Isolation of Salmonellae from Food Samples

V. Determination of the Method of Choice for Enumeration of *Salmonella*

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ABSTRACT

TAYLOR, WELTON I. (Children's Memorial Hospital, Chicago). Isolation of salmonellae from food samples. V. Determination of the method of choice for enumeration of *Salmonella*. Appl. Microbiol. **9**:487–490. 1961.—Comparison of three methods by which salmonellae may be isolated and enumerated from dried albumen, direct inoculation of enrichment media, centrifugation of samples, and pre-enrichment in noninhibitory media, reveals pre-enrichment to be the method of choice.

The superiority of pre-enrichment manifests itself in replicate aliquots of the same sample by producing a statistically significant increase in numbers of isolations of salmonellae and in empirical use with various albumen samples by consistently higher values of most probable numbers (MPN).

The primary factor involved in this superiority appears to be the greater ability of small numbers of salmonellae to initiate growth in the nonselective mannitol purple sugar broth than in the inhibitory enrichment media.

The method of analysis recommended entails inoculation of mannitol broth pre-enrichment medium, transfer of 24-hr culture aliquots to tetrathionate broth, and streaking on brilliant green agar for isolation of salmonellae.

The effectiveness of pre-enrichment as a method of securing a greater number of positive *Salmonella* isolations from albumen samples has been established (Taylor and Silliker, 1961). Likewise, a similar improvement over direct inoculation was shown to be possible with centrifugation (Silliker and Taylor, 1958). In the present experiments comparison is made of all of the methods for the detection of salmonellae used heretofore and of combinations of them to determine the method of choice for use in the analysis of foods such as albumen.

MATERIALS AND METHODS

Naturally contaminated dried albumen samples from many sources were used. Reconstituted albumen (10%), mechanically shaken to reduce sampling error, was the inoculum for pre-enrichment broth, enrichment broth, or water for centrifugation. All *Salmonella* isolations, therefore, were from those naturally occurring strains which had survived the processes used in the manufacture of dried albumen. No stock cultures were used.

Cystine-selenite F broth (selenite) and brilliant green-tetrathionate broth (tetrathionate) were the enrichment broths used. Mannitol (0.5%) purple sugar broth was used for pre-enrichment, and sterile buffered water for centrifugation.

When pre-enrichment medium was used, an aliquot of 0.2 ml of the 18 to 24-hr growth was transferred to 10-ml tubes of enrichment broth. After incubation, brilliant green agar was streaked for isolation of salmonellae. Suspect *Salmonella* colonies were picked to dulcitol lysine lactose iron agar, a newer modification of dulcitol lactose iron agar (Taylor and Silliker, 1958), and a modified lysine broth (Falkow, 1958). These media established the biochemical identity of the salmonellae through fermentation of dulcitol, H_2S production, and lysine utilization and have been proven by serological typing by the State of Illinois Public Health Laboratories, Chicago, to be valid indices of generic identification in over 98% of the more than 300 cultures submitted to typing procedures. The formulas are given in Tables 1 and 2. The pH of solution A (Table 1) is adjusted to pH 7.3 with NaOH. Solution B is heated to dissolve the ingredients and 2 ml added to screw-capped tubes (16 × 125 mm). Aluminum foil-

TABLE 1. *Composition of dulcitol lysine lactose iron agar*

Dulcitol lysine lactose iron agar	
Solution A (slant)	
Lactose	5 g
L-Lysine monohydrochloride	5 g
Dulcitol	2 g
Glucose	0.1 g
Ferrous sulfate, anhydrous	0.2 g
Sodium thiosulfate, anhydrous	0.3 g
Agar	15 g
Phenol red (2.5% aqueous soln)	1 ml
Water, distilled	1 liter
Solution B (butt)	
Phenol red agar base	30 g
Water, distilled	1 liter

covered baskets of uncapped tubes are autoclaved and allowed to solidify. Solution A is sterilized in a flask and dispensed aseptically from a sterile separatory funnel or by means of an automatic pipetting machine with a sterile syringe-valve assembly, 3 ml per tube. Tubes are capped tightly with sterile caps, slanted, and cooled. The medium keeps indefinitely at either room or refrigerator temperatures. The lysine broth (Table 2) is adjusted to pH 6.6, tubed in screw-capped tubes (16 × 125 mm) in 5 ml amounts, and autoclaved.

Members of the genus *Salmonella* with but few exceptions do not ferment lactose or sucrose, do ferment dulcitol, produce H_2S, and utilize L-lysine. A characteristic reaction of the genus, then, on dulcitol lysine lactose iron agar is an alkaline (red) slant, H_2S blackening in the middle, and a fractured acid (yellow) butt. Sucrose is omitted from the formula because *Proteus* species which ferment sucrose are both dulcitol and lysine negative and so are excluded from consideration by this medium. Arizona group paracolons (Kauffmann, 1954) do not ferment dulcitol, and therefore a red butt excludes them; Bethesda-Ballerup group paracolons may ferment dulcitol but are lysine negative and so produce a yellow slant, as does *Escherichia freundii*. The Providencia group ferments neither lactose nor dulcitol, does not produce H_2S, or utilize lysine; an unchanged (red) tube throughout. The small amount of glucose added to the medium enhances H_2S blackening. When Arizona strains were inoculated, it was observed that little or no blackening occurred in dulcitol lactose iron medium, whereas triple sugar iron agar slants showed profuse blackening. A trace of glucose, which is insufficient to cause a color change, restored the blackening.

