Evaluation of a Fluorescent Antibody-Enrichment Serology Combination Procedure for the Detection of Salmonellae in Condiments, Food Products, Food By-Products, and Animal Feeds

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The reliability of the enrichment serology (ES), fluorescent antibody (FA), and a combination of the FA and ES procedures for the detection of salmonellae were compared to the Salmonella cultural procedure outlined in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (BAM). A total of 126 subsamples from 22 different products were analyzed. By utilizing the BAM procedure as the reference standard, a total of 66 samples were positive for salmonellae. Within 44 h approximately 65% of the Salmonella-negative samples could be cleared by the FA test. At the end of 50 h 97% of the Salmonella-negative samples could be cleared by the combination FA-ES test. The FA procedure detected all 66 BAM positives but exhibited a high incidence of presumptive positives which were cultural negatives. The ES procedure detected 64 of the 66 BAM positives but exhibited a low incidence of presumptive positives which were cultural negatives. Incorporating positive FA and positive ES results in a combination FA-ES technique revealed that FA-ES positives were statistically equivalent to BAM positives.

More reliable and rapid test procedures for Salmonella detection would be beneficial to the food industry. The cultural procedure outlined in the Bacteriological Analytical Manual (BAM) specifies a 4- to 6-day time period to test food material for the presence of salmonellae (13). The occurrence of salmonellae contamination in various products from processing plants, however, is generally low (14). The use of rapid sensitive screening procedures are justified as long as the procedure permits proper testing of raw materials, in-process samples, and finished products to assure that Salmonella contamination has not occurred.

Several procedures for the rapid detection of Salmonella have been reported. The rapid procedures utilize enrichment broths of one type or another and proceed to a poly "H" agglutination or a fluorescent-antibody (FA) reaction. One procedure for the rapid determination of Salmonellae was reported by Banwart and Kreitzer (1). This procedure requires 24 to 49 h of testing time and involves the use of a specialized glass apparatus in which carbohydrate fermentation and motility characteristics are used as the basis for Salmonellae detection.

Another accelerated (50 h) detection procedure, referred to as "enrichment serology" (ES), has been reported by Sperber and Deibel (17). This procedure was compared to the cultural procedure outlined in the Bacteriological Analytical Manual by Fantasia et al. (4). This method was as accurate and sensitive as the BAM method as well as being more rapid. However, Sperber and Deibel also reported that three species were not detectable by the suggested pooled "H" antisera. These are S. agona (group B), S. quinhon (group x), and S. pullorum gallinarum (group D). Because the ES procedure depends upon the flagellar antigen which is produced almost exclusively by motile Salmonella, it is most likely that a nonmotile
variant will not be detectable by utilizing the ES procedure alone (17).

The FA technique has also been proposed in many screening procedures for detecting Salmonella organisms in meat (7, 8), eggs (16), nonfat dry milk (14, 15), and other foods (9, 10). The FA procedure has undergone evaluations whereby its inherent problems have been disclosed (6, 8, 9). Thomason reported that, when a direct FA procedure was applied to 304 environmental, food, and feed samples, a level of 3.1% false negatives occurred (18).

An improved FA system utilizing a Zeiss immunofluorescent microscope with a quartz-iodine (Halogen) light source combined with a “Fluoro-kit” (Clinical Sciences, Ind., Whippany, N.J.) has been made commercially available.

This study was designed to compare the ES and FA techniques for detection of salmonellae with the cultural method and to evaluate the combination of ES and FA results as a potential means for eliminating the weaknesses inherent in each of the systems when used alone.

MATERIALS AND METHODS

Subsamples (126) representing 22 different naturally contaminated condiments, food products, food by-products, and animal feeds were used in this investigation. The naturally contaminated samples were obtained from R. H. Deibel, University of Wisconsin, Madison, Wis., and from N. F. Insalata, General Foods Corp., Post Division Research, Battle Creek, Mich. All samples were stored in separate containers at 4 to 5 C to help maintain any existing populations of salmonellae.

