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Comparative detection of *Cryptosporidium parvum* oocysts from apple juice

Ming Qi Deng, Dean O. Cliver*

Department of Population Health and Reproduction, and World Health Organization Collaborating Center for Food Virology, School of Veterinary Medicine, University of California–Davis, One Shields Avenue, Davis, CA 95616-8743, USA

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Abstract

Drinking unpasteurized apple juice (or cider) has been associated with cryptosporidiosis, the diarrheal disease caused by the small protozoan parasite, *Cryptosporidium parvum*. This report compares detection of *C. parvum* oocysts from apple juice by acid-fast staining (AFS), direct immunofluorescence assay (DIFA), and polymerase chain reaction (PCR), following sample concentration by formalin–ethyl acetate sedimentation or sucrose flotation. Flotation was more efficient than sedimentation in recovering oocysts, and DIFA consistently detected lower numbers of oocysts than AFS. In combination, flotation–AFS could detect 3000 to 10 000 oocysts inoculated into 100 ml of apple juice while flotation–DIFA was able to detect as few as 100 oocysts. The highest sensitivity, 10 to 30 oocysts per 100 ml of apple juice, was achieved by DIFA following immunomagnetic capture (IC) of oocysts from samples concentrated by the flotation method. The detection limit of PCR following flotation or flotation–IC was 30 to 100 oocysts; sequence analysis of the amplicon demonstrated that the PCR amplicon was *C. parvum*-specific.

Keywords: *Cryptosporidium parvum*; Acid-fast staining; Apple cider; Apple juice; Immunofluorescence assay; Immunomagnetic capture; Polymerase chain reaction

1. Introduction

Apple juice (often called cider in the US) is a traditional beverage produced in the Fall. It is commonly manufactured locally at small cider mills where apples are crushed in presses. The presence of animals in orchards or nearby pastures can result in inadvertent contamination of apples on the ground with manure. As the practice of using drop apples for making apple juice is common (Besser et al., 1993), pathogens can be introduced from this source. Apples can also become contaminated when transported or stored in areas that contain manure, when rinsed with contaminated water, when pressed against contaminated equipment surfaces, or by workers' hands that are contaminated with feces containing pathogens. Because apple juice is frequently consumed unpasteurized, contamination with such pathogens

*Corresponding author. Tel.: + 1-530-754-9120; fax: + 1-530-752-5845.

E-mail address: docliver@ucdavis.edu (D.O. Cliver)

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has the potential to cause foodborne diseases in humans, as has been evidenced by its association with a variety of foodborne pathogens, including *Salmonella* (Centers for Disease Control, 1975), enterohemorrhagic *Escherichia coli* (Besser et al., 1993; Centers for Disease Control, 1996a), and the small protozoan parasite *Cryptosporidium parvum* (Millard et al., 1994; Centers for Disease Control, 1997).

Since the mid-1980s, *C. parvum* has been recognized worldwide as an important human pathogen causing watery diarrhea (Current and Garcia, 1991; MacKenzie et al., 1994; Fayer et al., 1997). Although cryptosporidiosis in immunocompetent individuals is self-limiting, it can become persistent and life-threatening in persons with deficient immune systems, such as in AIDS patients (Fayer et al., 1997). While most cases of cryptosporidiosis are attributed to drinking water contaminated with oocysts, the environmentally resistant, transmissible and infective stage of *C. parvum*, the high prevalence of oocysts in many species of animals and the ubiquity of oocysts in the environment suggest possible transmission in foods (Smith, 1993; Laberge and Griffiths, 1996). Several foods have been suspected as vehicles in cryptosporidiosis transmission: raw cow or goat milk, sausage, chicken salad, frozen tripe, green onions and apple juice (Smith, 1993; Centers for Disease Control, 1996b, 1997; Fayer et al., 1997).

