Isolation of *Salmonella* from Fluid Milk with the Use of Rappaport-Vassiliadis Medium

PETER VASSILIADIS¹, VICTORIA KALAPOTHAKI*, and DIMITRIOS TRICHOPOULOS²

¹Department of Hygiene and Epidemiology, University of Athens Medical School, Goudi, Athens 11527, Greece, and
²Department of Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Boston, Massachusetts 02115, USA.

(Received for publication July 11, 1990)

**ABSTRACT**

The performances of Rappaport-Vassiliadis (RV) medium and tetrathionate brilliant green broth (TT) for the detection of salmonellae in pasteurized fluid whole milk, artificially contaminated, were compared. The RV medium was found to be more sensitive and more selective than the TT medium, as far as *Salmonella typhimurium* was concerned. From the 100 samples contaminated with *S. typhimurium* only, 97 were found positive with RV medium, while 86 were found positive with TT medium (P<0.001). From the 100 samples contaminated with *S. typhimurium* plus gram-negative competing organisms, 79 were found positive with RV medium, while only 41 with TT medium (P<10⁻⁶). The mean values of growth of competing organisms in RV medium after 24 and 48 h of incubation at 43°C were 0.81 and 0.92, respectively, while the corresponding values in TT medium were 1.81 and 1.87.

The Rappaport-Vassiliadis (RV) medium has been shown in several studies to be superior to tetrathionate brilliant green broth (TT) in the isolation of *Salmonella* from many naturally contaminated types of samples, including food products (3,14). In these studies pasteurized milk products were not included, as it is very rare for these to be naturally contaminated with gram-negative organisms. In 1985, Northolt et al. (9) compared Muller-Kauffmann tetrathionate broth (MK) and RV medium incubated at 43°C for the detection of salmonellae in caseinate and skim milk powder, artificially contaminated. They found that the performance of MK medium was poor compared to that of the RV medium. Recently, Wilson et al. (19) compared RV and TT media to determine the optimal conditions at the selective enrichment step of the isolation procedure. Whole fluid milk contaminated with three different *Salmonella* serotypes was used. The data indicated an apparent superiority of RV over TT medium in the recovery of *S. typhimurium*, and a clear superiority of TT over RV medium in the recovery of *S. tennessee*. The third serotype, *S. dublin*, behaved poorly on both media but less so in RV medium. The authors concluded that, before a final decision is made about the efficiency of RV medium relative to that of TT broth in products with low level of competing microflora, further studies are needed. For this reason, this study was undertaken using fluid whole milk artificially contaminated with *S. typhimurium* and gram-negative competing organisms. The results are reported in this paper.

**MATERIALS AND METHODS**

**Samples**

From October 1989 until April 1990, 100 samples of pasteurized cow whole milk artificially contaminated were examined for the presence of *Salmonella*. Equal volumes of 25 ml from each milk carton were contaminated in two different ways; one by adding two gelatin capsules containing *S. typhimurium* and the other by adding the same *Salmonella* inoculum plus two strain of different species of competing organisms. To achieve a representative competitive flora of the bacteria naturally contaminating food, 200 human isolates of nine species of gram-negative organisms, including strains of *Esherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella ozonae*, *Proteus vulgaris*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were used. Each of these 200 isolates was used only once throughout the study, to avoid repeated assessment of the same possible antagonistic influence of a certain isolate on the recovery of salmonellae. Nutrient broth cultures of each isolate were incubated at 37°C for 24 h. Serial 10-fold dilutions were made from each suspension in order to obtain 5 to 100 colonies on a MacConkey agar plate culture, streaked with a loop calibrated to 0.01 ml. Gelatin capsules with 0.2 g milk powder contaminated with *S. typhimurium*, sublethally injured by spray-drying, were prepared and kindly provided by Dr. P. in’t Veld, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. The mean number of salmonellae per capsule was 5.0. The method of preparation and the principle of the use of these capsules has been described by Beckers et al. (1).

