Accelerated Procedure for *Salmonella* Detection in Dried Foods and Feeds Involving Only Broth Cultures and Serological Reactions[1]

W. H. SPERBER AND R. H. DEIBEL

*Department of Bacteriology and The Food Research Institute, The University of Wisconsin,
Madison, Wisconsin 53706*

A procedure has been developed in which *Salmonella* can be detected in dried foods and feeds within 50 hr. This includes preenrichment (18 hr), selective enrichment (24 hr), elective enrichment (6 to 8 hr), and serological testing (2 hr). The procedure is as sensitive as the more time-consuming, traditional (plating, biochemical, and serological) procedure which may require at least 4 to 5 days. The new procedure is rapid and accurate in comparison to traditional procedures. Moreover, it is simple and inexpensive to perform. As described, the procedure does not necessitate the isolation of pure cultures, but this step is performed easily if desired.

The food and feed industries are currently subjecting their products to an intensive scrutiny for *Salmonella* contamination. The recall of contaminated product from the market, actual loss of product, marred product prestige, and economic consequences of increased control programs have and will continue to have serious ramifications in the affected industries.

One of the major problems confronting industry today is the long time needed to dermine whether a product is free of *Salmonella* contamination. An absolute minimum of 4 days is required (2), and, if the presumptive test is positive, an additional 2 to 3 days are necessary. In general, 4 to 6 days must be allotted for this determination. This forces the manufacturer to consider two alternatives. One is to ship the product without waiting for laboratory clearance; the other, to warehouse the product until laboratory clearance is obtained. Neither alternative is economically appealing. In the first alternative, microbiological safety can be violated and the product may be subject to recall or seizure. The other alternative involves costly additional handling and storage of product. These considerations lay the foundation for one of the food and feed industries' most pressing needs: an accelerated procedure for *Salmonella* detection.

The accelerated procedure described herein necessitates both preenrichment and selective enrichment by the same methods as practiced in

the traditional procedure. However, instead of subsequently plating in selective agar media to effect isolation followed by biochemical screening and ultimate serological confirmation, the proposed procedure involves a 6-hr incubation in an elective broth medium and direct serological testing. For the most part, the definitive identification of salmonellae is serological, and, the sooner these specific immunochemical reactions can be employed in a given procedure, the less time will be required for its completion.

MATERIALS AND METHODS

Samples. Only foods and feeds that were contaminated naturally with salmonellae were used in this study. Most of these samples were supplied through the generosity of various food companies. Also, some samples were obtained from the Food and Drug Administration Laboratories. Some animal feed samples used in preliminary experiments were supplied by J. H. Silliker, Chicago Heights, Ill. To enhance longevity of the contaminating organisms, all samples were stored in plastic containers at 4 C.

Preenrichment culture procedures. Usually, 100 g of sample was added to 1 liter of Lactose Broth (Difco) as recommended by North (6). In some instances, the amount of sample examined was decreased [as with dried egg samples or in the most-probable-numbers (MPN) procedure], but the 10% (w/v) ratio of sample to Lactose Broth was maintained. Some samples, such as chocolate, that contained large amounts of water-insoluble material, were mixed with the lactose broth in a mechanical blender. The preenrichment cultures were usually incubated for 18 hr at 37 C.

Selective enrichment. Tubes containing 9 ml of

either Selenite-Cystine Broth (Difco) or Tetrathionate Broth (Difco) were inoculated with 1-ml volumes of the preenrichment broth. These media were prepared with only 90% of the recommended amount of water so that, when the 1 ml of inoculum was added, the enrichment components were present at full strength. After shaking to insure distribution of the inoculum, the cultures were incubated at 37 C for 24 hr.