In 1993, a large cryptosporidiosis outbreak was related to drinking unpasteurized, fresh pressed apple juice at a country fair (Millard et al., 1994). During the investigation, *C. parvum* oocysts were detected in the leftover apple juice, on the surface of the cider press, and in stool specimens from two calves at the farm. It was believed that apples used for the juice were contaminated when they fell to the ground, which was grazed by cattle shedding *C. parvum* oocysts. In 1996, another outbreak was associated with drinking commercially produced, unpasteurized apple juice, in which the apples used for the juice may have become contaminated when they were washed with well water that was fecally contaminated (Centers for Disease Control, 1997). Although these are the only reports of direct apple juice association with cryptosporidiosis, the true incidence could be much higher because many sporadic cases are not investigated and food samples are usually unavailable for analysis by the time that they have been traced and related to the outbreak, due to the long incubation period of the disease. Furthermore, because a procedure equivalent to bacterial enrichment culture to increase oocyst number is not available, detection of oocysts, which are usually present at a relatively low number in the food, is hampered by the lack of sensitive detection methods. In this study, the effectiveness of different procedures in detecting *C. parvum* oocysts from apple juice was evaluated. We hope that such information will facilitate future investigation of the role of apple juice in a cryptosporidiosis outbreak.

2. Materials and methods

2.1. Source of apple juice and C. parvum oocysts

Unpasteurized apple juice (freshly pressed, not from concentrate) was purchased from a local farmer's retail store, it was unfiltered, contained no preservatives, and had a pH of 3.7. Bovine-origin *C. parvum* oocysts were concentrated from infected calves, enumerated, and stored in $1\times$ Hanks balanced saline solution as described previously (Deng and Cliver, 1998). The oocyst stock was diluted in deionized distilled water to $5.0 \cdot 10^5$, $5.0 \cdot 10^3$, and $5.0 \cdot 10^1$ oocysts per ml. The same oocyst dilutions were used throughout the study to ensure consistency.

2.2. Inoculation of apple juice

To determine the sensitivities of different detection procedures, selected quantities of the above three oocyst dilutions were added to apple juice to achieve concentrations of 100 000, 30 000, 10 000, 3000, 1000, 300, 100, 30, 10 and 1 oocysts per 100 ml. Inoculated apple juice was mixed and stored at 4°C before being processed as described below. All inoculations, sample processing procedures, and *C. parvum* detection tests were repeated three times; uninoculated apple juice was always included as a negative control.

2.3. Oocyst concentration

Two commonly used oocyst concentration methods were compared; both were performed at room temperature. The formalin–ethyl acetate sedimenta-

tion procedure was adapted from one described previously (Weber et al., 1992). A 50-ml portion of inoculated apple juice was first centrifuged at 1000 *g* for 10 min, the pellet was resuspended in 30 ml of 10% formalin; mixed throughly with 12 ml of ethyl acetate, and centrifuged at 500 *g* for 10 min. Four layers (from top to bottom) were formed: ethyl acetate, debris, formalin and a small pellet. The compact debris layer was loosened with a wooden stick, and the top three layers (ethyl acetate, debris and formalin) were carefully decanted. The sediment was then resuspended in ~100 μl of 10% formalin and stored at 4°C until examination.

Sheather's sucrose flotation was performed as described previously (Deng and Cliver, 1998). Briefly, a 50-ml portion of apple juice was centrifuged at 1000 *g* for 10 min, the precipitate formed was washed and resuspended in 5 ml of Tween-water (distilled water containing 0.2% Tween-20), overlaid over 20 ml of sucrose solution (1.20 g/ml), and centrifuged again at 1000 *g* for 10 min. Two layers were formed; and the oocyst-containing upper layer was removed, washed, resuspended in ~100 μl of Tween-water, and stored at 4°C.

