An Improved Method for the Recovery of *Salmonella* Serovars from Orange Juice Using Universal Preenrichment Broth

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ABSTRACT

The relative effectiveness of three methods for the recovery of *Salmonella* serovars from orange juice was determined. One method, a modified *Bacteriological Analytical Manual* (BAM) procedure consisted of preenrichment in lactose broth at 35°C for 24 h, selective enrichment, and selective plating. Another method, a National Centers for Disease Control and Prevention (CDC 1) procedure, consisted of direct enrichment in tetrathionate broth at 35°C for 24 and 48 h, followed by selective plating. The third method (also from CDC and designated CDC 2) consisted of preenrichment in Universal Preenrichment (UP) broth at 35°C for 24 h, selective enrichment, and selective plating. In 10 experiments encompassing five different *Salmonella* serovars and 200 test portions per broth, the CDC 1 method recovered 141 *Salmonella*-positive test portions, the BAM method recovered 151, and the CDC 2 method recovered 171. In 2 of the 10 experiments, with two different *Salmonella* serovars, the BAM recovered significantly fewer (P < 0.05) *Salmonella*-positive test portions than did the CDC 2 method. On the basis of the above results, the second phase of this study focused on a comparison of the effectiveness of the BAM-recommended lactose broth and the CDC 2-recommended UP broth as preenrichment media for the recovery of *Salmonella* serovars from pasteurized and unpasteurized orange juice. Subsequent culture treatment of the two preenrichments was identical so that the effect of other variables (e.g., different selective enrichment media, various incubation temperatures, and different selective plating agars) on the relative performance of these two preenrichment media was excluded. In one of nine experiments, with pasteurized orange juice, lactose broth recovered significantly fewer (P < 0.05) *Salmonella*-positive test portions than did UP broth. For the combined results of the nine pasteurized orange juice experiments (180 test portions per broth), lactose broth recovered 99 *Salmonella*-positive test portions, and UP broth recovered 116. In three of seven experiments, with unpasteurized orange juice, lactose broth recovered significantly fewer (P < 0.05) *Salmonella*-positive test portions than did UP broth. For the combined results of the seven unpasteurized orange juice experiments (140 test portions per broth), lactose broth recovered 73 *Salmonella*-positive test portions, and UP broth recovered 117. For both pasteurized and unpasteurized orange juice, the total number of *Salmonella*-positive test portions recovered with UP broth was significantly greater than the number recovered with lactose broth. These results indicate that UP broth is a more effective enrichment broth for the recovery of *Salmonella* from orange juice than is lactose broth.

Due to its low pH, orange juice has not been considered a likely vehicle for *Salmonella* serovar outbreaks. However, *Salmonella* outbreaks associated with this juice have been reported (3, 4, 6, 8, 9). In 1995, in an outbreak in which unpasteurized orange juice was implicated, the U.S. Food and Drug Administration (FDA) did not detect *Salmonella* organisms when the *Bacteriological Analytical Manual* (BAM) culture method was used (24). The National Centers for Disease Control and Prevention (CDC), however, recovered *Salmonella* Gaminara from the orange juice with its culture procedure. There are two possible reasons for these discordant results: (i) the FDA’s samples may not have contained viable *Salmonella* Gaminara; or (ii) the samples may have contained injured *Salmonella* Gaminara at levels too low to be detected with the BAM culture method.

The BAM method and variations on it were used for this study. The variations consisted of deleting selenite cystine (SC) broth, adding tetrathionate broth (TT) incubated at 43°C, and including Rappaport-Vassiliadis (RV) medium incubated at 42°C. Thus, the modified BAM selective enrichments were TT (35 and 43°C) broth and RV (42°C) medium. The selective enrichments used by FDA analysts for the analysis of orange juice during the 1995 outbreak were SC (35°C) broth, TT (35°C) broth, and RV (42°C) medium. The BAM method was modified because, at the time this research was initiated, it was believed that SC broth would be deleted from the method in the near future. This belief was based on data, generated by this laboratory, that demonstrated equivalence between SC broth and RV medium for use with foods with low levels of competitive microflora (13). In addition, RV medium had already replaced SC broth for use with foods with high levels of competitive microflora (16, 24). The final factor that led to the modification of the method is the toxicity of selenium, a key constituent of SC broth. Spent SC broth contains selenium at a level that causes it to be designated as a hazardous waste by the Environmental Protection Agency (5). Because the use of SC broth in the BAM method was unlikely in the future, it was determined that its use in this study would be unnecessary. An Association of Official
Analytical Chemists International collaborative study has since been completed, and the use of SC broth has been removed from the BAM for use with all foods, except guar gum (12, 25).

