Rapid, Direct Fluorescent-Antibody Method for the Detection of Salmonellae in Food and Feeds

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Received for publication 11 July 1972

An improved immunofluorescent-antibody (FA) method for the detection of salmonellae in foods and feeds was developed. This FA method combines a rapid cultural phase and a serological phase that allow for propagation of salmonellae in a minimum time, employing the industrial 8-hr work day as a guide. Two hundred fifty naturally contaminated human food and animal feed samples, representing 647 trials, were tested by the FA method. A total of 18 different food and feed samples was used. The method used by the Association of Official Analytical Chemists (AOAC) for the detection of salmonellae was the control method. The percent agreement when comparing the FA slide method to the AOAC method ranged from 87.1 to 96.3%, depending upon the conjugated antisera used in comparative studies.

The need for a faster method for the detection of salmonellae led to the development of the fluorescent-antibody (FA) technique. The FA technique can serve as a useful tool in screening raw materials, environmental samples, and finished products (1-4, 6-9, 11-14). Many organizations now testing for salmonellae employ the time-consuming (5-7 days) and laborious method of the Association of Official Analytical Chemists (AOAC).

The objectives of this study were segmented into three phases. The first phase dealt with the development of a cultural method designed to enhance the rate of growth of low numbers of salmonellae and suppress the growth of organisms other than salmonellae. The following three-step cultural method was designed to satisfy these criteria: (i) a 7-hr pre-enrichment, (ii) a 17-hr selective enrichment, and (iii) a 5-hr modified M broth (Difco) elective enrichment.

The second phase of the method was the development of a staining procedure which enhanced the fluorescence of the stained salmonella cell while retaining the maximum number of cells and flagella on the microscope slide. To facilitate reading the slide, the staining procedure was also designed to minimize background fluorescence.

The third phase consisted of designing a serological scheme for the preparation of a commercially available conjugated salmonellae antisera containing all 79 somatic and flagellar antigens for the 31 salmonellae serotypes identified in food poisoning in the United States for the years 1965 to 1970 (15).

MATERIALS AND METHODS

FA antisera. With the assistance of the Center for Disease Control and the approval of the Food and Drug Administration, we designed the coverage of the conjugated antisera necessary for the task (Table 1). As a professional courtesy, the Difco Corp. of Detroit, Mich., prepared and submitted two individual pools of conjugated salmonellae antisera conforming to the scheme. The first pool of conjugated antisera (Difco O) contained antibodies to the somatic factors of the 31 serotypes. The second pool (Difco H) contained antibodies to the 48 flagellar antigens. These antisera were rehydrated by adding 5 ml of sterile distilled water, as directed by the manufacturer. Quantities of 0.5 ml were dispensed into sterile vials and frozen until used. Before staining, the "O" and "H" antisera were thawed at room temperature and diluted to a working dilution of 1:8 with pH 7.5 phosphate-buffered saline. A combination of "O" and "H" antisera (O + H) was prepared in this laboratory at a dilution of 1:16 for additional evaluation. This was later supplied by Difco and known as FA Salmonella Poly.

The Sylvana Corp. of Millburn, N.J., submitted a conjugated antisera having the same flagellar and somatic antigen coverage as Table 1, with the exception of the Vi antigen. This antisera was tested at a 1:8 dilution.

Another salmonella-conjugated antisera was received for evaluation, as a professional courtesy, from E. M. Ellis of the National Animal Disease Center. The antisera was kept under refrigeration and used at a 1:1 dilution.
Table 1. Scheme of the antigens needed in the conjugated salmonellae antiserum