2.4. Detection of C. parvum oocysts from concentrated samples

2.4.1. Modified acid-fast staining (AFS)

The modified cold Ziehl–Neelsen AFS (Boufassa-Ouzrout et al., 1986) was performed with slight alteration, using laboratory-prepared reagents. Briefly, a smear from a concentrated sample (by sedimentation or flotation) was made on a glass slide, allowed to air-dry, fixed in absolute methanol for 5 min, stained for 1 h (at room temperature) in Ziehl–Neelsen's carbol–fuchsin solution (prepared by mixing one volume of 15% basic fuchsin in absolute ethanol with nine volumes of 5.0% phenol in deionized water), destained in 10% sulfuric acid–alcohol for 20 s, stained with 1.5% malachite green solution (at room temperature) for 5 min, and examined under a normal light microscope. Positive controls using oocyst-containing fecal material were also included.

2.4.2. Direct immunofluorescence assay (DIFA)

Concentrated sample (50 μl) was subjected to DIFA using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (Meridian Diagnostics, Cincinnati, OH, USA), following the manufacturer's instructions. The stained sample was examined with an epi-fluorescence microscope under a FITC filter, with excitation bands at 450 to 490 nm and emission bands at 510 to 550 nm. A control using 50 μl of purified *C. parvum* oocyst suspension was also included. A sample was recorded as positive when a fluorescently stained object, of the correct size and morphology of fluorescently stained *C. parvum* oocysts in the positive control, was identified.

2.4.3. DIFA following immunomagnetic capture (IC) of oocysts

Polyclonal immunoglobulin (Ig) G was purified from rabbit antiserum raised against purified *C. parvum* oocysts, labeled with biotin, and attached onto streptavidin-coated paramagnetic particles (Promega, Madison, WI, USA), following previously described protocols (Deng et al., 1997). To monitor IgG coating efficiency, a 10 μl (10-μg) portion of magnetic particles was then incubated with FITC-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) at 37°C for 30 min, washed twice with 0.1 M phosphate-buffered saline (PBS, pH 7.2), and examined with a epi-fluorescence microscope. Rabbit IgG-coated particles showed bright green fluorescence whereas non-coated particles did not fluoresce.

IC–DIFA was performed using the concentrated samples from flotation. The concentrates were further washed and resuspended in 1 ml of PBS, mixed with 50 μl (50 μg) of anti-*C. parvum* IgG-coated paramagnetic particles, and incubated at 37°C for 2 h, with the tubes being gently inverted 2–3 times every 30 min. The magnetic bead–oocyst complex was separated from other materials by using a magnetic stand (Promega), gently washed three times and resuspended in 100 μl of PBS. A 50-μl portion was used directly for DIFA as described above, since dissociation of oocysts from these magnetic beads was found to be unnecessary in a preliminary experiment.

2.4.4. Polymerase chain reaction (PCR) following flotation or flotation–IC

The concentrated sample following flotation was washed and resuspended in 250 μl of 1× Tris–EDTA buffer (TE, pH 8.0) containing proteinase K (200 μg/ml, Promega) and 1.0% *N*-lauroyl sarcosine (Sigma). After digestion at 60°C (in a waterbath) for 1 h and centrifugation at 10 000 *g* for 5 min, genomic DNA was extracted from the lysate by

using a QIAamp blood kit (Qiagen, Santa Clarita, CA, USA), eluted in 30 μl of deionized distilled water pre-heated to 70°C, and subjected to PCR using a pair of previously described *C. parvum*-specific primers (Laberge et al., 1996) as below.

For PCR following flotation–IC, captured oocysts were dissociated by mixing with 100 μl of 0.1 μ HCl and neutralizing with 10 μl of 1.0 μ NaOH; and the beads were separated magnetically. The oocysts that remained were washed with TE and the DNA was extracted as above and used for PCR.

In addition to 30 μl of template DNA, the PCR mixture contained 1× PCR buffer II, 2.0 mM of $MgCl_2$, 0.2 mM of each of the four deoxynucleoside triphosphates, 0.5 μM of each of the two primers (forward primer: 5′-GCC CAC CTG GAT ATA CAC TTT-3′, bp 527 to 548; reverse primer: 5′-TCC CCC TCT CTA GTA CCA ACA GGA-3′, bp 861 to 884), 2.5 U of Taq DNA polymerase, and deionized distilled water to bring the final volume to 50 μl. The primers were synthesized by GeneMed Biotechnologies (San Francisco, CA, USA); whereas all other PCR reagents were purchased from Perkin-Elmer (Norwalk, CT, USA).