The selection of the pre-enrichment broth media was dependent on the type of product or food prototype to be analyzed. All milk products and milk by-products were pre-enriched in sterile distilled water containing 0.002% brilliant green dye and 0.6% tergitol anionic 7 (3, 12, 13). Baker’s yeast samples were pre-enriched in nutrient broth (BBL). All other samples were pre-enriched in lactose broth (BBL) (3, 13). A 10% (wt/vol) ratio of sample material to pre-enrichment broth was maintained, and all pre-enrichment media were tempered to 40 C prior to use. All samples undergoing pre-enrichment were incubated for 18 to 24 h at 35 C. The pH levels of all pre-enriched cultures after 18 to 24 h of incubation were between pH 4.5 and 6.4.

Samples (1 ml) of each pre-enrichment culture were inoculated into separate tubes containing 9.0 ml of 1.1 strength selenite cystine (BBL) and tetraethionate broths (BBL) (17) tempered at 40 C. The selectivity of the tetraethionate broth was enhanced with a final concentration of 0.002% brilliant green dye (12, 17). All samples of pre-enriched cultures inoculated into selective enrichment media were incubated at 35 C for 22 to 26 h.

FA procedure. The “CSI, Fluoro-kit for Salmonella screening” (Clinical Sciences, Inc.) was utilized. Salmonella “OH” somatic antisera conjugate A through S (prepared by CSI, Inc.) and FA Salmonella poly antisera conjugate A through S, including 0 factors 1 through 25, 27, 28, 30, 34 through 41, 45, and Vi (Difco), were tested on formalinized (0.15% final concentration of formalinized saline) 0.001-ml samples of each selective enrichment broth culture. Formalin was used as a safety factor for protection of laboratory personnel. Slides were prepared, fixed, rinsed, stained, rinsed, washed twice in phosphate buffer, rinsed, dried, and mounted with a cover slip in complete compliance with the instructions supplied by the manufacturer (Clinical Sciences Inc., Whippany, N.J.). All wells of the Clinislide were examined with the use of a Zeiss immunofluorescent microscope equipped with a 12-V, 100-W halogen lamp, ×40 and ×100 oil immersion Planapo and Plan iris diaphragm objectives, respectively, a 490-nm interference FITC filter, a KG1 heat absorption filter, a BG 38 red suppressing filter, a Zeiss no. 53 barrier filter, a dark-field ultracondenser, and ×8 narrow-angle eyepieces.

ES procedure. Tubes containing 10 ml of M broth (Difco) were inoculated with 0.05-ml samples of each selective enrichment broth culture. The tubes containing M broth were tempered at 40 C prior to use. All inoculated M broths were incubated at 35 C for 7 h. After 7 h of incubation, H-agglutination tests were performed on all M broth cultures by the procedure outlined by Sperber and Deibel (17). Nonspecific agglutination tests (ES controls) were also performed on all M broth cultures by using 0.1 ml of physiological saline in place of the 0.1 ml of poly H antisera for the H-agglutination procedure.

Cultural procedure. The salmonellae cultural procedure outlined in the U.S. Food and Drug Administration’s Bacteriological Analytical Manual was utilized with minor modifications (13). Differential selective plates of brilliant green agar (BBL), bismuth sulfite agar (BBL) aged 3 days at 4 to 5 C, and salmonella-shigella agar with additions of 1% sucrose and 0.65% agar (17) were streak-inoculated with one loopful (0.01 ml) of each selective enrichment culture. The streaked differential selective agar plates were incubated at 35 C for 24 to 48 h. After 24 h of incubation elapsed, the plates were examined for suspect salmonellae colonies. Negative plates were reincubated at 35 C for an additional 24 h and subsequently re-examined for suspect salmonellae colonies. Several colonies which were considered suspect on any given plate after 24 and 48 h of incubation were picked and inoculated into and onto triple sugar iron (TSI) agar slants and lysine iron agar (LIA) slants (2, 5). Inoculated slants of TSI and LIA were incubated at 35 C for 18 to 24 h. Cultures which exhibited typical Salmonella-positive reactions with TSI or LIA were transferred to brain-heart infusion (BHI) broth (BBL) and incubated at 35 C for 6 to 8 h. Flagellar (H) antigen tests were performed by using the poly H antiseria pool reported by Sperber and Deibel (17) for the ES test, plus Z, which was added to detect S. quinshin.

Analyses were performed on the same sample by using the FA, ES, and combination FA and ES procedures. Applying the BAM procedure as a refer-
ence standard, FA positives and negatives, ES positives and negatives, as well as FA and ES combination test positives and negatives were determined. Various statistical tests were applied to the data to ascertain the reliability of the FA and ES procedures as compared to BAM procedure for detecting salmonellae.