Traditional cultural procedure. Essentially, the method of the U.S. Food and Drug Laboratories was employed (2). Dried plates of Salmonella-Shigella (SS) Agar (Difco), Brilliant Green Agar (Difco), and Bismuth Sulfite Agar (Difco) were inoculated by streaking one loopful of the selective enrichment culture. Each enrichment was streaked on all three media. Thus, each sample ultimately required a total of six plates. Sucrose at 1% strength was added to the SS Agar to differentiate sucrose-fermenting colonies on the plate. Also, 0.65% agar was added to SS Agar to obtain a firm medium with superior streaking characteristics. The inoculated plates were incubated at 37 C for 24 hr, whereupon two to four suspect colonies from each plate were picked to slants of Triple Sugar Iron (TSI) Agar (Difco). The slants were incubated overnight at 37 C. Presumptively *Salmonella*-positive cultures were transferred into Brain Heart Infusion (BHI; Difco) and incubated at least 8 hr at 37 C, or until densely turbid, for flagellar antigen tests using pooled (8) Spicer-Edwards antisera (Difco). The remaining growth on the TSI slant was used for somatic (O) antigen analysis if the culture gave a positive test with pooled Spicer-Edwards antisera. If no reaction was obtained with Spicer-Edwards antiserum, a polyvalent O test was performed. If the culture was also polyvalent O-negative, it was not considered to be a *Salmonella*. To avoid overlooking mutant salmonellae, serological tests were performed on all TSI slants which were H_2S-positive or which exhibited an alkaline slant and acid butt. This procedure yielded two H_2S-negative salmonellae which otherwise would have been ignored.

RESULTS

Spicer-Edwards test modification. Traditionally, 2.0 ml of a Formalin-salt mixture (0.6 ml Formalin, 0.85 g of NaCl, and 100 ml of water) is added to 2.0 ml of a BHI culture which has grown to the requisite turbidity. After gentle mixing, 0.5 ml of this mixture is placed in a Kahn tube (10 by 75 mm) and 0.5 ml of diluted (1:500) Spicer-Edwards antiserum is added. Thus, in this test, the original culture is diluted fourfold but, since pure cultures are used, this has never been a matter of importance.

To circumvent the dilution of the original culture and thereby increase the sensitivity of the test, the following modifications were employed. (i) The volume of culture was increased by dispensing 0.85 ml of the turbid broth into Kahn tubes (10 by 75 mm). (ii) The concentration of the Formalin-salt solution was increased. One drop, or about 0.05 ml (3.0 ml of Formalin, 4.2 g

of NaCl, 100 ml of water), was added to the culture in the Kahn tube. (iii) A more concentrated Spicer-Edwards antiserum preparation was employed. The Spicer-Edwards antiserum was diluted 1:100 (in contrast to 1:500) and 0.1 ml was added to the Formalin-culture mixture.

The tubes were incubated at 50 C for at least 1 hr before being observed for agglutination. Pure cultures of salmonellae grown in BHI or the Tween 80-containing medium, APT Broth (Difco), were tested by the traditional and modified tests. With the traditional test, 2×10^8 cells/ml of original culture were necessary for agglutination. In comparison, only 5×10^7 cells/ml of original culture were necessary to give a positive result with the modified test. (This latter value is similar to the minimum number of cells necessary to give a positive reaction by fluorescent microscopy.) All subsequent flagellar agglutination tests were performed by using the modified test.

Spicer-Edwards tests with enrichment cultures. Attempts to shorten the traditional *Salmonella* isolation procedure involved the coupling of the modified Spicer-Edwards test directly to the broth cultures used in *Salmonella* isolation, thereby eliminating the array of plating and biochemical test media necessary to identify presumptive salmonellae. Neither the traditional nor the modified Spicer-Edwards test gave accurate results when cultures of either enrichment medium used in this study were examined. In some instances, this could be associated with the carryover of precipitates to the agglutination tests ($CaCO_3$ from Tetrathionate broth or selenium from selenite-Cystine broth). Another and somewhat common occurrence was the absence of agglutination which perhaps reflects poor antigen development in these relatively toxic media. These results indicated the necessity of a subsequent growth period in a medium that favors antigen or flagellar development.