A hot-start PCR was applied to avoid possible non-specific amplification: PCR reagents other than Taq DNA polymerase were mixed in 0.5-ml PCR tubes, topped with 30 μl of mineral oil, and incubated at 80°C on a Progene 120 thermal cycler (Techne, Princeton, NJ, USA) for at least 5 min before Taq was added. The PCR mixture then underwent initial denaturation at 94°C for 3 min and was subjected to 35 cycles of denaturation (94°C for 1 min), primer annealing (50°C for 1 min), and extension (72°C for 1 min). Following an additional period of extension at 72°C for 7 min, the PCR mixture was kept at 4°C and portions of 20 μl were analyzed by electrophoresis on 1.8% agarose gels and staining with ethidium bromide, with a 1-kb DNA ladder (GIBCO BRL Life Technologies, Gaithersburg, MD, USA) serving as a DNA size marker. Based on previously determined PCR specificity (Laberge et al., 1996), the detection of a product with the expected length (358-bp) was interpreted as demonstrating the presence of *C. parvum* in the sample. A positive control using 1000 oocysts and a negative control containing no template were included to monitor the efficiency of DNA extraction, PCR efficiency and cross-contamination during the manipulation.

To further confirm the specificity of PCR amplification, the 358-bp product was separated from PCR reagents by using a QIAquick PCR purification kit (Qiagen), resuspended in distilled deionized water at 7.0–8.0 ng/μl, and subjected to automated sequencing using the ABI automated sequencing system (Davis Sequencing, Davis, CA, USA). Both the forward primer and the reverse primer were used, and the obtained sequences were searched against the genome databases at the National Center for Biotechnology Information (NCBI) by using the BLAST 2.0 homology search program (NCBI, National Institutes of Health).

3. Results

3.1. Comparison of sample concentration methods and oocyst detection methods

The sensitivities of different detection methods following sedimentation or flotation are presented in Table 1. As is shown, AFS following sedimentation was the least sensitive – 10 000 oocysts were required for detection. DIFA following IC of a sample concentrated by flotation was the most sensitive, capable of detecting 30 (two occasions) or even 10 (one occasion) oocysts. The detection limit of PCR following flotation or flotation–IC was 100 oocysts (one occasion) to 30 oocysts (two occasions).

Of the sample concentration methods compared, sucrose flotation was generally more efficient in recovering oocysts from apple juice, since AFS and DIFA following flotation had consistently higher sensitivities than their counterparts following sedimentation. Similarly, DIFA demonstrated higher sensitivities than AFS, because lower numbers of oocysts were detectable following either sedimentation (300 vs. 10 000) or flotation (100 vs. 3000). Furthermore, DIFA slides were much easier to read than AFS smears, because of the limited areas to be examined and the superior contrast between fluorescently stained oocysts against a dark background in DIFA. Compared to sedimentation–DIFA and flotation–DIFA, smears of IC–DIFA were easier to read due to the reduced presence of debris, and oocysts were stained somewhat more brightly (data not shown).

Flotation–PCR and flotation–IC–PCR were slightly less sensitive than IC–DIFA since 30–100

Table 1
Sensitivities of different methods in detecting *C. parvum* oocysts from apple juice

Concentration method	Detection method	Number of inoculated oocysts per 100 ml										
		100 000	30 000	10 000	3000	1000	300	100	30	10	1	0
Sedimentation	AFS[a]	+	+	+	−	−	−	NT	NT	NT	NT	−
	DIFA[b]	NT[c]	NT	NT	+	+	+	−	NT	NT	NT	−
Flotation	AFS	NT	+	+	+	±[d] (1)	−	−	NT	NT	NT	−
	DIFA	NT	NT	NT	+	+	+	± (2)	−	NT	NT	−
	PCR[e]	NT	NT	+	+	+	+	+	± (1)	−	−	−
Flotation–IC[f]	DIFA	NT	NT	NT	+	+	+	+	+	± (2)	−	−
	PCR	NT	NT	NT	+	+	+	+	± (2)	−	−	−

[a] Acid-fast staining.
[b] Direct immunofluorescence assay.
[c] Not tested.
[d] Not all three replicate tests were positive; the number in the parentheses indicates the number of positive replicates.
[e] Polymerase chain reaction.
[f] Immunomagnetic capture after concentration by flotation.

oocysts were required for detection. However, they were easiest to interpret among all detection methods, because only the product of expected length (358-bp) was obtained (Fig. 1).