RESULTS AND DISCUSSION

Responses to the various detection procedures for a total of 126 samples, representing 22 types of finished product, are shown in Table 1. The BAM procedure detected 66 Salmonella-positive samples. The same 66 positive samples were detected by the FA procedure. This is in agreement with results which would be expected from publications of Georgala and Boothroyd (7, 8), Geopfert and Insalata (6), Harrington et al. (9), Insalata et al. (10), Silliker et al. (16), and Thomason (18). These investigators reported that the problem of the FA method lies in its tendency to indicate the presence of salmonellae in sample which are cultural negatives.

The ES procedure detected 64 of the 66 positive samples after interacting the results from both selective enrichment broths and considering any positive response to be Salmonella. ES from selenite cystine broth gave higher numbers of false negatives than ES from tetrathionate broth. If it were not for one egg noodle product in which the tetrathionate gave 8 out of 10 false negatives, the decision to drop the use of selenite completely might have been made. The apparent anomalous result demonstrates the need to recommend the use of both selenite and tetrathionate and to read the ES test as positive if either broth yields a positive response. The two salmonella-positive samples missed by the ES procedure in this investigation were both lactalbumin. In both samples the salmonellae appeared to be outgrown by a competing organism in the natural microflora of the lactalbumin.

The remaining 60 of the 126 samples which were analyzed for the presence of salmonellae were negative by the BAM procedure. The FA fluoro-kit with CSI FA Salmonella conjugate sera yielded 37 negatives and 23 presumptive positives corresponding to the 60 BAM negatives. When Difco FA Salmonella poly sera was used in place of the CSI conjugate with the FA fluoro-kit procedure, 46 negatives and 14 presumptive positives which were cultural negatives were detected. One explanation for this result could be that the FA procedure is more sensitive than the BAM procedure and consequently detects positives that the cultural procedure has not detected. This would appear as false alarms in the test data, since the BAM procedure was chosen as the standard and

| Product                  | No. of samples | Sample size (g) | ES without control | ES with control | FA (CSI sera) | FA (Difco sera) | BAM |
|--------------------------|----------------|-----------------|--------------------|-----------------|---------------|-----------------|-----|
| Baker's yeast            | 6              | 100             | + - @ &           | + - @ &         | + - @ &       | + - @ &         | @  |
| No. 1 egg noodles       | 10             | 10              | 2 4 0 0          | 2 4 0 0         | 2 1 3 0       | 2 4 0 0         | 2 4 |
| Black pepper             | 10             | 10              | 7 3 0 0          | 7 3 0 0         | 7 3 0 0       | 7 3 0 0         | 7 3 |
| Skim milk powder         | 6              | 100             | 6 0 0 0          | 6 0 0 0         | 6 0 0 0       | 6 0 0 0         | 6 0 |
| Mixed egg noodles        | 10             | 10              | 10 0 0 0         | 10 0 0 0        | 10 0 0 0      | 10 0 0 0        | 10 0|
| No. 3 egg noodles        | 10             | 10              | 10 0 0 0         | 10 0 0 0        | 10 0 0 0      | 10 0 0 0        | 10 0|
| Powdered egg yolk        | 8              | 100             | 0 7 1 0          | 0 7 1 0         | 0 7 1 0       | 0 7 1 0         | 0 7 |
| Wheat flour              | 10             | 25              | 0 10 0 0         | 0 10 0 0        | 0 10 0 0      | 0 10 0 0        | 10 |
| Dry pet food             | 3              | 100             | 0 3 0 0          | 0 3 0 0         | 0 1 2 0       | 0 1 2 0         | 0 3 |
| Orange meal              | 3              | 10              | 1 2 0 0          | 1 2 0 0         | 1 2 0 0       | 1 2 0 0         | 1 2 |
| Canada meat meal         | 3              | 10              | 2 1 0 0          | 2 1 0 0         | 2 1 0 0       | 2 1 0 0         | 2 1 |
| Bone meal                | 3              | 10              | 1 2 0 0          | 1 2 0 0         | 1 2 0 0       | 1 2 0 0         | 1 2 |
| Multi meal               | 5              | 10              | 0 2 3 0          | 0 2 3 0         | 0 2 3 0       | 0 2 3 0         | 0 3 |
| Black meat meal          | 3              | 10              | 2 0 1 0          | 2 0 1 0         | 2 0 1 0       | 2 0 1 0         | 2 1 |
| Washington bone meal     | 3              | 10              | 3 0 0 0          | 3 0 0 0         | 3 0 0 0       | 3 0 0 0         | 3 0 |
| Brown meat meal          | 5              | 10              | 1 4 0 0          | 1 4 0 0         | 1 4 0 0       | 1 4 0 0         | 1 4 |
| Tan meat meal            | 5              | 10              | 1 4 0 0          | 1 4 0 0         | 1 4 0 0       | 1 4 0 0         | 1 4 |
| SR meat meal             | 3              | 10              | 3 0 0 0          | 3 0 0 0         | 3 0 0 0       | 3 0 0 0         | 3 0 |
| Dry beef                 | 5              | 10              | 0 5 0 0          | 0 5 0 0         | 0 5 0 0       | 0 5 0 0         | 0 5 |
| Red meal                 | 5              | 10              | 1 3 1 0          | 1 3 1 0         | 1 3 1 0       | 1 3 1 0         | 1 4 |
| Soya flour               | 5              | 10              | 1 4 0 0          | 1 4 0 0         | 1 4 0 0       | 1 4 0 0         | 1 4 |
| Lactalbumin              | 5              | 10              | 3 0 0 0          | 3 0 0 0         | 3 0 0 0       | 3 0 0 0         | 5 0 |

