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Improved Isolation of Salmonellae from Naturally Contaminated Meat Products by Using Rappaport-Vassiliadis Enrichment Broth

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A total of 454 specimens of meat products were examined for salmonellae by using five procedures of enrichment. The use of a selective motility medium, inoculated from enrichment in Muller-Kauffmann broth, resulted in an increase in the number of positive specimens. However, simple enrichment in Rappaport-Vassiliadis broth, after preenrichment, was more sensitive and specific for recovering salmonellae than the selective motility medium-Muller-Kauffmann broth method.

There is now a tendency to utilize preenrichment, followed by enrichment, in the isolation of salmonellae from food and water (1, 4, 7, 8, 10). In addition to the fluid enrichment procedures in use, motility media may be employed both for primary enrichment (2) and secondary enrichment (3, 7).

Two important modifications (9, 11, 12-15) in the original broth described by Rappaport et al. (6) (the modified medium formerly called R10 medium and now named Rappaport-Vassiliadis malachite green-magnesium chloride [RV] enrichment medium [5]) have made the medium significantly more efficient in the isolation of salmonellae than the Muller-Kauffmann tetrathionate (MK) broth recommended as a reference medium by the International Standards Organization (1).

Recently, Smeltzer and Duncalfe (7) applied enrichment in MK medium, followed by secondary enrichment in the selective motility medium (SMM) of Harper and Shortridge (2). This technique results in a significant increase in the number of specimens found to contain salmonellae. These findings prompted a comparison between the technique used by Smeltzer and Duncalfe and the technique with RV enrichment broth, which is much simpler. This paper describes the results of this comparison.

MATERIALS AND METHODS

During 1980, 454 samples of meat products were examined for the presence of salmonellae. These samples included 100 specimens of pork sausages, 50 chicken carcasses, 217 samples of bovine minced meat, and 87 samples of pig mesenteric glands. All samples were preenriched in buffered-peptone water (P medium) (1) by adding 25 g of pork sausages, 25 g of bovine minced meat in 225 ml of P medium, or 15 g of finely cut mesenteric glands in 135 ml of the same medium and were incubated at 37°C for 20 to 22 h. The chicken carcasses were washed in sterile plastic bags with 500 ml of P medium and removed, and the wash was incubated as described above.

In 100 ml of MK broth, prepared by the method described by the International Organization for Standardization (1), 10 ml of preenrichment P medium was added, and then the MK broth was incubated at 43°C for 48 h. This period of incubation for MK broth was found by Smeltzer and Duncalfe (7) to be the optimum in any single method of enrichment used by them. After this incubation, the MK broth was subcultured onto brilliant green deoxycholate agar (BGDA). This modified agar (14) was prepared by adding, before boiling, 2.5 g of sodium deoxycholate per liter of Oxoid brilliant green agar. The BGDA contains very little brilliant green and is sterilized by boiling for about 30 s over a flame.

For the preparation of RV broth the following three solutions are necessary. Solution A contained tryptone (Difco), 5 g; NaCl (pure), 8 g; KH_2PO_4 (pure), 1.6 g; and distilled water, 1,000 ml. Solution B contained 400 g of analytically pure $MgCl_2 \cdot 6H_2O$ in 1,000 ml of distilled water. Solution C contained 0.4 g of analytically pure malachite green oxalate (Merck) in 100 ml of distilled water.

Solution A must be prepared on the day of the preparation of the medium. The solution is heated to about 70 to 80°C to dissolve the ingredients completely. Solution B can be kept in a dark bottle at room temperature for at least 1 year. To prepare this solution, it is advisable to dissolve the entire contents of $MgCl_2 \cdot 6H_2O$ from a newly opened container according to the formula because this salt is very hygroscopic. Solution C can be kept for at least 6 months at room temperature in a dark bottle. It is recommended

that Merck analytically pure malachite green oxalate be used (no. 1398) because other brands may not be equally effective. In the preparation of RV broth, 100 ml of solution B and only 10 ml of solution C were added to 1,000 ml of solution A (total volume of final medium, 1,110 ml). The medium was distributed in test tubes in 10-ml quantities (or in screw-capped bottles). It was sterilized at 115°C for 15 min, kept in a referigerator, and used within 1 month. This medium, when inoculated, must be incubated at 43°C.