**Media**

All samples were preenriched in buffered peptone water (BPW) (4). The Rappaport-Vassiliadis medium was prepared from the three necessary solutions (A, B, C) according to Vassiliadis et al. (14,15). It should be noted that the weight of MgCl₂ 6H₂O in solution B should be about 31.5 g per 100 ml, leading to a final concentration of about 2.85% in the prepared RV medium. Some of the commercially available RV media have not conformed to the suggested formulation (10,13), but this is now being corrected. Furthermore, the RV medium should be sterilized at 115°C because it has been reported that exposure to temperature...
of 121°C affects the performance of this medium (9). For the preparation of tetrathionate broth (TT), the commercially available dehydrated base from Difco (Detroit, MI) was used, with the addition of brilliant green in a final concentration of 1/100,000. The solid selective medium used was the brilliant green deoxycholate agar (BGDA) (18).

**Method**

Every Monday, seven cartons of pasteurized cow whole milk were brought into the laboratory. From each carton, 25 ml of milk was transferred in each of two Erlenmeyer flasks with 225 ml of BPW; in the first, two gelatin capsules with *S. typhimurium* were added, while in the second, two capsules with *S. typhimurium* plus 0.01 ml of the final dilution giving 5 to 100 cells of each of two different gram-negative organisms were added. After 24 h incubation at 37°C, 0.1 ml of the preenrichment in BPW was inoculated in 10 ml of RV medium, while 1 ml was inoculated in 10 ml of TT medium. For the RV medium it is important not to use a heavier inoculum, otherwise the medium loses its sensitivity and specificity (11,12,16). Both RV and TT media were incubated at 43°C for 24 and 48 h; in RV medium 24 h of incubation is usually adequate for the detection of salmonellae, but in rare occasions 48 h may be required. All these enrichments were streaked on BGDA plates which were incubated at 37°C for 24 h. Two suspicious colonies from each plate were inoculated in Kligler iron agar tubes. The resulting suspicious growths were further examined with biochemical and serological tests.

In the present study, the growth of competing organisms on the selective medium was classified on a scale from 0-4; 0 indicates absence of growth, 1 indicates growth on one quarter of the plate, 2 indicates growth on the whole plate, and 2 and 3 indicate growths between these extremes.

Statistical analysis of the results was done with the use of paired—$X^2$ test (MacNemar's test).

**RESULTS AND DISCUSSION**

The performance of RV and TT enrichment media, in the isolation of salmonellae from fluid whole milk, is given in Tables 1 and 2.

Table 1 shows the recovery of salmonellae from the milk samples contaminated only with *S. typhimurium*. From the 100 samples examined, 97 were found positive for salmonellae with the RV medium, while only 86 were found positive with the TT medium. The difference was statistically highly significant ($X^2$=11.0; $P<$0.001). Salmonellae were recovered after 24 h of incubation from all the 97 positive with RV medium samples (100%), while with TT medium only 79 out of the 86 positive samples (92%) were recovered.

Table 2 shows the recovery of salmonellae from the milk samples contaminated with *S. typhimurium* plus two different gram-negative competing organisms. From the 100 samples examined 79 were found positive with the RV medium while only 41 were found positive with the TT medium. The difference was statistically highly significant ($X^2$=36.1; $P<$0.001). From the 79 RV positive samples 73 were positive after 24 h of incubation (92%), while from the 41 TT positive samples 31 were positive after 24 h of incubation (76%).

Some of the total positive samples were found positive either only after 24 h or only after 48 h of incubation. With samples contaminated only with *S. typhimurium*, one sample was positive after 24 h but not after 48 h of incubation when RV medium was used, whereas three samples were positive only after 24 h and seven only after 48 h of incubation when TT medium was used (Table 1). With samples contaminated with *S. typhimurium* plus two gram-negative competing organisms, six samples were positive only after 24 h and six other only after 48 h when RV medium was used, whereas three samples were positive only after 24 h and 10 others only after 48 h when TT medium was used (Table 2). It should be pointed out that 24 h incubation in RV medium is usually adequate for isolation of salmonellae (14), but a negligible number of strains requires an incubation for 48 h; the unusual pattern observed in the present study (six samples positive only after 48 h) may be due to the fact that the *Salmonella* strain used was subletally injured, whereas the competing organisms were not, a combination that could allow relative preponderance of competing organisms during the first 24 h of incubation (1).

