A Research Note

An Improved Enrichment Procedure for the Isolation of Yersinia enterocolitica and Related Species From Milk

MARIZA LANDGRAF*, SEBASTIAO T. IARIA², and DEISE P. FALCAO³

*Universidade de São Paulo - Faculdade de Ciências Farmacêuticas, Departamento de Alimentos e Nutrição Experimental.
C.P. 66355 - CEP 05389-970 - São Paulo, SP - Brazil; ²Universidade de São Paulo - Instituto de Ciências Biomédicas, Departamento de Microbiologia - São Paulo, Brazil; ³Universidade Estadual Paulista Júlio de Mesquita Filho - Faculdade de Ciências Farmacêuticas, Departamento de Ciências Biológicas - Araçariguama, SP - Brazil

(Received for publication July 21, 1992)

ABSTRACT

A new enrichment procedure is proposed to improve the isolation of Yersinia enterocolitica and related species from milk. This procedure uses tryptic soy broth plus Polymyxin (5 IU/ml) and Novobiocin (10 μg/ml) - TSPN broth - incubated at 18°C for 3 d. Using raw milk and pasteurized milk inoculated with Yersinia strains, the efficiency of this procedure was compared to that of SB broth (sorbitol bile salts broth) incubated at 4°C for up to 21 d. Despite of the presence of antibiotics in TSPN broth, there were difficulties in recovering Yersinia from raw milk samples due to the presence of high levels of other organisms. Nevertheless, TSPN broth incubated at 18°C for 3 d showed better efficiency than the other method. In pasteurized milk samples, TSPN medium at 18°C for 3 d gave better results than the SB broth at 4°C for 7 d, showing that the proposed procedure is the preferable one due to the shorter period of incubation.

The isolation of Yersinia enterocolitica and related species from food requires an enrichment step. Several enrichment procedures exploit the psychrotrophic characteristic of this microorganism by employing a 4°C incubation for up to 21-28 d (6,7,11,13); others, using higher temperatures, have a shorter incubation time (5,10,15,17). Sorbitol bile salts broth (SB) has been used worldwide by Wauters' biotype/serotype/phagotype, Y. enterocolitica 2/O9/X², Y. enterocolitica 2/O9/X, Y. enterocolitica 1/O5/X, and Yersinia intermedia O16a,16b/X³. The former three were from human sources, and the latter two from raw milk and pasteurized milk, respectively.

Milk samples

Six samples of pasteurized milk (1 L each) were collected from retailers and six samples of raw milk (1 L each) were collected from a dairy bulk tank at São Paulo, Brazil. They were transported in ice.

Inoculation of samples

Cultures of Yersinia strains grown on tryptic soy broth at 25°C for 24 h were diluted in 0.85% saline (10⁻⁶,10⁻⁷, and 10⁻⁸ dilutions were inoculated into 27 ml of pasteurized or raw milk samples in Erlenmeyer flasks, in order to achieve 10, 10⁻¹, and 10⁻² CFU/ml of milk. At the same time, in order to determine the CFU/ml inoculated, 0.1 ml from each dilution was spread-plated onto trypticase soy agar plates that were incubated at 25°C for 48 h.

Detection Procedures

Two aliquots of 10 ml each of the contaminated samples were transferred to 90 ml of TSPN broth and to 90 ml of SB broth. The TSPN broth was incubated at 18°C for 3 d and the SB broth at 4°C for up to 21 d, with samples removed at 7 and 14 d.

After the incubation period, 0.1 ml of 10⁴, 10⁵, and 10⁶ dilutions were streaked onto MacConkey agar (Difco Laboratories) (pasteurized milk) or Yersinia selective agar (Difco Laboratories) (raw milk). Yersinia selective agar was used for raw milk because of the high level of microbial contamination. The plates were incubated at 25°C for 48 h. Colonies of presumptive Yersinia were tested serologically, and when negative for their respective sera, they were identified as previously described (3,16).

MATERIALS AND METHODS

Media

The new medium contains tryptic soy broth (TSB -Difco) and the filter-sterilized antibiotics solutions: Polymyxin B sulphate (Pfizer) - 5 IU/ml and sodium novobiocin (Upjohn) 10 μg/ml (TSPN). It is incubated at 18°C for 3 d. SB broth was prepared according to Aulisio and Stanfield (I).