| O group | Strain          | O antigens | H antigens | Phase I | Phase II | Phase needed of H antigens |
|---------|-----------------|------------|------------|---------|----------|---------------------------|
| A       | S. paratyphi A  | 1,2,12     | a          |         |          | I                         |
| B       | S. typhimurium  | 1,4,5,12   | i          | 1,2     |          | I and II                  |
| B       | S. gloucester   | 1,4,12,27  | i          | 1,w     |          | II                        |
| C,      | S. cholera suis var. kunzendorf | 6,7 | (c) | 1,5 | | I |
| C,      | S. newport      | 6,8        | e,h        | 1,2     |          | I                         |
| C,      | S. tallahassee  | 6,8        | Z4,Z32     |          |          | I                         |
| D       | S. kentucky     | (8)20      | i          | Z6      |          | II                        |
| D       | S. typhi 2V     | 9,12,V1    | d          |         |          | I                         |
| D       | S. rostock     | 1,9,12     | g,p,u      |         |          | I                         |
| D       | S. berta       | 9,12       | f,g,t      |         |          | I                         |
| D       | S. fresno      | (9)46      | Z38        |         |          | I                         |
| E,      | S. london      | 3,10       | 1,v        | 1,6     |          | II                        |
| E,      | S. binza       | 3,15       | y          | 1,5     |          | I                         |
| E,      | S. illinois    | (3),(15),34| Z10        | 1,5     |          | I                         |
| E,      | S. simsbury    | 1,3,19     | —          | Z27     |          | I                         |
| F       | S. rubislaw    | 11         | r          | e,n,x   |          | I                         |
| G       | S. poona var. 37 | 1,13,22,36,37 | Z       | 1,6     |          | I                         |
| G       | S. cubana      | 1,13,23    | Z29        |         |          | I                         |
| H       | S. carrau      | 6,14,24    | y          |         |          | I                         |
| H       | S. florida     | (1),6,14,25| d          | 1,7     |          | II                        |
| I       | S. woodstock   | 16         | Z42        | 1,(5),7 |          | I                         |
| I       | S. michigan    | 17         | 1,v        | 1,5     |          | II                        |
| I       | S. cerro       | 18         | Z4,Z23     |         |          | —                         |
| I       | S. gambara     | 21         | Z35        | e,n,Z15 |          | I and II                  |
| I       | S. aderike     | 28         | Z38        |         |          | I                         |
| I       | S. morocco     | 30         | 1,Z13,Z28  | e,n,Z15 |          | I                         |
| I       | S. ealing      | 35         | g,m,s      |         |          | I                         |
| I       | S. lansing     | 38         | i          | 1,5     |          | II                        |
| I       | S. champagn    | 39         | k          | 1,5     |          | I                         |
| I       | S. johannesburg | 1,40       | b          | e,n,x   |          | I and II                  |
| I       | S. ipswich     | 41         | Z4,Z24     |         |          | —                         |

Samples. The 250 naturally contaminated samples tested in this study were supplied by the Food and Drug Administration. These included 116 human foods and 134 animal feeds. Table 2 lists the types of samples tested and their most probable number values.

Sampling procedure. The samples were aseptically divided into 1,000-g quantities, placed into presterilized, 1-gal receptacles, and sealed. The test material was mixed to achieve homogeneity by tumbling end-over-end 100 times. A 25-g test sample was retrieved.

Pre-enrichment phase. A 25-g sample was placed in 225 ml of preheated (35 ± 2 C) modified M broth. The modified M broth was composed of tryptone (Difco), 10 g; yeast extract (Difco), 5 g; glucose, 2 g; sodium citrate, 5 g; sodium chloride, 5 g; dipotassium phosphate, 5 g; manganese chloride, 0.14 g; magnesium sulfate, 0.8 g; ferrous sulfate, 0.04 g; Tween 80, 0.75 g; and distilled water, 1 liter. The pH was adjusted to 7.0 with 4 to 6 N HCl prior to autoclaving for 15 min at 15 psi. The pH of the modified M broth plus sample was adjusted to 6.8 to 7.2 with 5 N NaOH when necessary. Blending of samples was performed when necessary to obtain a homogeneous suspension. The pre-enrichments were incubated in a shaker water bath (ca. 34 oscillations/min) for 7 hr at 35 ± 2 C.

Selective enrichment phase. After incubation, the pre-enrichment broth was removed from the water bath, and the particulate matter was allowed to settle for 5 min. Fifty milliliters was withdrawn from the top one-third layer of the culture and transferred to 450 ml (35 ± 2 C) of selenite-F broth (BBL). The selenite-F culture was incubated at 35 ± 2 C for 17 hr in the shaker water bath.

AOAC cultural method. The remaining portion of the pre-enrichment culture was placed in a still-air incubator for an additional 17 hr at 35 ± 2 C for simultaneous evaluation by the AOAC method (10).

