum*-contaminated drinking water has been associated with both large and small outbreaks (1, 11). Indirectly, fresh produce, such as leafy vegetables, has also been implicated as a result of washing with suspected contaminated water (9, 20). Crop irrigation from contaminated water sources and direct soil contact may also be factors.

The ease of transmission through contaminated water sources and fresh produce, the rising number of documented cases of cryptosporidiosis, and the potential public health and economic consequences of outbreaks have highlighted the need for rapid, sensitive, and reliable protocols for the detection and differentiation of *Cryptosporidium* spp. in complex matrices (3). Newer generations of molecular techniques for the detection of *C. parvum* involving immunomagnetic adsorption and PCR have significantly reduced detection times and increased sensitivity (5, 6, 10, 15, 16, 19). The literature is replete with molecular detection protocols that focus on screening water or fecal samples (4, 8, 12, 13, 18, 21). However, relatively few PCR-based methods have been adapted to such complex matrices as foods and beverages (5, 19). The diversity of food matrices, their heterogeneous nature, and the inherent presence of PCR inhibitory substances that are carried over

during DNA template preparation all contribute to the difficulty of detecting low levels of *Cryptosporidium* oocysts.

In the present study, we combined the selectivity of immunomagnetic separation and the sensitivity of nested PCR to compare two techniques for preparing DNA templates: a filter-based method using the FTA Concentrator-PS filter (Whatman, Inc., Newton, MA) and a total DNA extraction method using a MasterPure DNA kit by Epicentre (Madison, WI). Six artificially contaminated foods (orange juice, apple cider, whole milk, strawberries, parsley, and lettuce) were chosen for their past association with food-borne illness attributed to *C. parvum* contamination or their potential for contamination by *C. parvum*.

All foods and beverages were obtained from local retailers (Laurel, MD). Orange juice was defined as “no pulp” or “high pulp,” as advertised by the manufacturer. *C. parvum* oocysts were provided courtesy of Ron Fayer at the USDA (Beltsville, MD). Oocyst stocks were diluted in water, and counts were verified by a hemacytometer. For juice and milk products, 10-ml samples were inoculated with 5, 50, 500, or 5,000 oocysts and then processed directly by immunomagnetic adsorption. Ten-milliliter aliquots of NET buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) were artificially contaminated and processed in the same manner as the beverage samples and served as positive controls. Oocysts were directly applied to the surfaces of 10 g of leafy vegetables and 50 g of fresh strawberries in small volumes (<10 μ l) and allowed to air dry for 10 minutes. The samples were then washed in a manner similar to that previously described by Ortega et al., using BagPage filter bags (Interscience, St. Nom, France) and 100 ml of NET buffer (17). The sealed samples were gently rocked for 15 min on each side. The filtrate was collected and centrifuged for 10 min at 1,500 $\times$ g. The supernatant was aspirated to a final volume of 10 ml, and the pellet was resuspended in this volume. All foods and beverages were tested in replicates of five. For each matrix, one uninoculated sample was processed identically to

* Corresponding author. Mailing address: U.S. Food and Drug Administration, CFSAN/OARSA/DVA, MOD 1 Research Facility, Rm. 3603 (HFS-025), 8301 Muirkirk Rd., Laurel, MD 20708. Phone: (301) 210-7937. Fax: (301) 210-7979. E-mail: palmer.orlandi@fda.hhs.gov.

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FIG. 1. Comparison of *C. parvum* detection in several matrices, using kit-driven DNA extraction to prepare the PCR template. An asterisk indicates that no oocysts were detected. LOJ, low-pulp orange juice; HOJ, high-pulp orange juice; W. Milk, whole milk.

the other samples and served as the negative control, ensuring that the samples were not previously contaminated.

C. parvum oocysts were selectively adsorbed from produce washes or beverages with a Dynabeads anti-*Cryptosporidium* kit (Invitrogen, Carlsbad, CA), using the manufacturer's protocol for water testing. Captured oocysts were eluted from the magnetic particles in a volume of 0.05 ml 0.1 N HCl for 5 min at room temperature. Acid eluates were neutralized with 5 μ l of 1 N NaOH.

