

Evaluation of a Semiautomated System for Direct Fluorescent Antibody Detection of Salmonellae

BERENICE M. THOMASON,* G. ANN HEBERT, AND WILLIAM B. CHERRY

Center for Disease Control, Atlanta, Georgia 30333

Received for publication 19 May 1975

A semi-automatic system under development by Aerojet Medical and Biological Systems for the direct fluorescent antibody detection of salmonellae was evaluated with various food, feed, and environmental samples. All samples were simultaneously examined by Automated Bioassay System (ABS), manual direct fluorescent antibody procedures and cultural procedures. The ABS gave satisfactory results with the processed samples. It detected all of the culturally positive powdered egg and candy samples with no false-negative results and gave only 6.6 and 5.3% false-positive rates, respectively. With meatmeal samples the ABS failed to detect one culturally positive specimen that was also positive by manual fluorescent antibody and gave one (1.1%) false-positive result. A high rate of false-negative results was obtained by ABS on unprocessed samples of creek water, poultry, and sausage. Adding another enrichment step to the protocol reduced the false-negative rate considerably but severely increased the false-positive rate. The instruments worked reasonably well, but research is needed to improve enrichment procedures for samples to be processed by the system.

The fluorescent antibody (FA) test has proven to be a rapid, sensitive method for screening various food, feed, and environmental samples for salmonellae. However, when large numbers of samples are screened and the proportion of FA-negative specimens is large, the fatigue of the microscopist becomes a limiting problem. The availability of an automatic readout device would greatly increase the capacity of any laboratory in which large numbers of samples are routinely processed.

Several years ago Aerojet Medical and Biologicals Systems (AMB), now associated with Organon, Inc., developed a slide processor for the indirect automated bioassay system test for syphilis (1). Recently this processor was modified to perform direct FA staining. In addition, the company developed a fluorometric reader that eliminates the need for the microscope.

In this report, we describe our experience with a prototype system employing both the slide processor and the reader.

MATERIALS AND METHODS

ABS. The Automated Bioassay System (ABS) consisted of a slide processor and a fluorometric readout device (Fig. 1, 2). The sample-receiving slides required the use of special membrane filter disks through which the liquid inocula were filtered (Fig. 3).

ABS calibration. The ABS reader was calibrated by following the manufacturer's recommendation.

A series of dilutions of a stock *Salmonella* culture were made in selenite-cystine broth. Slides were prepared in quadruplicate and run on the ABS processor. A smear of each dilution was stained by the manual FA procedure to compare the sensitivity and reproducibility of the two methods.

Manual FA procedure. Smears of the same enrichment broths that were used for the ABS tests were made on multiwell slides and stained as described previously (7). The same conjugate dilution that was prepared for use on the ABS slide processor was used to stain the smears. A Zeiss IF microscope fitted with a 100-W halogen lamp was used to read the smears. The exciter filter was the KP500 interference filter; the Zeiss 50 was used as the barrier filter. A sample was considered positive if at least 10 organisms with typical morphology and fluorescing with a 3 or 4+ intensity were seen during the examination of the smear.

FA reagent. The FA *Salmonella* Poly (A-S) reagent (Difco) was used throughout the study. The conjugate was diluted 1:8 with phosphate-buffered saline (PBS), pH 7.2, containing 2% Tween 80 and filtered twice through a 0.22- μ m membrane filter (Millipore Corp.) before use. This product will stain salmonellae of O groups A-S encompassing O antigens 1 through 41. The reagent has been thoroughly evaluated for both specific and cross-staining activity (7). It will stain a large proportion of *Arizonae* serotypes and much smaller percentages of serotypes of *Citrobacter* and *Escherichia coli* (7).

Cultural procedures. The cultural procedures for isolating salmonellae were as follows: all dehydrated products were pre-enriched in lactose broth for 24 h. One milliliter of the pre-enrichment broth



FIG. 1. ABS Slide processor.

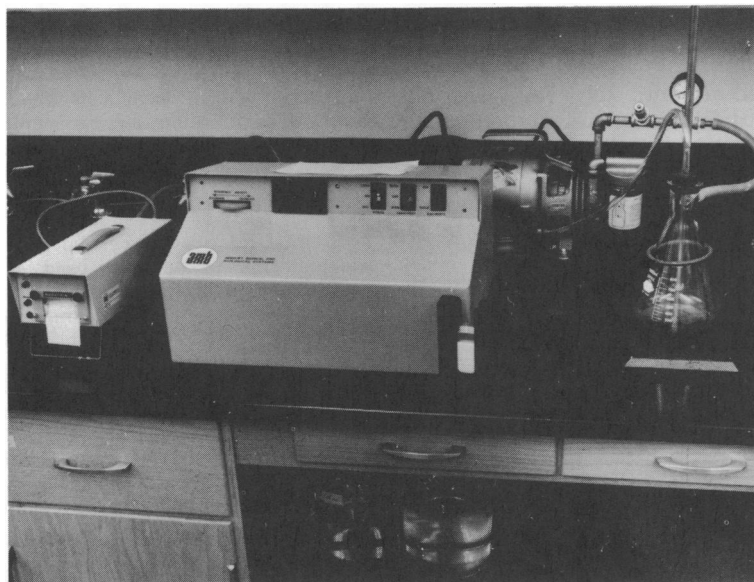


FIG. 2. ABS Slide reader.

was transferred to 10 ml of selenite-cystine or to tetrathionate broth with brilliant green dye (TET) for another 24-h incubation at 35 C. Twenty-five-gram samples of water, soil, vegetation, and raw poultry and meat products were grown in 225 ml of TET broth without pre-enrichment. After 24 h of incubation, all selective enrichment broths were streaked to brilliant green agar for isolating the salmonellae. After 24 h at 35 C, single colonies resembling *Salmonella* were picked to triple sugar iron agar, to lysine iron agar, and to urea medium.