Elective enrichment phase. After incubation, the selenite-F broth culture was removed from the water bath and allowed to stand for 5 min. Two milliliters was withdrawn from the top third of the culture and transferred to 18 ml (35 ± 2 C) of modified M broth. The pH of the elective modified M broth and sample was adjusted to 6.8 to 7.2 with 5 N NaOH prior to incubation for 5 hr in a water bath at 35 ± 2 C.

FA slide preparation. Nonfluorescing glass slides were washed with sudsol soap and rinsed in...
distilled water. Slides were soaked for 5 min in chromic acid, rinsed in distilled water, and air dried. A drop of glycerin was placed on each etched circle and immediately sprayed with Teflon. The slides were rinsed in tap water to remove the glycerin and placed in 0.5% NaOH for 5 min, followed by distilled-water rinses and air drying. The etched circles were coated with 0.3% purified agar using a water color brush, and the slides were dried at 55°C. The slides are stable in this form indefinitely, when stored in a dust-free environment. Duplicate smears were made on the prepared slides by withdrawing two 3-mm loopsful from the top one-third of the elective enrichment. The smears were dried on a slide warmer at 35 ± 2°C.

**FA staining procedure.** The dried smears were fixed for 3 min in modified Kirkpatrick ethyl alcohol-chloroform-Formalin (60:30:10) solution, followed by immersion in ethyl alcohol (95%) for 1 min, and then forced-air dried. The slides were placed in a staining chamber containing moistened filter paper, to prevent the stain from drying on the smear. The smears were stained by placing two drops of antiserum on each smear, allowing 30 min at 35 ± 2°C for the reaction to take place. The slides were then rinsed with phosphate-buffered saline (pH 7.5) followed by two 5-min soaks in phosphate-buffered saline (pH 7.5), 1 min in distilled water, 1 min in ethyl alcohol (95%), and finally air dried. All baths used in the staining procedure were changed daily.

All stained smears received one drop of mounting fluid (5 ml of Difco FA mounting fluid, 0.5 ml of carbonate buffer, pH adjusted to 9.2 with 5 N NaOH), a no. 1 cover slip, and one drop of Cargille, type A immersion oil.

**Microscopy examinations.** FA-stained smears were viewed on a Wild Heerburg microscope equipped with an Osram HBO-200 mercury arc burner, 50× and 100× immersion flourite objectives; BG-38 heat-absorbing filter; a BG-12 blue pass filter and an OG-1 blue absorbing eyepiece filter; a dark-field condenser and 15× wide-angle eyepieces. Dark-field microscopy was used to distinguish bacteria from debris.

In this work, the criteria for interpreting the degree of fluorescence of a smear were subjectively defined as follows: 0, no visibly fluorescing rods; 1+, faintly fluorescing rods without discernible lumen; 2+, faintly fluorescing rods with discernible lumen; 3+, strongly fluorescing rods with discernible lumen; and 4+, intensely fluorescing rods with discernible lumen.

The criteria for a positive reaction, as viewed on a prepared microscope slide, are (i) typical salmonella morphology, with or without attached flagella under darkfield and ultraviolet light and (ii) cells yielding a 3+ or 4+ degree of fluorescence under ultraviolet light.

**FA cultural.** After the smears were prepared from the modified M-broth elective enrichment, a cultural confirmation in addition to the AOAC procedure was performed by streaking a 3-mm loopful on selective media plates, as outlined in the AOAC method. The elective enrichment broth was then held under refrigeration. If the streak plates failed to detect salmonellae, 1 ml of the modified M broth was

| Table 2. Most-probable number of Salmonellae per gram in samples tested by the FA method and AOAC method |
|------------------------------------------------------------------------------------------------|
| **Samples** | MPN salmonellae per g | **No. of samples tested** |
|----------------|---------------------|-------------------------|
| Human food |                       |                         |
| Chicken (frozen) | 18.0               | 14                      |
| Distillers product | 0.45               | 8                       |
| Dried yeast       | 0.0091              | 16                      |
| Frog legs         | 0.36                | 8                       |
| Soya flour        | 0.20                | 20                      |
| Soya meal         | 0.20                | 14                      |
| Chocolate chunks  | NA                  | 16                      |
| Chocolate alba    | NA                  | 12                      |
| Rice              | NA                  | 8                       |
| Animal feed       |                     |                         |
| Black meat meal   | 0.45                | 20                      |
| Bone meal 1       | 0.2                 | 8                       |
| Bone meal 2       | 4.2                 | 8                       |
| Bone meal 42      | 4.6                 | 13                      |
| Canada meat meal  | 0.20                | 16                      |
| Meat meal         | 11.0                | 21                      |
| Washington fish meal | 0.0036            | 24                      |
| Brown meat meal   | 0.2                 | 8                       |
| Tan meat meal     | 0.2                 | 16                      |

* Total human food samples tested, 116; total animal feed samples, 134.