3.2. Sequence analysis of PCR product

Sequences of 323 nucleotides from the forward primer and 328 nucleotides from the reverse primer had 100% identity. The whole sequence of the 358-bp PCR product was then deduced. When searched against a total number of 404 657 sequences in the NCBI database, it had 100% identity with three sequences with access numbers M95743, Z17386 and Z22537. These three sequences are actually of the same origin – a gene for *C. parvum* oocyst wall protein of 190 000 Da, and PCR primers were designed based on the sequence of M95743 (Laberge et al., 1996). Other than five short regions (18 or 19 nucleotides long) within the PCR product that had 100% identities with gene segments of heterologous organisms, there was no other sequence, either of *C. parvum* or of other organisms, with noticeable homology with the PCR product. Therefore, by sequence analysis, the species-specificity of PCR amplification was further verified.

4. Discussion

Animals are the primary reservoir for the pathogenic organisms associated with foodborne outbreaks from consumption of unpasteurized apple juice (Centers for Disease Control, 1975, 1996a; Besser et al., 1993; Millard et al., 1994). It is known that *C. parvum* is highly prevalent in cattle and many other

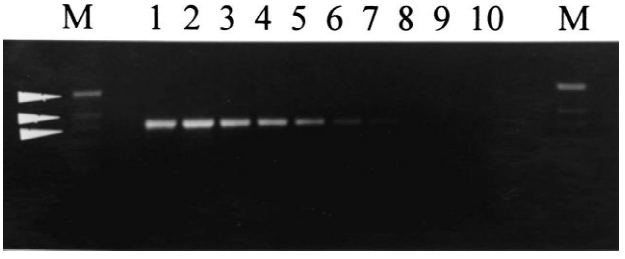

Fig. 1. Detection of *C. parvum* oocysts in apple juice by flotation–PCR. Lanes M, DNA size marker (1-kb ladder), bands indicated by arrows are (in descending order): 506 bp, 396 bp and 344 bp, respectively; lane 1, PCR positive control, using 1000 oocysts suspended in TE; lanes 2–9, apple ciders inoculated with 10 000, 3000, 1000, 300, 100, 30, 10 and 1 oocyst(s) per 100 ml; lane 10, negative control, no template in the PCR mixture.

animal species and that infected animals can shed numbers of *C. parvum* oocysts as high as 10^7–10^9 per ml of feces (Fayer et al., 1997). On the other hand, the infectious dose of *C. parvum* oocysts is as low as 30 oocysts (Dupont et al., 1995); therefore contamination of apple juice with manure is likely to cause consumer infections.

In this study, AFS was demonstrated to be unreliable, since its detection limit was 3000 oocysts, whereas far fewer oocysts can cause infection in a healthy person. The detection limit of 300–1000 oocysts per 100 ml suggested that a DIFA-negative apple juice could still cause human infection. Immunomagnetic separation has been developed in the past few years for a wide variety of applications, including separating pathogens from food samples (Seo et al., 1998) and *C. parvum* oocysts from environmental samples (Deng et al., 1997; Rochelle et al., 1999). In this study, IC–DIFA was able to detect as few as 10 oocysts from 100 ml of apple juice, the highest sensitivity of all the detection methods. Assuming that not more than a 300 ml portion of apple juice is consumed and considering that the minimal infectious dose of *C. parvum* is 30 oocysts (Dupont et al., 1995), our data suggest that IC–DIFA could be used in detecting *C. parvum* oocysts to the minimal infectious dose from apple juice. They also indicate that IC–DIFA could be applied for sensitive detection of *C. parvum* oocysts from other food samples.