These findings indicate that the RV medium is superior to the TT medium in the isolation of *S. typhimurium* from fluid whole milk artificially contaminated, when either medium is combined with BGDA. They are in agreement with the corresponding findings of Northolt et al. (9) who, in a study on calcium caseinate and milk powder, found that the productivity and selectivity of RV medium were better than those of the commercially available Muller Kauffmann medium (MK). We have indicated in a previous study (6) that the performance of Difco's and Oxoid's MK tetrathionate broths, in isolating salmonellae from contaminated food samples, is practically the same. The results of the present study are also compatible with the *S. typhimurium* findings of Wilson et al. (19) who, in a study on fluid...

**TABLE 1. Recovery of salmonellae from 100 fluid milk samples contaminated with *S*. typhimurium.**

<table>
<thead>
<tr>
<th>Selective enrichment procedures</th>
<th>Total positive samples</th>
<th>Positive after</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV/43°C</td>
<td>97</td>
<td>24 h only 1, 48 h only 0, 24+48 h 96</td>
</tr>
<tr>
<td>TT/43°C</td>
<td>86</td>
<td>24 h only 3, 48 h only 7, 24+48 h 76</td>
</tr>
</tbody>
</table>

RV = Rappaport-Vassiliadis medium incubated at 43°C. TT = Tetrathionate brilliant green broth incubated at 43°C.

**TABLE 2. Recovery of salmonellae from 100 fluid milk samples contaminated with *S*. typhimurium and gram-negative organisms.**

<table>
<thead>
<tr>
<th>Selective enrichment procedures</th>
<th>Total positive samples</th>
<th>Positive after</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV/43°C</td>
<td>79</td>
<td>24 h only 6, 48 h only 6, 24+48 h 67</td>
</tr>
<tr>
<td>TT/43°C</td>
<td>41</td>
<td>24 h only 3, 48 h only 10, 24+48 h 28</td>
</tr>
</tbody>
</table>

*a* Gram-negative organisms (*Enterobacteriaceae* and *Pseudomonas*).

*b* See footnote on Table 1.
whole milk, noted that the MPN/ml appears to be twice as high with RV/43°C (9.3 x 10³) than with TT/43°C (4.3 x 10³). However, these authors (19) have shown that the performance of TT medium is much better than that of RV medium with respect to S. tennessee.

The comparison of the results in Tables 1 and 2 shows that the sensitivity of both enrichment media is adversely affected by the presence of gram-negative microorganisms. This has also been reported by other investigators who have found that food and/or its competitive flora appear to have a negative influence on the isolation of salmonellae; the proportion of Salmonella isolations from reference samples decreased from 80 to 47% with TBB-ISO and from 95 to 63% with RV in the presence of food (1,2). In the present study, this is more evident in TT medium (86% positive samples in the absence, compared to 41% in the presence of gram-negative organisms) than in RV medium (97 and 79%, respectively). This phenomenon is probably due to the fact that the RV medium inhibits the growth of competing organisms much more than the TT medium, as we have shown in several studies (14). Such differences between RV and tetrahtionate brilliant green broths have been also observed in the isolation of salmonellae from pork sausages (17), chicken carcasses (8), and sewage (16). In the present study the mean value of growth of competing organisms in RV medium after 24 and 48 h of incubation at 43°C were 0.81 and 0.92, respectively. The corresponding values for TT medium were 1.81 and 1.87.

In this study the solid selective medium BGDA was used because we have found it at least as effective as other selective plating media for the isolation of salmonellae, after enrichment in either RV medium or in any of the tetrahtionate brilliant green broths (5-7). Use of other selective media such as bismuth sulfite agar, in addition to BGDA, probably could have increased the recovery.

The findings of the present study indicate that the sensitivity and specificity of RV medium, at least with respect to S. typhimurium, are superior to those of TT medium, not only in product with extensive competing microflora (e.g., raw meat) (11,12,16), but even when the microflora is at a substantially lower level.

ACKNOWLEDGMENTS

The authors are grateful to Dr P. in't Veld of the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands for providing us with the sublethally inured S. typhimurium gelatin capsules.

They also thank Ms. Demetra Menega for her skilful technical assistance.

REFERENCES