Testing various postenrichment media. BHI was used in preliminary trials since this medium affords good antigen development for use in the traditional Spicer-Edwards test. Although results with BHI were far superior to direct testing of enrichment cultures, one false-negative result was obtained when 73 samples were tested by the accelerated procedure (results compared with traditional cultural procedure). Moreover, difficulty was experienced in the agglutination tests, as spontaneous agglutination in the absence of antisera was observed occasionally. Often these results could be associated with a "rough" or sedimentary type of growth in the broth culture. In an attempt to avoid this type of growth, some experiments were undertaken with APT broth. Equivocal results were obtained; however, the

high concentration of glucose (1.0%) in the medium could have afforded a radical drop in pH value. Presumably, by omitting the glucose and allowing citrate in the medium to serve as the anaerobic energy source, it was speculated that a superior medium for *Salmonella* enrichment might result.

The results with BHI and APT broth cultures were not favorable. The use of APT broth minus glucose (APTMG) decreased the incidence of nonspecific agglutination, but enrichment cultures from 3 of 73 samples still gave false-positive or ambiguous results (controls spontaneously agglutinated).

In the course of this study, Old et al. (7) reported that fimbrial agglutination in *Salmonella* cultures could be eliminated by the addition of mannose to the medium. In the hope that these results might extend to other *Enterobacteriaceae*, and perhaps decrease further the incidence of nonspecific agglutination in the modified agglutination test, we added 0.2% D-mannose to APTMG broth.

Preliminary results with key samples, known to have caused difficulty previously, gave unequivocal results. An extended study using the mannose-containing medium (henceforth referred to as "M broth" for simplicity) was initiated. Various dried food and feed samples were cultured in preenrichment and enrichment media as described previously. Each enrichment medium was then further analyzed by the traditional (plating-biochemical-serological) and the accelerated enrichment serology (ES) procedures. In each of 105 individual tests (210 selective enrichment cultures), an absolute correlation between the two test procedures was obtained (Table 1). Significantly, 37 of the test samples contained *Salmonella* and some samples contained relatively low numbers (yeast powder and a food plant environmental sample) as evidenced by the frequency of positive results.

A number of MPN determinations were made on the milk chocolate, dried milk, and rosemary spice samples (Table 2). The sensitivity of both procedures was equivalent, since identical MPN values were obtained for each food tested. If an enrichment culture was positive by one procedure, it was positive by the other and, conversely, if an enrichment culture was negative by one procedure, it was also negative by the other.

Early in this phase of the study it was observed that a one-drop inoculum from the selective enrichment culture to M broth and 6 to 8 hr of incubation gave comparable results to a loop inoculum and overnight incubation. Most of the samples (Table 1) were tested by both of these

TABLE 1. *Comparison of traditional and ES procedures for the detection of Salmonella in dried foods and feeds*

Product	No. of lots	No. of samples	Sample size	*Salmonella* detection results	
				Traditional-positive; ES-positive[a]	Traditional-negative; ES-negative[a]
			g		
Rosemary spice........	8	8	100	4	4
Dried milk....	9	9	100	5	4
Egg albumen..	1	11	10	3	8
Food plant environmental sample......	1	20	10	1	19
Milk chocolate....	1	2	100	1	1
Yeast powder..	1	10	10	2	8
Gelatin........	5	10	10	0	10
Egg noodles...	8	25	100	11	14
Fish meal.....	10	10	50	10	0
Total	44	105		37	68

[a] Results obtained by each test procedure were the same for each individual sample.

TABLE 2. *Comparison of MPN results for Salmonella quantitation in dried foods using the traditional and ES detection procedures*

Product	No. of lots examined	MPN of salmonellae per 100 g of product[a]
Rosemary spice........	8	0.36 to 4.3[b] (1.7)[c]
Dried milk...........	1	9.3
Milk chocolate........	16	0.36 to 2.3 (0.78)

[a] Three-tube MPN.
[b] Identical results obtained with each procedure.
[c] Arithmetic mean in parentheses.

procedures; the procedures gave identical results. Thus, if time is a critical factor and schedules lend themselves to the shorter incubation period, results can be obtained somewhat earlier (about 50 hr; otherwise, 3 days with the overnight incubation period in M-broth).