In the first phase of this study, a comparison of the BAM method and two CDC procedures (20) for the detection of Salmonella serovars in artificially contaminated pasteurized orange juice was made. In the second phase of this study, the relative efficiencies of lactose broth and Universal Preenrichment (UP) broth (2) for the recovery of Salmonella serovars from artificially contaminated pasteurized and unpasteurized orange juice were compared.

MATERIALS AND METHODS

Sources of foods. Pasteurized (thermal process) and unpasteurized orange juice was purchased from retail outlets located in the Washington, D.C. area.

Source of inocula. The following Salmonella serovars were associated with the 1995 outbreak in central Florida (6, 9): Salmonella Gaminara (CDC orange juice isolate), Salmonella Hartford (CDC clinical isolate), Salmonella Braenderup (FDA environmental isolate), and Salmonella Muenchen (FDA environmental isolate). Two additional isolates, Salmonella Typhimurium (DT-104) and a second Salmonella Gaminara isolate, were obtained from the stock culture collection of the Division of Microbiological Studies, FDA.

Preparation of inocula. Brain heart infusion agar slants were inoculated with Salmonella serovars and incubated for 18 to 24 h at 35°C. Brain heart infusion broth was inoculated from these slants and incubated for 18 to 24 h at 35°C. Ten-milliliter aliquots from the broth were centrifuged at 3,090 g, and the pellets were suspended in 10 ml Butterfield’s phosphate buffer (pH 6.8 to 7.2). The cells were washed twice with 10-ml aliquots of Butterfield’s phosphate buffer. The cells were heat-shocked in borosilicate culture tubes placed in a thermostatically controlled water bath for 10 min at 55°C, and serial decimal dilutions were performed.

Inoculation of orange juice. Heat-shocked Salmonella serovar organisms were inoculated into 2.25-liter portions of orange juice (both pasteurized and unpasteurized) from dilutions estimated to give Salmonella serovar levels of 0.4 and 0.04 CFU/ml juice on the day sample analysis was initiated. The inoculated orange juice was mixed with a stirring bar for 15 min. Pasteurized orange juice was stored from 1 to 4 weeks at 2 to 5°C before initiation of analysis. Unpasteurized orange juice was stored from 5 to 10 days at 2 to 5°C before initiation of analysis. Neither pasteurized nor unpasteurized orange juice was analyzed more than 7 days after the sell-by date. Juice that appeared to be spoiled was not used in this study.

Examination of orange juice—phase 1. CDC method. Twenty 25-ml test portions from bulk contaminated orange juice were preenriched in 225 ml lactose broth for 24 ± 2 h at 35 ± 2°C (24) (Fig. 1). Five 25-g uninoculated control test portions were also preenriched in lactose broth to demonstrate the absence of Salmonella serovars in the uninoculated juice. From the incubated preenrichments, 1-ml aliquots were subcultured to two 10-ml portions of TT broth and incubated for 24 ± 2 h at 35 ± 2°C and at 43 ± 0.2°C, respectively, and 0.1-ml aliquots were subcultured to 10-ml portions of RV medium and incubated for 24 ± 2 h at 42 ± 0.2°C. Media at 35°C were incubated in an air incubator. Media at 42 and at 43°C were incubated in circulating, thermostatically controlled water baths. All preenrichments and selective enrichments used in this study were static cultures; no preenrichments or selective enrichments were shaken. Each tube of incubated selective enrichment was streaked to bismuth sulfite (BS), Hektoen enteric (HE), and xylose lysine deoxycholate (XLD) agar plates that were incubated for 24 ± 2 h at 35 ± 2°C. BS agar plates were reincubated for an additional 24 h at 35 ± 2°C (48 ± 2 h at 35 ± 2°C total incubation). Presumptive positive colonies were picked to triple sugar iron agar (TSI) and lysine iron agar (LIA) slants and incubated for 24 ± 2 h at 35 ± 2°C. Growth from presumptive-positive TSI slants was confirmed as Salmonella sp. with group-specific somatic antisera (Difco Laboratories, Detroit, Mich.).

Examination of orange juice—phase 1. BAM method. Twenty 25-ml test portions from bulk contaminated orange juice were enriched in 225 ml lactose broth for 24 ± 2 h at 35 ± 2°C (24) (Fig. 1). After 24 h, the TT broths were streaked to brilliant green (BG) and HE agar plates. The plates were incubated for 24 ± 2 h at 35 ± 2°C. A 1-ml aliquot from the 24-h TT broth was subcultured to a 10-ml portion of TT broth and incubated for 24 ± 2 h at 42 ± 0.2°C. The 24-h TT broth was reincubated for an additional 24 h at 35°C. The 48-h TT broth and the 24-h subculture were streaked to BG and HE agar plates. The plates were incubated for 24 h at 35°C. Presumptive-positive colonies were picked to TSI and LIA agar slants and confirmed as above.

