Evaluation of an Automated Fluorescent Antibody Procedure for Detection of \textit{Salmonella} in Foods and Feeds

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A prototype automated system using fluorescent antibody (FA) was evaluated for rapid detection of salmonellae in foods. Samples were enriched in selenite cystine and tetrathionate broths. After incubation, both were transferred into fresh selenite cystine for a 4-h "post-enrichment" to dilute possible background fluorescence from product. These cultures were then analyzed automatically, and results were compared with those obtained by the methods of the Association of Official Analytical Chemists (AOAC). Initially, 167 samples of milk powder, dried yeast, and imported frog legs were examined. The AOAC and automated FA methods correlated well with all samples but frog legs. Difficulty with the latter was caused by procedural and mechanical problems coupled with high numbers of competing microorganisms in post-enrichment cultures. Modification of procedure and partial redesign of equipment corrected these difficulties, and excellent correlation was obtained with another 116 frog leg samples. All 89 AOAC-confirmed positives were also detected by the automated FA method, and there were only 4\% false FA positives. The system shows potential for screening products for salmonellae; however, all positives should be confirmed by manual biochemical and serological methods.

Testing of foods and feeds for \textit{Salmonella} by the cultural method (10) of the Association of Official Analytical Chemists (AOAC) is a laborious process requiring 8 to 10 h of analytical time for each confirmed positive plus several days for preparation and incubation of media. Fluorescent antibody (FA) procedures (3, 5, 6, 7, 9, 11, 13, 14) offer considerable time savings and have been gaining acceptance for screening samples for salmonellae. Recently a direct FA procedure was approved by AOAC on the basis of results found in a collaborative study in 22 laboratories (2, 4). In our laboratory this manual FA method was used to test about 4,000 product samples, and results (4) were highly comparable with the AOAC cultural method.

Of the 619 samples confirmed positive by the AOAC cultural method, only four were negative by the FA method. The false positive rate was about 7\%, due to cross-over of the FA conjugate with some strains of \textit{Citrobacter} and \textit{Escherichia coli}. Therefore, the FA method should be used only to screen samples and all positives should be confirmed by conventional cultural, biochemical, and serological tests. Nevertheless, a considerable savings in analytical time is accomplished particularly with FA negative products, which can be immediately certified free of \textit{Salmonella}. The FA method is a qualitative technique and requires experience on the part of the microscopist in examining and evaluating FA slides. Normal individual variation in reading slides as well as eye fatigue occasionally cause difficulties in interpreting FA results. Organon Diagnostics (formerly Aerojet Medical and Biologicals Systems), El Monte, Calif., has developed an automated system that does not depend on microscope optics for measuring fluorescence in a stained specimen.

A prototype model of the system was evaluated by the Food and Drug Administration (FDA), New York District laboratories. An operational procedure was developed for automated screening of salmonellae in foods and feeds, and results were compared with those obtained by AOAC methods. After submission of this manuscript, a paper by Thomason et al. (15) was published on results of a similar evaluation of this equipment conducted at the Center for Disease Control.

**MATERIALS AND METHODS**

Cultures, media, and antisera. \textit{Salmonella typhimurium} (ATCC 14028) was used for the positive controls with the automated system. Cultures were maintained in FAS broth (Difco) and transferred daily to provide a ready source of actively growing cells. Enrichment, plating, and all other media were...
obtained from both Difco and BBL and were used interchangeably regardless of the source. The antisera used to determine the Salmonella group for isolates were obtained from Difco.

Fluorescent antibody. Lyophilized polyvalent fluorescent antibody was obtained from Difco (Bacto-FA Salmonella Poly) and was rehydrated in distilled water according to the manufacturer's instructions. For the manual FA procedure (i.e., analysis of culture smears by fluorescence microscopy), this stock solution was diluted with phosphate-buffered saline, pH 7.2 (FA buffer, Difco), to a working titer of 1:8 as described by Goldman (8). The same titer was used for the automated procedure except that the phosphate-buffered saline contained 2% Tween 80 to prevent adsorption of the fluorescent antibody onto the plastic filter slides used in the processor. The diluted fluorescent antibody was passed twice through a 0.22-μm membrane filter and stored under refrigeration. Before use, the antibody was filtered again and placed directly into the reservoir on the slide processor.