**RESULTS AND DISCUSSION**

Tables 3 and 4 compare the FA slide results, FA cultural results, and the standard AOAC method results. Table 3 reports the results obtained on all of the samples tested with each antiserum. For the small number of samples tested, the separate Difco “O” and Difco “H” antisera gave high percent recoveries, with only 3 slide false positives each. The Ellis antiserum, also tested on a relatively low number of samples, yielded 95.3% agreement with only 5 slide false positives. The Sylvania antiserum was tested 209 times, resulting in 87.1% agreement, 24 slide false positives, and 3 slide false negatives. The Difco “O + H” antiserum was tested a total of 250 times for 90.8% agreement, 17 slide false positives, and 6 slide false negatives. The slide false positives and false negatives with the Difco “O + H”
yielded smear tive, cultural M-broth elective enrichment Difco currently; or forming 648 Difco AOAC conjugated Ellis 54 Difco antiserum may be tested on each antiserum. Not antiserum. The antiserum yielded a high 95.4% agreement with only 3 slide false positives. The Difco "O + H" antiserum yielded 90.8% agreement with 6 slide false positives. The Sylvania antiserum yielded 86.2% agreement with 9 slide false positives. No

Table 3. FA slide results using each antiserum on all samples tested

| Antiserum used on FA slide | No. of FA slide trials in agreement with cultural confirmation | FA slide false positives | FA slide false negatives | No. of trials with each antiserum | Percent agreement | Percent slide false positives | Percent slide false negatives |
|---------------------------|---------------------------------------------------------------|--------------------------|------------------------|----------------------------------|------------------|-----------------------------|-----------------------------|
| Difco O                   | + + ++ | 25 | 11 | 2 | 0 | 3 | 0 | 0 | 0 | 0 | 41 | 38/41, 92.7% | 3/41, 7.3% | 0/41, 0% |
| Difco H                   | + + ++ | 25 | 11 | 2 | 0 | 3 | 0 | 0 | 0 | 0 | 41 | 38/41, 92.7% | 3/41, 7.3% | 0/41, 0% |
| Difco "O + H"            | + + ++ | 102 | 108 | 16 | 1 | 17 | 0 | 0 | 6 | 250 | 227/250, 89.8% | 17/250, 6.8% | 6/250, 2.4% |
| Sylvania                  | + + ++ | 77 | 87 | 17 | 1 | 24 | 0 | 0 | 3 | 21.9 | 182/209, 87.1% | 24/209, 11.5% | 3/209, 1.4% |
| Ellis                     | + + ++ | 54 | 42 | 5 | 0 | 5 | 0 | 0 | 0 | 106 | 101/106, 95.3% | 5/106, 4.7% | 0/106, 0% |

Abbreviations: AOAC+, any sample producing an isolate that exhibited cultural, biochemical, and unconjugated serological reactions typical for salmonellae in accordance with the AOAC published method; AOAC−, any sample confirmed as salmonellae-negative following AOAC published methodology; FAC+ (FA cultural positive), any modified M-broth elective enrichment (from which the slides are made) confirmed as containing salmonellae positive following AOAC published methodology; FAC− (FA cultural negative), any modified M-broth elective enrichment negative for salmonellae following AOAC published methodology; Slide+, any smear showing rods (i) proper morphology (with or without attached flagella) under darkfield and ultraviolet light, and (ii) a 3+ or 4+ degree of fluorescence under ultraviolet light; Slide−, any smear not conforming to the above; FA slide agreement, occurs when (i) a positive slide is confirmed by an AOAC positive or FAC positive; and (ii) when the FA slide and AOAC and FAC cultural methods are negative concurrently; FA slide false positive, any slide positive which cannot be culturally confirmed; FA slide false negative, any instance in which the slide was negative and the AOAC culture or FA cultural method, or both, yielded salmonellae.