In addition to having a sensitivity superior to AFS, DIFA is less time-consuming and less error-prone. In the AFS procedure, it took at least 60 min to stain the smear and about 30 min to examine it completely. Microscopic examination would become even more tedious if there were multiple samples to be examined. In the DIFA procedure, staining of the smear could be completed within 35 min (the smear could be air-dried and stored for next day staining), and examination of the sample could be done within 10 min. Moreover, because of the distinct fluorescence of oocysts against the dark background, it requires less expertise, and oocysts could more easily catch the examiner's attention than in AFS. As a result, there will be many fewer false negatives or false positives in routine analyses resulting from the examiner's fatigue and insufficient level of experience. Although the IC–DIFA procedure requires an additional 30–40 min, it is still less tedious than AFS-based methods.

Theoretically, PCR can detect one single copy of DNA; therefore, the detection limit could be as low as one oocyst. But such sensitivity was not achieved in flotation– or IC–PCR, probably because DNA extraction and/or PCR efficiency was affected by the presence of some inhibitory substances in apple juice. Although PCR takes the most time among all detection methods, the result is the easiest to interpret and requires no familiarity of the examiner to *C. parvum* oocysts. The superior specificity of PCR also presents a distinct advantage over other detection methods. Although *C. parvum* is the only *Cryptosporidium* species infectious to humans, many animal species can harbor other non-human pathogenic *Cryptosporidium* species that are indistinguishable from *C. parvum* by either AFS or DIFA. Specificity testing in a previous study (Laberge et al., 1996) and sequence analysis of the amplicon in this study confirmed that the PCR procedure is species-specific and thus can be relied upon to differentiate *C. parvum* from other *Cryptosporidium* species. Furthermore, when there is more than one possible source of oocysts associated with a cryptosporidiosis outbreak, it will help greatly in epidemiological study if the small number of oocysts detected from apple juice or other food samples can be differentiated from or matched with oocysts detected from patient specimens or field samples. Whereas this can not be achieved by using traditional methods, PCR has the potential to be used for such comparisons, providing that diagnostic primers are available to differentiate human isolates and animal isolates by amplifying a *C. parvum* specific gene fragment and then digesting it with selected restriction endonucleases (Bonnin et al., 1996; Spano et al., 1997).

C. parvum oocysts can survive for a long period in the environment (Robertson et al., 1992; Chauret et al., 1995). Although apple juice is acidic (pH of 3 to 4), *C. parvum* is acid-tolerant and can survive in apple juice for up to 4 weeks (Zhao et al., 1993; Millard et al., 1994). Therefore *C. parvum* oocysts detected in unpasteurized apple juice should be considered infectious to humans. The addition of preservatives is likely to be of little use since oocysts are resistant to most common disinfectants (Campbell et al., 1982). Investigations of previous outbreaks of foodborne cryptosporidiosis suggested recommendations to reduce the risk of producing oocyst contaminated apple juice, including preventing the introduction of animal manure into orchards

(via access to animals or fertilization with manure), avoiding use of apples that have fallen to the ground, washing and brushing apples before pressing, routine hand-washing for workers, and pasteurization (Millard et al., 1994; Centers for Disease Control, 1996a). While animal grazing in or around orchards and use of fallen apples are still common practices and will likely continue, pasteurization might be the ultimate measure. According to one study, *C. parvum* oocysts suspended in water and whole milk were rendered noninfectious when held at 71.7°C for 5, 10 and 15 s (Harp et al., 1996). Current practices of flash pasteurization of apple juice entail exposing apple juice to 71.1°C to 73.8°C for 15–20 s and quickly cooling it down. Such conditions are designed to eliminate harmful bacteria – such as *E. coli* 0157:H7 – while retaining fresh flavors and nutrients. We are currently evaluating the effects of these treatment conditions on viability of oocysts in apple juice.

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