Inactivation of Cryptosporidium parvum Oocysts in Cider by Flash Pasteurization

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ABSTRACT

Cryptosporidium parvum is a well-recognized pathogen of significant medical importance, and cider (apple juice) has been associated with foodborne cryptosporidiosis. This study investigated the effect of flash pasteurization on the viability of contaminant C. parvum oocysts. Cider inoculated with oocysts was heated at 70 or 71.7°C for 5, 10, or 20 s, and oocyst viability was measured by a semiquantitative in vitro infectivity assay. By infecting multiple wells of confluent Madin-Darby bovine kidney cells with serial dilutions of heat-treated oocysts and examining infected cells by indirect fluorescent antibody staining, the most probable number technique was applied to quantify log reduction of oocyst viability. Heating for 10 or 20 s at either temperature caused oocyst killing of at least 4.9 log (or 99.999%), whereas oocyst inactivation after pasteurization for 5 s at 70 and 71.7°C was 3.0 log (99.9%) and 4.8 log (99.998%), respectively. Our results suggested that current practices of flash pasteurization in the juice industry are sufficient in inactivating contaminant oocysts.

The small protozoan parasite Cryptosporidium parvum was first identified as a human pathogen in 1976 and is now recognized as a significant cause of severe gastrointestinal diseases in both immunocompetent and immunodeficient individuals (12, 19, 21). Although most cases of cryptosporidiosis are attributed to oocyst-contaminated drinking or recreational water, cryptosporidiosis associated with contaminated foodstuffs has been occasionally reported (7–9, 20).

Cider (apple juice) has been recognized as a novel vehicle in C. parvum transmission, and much attention has been focused on this issue. In 1993, a cryptosporidiosis outbreak was related to drinking unpasteurized, fresh-pressed cider at an agricultural fair in which 160 people were infected (20). It was the first large cryptosporidiosis outbreak in which foodborne transmission was documented, and it was believed that apples used for the cider were contaminated when they fell on ground grazed by cattle shedding C. parvum oocysts. In 1996, another cryptosporidiosis outbreak associated with drinking unpasteurized cider was reported, in which apples used for making cider may have become contaminated when washed with fecally contaminated well water (8).

Apple juice and apple cider are prepared in the fall from fresh, whole apples. Apple juice is often clarified or filtered to remove leftover solids from the pressing, whereas apple cider is usually less highly filtered and more of the apple solids are left in, giving a slightly cloudy appearance that is in contrast to the sparkling amber color of apple juice. In addition, apple juice is usually pasteurized, whereas preservation of apple cider has usually relied on the product’s acidity, refrigeration, and the addition of chemical preservatives. However, such distinction may be unreliable, and the two terms are thus almost interchangeable.

Cider is frequently manufactured locally at small cider mills. The presence of animals in orchards or nearby pastures and the practice of using drop apples for making cider can result in inadvertent contamination of apples with pathogens shed in animal feces (2). In addition to cryptosporidiosis, unpasteurized cider has been associated with outbreaks of a variety of foodborne pathogens, including enterohemorrhagic Escherichia coli and Salmonella (2, 6, 8, 26). To enhance the safety of unprocessed packaged fruit and vegetable juices, the U.S. Food and Drug Administration required that, starting September 8, 1998 (the start of the cider season), packaged, unpasteurized, fresh cider should be labeled to inform consumers of the potential risks posed by drinking unprocessed juices. Yet, as far as pasteurization of cider is concerned, to date there are no specific regulations on the time and temperature combinations to be applied. One source (14) says that cider is rapidly heated at a temperature in the range of 71.1 to 73.8°C for 10 to 20 s and quickly cooled. This high-temperature, short-time process is called “flash pasteurization” and is intended by the juice industry to eliminate pathogenic bacteria while retaining fresh flavors and nutrients of cider. Uljas and Ingham (27) allude to a cider pasteurization process at 71.1°C for 6 s, but their own work was done at 61°C for 2 to 4 min (15). Another article on the costs of cider pasteurization is based on 87.8°C for 5 s (18). This study was conducted to investigate the effects of flash pasteurization of cider at selected temperatures for various periods on viability of contaminant C. parvum oocysts.
MATERIALS AND METHODS

Sources of cider and *C. parvum* oocysts. Unpasteurized cider (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. Oocysts of *C. parvum* were isolated from naturally infected bovine calves on a local dairy farm, using methods described previously (10). The oocysts were purified from feces of infected calves by sucrose flotation and CsCl gradient centrifugation, enumerated, and stored at 4°C in 1× Hank’s balanced saline solution (Sigma Chemical Co., St. Louis, Mo.). Oocysts were enumerated microscopically with a hemacytometer following the standard counting procedure supplied with the instrument.