Volumes of 10 and 100 ml of RV medium were inoculated with 0.1 and 1 ml of P medium, respectively. They were then incubated at 43°C for 24 h and subcultured onto BGDA.

The secondary enrichment technique of Smeltzer and Duncalfe (7) was followed by introducing 0.2 ml of the MK medium (48-h incubation) or 0.2 ml from 100 ml of the RV medium (24-h incubation) into the center tube of the SMM described by Harper and Shortridge (2). The SMM medium was incubated at 37°C and inspected daily until the growth, detected as either blackening due to H_2S production or turbidity, reached the top of the outer portion of the tube. Subcultures were made from the outer portion of the positive tubes into BGDA. The tubes were inspected for 6 days.

All BGDA plates were incubated at 37°C for 24 h (occasionally, for 48 h). From the plates showing a suspicious growth, two to three colonies were examined by biochemical and agglutination tests from an overnight growth on Kligler iron agar.

The statistical analysis was done by the chi-square test.

RESULTS

The results concerning the positive samples and the number of serotypes and strains isolated with the three different enrichments and the two secondary enrichments are summarized in Table 1.

In Table 2, the day that the SMM tubes became positive with each of the procedures used is shown. From Tables 1 and 2 it is evident that, with secondary enrichment in SMM tubes, colonies of salmonellae were obtained at the earliest on day 5 or more often on day 6 after the beginning of the preenrichment, whereas with the simple RV medium, this time was reduced to only 3 days.

The 159 specimens found to contain salmonellae (with at least one of the five methods used) included 46 pork sausages, 50 chicken carcasses, 16 bovine minced meat samples, and 47 pig mesenteric glands.

The performance of each of the various methods employed for the examination of the specimens naturally contaminated with salmonellae is shown in Table 3. In Table 4, the specificities of the five methods used are compared.

DISCUSSION

In this paper, five procedures of salmonella isolation from meat products were compared.

TABLE 1. *Isolation of salmonellae from 454 specimens of meat products after preenrichment followed by different methods of enrichment and secondary enrichment*

Enrichment method[a]	No. of positive samples[b]	No. of serotypes isolated[c]	No. of strains isolated[d]
MK	92 (20.3)[e]	21	98
MK-SMM	121 (26.7)	23	134
RV (100 ml)	144 (31.7)	24	166
RV-SMM	151 (33.3)	23	169
RV (10 ml)	149 (32.8)	23	166

[a] MK, Enrichment in 100 ml of standardized MK broth inoculated with 10 ml of preenrichment in P medium and incubated at 43°C for 48 h; MK-SMM, inoculation of 0.2 ml of MK medium into the center tube of SMM which was incubated at 37°C; RV, enrichment in 100- or 10-ml volumes inoculated with 1 or 0.1 ml, respectively, of P medium and incubated at 43°C for 24 h; RV-SMM, inoculation of 0.2 ml of the 100-ml RV medium into SMM which was incubated at 37°C.

[b] A total of 159 (35.0%) were positive by at least one method.

[c] A total of 25 were positive by at least one method.

[d] A total of 250 were positive by at least one method.

[e] Percent is shown within parentheses.

