A Research Note

Potential Role of Refrigerated Milk Packaging in the Transmission of Listeriosis and Salmonellosis

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ABSTRACT

Cultures of three Listeria monocytogenes serotypes and three Salmonella spp. were applied to the exterior surfaces of waxed cardboard or plastic milk containers. Contamination sites were sampled with premoistened cotton swabs during 14 d of refrigeration. Unstressed cells of Listeria survived up to 14 d on the surfaces of waxed (1 serotype) and plastic (3 serotypes) containers. Heat-stressed cells of all three serotypes of Listeria survived for 2 d on both types of containers. One serotype survived for 4 d, but only on plastic containers. Unstressed cells of all three Salmonella strains survived up to 14 d on both types of containers. Heat-stressed Salmonella strains survived up to 2 d (waxed containers) and 4 d (plastic containers).

In a 1982 milk-associated outbreak of illness, it was suggested that the causative agent, Yersinia enterocolitica, could have been transmitted from pigs to humans by contaminated exterior surfaces of milk containers (1). In subsequent experiments, Y. enterocolitica organisms applied to the outside of refrigerated milk containers were recovered up to 21 d after contamination (8). This study was made to determine if this type of contamination could also be a factor in outbreaks of listeriosis (3) and salmonellosis (4,5).

MATERIALS AND METHODS

Bacterial strains

Two strains of Salmonella typhimurium and one of S. tennessee were studied along with three Listeria monocytogenes strains, representing serovars 1a, 1b and 4b. One of the S. typhimurium strains was incriminated in the 1985 Chicago milk outbreak; the other (a nondairy isolate) was included to detect any differences between two strains of the same serotype. S. tennessee (an isolate from nonfat dry milk) was included as a different serotype. The three strains of L. monocytogenes [obtained from the Food and Drug Administration (FDA) Cincinnati District laboratory] were maintained on Trypticase soy agar (TSB-YE); the Salmonella cultures were maintained on brain heart infusion (BHI) agar slants. All cultures were stored in the dark at room temperature.

Preparation of inoculum

Each slant culture of L. monocytogenes was inoculated into Trypticase soy broth with 0.6% yeast extract (TSB-YE); the Salmonella spp. were subcultured to BHI broth. Incubated broth was centrifuged at 1020 × g for 20 min (L. monocytogenes) and 3090 × g for 10 min (Salmonella). Cell sediments were washed twice with Butterfield’s phosphate buffer (pH 6.8-7.2) and then reconstituted to 10 ml with the buffer. All cultures were divided into 5-ml duplicates. One set of washed cell suspensions was heat-shocked in a water bath at 56°C (L. monocytogenes) and one set at 48°C (Salmonella) for 30 min (7,9). The remaining set was not heat-treated.

Contamination of milk containers

Sampling sites consisting of circular areas 3 mm in diameter were delineated on the waxed cardboard and plastic (6) milk containers to accommodate both the thermally shocked and unshocked bacteria. Each container type was contaminated by dipping sterile cotton-tipped applicators into the appropriate bacterial suspension and thoroughly wetting each sampling site. Triplicate samples were taken immediately after inoculation and at 2, 4, 7 and 14 d. One uninoculated 3-mm site on each carton was used as a negative control for the container. Contaminated containers were stored at -0.8 to 6.6°C for the 14-d test period.

Sampling for L. monocytogenes

A sterile cotton-tipped applicator premoistened with TSB-YE was used to sample the entire area of each of the triplicate contamination sites. The swab was rubbed over the surface in a circular, rotating motion and then placed in tubes containing 5 ml of TSB-YE and incubated at 35°C for 18-24 h. On the following day, gram stains were made of cultures from turbid tubes. These tubes were also streaked to TSA-YE agar plates. Growth after 18-24 h at 35°C was subjected to further testing. Cultures were considered to be L. monocytogenes if they produced blue to blue-gray colonies in the illumination test (2,6), a clear hemolytic zone around the inoculum stab in sheep-blood agar, and acid reactions with no gas in both the slant and butt of triple sugar iron agar; and if they demonstrated umbrella motility in SIM agar (Difco Laboratories, Detroit, MI); appeared as gram-positive, small nonsporeforming rods with palisade formation; fermented dextrose, maltose and rhamnose;

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failed to ferment mannitol and xylose; and gave positive reactions for the methyl-red and Voges-Proskauer test.