* Procedural responses: +, positive; -, negative; @, false positive; @, false negative.
positives not detected by this procedure are classified as false alarms.

When results of the ES procedure without nonspecific agglutination controls were compared with the 60 BAM negatives, there were 54 samples in agreement and 6 presumptive positives. When the nonspecific agglutination controls were included, the response improved to 59 negatives and only 1 presumptive positive in comparison to the 60 cultural negatives.

A summary of the data obtained from the materials analyzed for salmonellae by the BAM, ES, and FA procedures is shown in Table 2. The decision strategy applied to the ES and FA reaction columns (Rₙ) shown in Table 2 incorporated results obtained from both selenite cystine selective enrichments and tetrathionate selective enrichments. A positive response which occurred in any one of the selective enrichments was read as an overall positive reaction. For a negative reaction, both the selective enrichments must show negative responses. A control reaction was applied to the serological procedures of the ES test to negate positive nonspecific agglutination reactions. If the serological test of the ES procedure yielded a positive response and the serological control did not show nonspecific agglutination, this was recorded as a positive serological response for salmonellae.

Applying the statistical chi-square test to the data indicates that the 44-h FA and 50-h ES test procedures are adequate in detecting BAM positives. All BAM-positive samples were FA positive. The BAM and ES results were not identical, but, with 95% confidence, there was no significant difference in their ability to detect Salmonella.

The ES test procedure without nonspecific agglutination control reactions was significantly different at the 95% confidence level from the BAM procedure in detecting negatives. However, no significant differences were found (at the 95% confidence level) between the ES test procedure with nonspecific agglutination control reactions and the BAM procedure in detecting negatives. Running the ES test procedure with nonspecific agglutination control reactions materially reduced the ES false-alarm probability without reducing the probability of detecting BAM positives.

There was a significant difference (at a 95% confidence level) between the BAM procedure and the FA procedure using either CSI or Difco FA Salmonella sera conjugate in detecting negatives. No significant differences were found

| TABLE 2. Data summary of the ES, FA, and BAM procedures for detection of Salmonella |
|-----------------------------------------------|
| Reaction                                      |
| ES Without control | ES With control | FA CSI | FA Difco | BAM |
| SEL  | TET  | Rₙ | SEL  | TET  | Rₙ | SEL  | TET  | Rₙ | SEL  | TET  | Rₙ | SEL  | TET  | Rₙ | SEL  | TET  | Rₙ | SEL  | TET  | Rₙ |
| Positive, given BAM positive                  |
| 53   | 54   | 64 | 53   | 54   | 64 | 66   | 66   | 66 | 66   | 66   | 66 |
| False negative, given BAM positive            |
| 13   | 12   | 2  | 13   | 12   | 2  | 0    | 0    | 0  | 0    | 0    | 0  |
| Negative, given BAM negative                  |
| 56   | 56   | 54 | 59   | 60   | 59 | 45   | 42   | 37 | 52   | 50   | 46 |
| False positive, given BAM negative            |
| 4    | 5    | 6  | 1    | 0    | 1  | 15   | 18   | 23 | 8    | 10   | 14 |