TABLE 2. *Results of SMM tubes by day after inoculation*

Enrichment method	% Positive tubes on following day:					
	1	2	3	4	5	Total
MK-SMM	5.1	14.5	4.6	1.8	0.7	26.7
RV-SMM	2.6	26.2	4.0	0.4		33.3

Specifically, the RV medium (11) in 10- and 100-ml volumes was compared with the standardized MK broth (1) in a 100-ml volume and with SMM (2, 7) inoculated from either MK or RV broths. In the examination of 454 samples of meat products, we found that, after preenrichment, the use of an enrichment in MK broth, incubated at 43°C for 2 days, 92 specimens were found to be positive for salmonellae, whereas with a secondary enrichment in SMM made from the MK broth, 121 samples were found to be positive ($P < 0.05$). Thus, we were able to confirm the observations of Smeltzer and Dulcalfe (7). However, after preenrichment, the simple enrichment in 10 ml of RV broth at 43°C for 1 day yielded 149 positive specimens, instead of 121 positive specimens found after secondary enrichment in SMM ($P \sim 0.05$) and 92 positive specimens detected by MK broth ($P < 0.001$) (Table 1). Table 1 also shows that with at least one of the five methods used, 159 specimens were positive (100%), whereas none of the five methods alone was able to isolate all of the salmonellae that were isolated by the complete procedure.

TABLE 3. *Performance of enrichment methods used for the examination of naturally contaminated samples for salmonella*

Enrichment method[a]	No. of positive specimens[b]	%
MK	92	57.9
MK-SMM	121	76.1
RV (100 ml)	144	90.6
RV-SMM	151	95.7
RV (10 ml)	149	93.7

[a] See Table 1, footnote *a*.

[b] All 159 specimens were positive by at least one method.

TABLE 4. *Specificities of five enrichment methods for salmonella isolation*

Enrichment method[a]	No. of colonies examined	No. of salmonella colonies	No. of false-positive colonies
MK	544	238 (43.7)[b]	306 (56.3)
MK-SMM	565	321 (56.8)	244 (43.2)
RV (100 ml)	450	412 (91.6)	38 (8.4)
RV-SMM	462	384 (83.1)	78 (16.9)
RV (10 ml)	463	431 (93.1)	32 (6.9)

[a] See Table 1, footnote *a*.

[b] Percent is shown within parentheses.

The closest results were obtained with the simple enrichment in 10 ml of RV medium (149 positive samples) and the SMM tubes inoculated from the enrichment in 100 ml of RV medium (151 positive samples), which resulted in the detection of 93.7 and 95.0%, respectively, of all the positive specimens (Table 3). Almost equally efficient was the simple enrichment in 100 ml of RV medium (90.6% detection of all positive samples). The method involving a simple enrichment in MK broth resulted in the detection of only 57.9%, and the SMM secondary enrichment inoculated with MK broth, as advocated by Smeltzer and Duncalfe (7), resulted in detection by 76.1% of the total positive specimens. Consequently, the enrichment in 10 ml of RV broth resulted in 17.6% more isolations of salmonellae than did the SMM procedure recommended by Smeltzer and Duncalfe.

Analysis of the results of the four types of specimens revealed that the performance of each of the methods was independent of the type of the specimen, except with the samples of mesenteric glands, for which the MK or SMM method was almost as efficient as the RV method. This result may be explained by the fact that, in a quantitative study of eight of the mesenteric gland specimens, we found them to be highly contaminated with salmonellae, with some containing a most probable number of $\geq 2.5 \times 10^6$ organisms per 100 g of lymphatic tissue.

In the procedure involving SMM, the same serotypes were usually isolated as with the other techniques, but exceptions also occurred.

Of 25 *Salmonella* serotypes isolated during this investigation, 2 (*S. banana* (pork sausage) and *S. mbandaka* [minced meat]) were isolated for the first time in Greece.

In this trial, the methods involving enrichment in RV medium inhibited the lactose- and sucrose-negative competing organisms, which often produce salmonella-like colonies on BGDA, more strongly than the methods involving MK broth (Table 4).

The RV medium has the added advantage that it is very simple to prepare and, once prepared, can be kept ready for use for at least 1 month. Furthermore, it is much cheaper than either the MK medium or SMM.

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