Encephalitozoon cuniculi in Raw Cow’s Milk Remains Infectious After Pasteurization

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Abstract

This study describes the prevalence of Encephalitozoon cuniculi in raw cow’s milk and evaluates the effect of different milk pasteurization treatments on E. cuniculi infectivity for severe combined immunodeficient (SCID) mice. Using a nested polymerase chain reaction approach, 1 of 50 milking cows was found to repeatedly shed E. cuniculi in its feces and milk. Under experimental conditions, E. cuniculi spores in milk remained infective for SCID mice following pasteurization treatments at 72°C for 15 s or 85°C for 5 s. Based on these findings, pasteurized cow’s milk should be considered a potential source of E. cuniculi infection in humans.

Introduction

Parasitic protozoans can cause food- and waterborne disease in humans. Microsporidia are ubiquitous parasitic protozoans that cause human microsporidiosis, a disease that can range in severity from subclinical to lethal. Microsporidia are considered neglected parasites, and their role in foodborne disease is poorly understood (Sak et al., 2011). In studies to date on microsporidia in food, two genera—Enterocytozoon and Encephalitozoon—have been reported (Robertson et al., 2014). Encephalitozoon cuniculi, which was identified as the likely cause of a 2012 foodborne outbreak in Sweden in individuals consuming sandwiches and salad containing cucumber slices (Decraene et al., 2012), is among the most frequently detected microsporidia species in immunocompetent and immunodeficient humans. E. cuniculi infects most cell types, including phagocytes, and produces systemic infections that may be due in part to the trafficking of infected macrophages (Didier and Khan, 2014). Various microbial pathogens have been identified in raw milk and milk-derived products (Claeys et al., 2013; Domenec et al., 2013; Kalmus et al., 2015), but microsporidia in milk have not been reported. It is conceivable that milk could become contaminated in the mammary gland, particularly if there is macrophage infiltration of the gland during an infection or by contamination during milking (Claeys et al., 2013). This study aimed to determine the occurrence of E. cuniculi in raw cow’s milk and its infectivity following pasteurization for microsporidial animal model hosts—severe combined immunodeficient (SCID) mice.

Materials and Methods

Fifty lactating multiparous Holstein cows (Czech Republic) were included in the study. Five samples each of feces (3 g), urine (50 mL), and milk (50 mL; 12.5 mL from each quarter) were obtained from each cow during five consecutive milking periods. Samples were placed in sterile tubes and stored at 4°C until screening. A somatic cell count (SCC) was determined for each milk sample using a Fossomatic 90 instrument (Foss Electric, Hillerod, Denmark). Prior to DNA extraction, milk and urine were centrifuged for 20 min at 3000·g and 4°C. Two hundred milligrams of feces, 200 mg of milk sediment, or the entire sample of urine sediment were homogenized by bead disruption using 0.5-mm glass beads (Biospec Products, Inc., Bartlesville, OK) in a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA) at a speed of 5 m/s for 1 min. DNA was extracted using the QIAamp® DNA Stool Kit (Qiagen, Hilden, Germany) and a nested polymerase chain reaction (PCR) protocol was used to amplify the ribosomal internal transcribed spacer (Katzwinkel-Wladarsch et al., 1996). PCR amplicons were sequenced directly in both directions with an ABI 3130 sequence analyzer (Applied Biosystems, Foster City, CA). The identities of the obtained sequences were determined by a BLAST search (www.ncbi.nlm.nih.gov/blast). The real-time quantitative PCR was performed according to

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the protocol of Wolk et al. (2002). Each set of PCR assays contained external standard presented as DNA isolated from a specific amount of *E. cuniculi* spores. A standard curve was established between the Ct values and the spore numbers ranging from 10 to 10⁷. For each specimen, this standard curve was used to estimate the spore number by interpolation of the Ct value obtained by real-time PCR. Negative control (distilled water) was included in each set of experiments.

Spores of *E. cuniculi* strain II, cultivated at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences and purified from cells by centrifugation over 50% Percoll (Sigma-Aldrich, St. Louis, MO), were used for pasteurization experiments. Six groups of 8-week-old Severe Combined Immunodeficient (SCID) mice (three animals per group) (Charles River, Sulzfeld, Germany) were bred in plastic cages with sterilized wood-chip bedding situated in IVC Air Handling Solutions (Techniplast, Buguggiate, Italy) with high-efficiency particulate air filters. All mice were supplied with a sterilized diet (TOP-VELAŻ, Praha, Czech Republic) and sterilized water *ad libitum*. Mice were orally inoculated by intragastric gavage with 1 × 10⁶ spores of *E. cuniculi* in 200 µL of milk from the different pasteurization treatments (Table 1). Pasteurization treatments were simulated in a PCR cycler in a 100-µL volume. Tested pasteurization of raw milk satisfied the requirements laid down in Chapter XI of Annex II to Regulation (EC) No 852/2004. Milk pasteurization was verified by testing for activity of the enzymes alkaline phosphatase and peroxidase in accordance with Commission Regulation (EC) No 1664/2006 and Commission Decision No 91/180/EEC.

