Survey of Ontario Bulk Tank Raw Milk for Food-Borne Pathogens

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ABSTRACT

Raw (unpasteurized) milk can be a source of food-borne pathogens. Raw milk consumption results in sporadic disease outbreaks. Pasteurization is designed to destroy all bacterial pathogens common to raw milk, excluding spore-forming bacteria and possibly Mycobacterium paratuberculosis, but some people continue to drink raw milk, believing it to be safe. Current methods for assessing the bacteriological quality of raw milk, such as aerobic plate counts, are not usually designed to detect specific pathogens. The objective of this study was to estimate the proportion of pick-ups (loads of raw milk from a single farm bulk tank) from Ontario farm bulk tanks that contained Listeria monocytogenes, Salmonella spp., Campylobacter spp., and/or verotoxigenic Escherichia coli (VTEC). Samples from 1,720 pick-ups of raw milk were tested for the presence of these pathogens, and 47 L. monocytogenes, three Salmonella spp., eight Campylobacter spp., and 15 VTEC isolates were detected, representing 2.73, 0.17, 0.47, and 0.87% of milk samples, respectively. Estimates of the proportion of theoretical tanker truck loads of pooled raw milk contaminated with pathogens ranged from a low of 0.51% of tankers containing raw milk from 3 bulk tanks being contaminated with Salmonella spp. to a high of 34.41% of tankers containing raw milk from 10 bulk tanks being contaminated with at least one of the pathogens. Associations between the presence of pathogens and raw milk sample characteristics were investigated. The mean somatic cell count was higher among VTEC- or L. monocytogenes-positive samples, and the mean aerobic plate count was found to be higher among L. monocytogenes-positive samples. These results confirm the presence of bacterial food pathogens in raw milk and emphasize the importance of continued diligence in the application of hygiene programs within dairies and the separation of raw milk from pasteurized milk and milk products.

Key words: Raw milk, Salmonella, Listeria monocytogenes, Campylobacter, verotoxigenic Escherichia coli

Food-borne pathogens continue to cause significant human disease throughout the world. Raw and improperly pasteurized milk have been implicated in a number of disease outbreaks (3, 8, 14, 21, 25, 35, 38). Several of these outbreaks involved children (3, 21). Children, the elderly, and the immunocompromised are particularly susceptible to disease from food-borne pathogens (5). Listeria monocytogenes, Salmonella spp., verotoxigenic Escherichia coli (VTEC), and Campylobacter spp. are recognized as important agents of food-borne illness associated with the consumption of raw milk and milk products (3, 8, 14, 21, 25, 35, 38). L. monocytogenes outbreaks associated with pasteurized milk (14) and Mexican-style soft cheese (25) affected 49 people and 142 people, respectively, with mortality rates of 28.6 to 33.8%. Large outbreaks of salmonellosis have been associated with improperly pasteurized milk (38) and Cheddar cheese made from heat-treated but non-pasteurized milk (8). Smaller outbreaks have been reported among farm families who consume raw milk from their own farms (17, 18). Campylobacter spp. have been implicated as the causative agents of large and small milk-borne outbreaks (21, 35, 42). An outbreak affecting 2,500 school children was traced to Campylobacter-contaminated milk from one dairy (21). Raw milk has also been implicated as a source of VTEC in disease outbreaks (3, 29). An outbreak in Ontario involved a group of children who consumed raw milk during a visit to a local dairy farm (3). VTEC was isolated from 43 of 48 symptomatic cases and three children developed hemolytic uremic syndrome.

Most microbiological protocols that are routinely used to assess the quality of raw milk were not designed to detect specific pathogens. Raw milk with low aerobic plate counts or low somatic cell counts may or may not contain pathogenic bacteria capable of causing illness. Conversely, an elevated total bacterial count may or may not coexist with the presence of human pathogens. Pasteurization is designed to destroy all bacterial pathogens common to raw milk, excluding spore-forming bacteria (34) and possibly Mycobacterium paratuberculosis (19), and it has been used extensively as an effective and efficient method of preventing transmission of food-borne pathogens to consumers via milk and milk products. In Ontario it is illegal to sell raw milk (41).
There is a need for a better understanding of the current prevalence of bacterial pathogens in raw milk. This information could be used to assess the public health value of regulations banning the sale of raw milk and to educate the public concerning the probability of exposure to bacterial pathogens when consuming raw milk.