Flash pasteurization of oocyst-containing cider. Heat treatment was performed on a type 17600 Dri-Bath (Barnstead Thermolyne, Dubuque, Iowa), and temperature was measured with a 52 K/J thermometer (John Fluke Mfg. Co., Everett, Wash.). The Dri-Bath was preferred to a thermal cycler in that 1.5-ml microcentrifuge tubes, rather than 0.5-ml tubes, could be used for relatively large sample volumes, and temperature of the liquid in a centrifuge tube throughout the heating process could be more conveniently monitored.

Initially, microcentrifuge tubes containing 1.485 ml of cider were placed in the center wells of the Dri-Bath and the temperature was equilibrated to either 70 or 71.7°C by adjusting the temperature setting of the Dri-Bath. A 15-µl portion of oocyst stock suspension at 1×10⁶/ml of oocysts was then taken by a micropipet, the micropipet was submerged into cider, and the oocyst suspension was quickly released. The oocyst dilution factor was thus 100 (final oocyst concentration was, therefore, 1×10⁵/ml), and there was no measurable temperature drop after oocyst inoculation. Following 5-, 10-, or 20-s incubation, the tube was removed from the Dri-Bath, immediately chilled in ice-cold water, and stored at 4°C till oocyst viability assay.

To monitor temperature fluctuation during heat treatment, 15 µl of cider was added into another tube containing 1.485 ml of cider and the temperature was recorded during the 20-s incubation period. As the control in oocyst viability measurement, oocysts were inoculated into cider and kept at room temperature for 20 s before being chilled in ice-cold water and stored at 4°C. After preliminary trials, the experiment reported herein was performed twice.

In vitro infectivity assay. To measure oocyst viability, oocysts in heated cider were pelleted by centrifugation at 1,000×g for 10 min, washed with cell culture phosphate-buffered saline (CC-PBS; 0.8% NaCl, 0.115% Na₂HPO₄, 0.02% KCl, 0.02% KH₂PO₄, pH 7.2), resuspended in 1.0 ml of CC-PBS (final oocyst concentration of 1.5×10⁶/ml), and used in cell infection.

Cell culture. Madin-Darby bovine kidney cells (CCL 22, American Type Culture Collection, Rockville, Md.) were cultured in Eagle’s minimum essential medium (E-MEM; Sigma) containing nonessential amino acids, Earle’s salts, 2 mM of L-glutamine, 50 U/ml of penicillin, 50 µg/ml of streptomycin, and 50 µg/ml of gentamicin. E-MEM containing 10% of fetal bovine serum (FBS; Sigma) was used for cell growth, and E-MEM with 5% FBS was used for cell maintenance and *C. parvum* infection. Cells were first grown in 25-cm² tissue culture flasks (Corning Glass Works, Corning, New York, N.Y.) at 37°C in a normal incubator detached by incubation with 1 ml of 1× trypsin-EDTA (Gibco/BRL Life Technologies, Grand Island, N.Y.) for 2 min at 37°C, and distributed into wells on 16-well Lab-Tek glass chamber slides (Nalge Nunc International, Naperville, Ill.). The inoculum for each well was predetermined so that 70 to 90% confluency was achieved after 24-h incubation at 37°C in a desiccator-type candle jar. The candle jar was used herein and in subsequent incubation steps to achieve desirable atmospheres, mainly, preferred concentrations of oxygen and carbon dioxide (28). Immediately before being infected with *C. parvum*, growth medium was aspirated from the wells and the cell sheet was rinsed with CC-PBS prewarmed to 37°C in a water bath.