Effect of medium and other organisms. Throughout the course of this study, experiments were performed to determine the effect of relative numbers of competing organisms on the performance of the modified H agglutination test. Under actual test conditions (i.e., after preenrichment and selective enrichment), it was estimated that an average (or perhaps minimal) inoculum from the

selective enrichment broth would contain about 10^3 *Salmonella* cells per ml. Thus, the minimal inoculum level in the M broth would be approximately 100 *Salmonella* cells per ml. These conditions were approximated by inoculating a constant level of 10^6 non-*Salmonella* cells per ml, varying the numbers of *Salmonella* cells (from 10^6/ml to less than 100/ml) into M broth, and incubating for 18 hr. For comparative purposes, the three broth media described previously were used. After incubation, the modified H-agglutination test was employed for *Salmonella* detection. Both APTMG and M broth afforded *Salmonella* detection at all levels of inoculation (Table 3). At the lowest levels of *Salmonella* inoculation (about 100 cells per ml), the maximum ratio of non-*Salmonella* to *Salmonella* cells was approximately 10,000:1. Although both APTMG and M broth were effective equally, the use of APTMG was negated because of occasional spontaneous agglutination reactions when food samples were examined. Thus, relatively small numbers of *Salmonella* can be detected in the presence of overwhelming numbers of other *Enterobacteriaceae*.

M-broth cultures and reactions with somatic antisera. Some of the M-broth cultures ultimately derived from samples that were naturally contaminated with *Salmonella* were screened by the slide agglutination test with somatic grouping antisera. One drop of the turbid M broth was mixed with one drop of antiserum on a microscope slide, rotated, and observed in the usual

manner. Although extensive testing of this procedure was not attempted, its possible applicability is demonstrated in Table 4. In each instance, the group reactions obtained with M-broth preparation agreed with pure culture reactions obtained by the traditional plating procedures. In each sample, only one serological group of contaminating *Salmonella* was encountered. If multiple 0 groups were present, then corresponding reactions with the respective group antiserum would be expected.

Recommended ES procedure for Salmonella detection. (i) A 10% suspension of the food sample is prepared in sterile Lactose Broth and incubated at 37 C for 18 to 24 hr.

(ii) One milliliter of the preenrichment culture is transferred to 9 ml of Selenite-Cystine Broth and 1 ml to 9 ml of Tetrathionate Broth. The enrichment media are incubated at 37 C for 24 hr.

(iii) M broth is prepared by adding 1.0 g of Tryptone (Difco), 0.5 g of Yeast Extract (Difco), 0.5 g of NaCl, 0.5 g of K_2HPO_4, 0.5 g of sodium citrate, 0.2 g of D-mannose, 2.0 ml of salts C, and 0.75 ml of 10% Tween 80 (Baker) to 100 ml of distilled water. The pH is adjusted to 7.0 with several drops of 4 to 6 N HCl prior to sterilization. A 10-ml amount of the medium is dispensed into screw-cap tubes (16 by 125 mm) and autoclaved at 121 C for 15 min.

Salts C is prepared by dissolving 4.0 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.2 g of $FeSO_4 \cdot 7H_2O$, and 0.72 g of $MnCl_2 \cdot 4H_2O$ in 100 ml of distilled water. The stock solution is stored in a

TABLE 3. *Effect of competing organisms and broth composition on the detection of S. thompson by a modified Spicer-Edwards test*

S thompson/ml	Competing organisms and broth culture media[a]											
	E. coli				P. vulgaris				Citrobacter sp.			
	BHI	APT	APTMG	M	BHI	APT	APTMG	M	BHI	APT	APTMG	M
1,000,000	+[b]	+	+	+	+	+	+	+	+	+	+	+
10,000	+	−	+	+	+	−	+	+	−	−	+	+
5,000	−[c]	−	+	+	+	−	+	+	−	−	+	+
2,000	−	−	+	+	+	−	+	+	−	−	+	+
1,000	−	−	+	+	+	−	+	+	−	−	+	+
200	−	−	+	+	+	−	+	+	−	−	+	+
83	−	−	+	+	+	−	+	+				
62					+	−	+	+				
Negative control[d]	−	−	−	−	−	−	−	−	−	−	−	−
Positive control[e]	+	+	+	+	+	+	+	+	+	+	+	+

[a] Approximately 1,000,000 cells/ml of the competing organism were inoculated into each tube.
[b] Positive modified Spicer-Edwards test.
[c] Negative modified Spicer-Edwards test.
[d] Pure culture of competing organism.
[e] Pure culture of *S. thompson*.