Microorganisms

Yersinia strains utilized were Y. enterocolitica 4/O3/VIII (Wauters' biotype/serotype/phagotype), Y. enterocolitica 2/O9/X², Y. enterocolitica 2/O9/X, Y. enterocolitica 1/O5/X, and Yersinia intermedia O16a,16b/X³. The former three were from human sources, and the latter two from raw milk and pasteurized milk, respectively.

Milk samples

Six samples of pasteurized milk (1 L each) were collected from retailers and six samples of raw milk (1 L each) were collected from a dairy bulk tank at São Paulo, Brazil. They were transported in ice.

Inoculation of samples

Cultures of Yersinia strains grown on tryptic soy broth at 25°C for 24 h were diluted in 0.85% saline (10⁻⁶,10⁻⁷, and 10⁻⁸ dilutions were inoculated into 27 ml of pasteurized or raw milk samples in Erlenmeyer flasks, in order to achieve 10, 10⁻¹, and 10⁻² CFU/ml of milk. At the same time, in order to determine the CFU/ml inoculated, 0.1 ml from each dilution was spread-plated onto trypticase soy agar plates that were incubated at 25°C for 48 h.

Detection Procedures

Two aliquots of 10 ml each of the contaminated samples were transferred to 90 ml of TSPN broth and to 90 ml of SB broth. The TSPN broth was incubated at 18°C for 3 d and the SB broth at 4°C for up to 21 d, with samples removed at 7 and 14 d.

After the incubation period, 0.1 ml of 10⁴, 10⁵, and 10⁶ dilutions were streaked onto MacConkey agar (Difco Laboratories) (pasteurized milk) or Yersinia selective agar (Difco Laboratories) (raw milk). Yersinia selective agar was used for raw milk because of the high level of microbial contamination. The plates were incubated at 25°C for 48 h. Colonies of presumptive Yersinia were tested serologically, and when negative for their respective sera, they were identified as previously described (3,16).
Simultaneously with these procedures, detection of endogenous *Yersinia* and aerobic plate counts of those samples was carried out to verify the possible influence of the microbial load of raw and pasteurized milk samples on the recovery of *Yersinia*.

**Screening for endogenous *Yersinia* in milk samples.** Two portions of 25 ml each, from noninoculated milk samples, were added to 225 ml of TSPN broth and incubated at 18°C for 3 d, and to 225 ml of SB broth incubated at 4°C for up to 21 d. After the incubation period, a loopful of the incubated broth was streaked onto MacConkey agar and CIN agar plates with incubation at 25°C for 48 h. Suspect colonies were identified as described before.

Determination of aerobic colony count at 4 and at 25°C (4). The milk samples were serially diluted from 10^-6 to 10^0 into phosphate-buffered saline and two aliquots of 1 ml each from each dilution were added to Petri dishes. Tempered plate count agar was poured into each plate and mixed by rotating and tilting. After solidification, one set of plates was incubated at 4°C for 10 d and another at 25°C for 48 h.

**Statistical analysis**

The Kruskal-Wallis (14) and Dunn (9) analyses were used to compare results.

**RESULTS AND DISCUSSION**

Regarding SB broth, *Yersinia* strains were not recovered at all from six samples of artificially inoculated raw milk, during the first 7 d of incubation. The recoveries of inoculated *Yersinia* strains, inoculated at three different levels in three samples of raw milk, using TSPN broth incubated at 18°C for 3 d, and SB broth incubated at 4°C for 14 and 21 d. When TSPN broth was employed, all *Yersinia* strains except *Yersinia intermedia* at 10 CFU/ml, were recovered, at least, from one sample. The other two samples as well as the SB broth showed varying recoveries, with some strains being recovered and others not.

Though there were some difficulties in recovering the strains from raw milk using both procedures, recoveries using TSPN broth were better. The difficulties are a consequence of the presence of high numbers of psychrotrophic and mesophilic microorganisms in raw milk samples. Another factor that may have contributed to these results is the presence of endogenous strains of *Yersinia* (Table 2). Fukushima and Gomyoda (8) have reported the inhibition of *Yersinia enterocolitica* O3 when grown together with *Y. intermedia* biotype 1, *Y. enterocolitica* biotype 03, *Y. intermedia*, and *Y. enterocolitica* biotype 05.