Cell infection. The oocyst suspension was again centrifuged at 1,000×g for 10 min, and oocyst exocytosis was induced by using the trypsin–taurocholic acid method (17). Oocysts were incubated in CC-PBS containing 0.25% trypsin (type II-S, Sigma) and 0.75% taurocholic acid (sodium salt, Sigma) at 37°C for 60 min, with vortexing every 10 to 15 min. The mixture of oocysts and sporozoites was then centrifuged at 1,000×g for 10 min and the sediment resuspended in 500 µl of CC-PBS to achieve a final oocyst concentration of 3×10⁶/ml. Serial 10-fold dilutions were made in prewarmed E-MEM with 5% FBS, and a 100-µl portion of each dilution was added onto 16-well chamber slides. Three wells were used for each dilution of concentrations of 3×10⁶, 3×10⁵, 3×10⁴, 3×10³, and 300 oocysts per ml. Therefore, the oocyst inoculum for these wells was 3×10⁵, 3×10⁴, 3×10³, 300, and 30 oocysts, respectively. The remaining well on each tissue culture chamber slide was used as the negative control that received no oocyst inoculum. The chamber slides were incubated at 37°C in the candle jar for 3 h to allow maximal oocyst exocytosis and sporozoite attachment onto host cells. The inoculum was then removed, the cell sheet was washed three times with prewarmed CC-PBS, fresh maintenance medium was added, and cells were again incubated in the candle jar at 37°C.

Characterization of in vitro *C. parvum* development. Infected cells were examined at 48 h after infection by indirect fluorescent antibody staining (10). Briefly, the cell sheet was rinsed with prewarmed CC-PBS, fixed with absolute methanol at room temperature for 20 min, and stained sequentially with rabbit anti-anterior and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Sigma). The glass slides were then separated from media chambers by using the chamber slide separator (provided by the manufacturer of tissue culture chamber slides) according to the instructions, and the slides were examined with a fluorescence microscope using a filter with excitation wavelength of 450 to 490 nm and emission wavelength of 510 to 550 nm. Each individual well was recorded as positive or negative based on the presence or absence of fluorescently stained *C. parvum* life stages.

Calculation of oocyst inactivation by using the most probable number technique. The standard most probable number (MPN) technique (22) was adapted for calculating *C. parvum* oocyst inactivation using the MPN estimates and 95% confidence limits table (Table 1). The MPN index of a treated sample was obtained based on the positive and negative results of multiple wells used for three consecutive dilutions, and the MPN of the initial sample was calculated by multiplying the MPN index by the dilution factor. Oocyst inactivation was then determined by comparing the MPN of the sample with that of the control using untreated oocysts.

RESULTS AND DISCUSSION

The positive and negative results of cell culture–based infectivity assay and calculated log reduction of oocyst viability in one pasteurization experiment are presented in
TABLE 1. MPN estimates and 95% confidence limits based on results in each of three replicates at three serial 10-fold dilutions

<table>
<thead>
<tr>
<th>No. of positive wells per 3 wells</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution 1</td>
<td>Dilution 2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>1</td>
<td>0</td>
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</tbody>
</table>

a Adapted from Peeler et al. (19).

Table 2. The experiment was repeated once more, with identical results. Because serial 10-fold dilutions of an oocyst suspension were used for cell infection, log reduction of oocyst infectivity was calculated by comparing the MPN of a treated sample with that of the untreated control.

When unheated oocysts were used as inoculum for cell infection, three of three wells receiving as few as 30 oocysts were positive, as were all wells receiving more oocysts. The dilutions of 300 (dilution 1) and 30 (dilution 2) oocysts per well were used for obtaining the MPN index. Because it was predetermined that the system described was unable to detect cell infection caused by three oocysts (data not shown), the third dilution of three oocysts per well was also included and all wells were treated as negative. Therefore, by using the combination of 3-3-0, the MPN index of the control was 240. Because dilution 1 (300 oocysts per well) was a $10^{-3}$ dilution of the sample (dilution factor was 1,000), the MPN of the control was $240 \times 1,000 = 240,000$.

When oocysts treated at 70 or 71.7°C for 10 or 20 s were used as inoculum, all three wells receiving as many as 300,000 were negative. Oocysts dilutions of 300,000 (dilution 1), 30,000 (dilution 2), and 3,000 (dilution 3) oocysts per well were included and, based on the combination of 0-0-0, the MPN index was less than 3. Because dilution 1 was the lowest dilution used in cell culture (dilution factor was 1), the MPN was less than 3. By comparison with the MPN of the control, log reduction of oocyst infectivity was calculated as more than log (240,000/3), indicating that flash pasteurization at either temperature for 10 or 20 s caused oocyst killing of at least 4.9 log (equivalent to 99.999%).