Samples. Samples of yeast powder, frozen frog legs, and powdered milk were collected from commercial sources as routine surveillance samples by the FDA, New York District. Some samples had been tested previously by AOAC cultural methods and were stored at room temperature until analyzed with the automated system; all other samples were tested with the automated system immediately upon receipt.

Analysis for Salmonella by AOAC methods. All samples were prepared (i.e., pre-enrichment) according to the FDA Bacteriological Analytical Manual (12). These procedures vary, depending on product type. Pre-enriched samples were enriched in selenite cystine and tetrathionate broths and streaked on plating media, and suspected isolates were confirmed biochemically and serologically as Salmonella, all by procedures described in the AOAC Compendium (10), except that Hektoen enteric agar (1) was used as an additional plating medium. For brevity, the above methods will collectively be referred to simply as the AOAC cultural method.

Each sample was also analyzed for salmonellae by manual FA methods as described in a supplement to the AOAC Compendium (2). Samples were prepared and enriched as previously described. Selenite cystine and tetrathionate enrichment cultures were both transferred to fresh selenite cystine and incubated for 4 h at 35 C as a "post-enrichment" broth. This step is required to eliminate product carry-over that occasionally causes background fluorescence. FA-stained smears from post-enrichment cultures were stained with polyvalent fluorescent antibody and examined with an American Optical L-10 fluorescence microscope utilizing a mercury lamp.

Automated equipment. The automated equipment (Automated Bioassay System) was designed by Organon Diagnostics, El Monte, Calif., for the detection of salmonellae by the direct FA technique. This prototype system has two major components, a slide processor and a slide reader. Photographs of this equipment and a description of its operation were presented by Thomason et al. (15). Briefly, the automated equipment adds a sample of enrichment broth to membrane filters supported on plastic slides, removes spent broth, washes cells, and stains them with polyvalent FA conjugate at the rate of 120 slides/h. The degree of fluorescence, on an arbitrary scale of 0 to 200, is automatically determined in a slide reader at the rate of 360 slides/h.

RESULTS AND DISCUSSION

Preliminary experiments. According to the manufacturer, the automated system requires at least 10^6 Salmonella cells per ml of culture broth to obtain fluorescence readings within a useful range (75 to 200). This was confirmed in our studies by subjecting a series of dilutions of a control culture of S. typhimurium to analysis by the automated procedure. The culture was grown for 18 h at 35 C in FAS broth, and a series of dilutions was prepared in phosphate-buffered saline. Cell counts were determined by a direct microscopic method (10), and 0.1 ml of each dilution was analyzed in the automated system. The entire experiment was repeated on 4 different days, and the composite results are shown in Fig. 1. Significant changes in the fluorescence readings were not observed until the number of cells was greater than about 8 x 10^6 per ml. At this concentration the fluorescence reading was approximately 50. At that point the response increased rapidly until the number of cells was about 7 x 10^6. A reading of 75 was obtained with about 10^6 cells/ml.

Studies were performed to develop a post-enrichment procedure to insure that a sufficient number of Salmonella cells would be present for automated FA analysis. These studies included an evaluation of various enrichment and post-enrichment media and incubation times to determine which culture conditions gave Salmonella cells capable of bright fluorescence in a medium with low levels of debris and background fluorescence. Four samples each of two naturally contaminated products (yeast powder and dried milk) suspected of containing Salmonella organisms were used to prepare enrichment cultures in selenite cystine and tetrathionate broths. After 18 to 24 h at 35 C, 0.5 ml of the enrichment cultures was used to inoculate tubes containing 10 ml of various broth media (selenite cystine, tetrathionate, FA salmonella, and tryptic soy tryptose) as post-enrichments. Tubes of each medium were incubated for 2, 4, and 6 h and then analyzed by the manual FA method. Two of the milk samples and three of the yeast samples were found to contain cells capable of fluorescence when stained. With these samples, fluorescent cells were detected at all three incubation times in each of the four post-enrichment media. In general, smears from FA salmonella and tryptic...
soy tryptose broths contained cellular debris and had high background fluorescence, which would likely interfere with the automated procedure. Smears from selenite cystine and tetra-thionate post-enrichments had less debris and much lower background fluorescence. Although actual numbers of fluorescing cells were not determined per milliliter, the count per field (×1000 magnification) was generally 5 to 10 times higher with selenite cystine than with tetra-thionate, particularly with the yeast samples. This was true at all of the incubation times tested. With selenite cystine, the numbers of fluorescing cells per field were approximately the same in tubes incubated at 4 or at 6 h and ranged between 10 and 20. In some samples at 2 h, only 4 to 10 fluorescing cells were obtained per field. On the basis of these studies, a 4-h post-enrichment step in selenite cystine broth was selected for use in evaluating the automated system. Results of an AOAC collaborative study (4) showed that this same post-enrichment procedure gave the best results for use with the manual FA method adopted by AOAC (2).