Examination of orange juice—phase 1. CDC method. Twenty 25-ml test portions from bulk contaminated orange juice were preenriched in 225 ml UP broth for 24 ± 2 h at 35 ± 2°C (Fig. 1). One-milliliter and 0.1-ml aliquots were subcultured from UP broth to 9- and 10-ml portions of TT broth, respectively, and 0.1-ml aliquots were subcultured from UP broth to 10-ml portions of RV medium. The TT broths and RV media were incubated for
was performed to determine the level of contamination in the bulk food on the day of initiation of analysis. MPN portions from orange juice were preenriched at a 1:9 ratio of test portion to lactose broth by swirling as recommended by the BAM.

<table>
<thead>
<tr>
<th>Salmonella serovar</th>
<th>MPN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BAM method</th>
<th>CDC 1 method with TT broth</th>
<th>CDC 2 method with UP broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaminara (from orange juice)</td>
<td>0.023</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gaminara (from casein)</td>
<td>&lt;0.003</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>Braenderup</td>
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<td>16</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>0.023</td>
<td>17&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>0.023</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
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<td>19</td>
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<td></td>
<td>0.231</td>
<td>20</td>
<td>18</td>
<td>20</td>
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<td></td>
<td>0.231</td>
<td>20</td>
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<td>20</td>
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<tr>
<td>Total number of Salmonella-positive test portions recovered</td>
<td>151&lt;sup&gt;B&lt;/sup&gt;</td>
<td>141&lt;sup&gt;B&lt;/sup&gt;</td>
<td>171&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Most probable number per ml orange juice preenriched in lactose broth.

<sup>b</sup> Values within a row not followed by a common letter are significantly different (P < 0.05).

24 ± 2 h at 35 ± 2°C and at 42 ± 0.2°C, respectively. After incubation, the TT broths and RV media were streaked to BG and HE agar plates. The plates were incubated for 24 ± 2 h at 35 ± 2°C. Presumptive-positive colonies were picked to TSI and LIA slants and confirmed as above.

Examination of orange juice—phase 2. Twenty 25-ml test portions from bulk contaminated orange juice were preenriched in 225-ml portions of lactose broth (24) and UP broth for 24 ± 2 h at 35 ± 2°C. Five 25-g uninoculated control test portions were also preenriched in lactose broth to demonstrate the absence of Salmonella serovars in the uninoculated orange juice. From the preenriched broth, 1-ml aliquots were subcultured to two 10-ml portions of TT broth and incubated for 24 ± 2 h at 35 ± 2°C and 43 ± 0.2°C, respectively, and 0.1-ml aliquots were subcultured to 10-ml portions of RV medium and incubated for 24 ± 2 h at 42 ± 0.2°C. Each incubated selective enrichment was streaked to BS, HE, and XLD agar plates that were incubated for 24 ± 2 h at 35 ± 2°C. BS agar plates were reincubated for an additional 24 h at 35 ± 2°C (48 ± 2 h at 35 ± 2°C total incubation). Presumptive-positive colonies were picked to TSI and LIA agar slants and confirmed as above.

Determination of levels of inoculation for phase I and phase II. A three-tube most probable number (MPN) analysis (24) was performed to determine the level of contamination in the bulk food on the day of initiation of analysis. MPN portions (100, 10, and 1 ml) from orange juice were preenriched at a 1:9 ratio of test portion to lactose broth by swirling as recommended by the BAM (24). The 0.1-ml portion of orange juice was preenriched in 9.9 ml of lactose broth.

The MPN preenrichments were incubated for 24 ± 2 h at 35 ± 2°C. One-milliliter portions from each incubated MPN preenrichment were subcultured to two 10-ml portions of TT broth and incubated for 24 ± 2 h at 35 ± 2°C and 43 ± 0.2°C, respectively, and 0.1-ml aliquots were subcultured to 10-ml portions of RV medium and incubated for 24 ± 2 h at 42 ± 0.2°C. The selective enrichment media were streaked to BS, HE, and XLD agar plates, and presumptive positive isolates were confirmed as previously described.

Media and reagents. RV medium was made from its individual ingredients as directed by the BAM (24). All media, except the selective plating agars (BG, BS, HE, XLD), were made from Difco dehydrated preparations. BG, BS, HE, and XLD agar plates were made from BBL (Cockeysville, Md.) dehydrated preparations.