For cell infections using oocysts treated by flash pasteurization at 71.7°C for 5 s, only one of three wells receiving 300,000 oocysts was positive, whereas the other two wells receiving 300,000 and all three wells receiving 30,000 or 3,000 oocysts were negative. Based on the 1-0-0 combination, the MPN index and the MPN were 4. Therefore, log reduction of oocyst viability was calculated as log (240,000/4) or 4.8 (equivalent to 99.998% inactivation). Similarly, for oocysts exposed at 70°C for 5 s, three wells receiving 300,000 and 30,000 oocysts were positive, whereas all wells infected with 3,000 or fewer oocysts were negative; therefore, the MPN index (3-3-0 combination) and the MPN was 240 and log reduction of oocyst inactivation was calculated as log (240,000/240) = 3.0 (equivalent to 99.9% inactivation).

In a prior study, oocysts suspended in water and whole milk became noninfectious to suckling mice after being held at 71.7°C for 5, 10, and 15 s in a laboratory scale pasteurizer (13). In the present study, temperatures of 70 and 71.7°C—slightly below the temperatures currently used for flash pasteurization of cider—were chosen in the hope of establishing a tolerance level. The results demonstrated that pasteurization at 70°C for 10 s or longer could cause at least 4.9 logs of oocyst killing. These findings should provide a start toward design of a pasteurization or flash pasteurization process sufficient to ensure the required 5-log kill of Cryptosporidium. Further work would be needed to yield formal D- and z-values. Splittstoesser et al. (25),

TABLE 2. Inactivation of C. parvum oocysts in apple juice by flash pasteurization

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Exposure time (s)</th>
<th>No. of inoculated oocysts per well</th>
<th>Reduction of oocyst viability (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>300,000</td>
<td>30,000</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>70</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>71.7</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
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<td></td>
<td>10</td>
<td>0</td>
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<td></td>
<td>20</td>
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</table>

a Time at the specified temperatures.
b Apple juice was held at room temperature after oocyst inoculation.
c Number of positive wells (C. parvum intermediate stages were detected by indirect fluorescent antibody staining) among three inoculated wells.
d C. parvum intermediate life stages not detected.
extrapolating from their findings, say that 4-log inactivation of *E. coli* should occur in 0.013 min at 70°C; this obviously would not kill an equal amount of *Cryptosporidium*.

One critical factor in determining inactivation or disinfection of *C. parvum* oocysts is the viability criterion. Although in vivo infectivity assays using small animal models such as suckling mice are considered to be the gold standard, they are time-consuming and expensive to perform because of the inconvenience and high cost of animal maintenance. In vitro excystation and staining by fluorogenic vital dyes 4',6'-diamidino-2-phenyl-indole (DAPI) and propidium iodide (PI) have been routinely used for oocyst viability determination (5, 16). Although they correlate with each other relatively well, the reliability of such assays has been questioned. In one study, DAPI/PI staining and in vitro excystation were compared with mouse infectivity assay in assessing oocyst viability after chemical disinfection (3). It was found that in vitro excystation and DAPI/PI staining provided similar results, but both significantly overestimated oocyst viability when compared with animal infectivity. Two nucleic acid intercalators, SYTO-9 and SYTO-59, were shown to correlate well with infectivity to neonatal mice but not with in vitro excystation (1). However, a more recent study of oocyst inactivation by ozone reported that all three methods overestimated infectivity when compared with infection of suckling mice (4). In addition, none of these three methods can be used for small numbers of oocysts. In the past few years, cell culture–based infectivity assays have been shown to be applicable for detection of low numbers of viable (infectious) oocysts from water samples (11, 24, 26). The present study demonstrated that such a method is also applicable in studies of oocyst inactivation and/or survival by using the MPN technique. Although the MPN is not a precise measure, a specific value can be obtained for a sample, yielding a maximum likelihood estimate (22). The application of this technique made it possible to semiquantitatively assess oocyst inactivation, which is of particular importance for evaluating a disinfection method in eliminating *Cryptosporidium* oocysts from the transmission cycle. Since the cell culture–based infectivity assay measures oocyst ability to cause cell infection in a manner that mimics the in vivo infection process but without the use of animal models (23), it is superior to in vitro excystation and vital dye staining methods and will be useful in designing novel methods for oocyst inactivation and disinfection.

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