Evaluation of the automated system. A summary of the procedures used for evaluation of the automated system is shown in Fig. 2. The same enrichment cultures were used as a source of cells to confirm Salmonella by the AOAC cultural method and to inoculate selenite cystine broth as a post-enrichment for the manual and automated FA methods. The post-enrichment cultures were streaked onto Hek-toen enteric agar as a control for viable cells. Cells in the post-enrichment cultures were then killed by addition of formaldehyde to a final concentration of 0.6%. This step was necessary to prevent contamination of equipment. After 30 min, samples were loaded into the slide processor and analyzed automatically.

The following controls were included. Each time enrichment cultures were prepared, tubes of selenite cystine and tetra-thionate were also inoculated with 0.1 ml of an 18- to 24-h culture of the control Salmonella strain. These tubes were processed along with the enrichment cultures and were used as positive controls for both the manual and automated FA analysis. A sterile tube of selenite cystine broth was included as a negative control. Negative and positive food samples were used as controls for each different type of product tested. The positive control was a sample of food inoculated with a 24-h broth culture of the control organism (0.1 ml/25 g) and processed through all the enrichment steps along with the test samples. The negative food control consisted of the uninoculated product appropriately diluted in the pre-enrichment media, immediately inoculated into enrichment media, and from there directly inoculated into post-enrichment media without incubation between each transfer. The above controls indicated whether product or broth inhibited growth of salmonellae and whether product carry-over caused false FA readings. Phosphate-buffered saline controls were also included after every eight samples to see whether the automated system itself was contributing any background fluorescence that would change the zero reading.

Initially, the automated system appeared to perform well with products containing a high level of salmonellae and few competitive bacte-

![Fig. 1. Fluorescence readings exhibited by various concentrations of S. typhimurium cells processed through the automated system. Each type of symbol represents results from a separate series of dilutions. Each dilution series was prepared and processed on a different day.](http://aem.asm.org/ Downloaded from)
AUTOMATED FLUORESCENT ANTIBODY PROCEDURE

FIG. 2. Flow diagram of the tests used in evaluation of the automated FA system. Abbreviations: SC, Selenite cystine broth; TT, tetrathionate broth; BG, brilliant green agar; BS, bismuth sulfite agar; SS, Salmonella-Shigella agar; HE, Hektoen enteric agar; TSI, triple sugar iron agar slants; and LIA, lysine iron agar slants.

FIG. 3. Fluorescence readings obtained by analysis of 36 samples of dry milk powder for salmonellae by the automated procedure. Duplicate slides were prepared for each post-enrichment broth, and the fluorescence readings were averaged. For comparison, the number of samples confirmed as positive for salmonellae by the AOAC procedure is shown above the center line, and the number of confirmed negative samples is shown below the line.
ria. Dry milk powder and yeast powder were examples of this class of products. Figure 3 shows the number of dry milk samples that were confirmed either positive or negative by AOAC cultural methods and their corresponding readings (average of duplicates) by the automated FA method. For easy comparison, the numbers of confirmed positive samples at each reading are shown above the center line and the numbers of negative samples are shown below the line. Note that the lowest FA readings for the positive samples by the AOAC cultural method were about 20 units higher than the highest readings for the negative samples. Similar results were obtained with yeast powder (Fig. 4). The majority of these samples were obtained over a 2- to 3-month period from a single manufacturer experiencing a severe Salmonella problem, and only three were found to be free of salmonellae. About 5 fluorescence units separated the confirmed positive from the confirmed negative samples.