Statistical analysis. Data were analyzed with McNemar’s chi square test (23). Differences in recovery of Salmonella sp. were significant at P < 0.05.

RESULTS AND DISCUSSION

Salmonella serovars, used in this study, were heat shocked so that they would sustain an injury similar to that sustained by Salmonella organisms that have gone through an incomplete or failed thermal pasteurization process. Because the time and temperature parameters that might exist during a failed pasteurization process are virtually limitless, an arbitrary heat-stress protocol (55°C for 10 min) that has been used successfully in the past was chosen (1, 14, 15). Heat-injured Salmonella organisms were then inoculated into pasteurized (pH 3.8 to 3.9) and unpasteurized orange juice (pH 3.5 to 3.6) and stored at refrigerated temperatures before analysis. Cells were also heat shocked, before inoculation, into unpasteurized orange juice, even though there is no expectation that heat-stressed Salmonella would be found in unpasteurized juice, because this added stress would further test the relative efficacies of lactose and UP broths. Moreover, naturally contaminated unpasteurized orange juice may contain Salmonella organisms that have been stressed in other ways (e.g., drying), so that further stress of the organism, beyond acid-stress of the juice, was warranted.

The results in Table 1 show that the CDC 2 method was the most productive of the three methods examined for the recovery of Salmonella serovars from orange juice in the first phase of this study. In all cases, this method was equally or significantly more effective (P < 0.05) than either the CDC 1 or the BAM method. The CDC 2 method also recovered the greatest number of Salmonella-positive...
Salmonella test portions overall: the CDC 1 method (141), the BAM method (151), and the CDC 2 method (171). The different recoveries of Salmonella organisms would be acid-stressed due to the low pH of orange juice. Thus, nonselective preenrichment of the samples would be indicated. The CDC 2 method preenriches samples in nonselective enrichment media to allow injured Salmonella to resuscitate. In this case, the Salmonella organisms would be acid-stressed due to the low pH of orange juice. The CDC 2 method and the BAM method both preenrich samples in nonselective enrichment media to allow injured Salmonella to resuscitate. In this case, the Salmonella organisms would be acid-stressed due to the low pH of orange juice. Thus, nonselective preenrichment of the samples would be indicated. The CDC 2 method preenriches samples in UP broth, a highly buffered (pH 6.3) enrichment medium used for the detection of Salmonella and Listeria in foods (2, 20). The BAM method preenriches samples in lactose broth, a weekly buffered (pH 6.8) enrichment medium used for the detection of Salmonella in foods (19, 24). The orange juice, with concomitant acid-tolerant competitive microflora, may have caused the lactose broth to acidify too quickly, before the injured Salmonella could be fully resuscitated, thereby producing false-negative results. It has been reported that apple cider, another acidic fruit juice, acidifies weakly buffered enrichment media (11).

An additional factor that may account for the enhanced recovery of Salmonella from orange juice with UP broth is sodium pyruvate. UP broth contains sodium pyruvate while lactose broth does not. Sodium pyruvate has been reported to aid in the resuscitation of injured Salmonella (7, 17, 18, 22). In this study, injured (heat-stressed) Salmonella was inoculated into acidic orange juice. Under such circumstances, the resuscitative qualities of preenrichment media would be rigorously tested.

The relatively low recovery of Salmonella by the CDC 1 method was probably due to the lack of a nonselective preenrichment medium in the method. It has been reported that the growth of nonresuscitated injured or stressed cells is inhibited by selective media such as TT broth (1, 10, 21).

In the second phase of this study, the BAM method with lactose broth (BAM-LAC) and a modified BAM method that utilized UP broth (BAM-UP) instead of lactose broth, were compared with both pasteurized and unpasteurized orange juice. As with the first phase, the method employing the use of UP broth was the most productive method for the recovery of Salmonella from pasteurized orange juice (Table 2). In one of nine experiments, the BAM-UP method recovered significantly more Salmonella-positive orange juice test portions than did the BAM-LAC method. The BAM-UP method also recovered the greatest overall number of Salmonella-positive test portions. The BAM-UP method recovered 116 Salmonella-positive test portions, while the BAM-LAC method with lactose broth recovered 99 Salmonella-positive test portions. The results were similar with unpasteurized orange juice (Table 3). In three of seven experiments, the BAM-UP method recovered significantly more Salmonella-positive test portions than did the BAM-LAC method. Moreover, the BAM-UP method recovered a significantly greater number of Salmonella-positive test portions than did the BAM-LAC method (117 versus 73 Salmonella-positive test portions). These results indicate that lactose broth should be replaced with UP broth for the Salmonella analysis of orange juice.

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REFERENCES