Comparison of two enrichment procedures in the recovery of *Yersinia* spp. from inoculated raw milk. Besides the greater number of isolations from the cryo-enrichment, it must be pointed out that only three strains: *Yersinia* O10,34/X (SB 4°C/21 d), *Yersinia* 052/X (TSPN 18°C/3d) and *Yersinia* NAG/X (SB 4°C/14 and 21 d) were isolated by streaking a loopful from enrichment media onto selective media plates. The others were isolated from spread-plates of the 10^4 and 10^6 dilutions of contaminated milk. These plates showed colonies that resembled *Yersinia* but did not react with the sera against the inoculated strain. These colonies were identified as *Yersinia* by biochemical and serological tests and by phagotyping.

Statistical analysis was not performed on the data obtained from raw milk samples as some of the strains were not detected. However, this does not mean that inoculated *Yersinia* strains were not present at lower dilutions of the milk. These plates were too crowded that colonies of presumptive *Yersinia* could easily have gone undetected.

The recoveries of inoculated *Yersinia* from pasteurized milk samples are shown in Table 3. The statistical analysis performed on the data obtained from pasteurized milk showed that TSPN broth incubated at 18°C for 3 d and SB broth incubated at 4°C for 21 d resulted in significantly better recoveries than SB broth incubated at 4°C for 7 d.

These results are probably due to less bacterial competition in pasteurized milk than in raw milk. Psychrotrophic bacteria as well as endogenous *Yersinia* spp. were absent in all samples of pasteurized milk and mesophiles were not present in high numbers. Even in the sample with the highest level of contamination, 1.7 x 10^5 CFU/ml, the *Yersinia* strains developed well. Moreover, substances in milk such as bacteriocins, agglutinins, antibiotics, and pesticides can interfere with the growth of bacteria in raw and pasteurized milk (2).

**CONCLUSION**

None of the existing methods allows the simultaneous recovery of all pathogenic strains of *Y. enterocolitica* from milk, using TSPN broth incubated at 18°C for 3 d, and SB broth incubated at 4°C for 21 d resulted in significantly better recoveries than SB broth incubated at 4°C for 7 d.

These results are probably due to less bacterial competition in pasteurized milk than in raw milk. Psychrotrophic bacteria as well as endogenous *Yersinia* spp. were absent in all samples of pasteurized milk and mesophiles were not present in high numbers. Even in the sample with the highest level of contamination, 1.7 x 10^5 CFU/ml, the *Yersinia* strains developed well. Moreover, substances in milk such as bacteriocins, agglutinins, antibiotics, and pesticides can interfere with the growth of bacteria in raw and pasteurized milk (2).

**CONCLUSION**

None of the existing methods allows the simultaneous recovery of all pathogenic strains of *Y. enterocolitica* from milk, using TSPN broth incubated at 18°C for 3 d, and SB broth incubated at 4°C for 21 d resulted in significantly better recoveries than SB broth incubated at 4°C for 7 d.

These results are probably due to less bacterial competition in pasteurized milk than in raw milk. Psychrotrophic bacteria as well as endogenous *Yersinia* spp. were absent in all samples of pasteurized milk and mesophiles were not present in high numbers. Even in the sample with the highest level of contamination, 1.7 x 10^5 CFU/ml, the *Yersinia* strains developed well. Moreover, substances in milk such as bacteriocins, agglutinins, antibiotics, and pesticides can interfere with the growth of bacteria in raw and pasteurized milk (2).

**TABLE 1. Comparison of two enrichment procedures in the recovery of *Yersinia* spp. from inoculated raw milk.**

<table>
<thead>
<tr>
<th><em>Yersinia</em></th>
<th>1 - 10^6</th>
<th>10 - 100^6</th>
<th>100 - 1000^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y.e.03</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y.e.05</td>
<td>-</td>
<td>7.00</td>
<td>-</td>
</tr>
<tr>
<td>Y.e.09</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y.e.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Y.e. = *Y. enterocolitica* O3, Y.e.03 = *Y. enterocolitica* O3, Y.e.05 = *Y. enterocolitica* O5, Y.e.09 = *Y. enterocolitica* O9, Y.e.08 = *Y. enterocolitica* O8.
* Level of inoculum (CFU/ml).
* Sorbitol bile salts broth incubated at 4°C for 14 and 21 d.
* Tryptic soy broth + Polymyxin + Novobiocin incubated at 18°C for 3 d.
* Milk samples M1, M2, M3.
* Not detected.
* Log CFU/ml.
TABLE 2. Aerobic plate count at 4 and 25°C and endogenous Yersinia spp. isolated from noninoculated raw milk samples.