A problem was encountered with automated FA analysis of frog legs. The fluorescence readings for confirmed positive samples ranged throughout the entire scale (Fig. 5). However, all confirmed positive samples but no confirmed negative samples were positive by manual FA methods. Many of these frog leg samples produced large numbers of non-salmonellae colonies on plates of selective media. Apparently, the competitive bacteria prevented the Salmonella in such samples from reaching sufficient levels in enrichment cultures to produce high automated FA readings. However, these difficulties were not entirely related to the presence of competitive bacteria. During the initial phases of the evaluation study, it was noticed that sometimes there was uneven automatic delivery of the sample cell suspension onto the filter slides. Also, some slides became flooded with wash solution, indicating that either excessive solution was applied at the wash stations or the slide filters were clogged and not flowing properly. These problems caused variation of as much as 50 units between duplicate readings of the same post-enrichment culture, particularly with frog legs. Although these problems existed with milk and yeast powder, there was not as much variation in duplicate readings, apparently because Salmonella was the predominant organism. The evaluation study was interrupted and modifications were made to both the mechanical equipment and the operating procedures.

Uneven sample delivery was caused by ad-

![Figure 4](http://aem.asm.org/)

**Fig. 4.** Results for the automated FA analysis of 63 samples of yeast powder. Data are displayed as described in the legend to Fig. 3.
herence of cells of the sample holders in the slide processor coupled with occasional incomplete delivery of the entire sample volume onto the slide filters. The former was corrected by the addition of 0.05 ml of wash solution (which contained 0.05% Tween 80) to each 0.1-ml sample of post-enrichment broth. Modification of the equipment by the manufacturer corrected the mechanical delivery problem. Also, the sensitivity of the reader was modified so that fewer Salmonella cells were required for readings greater than 75. At this time, however, a solution to the occasional flooding problem had not been found.

The modified equipment was retested with another group of frog leg samples (Fig. 6). Considerably better separation on the fluorescence scale was obtained between the confirmed positive and negative samples (minimum of 15 units of separation). The variation in duplicate samples was lowered to a maximum of about 20 units (average, 10 units). Eight of 27 confirmed negative samples of frog legs were found to give an average of the duplicate readings higher than 50. In four of the eight false positive samples, the FA-stained organisms were identified as Citrobacter. The lowest average duplicate reading for confirmed positive samples was 65. All of the positive samples by the AOAC method were also found positive by the manual FA procedure.

The results of these studies indicate that the automated system shows potential as a rapid screening method for Salmonella in foods. All FA positive samples (i.e., samples giving fluorescence readings above, say, 50) would have to be confirmed as positive by AOAC methods. A considerable amount of time and labor would be eliminated by exclusion of the FA negative samples (say, readings lower than 50) from the extensive cultural, biochemical, and serological testing now required for confirmation of all isolates from plates of selective media.

Results from the evaluation performed by Thomason et al. (15) are not directly comparable with those presented here because different product types were analyzed and different cultural procedures were used. In general these workers found that the system performed satisfactorily on processed foods but was unsatisfactory for environmental samples and unprocessed foods such as poultry and sausage. Our initial observation indicated a similar problem in regard to unprocessed frog legs. In our studies, this problem was apparently solved by modification of the equipment and by addition...
of Tween 80 to the formolized cultures. Thomason (personal communication) also used Tween 80 and the modified equipment. It seems likely, therefore, that their difficulties with unprocessed or highly contaminated foods is relative to the cultural procedure used in preparing samples for automated analysis.

Other studies are planned for testing a wider variety of foods and feeds by the automated system, and the possibility of adapting the equipment for the analysis of other food-borne pathogens is also being considered. The manufacturer has redesigned the slides and made improvements to the conjugate and wash solution delivery systems, which may further increase the difference in fluorescence readings between confirmed positive and confirmed negative samples.

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LITERATURE CITED