DNA templates were then prepared from the acid-eluted oocysts. Separate trials were done for each DNA template preparation (i.e., individual samples were not split between FTA and DNA extraction). In a procedure adapted from Orlandi and Lampel (16), oocysts captured by immunoadsorption were diluted into 10 ml of NET buffer, passed through FTA Concentrator-PS units (16) under vacuum, and washed twice with 10 ml of FTA purification buffer (Whatman, Inc., Newton, MA), followed by two washes with 10 ml of a 1×10^{-4} dilution of Tris-EDTA. Filter units were disassembled, and the disk was dried on a 56°C heating block. Prior to PCR amplification, a 6-mm single-hole punch was used to excise three areas of the concentrated template. Three areas were used in order to avoid concentrating PCR inhibitors and to avoid overwhelming the capacity of the filter, as is possible when only a single punch is used (data not shown). Thus, all three punches from each FTA Concentrator-PS unit were amplified by PCR and samples were scored as positive when any of the three punches produced the appropriate amplicon. Alternately, DNA templates were prepared by extraction of total DNA (MasterPure DNA extraction kit) from the neutralized oocyst concentrates. The final DNA pellet was resuspended in 10 μ l Tris-EDTA, which was used directly as the template for PCR. For each set of extractions, a negative control was done in order to ensure that the kit remained free of contamination.

Nested PCR primer pairs that targeted the 18S rRNA gene were previously described by Sturbaum et al. (22). First-round reactions were conducted with a total volume of 100 μ l, with either the FTA filter or 10 μ l of extracted DNA as the template. The reaction mixture consisted of HotStarTaq master

FIG. 2. Comparison of *C. parvum* detection in several matrices, using FTA Concentrator-PS units to prepare the PCR template. An asterisk indicates that no oocysts were detected. LOJ, low-pulp orange juice; HOJ, high-pulp orange juice; W. Milk, whole milk.

mix (Qiagen, Valencia, CA) containing 200 μ M of each nucleotide, an adjusted final concentration of 2 mM $MgCl_2$, and 0.2 μ M each of the first-round primers. All PCR amplifications were performed with a PTC-200 DNA engine (MJ Research, Waltham, MA). For each set of PCRs, a sample in which sterile water was used as the template in order to ensure that the PCR reagents were uncontaminated was included. Nested PCR was conducted with a reaction volume of 25 μ l, using the same reaction component concentrations, with 1 μ l of the primary amplicon as the template. The reaction conditions for both rounds of amplification were as previously described (15). All second-round (nested) PCR products were separated by agarose (1.5%) gel electrophoresis gel containing ethidium bromide (0.2 μ g/ml) and visualized on a UV transilluminator.

Figure 1 summarizes the results observed for DNA templates prepared by kit-driven extraction. Oocysts were detected in 100% of all high-inoculum-level (500- and 5,000-oocyst) samples and in 59% of low-inoculum-level (50- and 5-oocyst) samples. At the 50-oocyst inoculum level, only lettuce (two positive replicates among five replicates, or 2/5) and parsley (4/5) were not positive for all five replicates. At the five-oocyst level, the NET control performed the best (3/5) and cider the poorest (0/5).

The FTA Concentrator-PS units (Fig. 2) performed comparably to the kit-driven template preparation at the higher *C. parvum* oocyst levels (88% detection), although slight decreases in detection sensitivity were observed for the two highest levels in cider and both high- and low-pulp orange juice (4/5). In contrast, inocula at the 50-oocyst level in cider, high-pulp orange juice, and parsley were undetected. Only high-pulp orange juice and parsley were detected (1/5) at the lowest level of oocyst inoculum. Overall, oocysts were detected in 15% of the low-inoculum-level samples. None of the uninoculated matrices were positive for *C. parvum* when either method of DNA template preparation was used.