Animal groups inoculated with microsporidia-free milk or spores in milk without pasteurization treatment were used as negative and positive controls, respectively. Each group of animals was housed separately in individually ventilated cages (Tecniplast) and fed sterile food and water *ad libitum*. Each mouse was sacrificed 21 days postinoculation and kidney, liver, spleen, and brain tissues were examined for the presence of *E. cuniculi* DNA. DNA extraction and PCR analysis were performed as described earlier with the exception that DNA was extracted using a QIAamp® DNA Tissue Mini Kit. If at least one tissue specimen was *E. cuniculi* positive, the spores were considered infective.

All housing, feeding, and experimental procedures were conducted under protocols approved by the Institute of Parasitology, Biology Centre and Central Commission for Animal Welfare, Czech Republic (#152/2012).

**Results and Discussion**

*E. cuniculi* was detected in only 1 of 50 cows examined in this study, which is consistent with the findings of Halanova et al. (1999) and Abu-Akkada et al. (2015), and suggests that cattle are less frequently infected by *E. cuniculi* than are other domestic animals and humans, where prevalence can reach 80% (Snowden, 2014). Detection of *E. cuniculi*–specific DNA in multiple samples of feces (three of five positive samples) and milk (two of five positive samples) collected on different days from one animal demonstrated an active infection. Previous studies have shown that *E. cuniculi* infections can persist for months (Sak et al., 2011); therefore, a single infected cow could produce a considerable amount of contaminated milk (i.e., our cow produced 1.2 × 10⁷ spores/L of milk). Taking into consideration the dilution in tank milk on the particular farm, which had 100 cows, there was a burden of 1.2 × 10⁷ spores/L of tank milk.

Given that *E. cuniculi* frequently infects phagocytes serving as vehicles of infection within a host body (Didier and Khan, 2014), we hypothesized that milk from a cow with evidence of mastitis, thus those with a SCC >100,000, would be more frequently contaminated than milk from a healthy mammary gland, because >90% of SCC are composed of leukocytes (Bradley and Green, 2005). Although SCCs in particular milk samples in our study varied between 13,000 and 364,000 with the mean of 141,000 ± 95,000, the finding of *E. cuniculi* in milk with a SCC <80,000/mL did not support that hypothesis.

Raw milk represents a potential source of various pathogens including bacteria, protozoa, and viruses originating from blood, mastitis, or contamination of milk from feces, skin, or environment (Claeys et al., 2013). Therefore, raw milk is pasteurized by heating to kill these pathogens (Langer et al., 2012). Contrary to results of Domenech et al. (2013), who did not detect any pathogenic bacteria in cheese and ice cream despite positive findings of a microorganism indicator of unhygienic processing practices implying correct pasteurization and a possible postrecontamination during processing, we detected microsporidial DNA in raw milk. Moreover, the experiments performed in this study showed that after pasteurization at 72°C for 15 s or 85.0°C for 5 s, spores of *E. cuniculi* DNA in SCID mice tissue.

**Table 1. Effect of Different Pasteurization Treatments on Infectivity of *Encephalitozoon cuniculi* Spores for Severe Combined Immunodeficient (SCID) Mice Including Verification of Pasteurization Process**

<table>
<thead>
<tr>
<th>Pasteurization treatment (°C/time)</th>
<th>Verification of pasteurization process</th>
<th>Presence of <em>E. cuniculi</em>–specific DNA in SCID mice tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Low temperature, long time (63°C/30 min)</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>High temperature, short time (72°C/15 s)</td>
<td>Inactive</td>
<td>Active</td>
</tr>
<tr>
<td>Higher heat, shorter time (85°C/5 s)</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>Higher heat, shorter time (95°C/5 s)</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>Negative controlb</td>
<td>Active</td>
<td>Active</td>
</tr>
<tr>
<td>Positive controlb</td>
<td>Active</td>
<td>Active</td>
</tr>
</tbody>
</table>

AAnimal inoculated with *E. cuniculi*–free raw milk.
bAnimal inoculated with *E. cuniculi* in raw milk without any treatment.
remained infective for SCID mice (Table 1), leading to acute infection spread within mice. High-temperature short-time pasteurization at 72°C and 85°C is used for the production of drinking milk, yogurts, or cheese. Although the prevalence of E. cuniculi in cow’s milk is low, drinking milk pasteurized at 72°C and 85°C could be sources of E. cuniculi infection in humans.

Although European regulations on foodstuffs demand good manufacturing practices and standard sanitation operating procedures within Hazard Analysis and Critical Control Point (HACCP) from all food manufacturers (Domenech et al., 2007, 2009), microsporidia are not listed as controlled pathogens. Our results suggest that examination for the presence of microsporidia should be implemented into HACCP in the milk industry, as implementation of HACCP was shown to be an adequate cost–benefit relationship in yogurt production despite additional costs (Cusato et al., 2014).

Future studies should address the influence of post-pasteurization processes such as fermentation and maturation of yogurt and cheese on the survivability of E. cuniculi.

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Disclosure Statement

No competing financial interests exist.

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