Sampling for Salmonella

The entire area of a contaminated site or control site was swabbed with a sterile cotton-tipped applicator moistened in lactose broth. The applicator was rubbed over the surface in a circular, rotating motion and then placed in 10 ml of lactose broth and incubated for 24 ± 2 h at 37°C. The lactose broth tubes were mixed and 1-ml portions were subcultured into 10-ml amounts of selenite cystine (SC) broth and tetrathionate (TT) broth with added brilliant green dye (10.0 mg/L final concentration). The SC and TT tubes were incubated for 24 ± 2 h, and a 3-mm loopful of each selective broth was streaked onto plates of xylose lysine desoxycholate, Hektoen enteric and bis-muth sulfite (BS) agars. After incubation for 24 ± 2 h, colonies typical of the genus Salmonella were transferred to triple sugar iron (TSI) and lysine iron agar (LIA) slants. BS agar plates with no typical Salmonella colonies were reincubated and examined again after total incubation time of 48 ± 2 h. After 24 ± 2 h, isolates having TSI and LIA reactions typical of Salmonella were subjected to biochemical testing and somatic serological confirmation.

RESULTS

Viable test organisms, both Listeria and Salmonella, were isolated from both carton types shortly after the contamination procedure (Table 1). Neither genus was recovered from any control sites.

Each of the three serotypes of L. monocytogenes, when not heat-shocked, was recovered from at least one site on the exterior of the plastic container at day 14. Only serotype 4b was recovered at day 14 from the waxed cardboard container; final recovery of serotypes 1a and 1b from waxed cardboard containers was, respectively, at 7 and 4 d after contamination. All heat-shocked cultures of L. monocytogenes serotypes survived for at least 2 d, regardless of the carton type. Only serotype 1b was isolated as late as day 4, but from only 1 of 3 sites on the plastic container. All further testing for heat-shocked L. monocytogenes was negative after day 4.

Salmonella cultures not heat-treated were recovered from all contamination sites at each sampling interval through day 14 regardless of the container type. TriPLICATE tests of each carton type at each test interval resulted in the examination of 90 sites during the 2-week period. Only one site failed to yield a positive result. This occurred on day 14, when one of three sites on the waxed cardboard container was negative for the Salmonella strain incriminated in the Chicago milk outbreak.

When heat-shocked Salmonella cells were tested on waxed cardboard cartons, the S. typhimurium isolated from milk had the longest survival period, 2 d. The remaining Salmonella strains were detected only at time zero. When heat-shocked cells of S. typhimurium were tested on the plastic container, the S. typhimurium originally isolated from milk was recovered from 2 of 3 sites.

<table>
<thead>
<tr>
<th>TABLE 1. Survival of L. monocytogenes and Salmonella spp. on refrigerated milk cartons.</th>
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<tr>
<td>Bacteria</td>
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<tr>
<td>Listeria serotype</td>
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<td>Unshocked</td>
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<td>1a</td>
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<td>1b</td>
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<td>4b</td>
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<td>Heat-shocked</td>
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<td>1a</td>
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<td>1b</td>
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<td>4b</td>
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<tr>
<td>Salmonella spp.</td>
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<td>Unshocked</td>
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<tr>
<td>S. typhimurium (Chicago outbreak)</td>
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*a, recovered; -, not recovered.

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after 4 d, but the 2-d sampling was negative. The nondairy *S. typhimurium* culture was not detected beyond the initial sampling. The third culture, *S. tennessee*, was recovered from 1 of 3 sites and survived for 4 d.

**DISCUSSION**

This study demonstrates that both heat-shocked and unshocked cells of *L. monocytogenes* and *Salmonella* can survive on the exterior surfaces of milk containers when inocula are fairly heavy. The prolonged survival of unshocked cells of both genera on an inert surface demonstrates the hardness and durability of environmental strains. Our results suggest that unless some adverse treatment is used to weaken or kill the cells, they could pose a health hazard by gaining access to the exteriors of food containers, subsequently contaminating the consumer's hands and/or the food. The limited recovery of heat-shocked bacteria of both genera implies that for their survival, a protective or surface-adhering substance, such as moisture or a residue of dried milk, may be needed.

Survival time was somewhat longer on the plastic containers, possibly because of the textured surface of this material. It was concluded that environmental sampling in investigations of foodborne illness should include the external surfaces of food containers.

**ACKNOWLEDGMENTS**

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**REFERENCES**


**Rosenow and Marth, con't. from p. 729**