The objectives of this study were (i) to estimate the prevalence of contamination of raw milk samples from farm bulk tanks in Ontario with \textit{L. monocytogenes}, \textit{Salmonella} spp., \textit{Campylobacter} spp., and/or VTEC; (ii) to calculate the probability of theoretical tanker truck loads of raw milk pooled from three, five, or 10 farm bulk tanks containing the above pathogens; and (iii) to identify associations observed between the presence of pathogens in raw milk and aerobic plate counts, somatic cell counts, and county of origin of the milk.

**MATERIALS AND METHODS**

**Sample acquisition and processing**

In Ontario, prior to loading raw milk from each farm's bulk tank, raw milk samples are collected by milk tanker drivers by aseptically transferring a 35- to 40-ml aliquot of the raw milk into sterile safety-latch vials. The samples are delivered within 2 days of sampling, in refrigerated trucks (4°C), to the Agricultural and Food Laboratory Services Center (AFLSC) in Guelph, Ontario, for milk quality and milk composition testing. Three-day-old raw milk samples from 1,720 independent farm bulk tanks were intercepted following bacteriological quality testing at the AFLSC between October 1995 and May 1996 for use in this study. Bacteriological quality tests were performed using aseptic procedures. Raw milk samples (approximately 40 ml) were selected for the study according to a randomized list of milk producer numbers. Aerobic plate counts (28) were performed on the same samples used in this study. Somatic cell counts were not performed on the actual raw milk samples tested in this study, but the average monthly values of these tests, performed regularly at AFLSC by using a Fossomatic 400 Somatic Counter (Foss Electric, Hillerod, Denmark) (16), for the same producers were included in the statistical analysis.

**Isolation of Campylobacter spp.**

Fifteen milliliters of each raw milk sample was added to 135 ml of Rosef's enrichment broth (24) and incubated at 30°C for 4 h; then 10 μg of vancomycin, 5 μg of trimethoprim, 0.7 μg of polymyxin B sulfate, 100 μg of cycloheximide, and 35.6 μg of cefoperazone (Sigma Chemical Co., St. Louis, MO) per ml were added. The enrichments were incubated at 37°C for 2 h and at 42°C for 44 h. An aliquot of each enrichment broth was used to streak Mueller Hinton blood agar plates (24) and the plates were incubated at 42°C under microaerophilic conditions (5% O2, 10% CO2, and 85% N2). The plates were examined after 48 to 72 h for the presence of \textit{Campylobacter}-like colonies which were tested for catalase and oxidase reactions, Gram stain, motility (observed under dark-field microscopy), and aerotolerance. Presumptive \textit{Campylobacter} spp. were sent to Health Canada in Ottawa, Ontario, Canada for serotyping (26).

**Isolation of Salmonella spp.**

A second 15-ml volume from the original milk sample was added to 135 ml of universal preenrichment broth (UPB) (Difco) and incubated for 24 ± 2 h at 35°C. The same UPB enrichments were used for the detection of \textit{Salmonella} spp., VTEC, and \textit{L. monocytogenes}. Modified semisolid Rappaport-Vassiliadis (MSRV) (Difco) agar was used to detect \textit{Salmonella}-containing cultures, as described (9). A loopful from positive MSRV plates was used to inoculate MacConkey agar (BBL; Cockeysville, MD) plates. A single colony from each MacConkey agar plate was used to inoculate slants of Simon's citrate agar, urea agar, and triple sugar iron agar (all from BBL). Strains which appeared typical of \textit{Salmonella} spp. on these media were tested by seroagglutination using \textit{Salmonella} O antisera poly A-I and Vi (Difco). Positive cultures were sent to Health Canada in Guelph, Ontario, for serotyping and tested by AmpliSensor polymerase chain reaction (PCR) \textit{Salmonella} assay (6).

**Isolation of \textit{L. monocytogenes}**

One milliliter of each UPB enrichment was added to tubes containing 9 ml of \textit{Listeria} broth (LEB) and incubated at 35°C for 48 ± 2 h. LEB (100 μl) was added to 10-ml tubes of modified Fraser broth plus 10 μl of Fraser broth additive per ml (all media from BBL). The modified Fraser broth tubes were incubated at 35°C for 24 ± 2 h and then an inoculum was transferred to both \textit{Listeria} plating medium and Oxford agar (both from BBL) plates, which were incubated at 30°C for 48 ± 2 h and 35°C for 48 ± 2 h, respectively. Three typical colonies per plate were tested by the CAMP reaction (using \textit{Staphylococcus aureus} ATCC 25923) (2). One CAMP-positive culture per sample was tested for tumbling motility (observed under dark-field microscopy), catalase reaction, Gram stain reaction, and rhannose, xylose, and mannitol fermentation patterns. Strains which resembled \textit{L. monocytogenes} by these criteria were tested by a fluorogenic 5' nucleic PCR assay (1).