* Abbreviations: SEL, selenite cystine selective enrichment; TET, tetrathionate selective enrichment; Rₙ, combined reaction. Any positive response makes result positive.

| TABLE 3. BAM reactions and data summary of combination procedures |
|-------------------------------------------------------------------|
| Reaction | BAM | FAₐ-ES-1* | FAₐ-ES-2* | FAₑ-ES-1* | FAₑ-ES-2* |
| Positive, given BAM positive | 66 | 64 | 64 | 64 | 64 |
| False negative, given BAM positive | 0 | 2 | 2 | 2 | 2 |
| Negative, given BAM negative | 60 | 59 | 60 | 58 | 60 |
| False positive, given BAM negative | 0 | 1 | 0 | 2 | 0 |

* FAₐ-ES-1, FA with CSI sera combined with the ES test without nonspecific agglutination controls.
* FAₐ-ES-2, FA with CSI sera combined with the ES test with nonspecific agglutination controls.
* FAₑ-ES-1, FA with Difco sera combined with the ES test without nonspecific agglutination controls.
* FAₑ-ES-2, FA with Difco sera combined with the ES test with nonspecific agglutination controls.
(at a 95% confidence level) between CSI or Difco FA Salmonella sera conjugate in detecting negatives.

The combination of FA and ES procedural results for the detection of salmonellae was also compared to results obtained by utilizing the Salmonella cultural procedure outlined in the U. S. Food and Drug Administration's Bacteriological Analytical Manual. The FA procedure using CSI sera combined with the ES test with and without nonspecific agglutination controls (FAc-ES-1 and FAd-ES-2, respectively), and the FA procedure using Difco sera combined with the ES test with and without nonspecific agglutination controls (FAd-ES-1 and FAd-ES-2, respectively) represent the possible combinations observed in this investigation. A summary of the data obtained by combining the FA and ES procedures is shown in Table 3.

The FAc-ES-1, FAc-ES-2, FAd-ES-1, and FAd-ES-2 combinations each exhibited two missed detections. However, the differences were not statistically significant. Specifically, no significant difference was found (at a 95% confidence level) between FA-ES combinations and the BAM procedure in detecting positives. This investigation revealed that, whenever presumptive positive results were obtained with the FA and ES procedures, the BAM procedure also revealed positive confirmation for the presence of salmonellae. Incorporating positive FA and ES results in a combination FA-ES technique revealed that the FA-ES positives were equivalent to BAM positives.

The FAc-ES-1 combination exhibited one false alarm. No significant difference was found (at a 95% confidence level) between FAc-ES-1 and the BAM procedure in detecting negatives. The FAc-ES-1 combination exhibited two false alarms. The differences were not statistically significant.

No significant difference was found (at a 95% confidence level) between the FAc-ES-1 combination or the FAd-ES-2 combination and traditional cultural procedure in detecting negatives. Results are arithmetically identical. The FA-ES-2 combination technique detected all 60 BAM negatives.

![Analytical scheme for Salmonella](http://aem.asm.org/Downloaded from http://aem.asm.org/on May 8, 2020 by guest)
Combining FA and ES results yielded greater accuracy than when either of the two rapid screening procedures was used alone. The suggested analytical scheme is shown in Fig. 1. In use, the 44-h FA results would be obtained first. If FA readings are negative, the sample is negative at this point and no further testing would be required. If the FA readings are positive, completion of the 50-h ES test along with nonspecific agglutination controls is necessary. If the ES test also yields a positive reaction and a negative nonspecific agglutination control reaction, the sample is deemed positive and no further testing would be required. If the ES test yields a negative reaction and the control remains negative, or if the ES test yields a positive reaction and the control indicates nonspecific agglutination (a nonspecific agglutination negates the ES test), then, with at least 92.7% probability (at the 95% confidence level), the sample may be considered negative. Since there is a high probability of the sample being negative, it is suggested that product from a manufacturing operation be released but held under recall control while the traditional cultural procedure (BAM) is being completed. The factors which may cause the apparent negative when the sample is truly positive would be the presence of nonmotile salmonellae or suppression of salmonella growth by competitive microorganisms, both of which situations would interfere with or preclude a positive ES response.

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