Unfortunately, the slant reaction is not completely reliable. Sometimes rapidly growing Bethesda-Ballerup or *E. freundii* strains will cause an alkaline reversion on the slant which mimics the lysine-positive reaction. Conversely, slowly growing salmonellae sometimes do not produce an alkaline reaction in 18 to 24 hr, although this rarely is the case at 24 hr. Verification in modified lysine broth settles these doubtful results with great accuracy. The original lysine broth formula (Falkow, 1958) was used to replace the ninhydrin test previously used (Carlquist, 1956), but was found to be less accurate mainly because *E. freundii* and its paracolon types frequently gave false positive results. It was reasoned that the peptone was a nitrogen source for liberation of ammonia even if lysine were not utilized. When peptone was omitted from the formula, a series of comparisons revealed the modified formula to be both valid and reliable, exceeding the accuracy of even the ninhydrin test, which sometimes gave false negative results because of poor extraction with chloroform. Commercially prepared lysine decarboxylase broth (Difco)[1] or Falkow-lysine broth (BBL)[2] are available but have not been subjected to comparison. An alternate formula using bromthymol blue instead of bromcresol purple and 1% lactose (Bachrach, 1959) has not been tested for accuracy; like the Falkow formula, it contains peptone.

TABLE 2. *Composition of lysine broth (modified)*

L-Lysine monohydrochloride	5 g
Yeast extract	3 g
Glucose	1 g
Bromcresol purple (1.6% alcoholic solution)	1 ml
Water, distilled	1 liter

Use of polyvalent antiserum for confirmation may be left to the discretion of the laboratory; in unpublished data we have demonstrated the biochemical identification to be more reliable than the serological with polyvalent antiserum. This inaccuracy was observed upon initial isolation of processed food strains of salmonellae which, seemingly, are serologically less reactive than isolates from clinical specimens. A longer reaction time is routinely necessary for even those organisms which were serologically typable.

Results and Discussion

The results of ten replicate experiments involving 1,000 samples and 261 *Salmonella* isolations and the statistical analysis of some of the more important comparisons are shown in Table 3. It is immediately obvious that those options which are most efficacious for isolation of salmonellae utilize pre-enrichment. The failure to produce a greater number of positives with centrifugation and the spectacular improvement with pre-enrichment are likely the results of two different

[1] Difco Laboratories, Inc., Detroit, Mich.
[2] Baltimore Biological Laboratory, Inc., Baltimore, Md.

TABLE 3. *Isolation of Salmonellae from albumen by various techniques*

Direct (D)		Centrifuged (C)		Centrifuged pre-enriched (CP)		Pre-enriched (P)		Pre-enriched centrifuged (PC)	
S*	T*	S	T	S	T	S	T	S	T
4†	3	1	2	30	37	38	54	36	56

Comparison of techniques	χ^2‡	*P value*
D-S vs. C-S 4-1	0.8	0.3
D-S vs. P-S 4-38	32.8	<0.0005
CP-S vs. P-S 30-38	1.1	0.2
PC-S vs. P-S 36-38	0.02	0.8
CP-S vs. CP-T 30-57	0.8	0.3
P-S vs. P-T 38-54	4.5	<0.05
CP-T vs. P-T 37-54	5.2	<0.05
PC-T vs. P-T 56-54	0.02	0.8

* S = Cystine-selenite F broth, T = brilliant green tetrathionate broth.

† Number positive *Salmonella* isolations from 100 aliquots of naturally contaminated dried albumen.

‡ Chi-square, 4-fold table, Yates correction.

phenomena: (i) restoration of selectivity—the ability to inhibit coliforms while permitting salmonellae to multiply, and (ii) sensitivity—the ability to initiate growth with a smaller number of organisms.

Centrifugation restores selectivity to the enrichment broth by removing from the sample that soluble material which acts as an adulterant to the enrichment medium. If the number of salmonellae in the sample aliquot is sufficient to guarantee the initiation of growth in the unadulterated enrichment broth, then centrifugation suffices to obtain the positive isolation.

Pre-enrichment contributes to sensitivity since a noninhibitory medium, such as mannitol purple sugar broth, will produce a greater population of salmonellae in 24 hr than will either of the inhibitory enrichment broths. If the numbers of salmonellae are small, it is probable that a number insufficient to guarantee growth of the strain in an aliquot of selenite or tetrathionate may be more than enough to initiate growth in the pre-enrichment broth. The failure of the pre-enriched centrifuged broth to be significantly better than the pre-enriched broth alone in either tetrathionate (54/100 vs. 56/100) or in selenite (38/100 vs. 36/100), is thus explained.