Table 4. *Somatic antigen grouping of salmonellae in M broth*

Product	Somatic antigen group											
	A	B	C₁	C₂	D	E₁	E₂	E₄	F	G	H	I
Rosemary spice..................	−[a]	−	+[b]	−	−	−	−	−	−	−	−	−
Dried milk (samples 1, 4, 5)...............	−	−	−	−	−	−	+	−	−	−	−	−
Dried milk (samples 2, 7)..................	−	−	−	−	−	−	−	−	−	+	−	−
Egg albumen.....................	−	−	+	−	−	−	−	−	−	−	−	−
Milk chocolate (samples 2, 4, 5, 6, 7)..........	−	−	−	−	−	+	−	−	−	−	−	−
Milk chocolate (sample 8).....................	−	−	−	−	−	−	+	−	−	−	−	−
Egg noodles (samples 3, 4, 6)..............	−	−	+	−	−	−	−	−	−	−	−	−
Egg noodles (sample 2).....................	−	−	−	−	+	−	−	−	−	−	−	−

[a] No agglutination.
[b] Agglutination.

refrigerator. A slight precipitate forms upon storage and must be resuspended before being added to the medium.

The 10-ml volume of broth facilitates not only the employment of a drop inoculum from the enrichment broths, but also the subsequent withdrawal of culture for serological tests such that no precipitates are included and actively motile cells are obtained more readily. The citrate in M broth has a dual function in that it chelates the inorganic salts and also affords enrichment of *Salmonella* by serving as an energy source.

In this laboratory, preenrichment cultures are prepared before 3:00 PM each day. On the following (second) morning, enrichment cultures are then inoculated at 8:00 to 9:00 AM. This permits at least an 18-hr incubation of the preenrichment culture. The M broth is inoculated with one drop of enrichment culture at 8:00 to 9:00 AM on the next (third) morning, shaken to distribute the inoculum, tempered, incubated for 6 hr at 37 C, and tested by the modified H-agglutination test. The M-broth cultures may be incubated overnight at 37 C if the modified agglutination test cannot be performed on the same day. In this event, the M broth should be inoculated with one loopful (0.01 ml) of the enrichment culture instead of one drop (0.05 ml). The primary purpose of inoculating the enrichment broths and M broth at 8:00 to 9:00 AM is to facilitate completion of the H-agglutination tests at the end of the day.

(iv) The modified H-agglutination test is performed as follows. To one drop of Formalin-salt solution (3.0 ml of Formalin, 4.2 g of NaCl in 100 ml distilled water) in a Kahn tube (10 by 75 mm) is added 0.85 ml of M-broth culture. It is important not to agitate the M broth prior to removal of the sample, since any precipitates present (e.g., $CaCO_3$ carried over from the Tetrathionate Broth) might be resuspended and cause confusion in the reading of the agglutination test. To this mixture is added 0.1 ml of pooled antisera (*see below*). The Kahn tube is gently shaken and incubated at least 1 hr at 50 C before observing for agglutination. The same criteria are employed as in reading other tube agglutination tests for H antigens in that any visible amount of floccular precipitate is regarded as a positive test for *Salmonella*.

The pooled antiserum is prepared by adding 0.5 ml of each properly rehydrated Difco H antiserum (Spicer-Edwards sera 1, 2, 3, 4, L complex, en complex, 1 complex; and H antisera z_6, poly D, and poly F) to 11.5 ml of 0.85% NaCl. The diluted serum is stored in a refrigerator for a maximum of 5 days before use. Extended storage of the preparation may be possible, but it was not attempted in this study.

It is imperative to temper all media to 37 C before inoculation. This is particularly critical with the M broth, which is usually incubated only 6 hr.

The M-broth culture can also be used to determine the somatic antigens of salmonellae present. One drop of culture is mixed with one drop of antiserum on a glass slide, gently rotated, and observed for agglutination. It will be necessary to agitate the M-broth culture if the cells

have settled out by the time these determinations are done. In this event, any $CaCO_3$ or other precipitates present must be allowed to resettle before a portion of the culture is removed.