**Isolation of verotoxigenic \textit{E. coli}**

One milliliter of the UPB enrichment was inoculated into tubes containing 9 ml of MacConkey broth (Difco) and incubated at 35°C for 18 to 24 h. The tubes were mixed with a vortex briefly and 100 μl from each tube was inoculated into a 1-ml volume of brain heart infusion broth (BHI) (BBL). After 6 to 8 h of incubation at 35°C, the BHI tubes were centrifuged for 2 min at 14,000 rpm, and the supernatants were tested for verotoxigenic (VT) activity by the routine Vero cell assay (7). VT-positive broths were inoculated onto MacConkey agar plates, and individual colonies were picked from these plates and tested for VT activity. If no VT strain was isolated, the screening process was repeated. VT strains were tested by a confirmatory Vero cell assay, identified by the Microscan Walkaway 40 Identification system (Dade Diagnostics Corp., Mississauga, ON), sent to Health Canada in Guelph, Ontario, for serotyping, and tested by a VTEC-specific PCR assay (37).

**Data analyses**

Test results were captured in an electronic database that consisted of one record per farm milk sample. Producer identification codes were used to electronically merge test results with AFLSC data, including aerobic plate count and county of origin for each raw milk sample and the monthly somatic cell count for the farm of origin. The prevalence of pathogens detected among pick-ups of raw milk from farm bulk tanks was calculated as the percentage of total pick-ups analyzed containing pathogens. The 95% confidence limits were calculated as described (13). The prevalence of pathogens in theoretical tanker trucks consisting of pooled raw milk from 3, 5, and 10 randomly selected farm bulk tanks was calculated by first estimating the probability that all contributing pick-ups would be negative (assuming independence between pick-ups) and then raising the complement of the probability of contamination of each farm bulk tank load (i.e., \(1 - P\)) to the power of \(n\) pick-ups making up a load (\(n = 3, 5,\) or 10) (30).
Subsequently, the probability of at least one contributing farm pick-up being contaminated was estimated by subtracting the probability that all contributing farm pick-ups were negative from one (30). Associations between the presence of pathogens and raw milk characteristics (county of origin, log of aerobic plate counts, and log of somatic cell counts) were calculated using chi-square analysis and Fisher’s exact test (13).

RESULTS AND DISCUSSION

Serotyping results

Table 1 summarizes the serotyping results for Salmonella spp., Campylobacter spp., and VTEC strains isolated in the study. One of the three Salmonella strains isolated was S. muenster, the predominant serotype isolated during previous studies in Ontario (31, 32). While O157:H7 has been the VTEC serotype most commonly associated with disease, several other serotypes have also been implicated in causing disease in humans (22, 23, 40). These include the O26:H11, O113:H21, and O163:H19 serotypes which were isolated during this study. The most frequently isolated Campylobacter serotype was HL7, which was reported previously to be one of the most common C. jejuni serotypes (26).

PCR results

Table 2 summarizes the results of polymerase chain reaction assays on isolates of L. monocytogenes, Salmonella spp., and VTEC. The L. monocytogenes PCR reaction showed positive results for all presumptive L. monocytogenes isolates tested and negative results for 58 of 58 strains which were isolated from selective media but which were not positive by the PCR assay.

CAMP negative. All three of the Salmonella isolates tested positive by the Salmonella PCR assay. The VTEC PCR assay showed a positive result for all eight of the VTEC strains. The VTEC PCR assay was designed to detect all types of VTEC (37), in contrast to many commercially available VTEC detection systems which detect only the O157:H7 serotype.

Estimates of pathogen prevalence among raw milk samples from farm bulk tanks

Table 3 summarizes prevalence estimates for (i) specific pathogens, (ii) the presence of any one of the pathogens, and (iii) the presence of more than one of the pathogens among farm bulk tank samples of raw milk in Ontario. Fifteen-milliliter sampling volumes were used so that the same milk sample could be tested for all four pathogens in the study. While this volume is comparable to that used in other surveys (10, 11, 27, 36), the use of a larger sample volume may have increased the sensitivity of the test.