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Aerobic plate count (CFU/ml)</th>
<th>Strain</th>
<th>Enrichment process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.7 x 10⁵</td>
<td>1.3 x 10⁷</td>
<td>No strain isolated</td>
</tr>
<tr>
<td>2</td>
<td>1.3 x 10⁵</td>
<td>1.7 x 10⁶</td>
<td>Yersinia O:16a,16b/X₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSPN - 18°C/3 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SB - 4°C/21 d</td>
</tr>
<tr>
<td>3</td>
<td>1.3 x 10⁵</td>
<td>2.3 x 10⁷</td>
<td>Yersinia O:10,34/X₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSPN - 18°C/3 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SB - 4°C/7, 14, 21 d</td>
</tr>
<tr>
<td>4</td>
<td>1.5 x 10⁵</td>
<td>2.2 x 10⁷</td>
<td>Y. intermedia O:16a,16b/X₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSPN - 18°C/3 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SB - 4°C/14 d</td>
</tr>
<tr>
<td>5</td>
<td>3.0 x 10⁵</td>
<td>1.3 x 10⁶</td>
<td>Y. intermedia O:52/X₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSPN - 18°C/3 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SB - 4°C/14, 21 d</td>
</tr>
<tr>
<td>6</td>
<td>8.4 x 10⁴</td>
<td>8.0 x 10⁵</td>
<td>Yersinia O:16a,16b/X₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSPN - 18°C/3 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SB - 4°C/14 d</td>
</tr>
</tbody>
</table>

* Colony forming units/ml.
b Tryptic soy broth plus polymyxin and novobiocin incubated at 18°C for 3 d.
* Sorbitol bile salts broth incubated at 4°C for up to 21 d.
* Nonagglutinating in somatic sera.

TABLE 3. Comparison of two enrichment procedures in the recovery of Yersinia spp. from inoculated pasteurized milk.

<table>
<thead>
<tr>
<th>Yersinia</th>
<th>1 - 10⁸</th>
<th>10 - 10⁹</th>
<th>100 - 10⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td>SB7⁣</td>
<td>SB14⁣</td>
<td>SB21⁣</td>
</tr>
<tr>
<td>Y. i.</td>
<td>2.76⁣</td>
<td>5.79⁣</td>
<td>7.62⁣</td>
</tr>
<tr>
<td>Y. e. O3</td>
<td>2.64⁣</td>
<td>5.26⁣</td>
<td>6.58⁣</td>
</tr>
<tr>
<td>Y. e. O5</td>
<td>3.16⁣</td>
<td>6.59⁣</td>
<td>7.57⁣</td>
</tr>
<tr>
<td>Y. e. O9</td>
<td>2.40⁣</td>
<td>5.00⁣</td>
<td>7.06⁣</td>
</tr>
<tr>
<td>Y. e. O8</td>
<td>2.30⁣</td>
<td>4.50⁣</td>
<td>6.90⁣</td>
</tr>
</tbody>
</table>

abcd Refer to Table 1.
* Log of geometric mean of CFU/ml.

foods. By this new method, the pathogenic serotypes O3, O8, and O9 could be recovered even at low numbers when inoculated into raw and pasteurized milk samples. The short incubation time required by this method is an additional advantage in a food microbiology laboratory. Therefore, the use of TSPN broth method as described is recommended as, at least, one of the enrichment methods for the isolation of Y. enterocolitica and related species from milk samples.

ACKNOWLEDGMENTS

We thank Dr. Neil F. Novo and Dr. Iara Juliano for the statistical analyses performed on our data and Pearl I. Peterkin for helpful advice in the preparation of the manuscript. We are also indebted to Edneia F. Correa for technical assistance.

This work was supported by grants FAPESP 81/0873-1 and 86/2116-7 from the Fundação de Amparo a Pesquisa do Estado de São Paulo.

REFERENCES


Editors Note:
Page 356 of the April 1993 issue of the Journal of Food Protection inadvertently transposed two photographs.

**Errata to Vol. 56, No. 2, February 1993**

**Journal of Food Protection**


**Figure 1.** Photograph of LPM agar streak plate of UVM-2 enrichment of compositied sample 3 from the first fecal sampling date. Note clear zone of inhibition surrounding inhibitory colony. Background growth was found to contain *L. innocua* (see Table 3).

**Figure 1.** Soldering iron with four brass rods used to mark the corners of samples to be excised from carcass surfaces.