Loopfuls of the M-broth culture can also be streaked onto appropriate selective agar medium to isolate the salmonellae in pure culture, if desired.

DISCUSSION

To be totally acceptable, any improved *Salmonella* detection procedure must not only facilitate a decreased time factor but it must also be at least as sensitive and accurate as procedures currently in use. The ES procedure proposed herein fulfills these criteria.

An outstanding merit of the ES procedure is that it can be performed easily at minimal cost. In both the traditional and ES procedures, the sample must be cultured in preenrichment and selective enrichment broths. It is well known that the many subsequent bacteriological manipulations in the traditional procedure are economically undesirable, but a definitive cost estimate is difficult to provide. However, in the ES procedure, an estimate can be made because the antisera constitute the major cost factor. For each H-agglutination test performed the cost of the antisera is approximately 5.5 cents. Thus, the economics associated with the ES procedure are extremely favorable not only in regard to cost of materials used in testing but also in savings associated with reduced laboratory labor and reduced product-warehousing costs.

Some shortcomings of minimal magnitude are inherent in the described ES procedure. Although most of the agglutination tests reported in this study were performed by using pooled Spicer-Edwards antisera, in the later phases the H antigens detectable were expanded by inclusion of additional H antibodies. According to the Kauffmann-White schema (4), 37 of 959 *Salmonella* sp. cannot be detected by the pooled Spicer-Edwards antisera. However, only 3 of the 959 species will not be detected by pooled Spicer-Edwards antisera supplemented with the commercially available H antisera z_6, polyvalent D, and polyvalent F (Difco).

The few samples examined indicated that the supplemented pooled Spicer-Edwards antisera neither aided nor detracted in the incidence of positive samples in comparison to unsupplemented pooled Spicer-Edwards antisera. Although definitive data is not available from this study to justify completely the inclusion of the three additional antisera, it is deemed prudent to obtain the additional coverage.

The three species not detectable by the suggested pooled antisera are *S. agona* (group B), *S. quinhon* (group X), and *S. pullorum-gallinarum* (group D). In the 3-year period, 1965 to 1967, *S. agona* and *S. quinhon* were never isolated from nonhuman sources and only one isolation was made from human sources (5). Moreover, the relative and real incidence of the avian pathogen, *S. pullorum-gallinarum*, appears to be steadily decreasing (Table 5). Thus, the major factor that cannot be evaluated with the statistical data available is the incidence of nonmotile variants. However, even in instances where motility cannot be detected, positive reactions with H antisera have been observed (1). Although it is estimated that these isolates are relatively rare, definitive data regarding their occurrence is lacking.

Previous studies have dealt with the utility of using flagellar antisera for rapid screening methods of pure *Salmonella* cultures. Years ago, Hajna and Damon (3) pioneered H agglutination as a rapid screening method for *Salmonella* detection. More recently, Silliker et al. (8) reaffirmed the usefulness of this procedure. The Food and Drug Administration Laboratories advocate the employment of both a polyvalent H *and* Spicer-Edwards H antisera in their screening procedure (9). However, all of these screening methods embody isolation of colonies on selective agar media (24-hr growth period) followed by biochemical screening in TSI Agar (24 additional hr) and ultimately serological tests employing 6- to 8-hr broth cultures. In essence, the ES procedure herein described obviates the bacteriological manipulations and reduces the time factor by 48 hr. With the accuracy and sensitivity of both procedures being equivalent, the economic benefits are in favor of the ES procedure.

Preliminary studies indicate that the ES procedure can be extended to other types of foods aside from dried foods. Also, it is anticipated that the procedure will have utility in detecting

TABLE 5. *Incidence of S. pullorum-gallinarum from nonhuman sources*[a]

Year	Total S. pullorum-gallinarum isolations	Total nonhuman Salmonella isolations	S. pullorum-gallinarum isolations
			%
1964	270	5461	5.0
1965	237	6864	3.5
1966	80	7709	1.0
1967	54	8794	0.6

[a] Annual *Salmonella* surveillance reports (5).

Salmonella in fecal specimens. These studies will constitute the subject of a subsequent report.

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