The day-to-day exposures to pathogens of farm families who drink raw milk from their own farm bulk tanks are not statistically independent events. The probability of exposure to pathogens of farm family members who drink raw milk from a persistently infected herd would be greater than that

<table>
<thead>
<tr>
<th>Species isolates No. samples</th>
<th>PCR positive No. (%)</th>
<th>PCR negative No. (%)</th>
<th>Target of PCR primers (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>46a</td>
<td>46 (100)</td>
<td>0 (0) hlyA gene (1)</td>
</tr>
<tr>
<td>Verocytotoxigenic E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a There were 47 L. monocytogenes isolates collected during this study. One of these isolates was lost during processing and was not tested by PCR assay.

<table>
<thead>
<tr>
<th>TABLE 3. Summary of prevalence of specific pathogens in raw milk among 1,720 random farm bulk tanks in Ontario</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species of pathogen&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>CA</td>
</tr>
<tr>
<td>SA</td>
</tr>
<tr>
<td>VTEC</td>
</tr>
<tr>
<td>LM</td>
</tr>
<tr>
<td>Any of CA, SA, VTEC, LM</td>
</tr>
<tr>
<td>More than one of CA, SA, VTEC</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reference (13).

<sup>b</sup> CA, Campylobacter spp.; SA, Salmonella spp.; VTEC, verocytotoxigenic E. coli; LM, Listeria monocytogenes.
for family members who drink raw milk from a noninfected or intermittently infected herd. Nevertheless, the statistics reported in Table 3 provide overall estimates of probability of exposure to selected pathogens from raw milk from random bulk tanks at a provincial level.

*L. monocytogenes* was found to be present in 2.73% of the raw milk samples tested in this study. There have been several studies from different countries that estimated the prevalence of *L. monocytogenes* in raw milk (11, 12, 20, 36, 39). Reported *L. monocytogenes* prevalences ranged from 1.3% of Ontario raw milk samples (11) to 45.3% of raw milk samples from a single dairy in Spain (39).

*Salmonella* spp. were found to be present in 0.17% of the samples tested. Surveys of raw milk from farms or transport trucks in different countries reported isolation rates of 0.17% to 4.7% of samples tested (31, 32, 33, 36). Previous surveys of Ontario raw milk bulk tanks reported prevalences of 1.1% and 4.3% of farms tested (31, 32). These values are slightly higher than that obtained from this study; however, both of the previous studies tested the same farms on multiple occasions (compared to a single testing per farm in this study) and both tested in-line milk filters versus the whole raw milk. It has been proposed that the use of in-line filters may increase isolation rates for *Salmonella* spp., as debris on the filter may allow the bacteria to adhere (31). It is also possible that bacteria may multiply on the filter during milking, leading to increased isolation rates. In the present study, the use of samples processed by the AFLSC was preferred because more samples could be tested that were more representative of raw milk from provincial bulk tanks than could be achieved through a cooperative study involving the collection of in-line filters.

Results of one Ontario survey suggested that the presence of *Salmonella* spp. in raw milk bulk tanks was intermittent (31), in contrast to the results of a farm study in England where *S. typhimurium* was found to persist in a dairy herd for 3.5 years (17).

*Campylobacter* spp. were isolated from 0.47% of the samples tested. In two US studies *Campylobacter* spp. were isolated from 1.5 and 0.9% of the raw milk samples tested (10, 27). The former study looked at individual farms while the latter examined multiple samplings of the same nine farms.

VTEC was isolated from 0.87% of samples in the present study. A previous survey of raw milk bulk tanks in Ontario isolated VTEC from 1.4% of dairy farms (7). In the previous survey, in-line milk filters were cultured and the farms were tested on more than one occasion.

Only 2 raw milk samples were found to contain more than one of the four pathogens investigated. Both *L. monocytogenes* and VTEC were isolated from one sample, and both *Campylobacter* spp. and *L. monocytogenes* were isolated from another sample.

### Table 4. Estimate of the probability of pathogens being present within theoretical tanker truck loads of raw milk pooled from 3, 5 or 10 randomly selected farm bulk tanks