FTA filters cause contact lysis of microorganisms and consequently trap released nucleic acids within the fiber matrix of the filter (16). With this protocol, approximately 20% losses were observed in previous studies, mainly during the initial filtration step (data not shown). This may reflect the capacity

of the FTA filter unit under these conditions as well as the pore size of the fiber matrix (~25 µm). These losses will significantly affect the lower limits of detection for the FTA Concentrator-PS units. This was most noticeable in the NET controls, where oocysts were not detected in either the 50- or the 5-oocyst inoculum. It may be that oocysts (4 to 6 µm) pass through the filters without lysing. Oocyst association with particulates in matrix samples may increase the odds of contact and subsequent lysis in the filter.

Regardless of DNA template preparation, the matrices from which the oocysts were derived appeared to have some influence on the quality of the DNA templates obtained. Matrix interference between beads and the magnet is the likely cause of poor recovery in orange juice samples, as observed by reduced bead recovery. Apple cider also proved to be a difficult matrix. Others report PCR inhibition, most likely due to polyphenolics, when attempting to detect pathogens in apple cider (5, 14). Losses in parsley and lettuce samples are attributed to the additional steps and the greater surface area. Another matrix consideration is pH. We observed marginally higher rates of recovery when the pH values of the matrices, when combined with the immunomagnetic separation buffers, were between 7.0 and 8.1 (data not shown).

Based on the observed results, we believe that *C. parvum* oocysts could be successfully recovered and detected from other commodities with properties similar to those demonstrated here, using either method of DNA template preparation. This includes milk products of various fat content levels (whole, skim, half and half, etc.), apple juice, apple sauce, and other herbs and leafy produce.

Detection of pathogens in food matrices associated with outbreaks or surveillance of commodities susceptible to contamination requires that a detection method balance sensitivity, specificity, sample size, and analysis time with the highest degree of reproducibility possible. Whereas the FTA Concentrator-PS units were less sensitive and introduced replicate variability with lower levels of inocula than the conventional DNA extraction kit, they required considerably less time and fewer sample manipulations. The determining factor as to which DNA preparation method is most appropriate will be dictated by the scope and aim of the intended analysis and whether it is beneficial for the researcher to sacrifice some sensitivity for more-rapid results.