Empirical testing of the three methods produced the results shown in Table 4. Mannitol purple sugar broth aliquots performed a dual function since they were streaked directly onto brilliant green agar (direct), and used to inoculate selenite and tetrathionate pre-enriched broths. In 7 out of the 9 samples analyzed, the direct mannitol purple sugar broth was equal or superior to the enrichment broths, sometimes spectacularly so (samples 5 and 6). Centrifuged samples likewise were superior to direct enrichment broths 7 out of 8 times; but when compared to pre-enrichment, they produced greater numbers 3 times out of 8, the same in 1 time out of 8 and fewer in 4 times out of 8. The magnitude of the high counts was greatest in the pre-enriched samples. Pre-enrichment proved superior to direct inoculation of all three broths 10 times out of 12 and was equal to them in the other 2.

TABLE 4. *Comparison of Salmonella MPN* obtained by different analytical methods*

Sample	Direct			Centrifuged		Mannitol pre-enriched	
	Selenite broth	Tetrathionate broth	Mannitol broth	Selenite broth	Tetrathionate broth	Selenite broth	Tetrathionate broth
1	9	0	—	—	—	7	15
2	0	23	—	—	—	43	95
3	0	23	—	—	—	93	150
4	0	9	7	—	—	21	150
5	2	1	2,300+	43	43	2,300+	2,300+
6	4	0	1,500	0	2	35	2,100
7	0.4	0	0.4	2	0.4	1	1
8	2	0	0.7	4	9	0.7	1
9	0	0	0	4	1	0	0
10	0	0	0.4	0.4	0	0	1
11	0	0	1	0.4	1	0.4	1
12	0	0	8	1	2	4	16

* MPN = most probable number of *Salmonella* per g of dried albumen.

Pre-enrichment becomes the method of choice for analysis of food samples such as albumen on the basis of data presented here. It requires no specialized equipment, training of personnel, or excessive manipulations such as are entailed in centrifugation. The introduction of an extra medium requires one more handling and an added day for its incubation. This may be partly offset by streaking the mannitol directly onto brilliant green agar plates at the same time that it is used to inoculate tetrathionate so that a "presumptive" *Salmonella* count may be obtained a day earlier than the "confirmed" count from the tetrathionate.

The choice of media to be used in the method of salmonella analysis is dictated by the results seen in previous experiments. Brilliant green agar is the most valuable selective plating medium (Taylor, 1958; Taylor and Silliker, 1958; Taylor, Silliker, and Andrews, 1958). Brilliant green-tetrathionate enrichment broth has proven superior to cystine-selenite in this and the preceding paper (Taylor and Silliker, 1961). Mannitol purple sugar broth becomes the pre-enrichment medium of choice for reasons best described as rational rather than from experimental data presented. In the preceding paper, no significant differences were observed whether dulcitol, mannitol, lactose, or no carbohydrate was added to purple broth base. North (1961) prefers lactose broth since both coliforms and salmonellae may be enumerated from the same tube, which serves as the presumptive medium for both analyses. This is an advantage which certainly should be considered by any laboratory which must perform both analyses. If, however, only salmonellae are considered, it would seem logical to use a carbohydrate assimilated by salmonellae rather than use lactose which stands to benefit coliforms, enterococci, paracolons, and other nonsalmonellae, all of which must be inhibited ultimately if one is to isolate and identify salmonellae. This reasoning has led others (Stokes and Osborne, 1955; Hajna, 1955) to choose mannitol for inclusion in media designed to enhance *Salmonella* isolation.

After the experiments presented herein were completed, Sugiyama, Dack and Lippitz (1960) demonstrated the superiority of both centrifugation and pre-enrichment with lauryl tryptose broth containing *Salmonella* antiserum, over direct inoculation of selenite broth. Unfortunately, there was no comparison with lauryl tryptose broth without antiserum so that the factor of agglutination as a means of enhancing salmonella isolation might be evaluated alone. Likewise centrifugation involving "minimal-centrifugation" designed to sediment only the flocculated organisms,

theoretically leaving non-salmonellae in the supernatant liquor to be discarded, was not compared with maximal centrifugation in which all organisms were sedimented. Without controls for pre-enrichment, antisera, and differential centrifugation, the net comparison is that of the combined factors against the routine method. Since in the process of pre-enrichment, the initial small numbers of salmonellae are swelled to millions per ml of broth, it is difficult to imagine a statistically significant improvement resulting from agglutination per se. Likewise, when the authors demonstrate the the ability to isolate salmonellae from selenite broth in which they were initially outnumbered by coliforms by a million to one, it becomes difficult to imagine that the small numerical superiority of salmonellae afforded by differential centrifugation of agglutinated cells would prove significantly better than total sedimentation of all cells for selenite inoculation. It would seem to be desirable to test these alternatives more exhaustively.

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