<table>
<thead>
<tr>
<th>Species of pathogen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Single</th>
<th>3 pooled</th>
<th>5 pooled</th>
<th>10 pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>0.47</td>
<td>1.40</td>
<td>2.33</td>
<td>4.60</td>
</tr>
<tr>
<td>SA</td>
<td>0.17</td>
<td>0.51</td>
<td>0.85</td>
<td>1.69</td>
</tr>
<tr>
<td>VTEC</td>
<td>0.87</td>
<td>2.59</td>
<td>4.27</td>
<td>8.37</td>
</tr>
<tr>
<td>LM</td>
<td>2.73</td>
<td>7.97</td>
<td>12.92</td>
<td>24.18</td>
</tr>
<tr>
<td>Any of CA, SA, VTEC, LM</td>
<td>4.13</td>
<td>11.89</td>
<td>19.01</td>
<td>34.41</td>
</tr>
<tr>
<td>More than one of CA, SA, VTEC, LM</td>
<td>0.12</td>
<td>0.36</td>
<td>0.60</td>
<td>1.19</td>
</tr>
</tbody>
</table>

<sup>a</sup>The probability of milk from at least one contributing farm bulk tank being positive, and thus contaminating the load, is equal to one minus the probability raw milk from all (<i>n</i>) contributing farm bulk tanks being negative (<i>P</i> of at least 1 among <i>n</i> = 1 - (1 - <i>P</i>)<sup>n</sup>) (30).


created from the pooling of raw milk from 3, 5, and 10 randomly selected farm bulk tanks. The point estimates ranged from 0.51% of loads consisting of raw milk from 3 random farm bulk tanks being contaminated with *Salmonella* spp. to 34.41% of loads consisting of milk from 10 random farm bulk tanks being contaminated with at least one of the four pathogens investigated.

The assumption of total independence between milk samples from farm bulk tanks contributing to theoretical truck loads is not completely valid because trucks must follow prescribed routes, and the status of milk from a given farm’s bulk tank on one day will be correlated with its status on subsequent days. Nevertheless, at the provincial level, Table 4 provides an indication of the amount of cross-contamination that can occur in random pooling of raw milk from farm bulk tanks. These estimates illustrate that although the prevalence of pathogens among milk samples from farm bulk tanks may be relatively low, the prevalence among truck loads of pooled raw milk can be relatively high (Table 4). Furthermore, depending on the number of farm bulk tanks and pooled truck loads that a dairy receives, the managers of such establishments would be wise to assume that pathogens do enter their processing systems via raw milk obtained from randomly selected individual farm bulk tanks or from truck loads of pooled raw milk. However, simple exposure to pathogens does not necessarily lead to human infection or disease. The health impact of exposure is influenced by the volume of milk consumed, the concentra-
tion of pathogens within that milk, the total number of organisms to which a person is exposed through various sources, and the dose-response of an individual to such exposure. These factors will vary between situations and individuals. This study does not attempt to adjust for these additional factors that will influence the probability of subclinical or clinical disease occurring and the resultant biological, social, and economic impacts on people.

Associations between pathogen presence and milk characteristics

No statistically significant associations were observed between the presence of any of the four pathogens and the county of origin, log of aerobic plate counts or log of somatic cell counts, with the following exceptions. The mean of the log of monthly somatic cell counts was higher among VTEC-positive than among VTEC-negative samples ($P = 0.03$), and higher among $L. monocytogenes$-positive than $L. monocytogenes$-negative samples ($P = 0.0002$). Also, the log of the aerobic plate count was higher among $L. monocytogenes$-positive than $L. monocytogenes$-negative samples ($P = 0.04$).

Because the somatic cell count was not performed on the same bulk tank load of raw milk that the pathogens were isolated from, the association between the presence of $L. monocytogenes$ and VTEC and higher somatic cell counts must be interpreted with caution. This association could, however, suggest mammary infection with these pathogens. Both $L. monocytogenes$- and $E. coli$-induced mastitis have been reported ($4$, $15$). The higher aerobic plate counts associated with the presence of $L. monocytogenes$ may indicate that the milk has come into contact with a source of $L. monocytogenes$ contamination, which may also contain high levels of other microorganisms.

Pasteurization of milk was originally implemented to prevent transmission of diseases caused by $Mycobacterium tuberculosis$ and $Brucella$ spp. While these pathogens are no longer prevalent in Canada, raw milk does contain other food-borne pathogens, and consumption of raw milk involves a risk of exposure to these pathogens. Although the proportion of farm bulk tank raw milk samples that were found to be contaminated with $L. monocytogenes$, $Salmonella$ spp., $Campylobacter$ spp., or VTEC was relatively low, the estimated proportion of truck loads of pooled raw milk entering dairies that were expected to be contaminated with at least one of the pathogens investigated was relatively high. In conclusion, these findings emphasize the importance of continued diligence in the application of hygiene programs within dairies and the separation of raw from pasteurized milk and milk products. These findings also supported the intent of regulations forbidding the routine sale of raw milk.

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