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REFERENCES

- Centers for Disease Control and Prevention. 1987. Cryptosporidiosis—New Mexico, 1986. *Morb. Mortal. Wkly. Rep.* **36**:561–563.
- Centers for Disease Control and Prevention. 1997. Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider—Connecticut and New York, October 1996. *Morb. Mortal. Wkly. Rep.* **46**:4–8.
- Centers for Disease Control and Prevention. 2007. Summary of notifiable diseases—United States, 2005. *Morb. Mortal. Wkly. Rep.* **54**:2–92.
- Chesnot, T., and J. Schwartzbrod. 2004. Quantitative and qualitative comparison of density-based purification methods for detection of *Cryptosporidium* oocysts in turbid environmental matrices. *J. Microbiol. Methods* **58**:375–386.
- Deng, M. Q., and D. O. Cliver. 2000. Comparative detection of *Cryptosporidium parvum* oocysts from apple juice. *Int. J. Food Microbiol.* **54**:155–162.
- Di Pinto, A., and M. G. Tantilillo. 2002. Direct detection of *Cryptosporidium parvum* oocysts by immunomagnetic separation-polymerase chain reaction in raw milk. *J. Food Prot.* **65**:1345–1348.
- Fayer, R., J. M. Trout, T. K. Graczyk, and E. J. Lewis. 2000. Prevalence of *Cryptosporidium*, *Giardia* and *Eimeria* infections in post-weaned and adult cattle on three Maryland farms. *Vet. Parasitol.* **93**:103–112.
- Kruger, P., A. Wiedenmann, and K. Botzenhart. 1998. Detection of *Cryptosporidium* oocysts in water: comparison of the conventional microscopic immunofluorescence method with PCR and TaqMan PCR, p. 1–9. In *Proceedings of the OECD Workshop Interlaken 1998*. OECD Workshop Molecular Methods for Safe Drinking Water. Organisation for Economic Co-operation and Development, Interlaken, Switzerland.
- Laberge, I., M. W. Griffiths, and M. W. Griffiths. 1996. Prevalence, detection and control of *Cryptosporidium parvum* in food. *Int. J. Food Microbiol.* **32**:1–26.
- 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.
- Mac Kenzie, W. R., N. J. Hoxie, M. E. Proctor, M. S. Gradus, K. A. Blair, D. E. Peterson, J. J. Kazmierczak, D. G. Addiss, K. R. Fox, J. B. Rose, et al. 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *N. Engl. J. Med.* **331**:161–167.
- McLauchlin, J., C. Amar, S. Pedraza-Díaz, and G. L. Nichols. 2000. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. *J. Clin. Microbiol.* **38**:3984–3990.
- Ochiai, Y., C. Takada, and M. Hosaka. 2005. Detection and discrimination of *Cryptosporidium parvum* and *C. hominis* in water samples by immunomagnetic separation-PCR. *Appl. Environ. Microbiol.* **71**:898–903.
- Ogunjimi, A. A., and P. V. Choudary. 1999. Adsorption of endogenous polyphenols relieves the inhibition by fruit juices and fresh produce of immuno-PCR detection of *Escherichia coli* O157:H7. *FEMS Immunol. Med. Microbiol.* **23**:213–220.
- Orlandi, P. A., Jr., C. Frazar, L. Carter, and D. M. Chu. 2004. Detection of *Cyclospora* and *Cryptosporidium* from fresh produce: isolation and identification by polymerase chain reaction (PCR) and microscopic analysis, p. 1–20. In G. J. Jackson, R. Merker, and R. Bandler (ed.), *FDA bacteriological analytical manual*. U.S. Food and Drug Administration, Rockville, MD.
- Orlandi, P. A., and K. A. Lampel. 2000. Extraction-free, filter-based template preparation for rapid and sensitive PCR detection of pathogenic parasitic protozoa. *J. Clin. Microbiol.* **38**:2271–2277.
- Ortega, Y. R., C. R. Roxas, R. H. Gilman, N. J. Miller, L. Cabrera, C. Taquiri, and C. R. Sterling. 1997. Isolation of *Cryptosporidium parvum* and *Cyclospora cayetanensis* from vegetables collected in markets of an endemic region in Peru. *Am. J. Trop. Med. Hyg.* **57**:683–686.
- Quintero-Betancourt, W., E. R. Peele, and J. B. Rose. 2002. *Cryptosporidium parvum* and *Cyclospora cayetanensis*: a review of laboratory methods for detection of these waterborne parasites. *J. Microbiol. Methods* **49**:209–224.
- Ripabelli, G., A. Leone, M. L. Sammarco, I. Fanelli, G. M. Grasso, and J. McLauchlin. 2004. Detection of *Cryptosporidium parvum* oocysts in experimentally contaminated lettuce using filtration, immunomagnetic separation, light microscopy, and PCR. *Foodborne Pathog. Dis.* **1**:216–222.
- Robertson, L. J., and B. Gjerde. 2001. Occurrence of parasites on fruits and vegetables in Norway. *J. Food Prot.* **64**:1793–1798.
- Rochelle, P. A., D. M. Ferguson, T. J. Handoyo, L. R. De, M. H. Stewart, and R. L. Wolfe. 1997. An assay combining cell culture with reverse transcriptase PCR to detect and determine the infectivity of waterborne *Cryptosporidium parvum*. *Appl. Environ. Microbiol.* **63**:2029–2037.
- Sturbaum, G. D., C. Reed, P. J. Hoover, B. H. Jost, M. M. Marshall, and C. R. Sterling. 2001. Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. *Appl. Environ. Microbiol.* **67**:2665–2668.