Table 4. FA results of each antiserum when tested using the same samples

| Antiserum used on FA slide | No. of FA slide trials in agreement with cultural confirmation | FA slide false positives | FA slide false negatives | No. of trials with each antiserum | Percent agreement | Percent slide false positives | Percent slide false negatives |
|---------------------------|---------------------------------------------------------------|--------------------------|------------------------|----------------------------------|------------------|-----------------------------|-----------------------------|
| Difco "O + H"            | + + ++ | 29 | 27 | 3 | 0 | 6 | 0 | 0 | 0 | 65 | 59/65, 90.8% | 9/65, 9.2% | 0/65, 0% |
| Sylvania                  | + + ++ | 29 | 24 | 3 | 0 | 9 | 0 | 0 | 0 | 65 | 56/65, 86.2% | 9/65, 14.8% | 0/65, 0% |
| Ellis                     | + + ++ | 29 | 30 | 3 | 0 | 3 | 0 | 0 | 0 | 65 | 62/65, 95.4% | 3/65, 4.6% | 0/65, 0% |

See footnote to Table 3.

antiserum may be due to the 1:16 dilution of the antiserum. Because all of the antisera were not tested an equal number of times on all the samples, the above percentage agreements for each antiserum from Table 3 cannot be directly compared.

Therefore, Table 4 offers the results of the three antisera tested the most extensively and reports only the results obtained with the antisera when tested on the same 65 samples concurrently. The Ellis antiserum yielded a high 95.4% agreement with only 3 slide false positives. The Difco "O + H" antiserum yielded 90.8% agreement with 6 slide false positives. The Sylvania antiserum yielded 86.2% agreement with 9 slide false positives. No
false-negatives were obtained with any of the antisera on these 65 samples.

This study helped to demonstrate that the FA technique may be successfully used in rapid screening for salmonellae in foods and feeds. The advantages of the application of the FA method are: (i) 32-hr elapsed time, (ii) increased sensitivity over the cultural method through the detection of low numbers of salmonellae, (iii) increased specificity through the use of a designed antiserum, (iv) an economic saving by releasing product sooner, thereby freeing expensive storage space, (v) the ability to rapidly determine the effectiveness of sanitation procedures in industrial processes, and (vi) a shorter testing time in which more samples can be checked, thus giving greater protection to the consumer. With at least two commercial sources of conjugated salmonellae sera for use in the direct procedure, the application of the FA technique may increase in industry.

There are, however, several aspects of the procedure that require further development. These include: (i) increasing the specificity and sensitivity of the antiserum, (ii) cultural improvement, and (iii) microscopy definition in the diagnostic phase, to minimize subjective interpretations. The somatic antigens of the salmonellae are shared, to some extent, with other Enterobacteriaceae. Concomitant microflora-possessing antigens common to salmonellae may produce FA slide false positives in some sample types. Intensification of the selective and elective enrichment phases to stimulate salmonellae growth with the suppression of nonsalmonellae may be required to reduce the number of false positives. Also, the number of false positives can be minimized by use of higher dilutions of the antiserum.

The sensitivity and selectivity of the FA method may vary, as slide false negative results seem to occur more frequently in various specific sample types. These false-negatives may be due, in part, to the degree of background fluorescence of some foods which tend to mask the fluorescence of the salmonellae cells.

A uniform nomenclature and criteria for the microscopy detection of salmonellae should be established. The relationships between the number of cells and the culturally positive samples should also be established.

We hope to continue our efforts in studying the advantages of the combination or the integration, or both, of microbiological samples for use as a broad-base screening technique, fortified by ultimate confirmation with the standard AOAC method.

ACKNOWLEDGMENTS

We express our sincere thanks to E. M. Bunner of the Difco Corp. for gratuitously producing and supplying a conjugated antiserum, and acknowledge the efforts of the Sylvana Co., Millburn, N.J., for their cooperation. It is a pleasure to acknowledge the helpful discussions and technical assistance of B. M. Thomason of the Center for Disease Control and of J. M. Goepfert of the Food Research Institute. Thest studies were supported by the U.S. Food and Drug Administration through contract CPF 69-33. We are grateful for their support.

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