The identification of all salmonellae was confirmed by O slide agglutination and by H tube agglutination tests.

ABS-FA procedure. After 24-h incubation of the inoculated selective enrichment broths, 0.1 ml of culture was transferred to 2 ml of selenite-cystine broth or to 2 ml of a medium prepared by mixing equal amounts of Trypticase soy and tryptose broths. The selenite-cystine broths were incubated in the 37 C waterbath for 4 h, whereas the Trypticase soy-tryptose broths received 2 h of incubation.

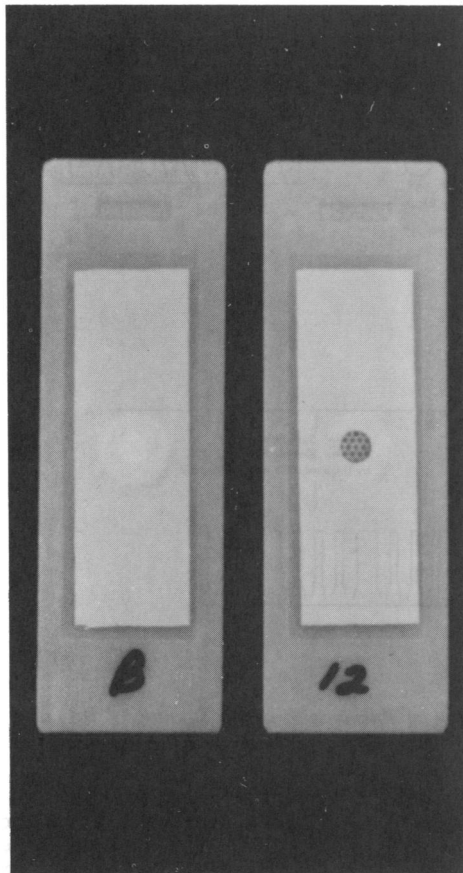


FIG. 3. AMB filter slides: B, blank slide; 12, processed slide that received selenite broth specimen.

The broth cultures were killed by the addition of 0.05 ml of 37% formaldehyde. After 30 min, 0.1 ml of 2% Tween 80 was added, and the cultures were stirred on a Vortex mixer for 10 s. Each well of the plastic specimen blocks supplied with the slide processor was loaded with 0.13 ml of sample (Fig. 4). In the latter part of the study, the procedure was modified by first pipetting 0.1 ml of a formalinized sample into each specimen block and overlaying it with 0.05 ml of a 0.05% solution of Tween 80 in PBS. This modification was designed to decrease sample retention within the wells of the specimen block. The procedure of using 4-h selenite-cystine broth cultures for the ABS-FA system will be referred to as ABS-FA Protocol 1 (Fig. 4).

The loaded specimen blocks were placed on the processor where the samples were sequentially and automatically deposited onto the filter slides. The liquid was pulled through the filter by suction, leaving the cells deposited on the surface. The slides then moved through various stations on the processor as follows. First, the filters were rinsed under suction to free the cells of debris; next, conjugate was added and the slides were incubated in a humidified chamber (35 C) for 15 min. Then each slide was

rinsed twice under suction to remove excess conjugate, dried by a stream of air, and loaded onto a slide magazine. About 26 min was required for a slide to complete the cycle. A stained slide was unloaded every 30 s after the initial 26 min.

Each run included at least three controls consisting of membrane slides through which (i) PBS, (ii) uninoculated selenite-cystine broth, and (iii) a stock *Salmonella* broth culture were filtered. These were processed along with the test samples. Each run also contained a sample of the test material which was inoculated with *Salmonella* to serve as a positive sample control. Controls for false-positive results consisted of samples which were negative in all tests when they were included as part of each experiment with a food or environmental specimen group.

The magazine containing the processed slides was removed from the processor and the slides were unloaded and moistened with PBS. The magazine was then placed in the reader which was calibrated by noting appropriate readings for the blank, PBS, and broth control slides. A digital readout between 0 and 200 represented the cumulative fluorescence intensity of the 4-mm diameter membrane filter (Millipore Corp.) on which the bacteria were deposited and stained. On the basis of results of a previous evaluation as well as preliminary experiments, all readings above 50 units of fluorescence were considered positive.

RESULTS

When twofold dilutions of a stock *Salmonella* broth culture that contained approximately 10^8 organisms per ml were processed and read by the ABS reader, variation of as much as 100 points between quadruplicate samples was observed (Table 1). These replicate samples were from well vortexed dilutions that were dispensed from the same pipette so that the variations must have occurred because of one or more of the following factors: (i) differences in numbers of cells deposited on the membrane, (ii) lack of uniformity of deposition of cells on the membrane, (iii) variable losses of cells during washing, (iv) nonuniform conjugate concentration due to dilution by residual wash fluids or by condensate during incubation. The experiment was repeated several times with similar results. The ABS reader required a minimum of 10^6 *Salmonella* organisms per ml to give a significant response. The manual FA test was positive through the 1:512 dilution, whereas the ABS gave negative responses for half of the 1:256 dilutions tested and, in one case, for a 1:128 dilution (Table 1).

ABS-FA Protocol 1. Powdered eggs. A total of 100 samples of powdered eggs were examined by the ABS-FA Protocol 1 (Fig. 4) and by manual FA and cultural techniques. Of the 100 samples, nine were positive by culture. The ABS test gave readings above 50 on all of the culturally positive samples and on 6 of the 91

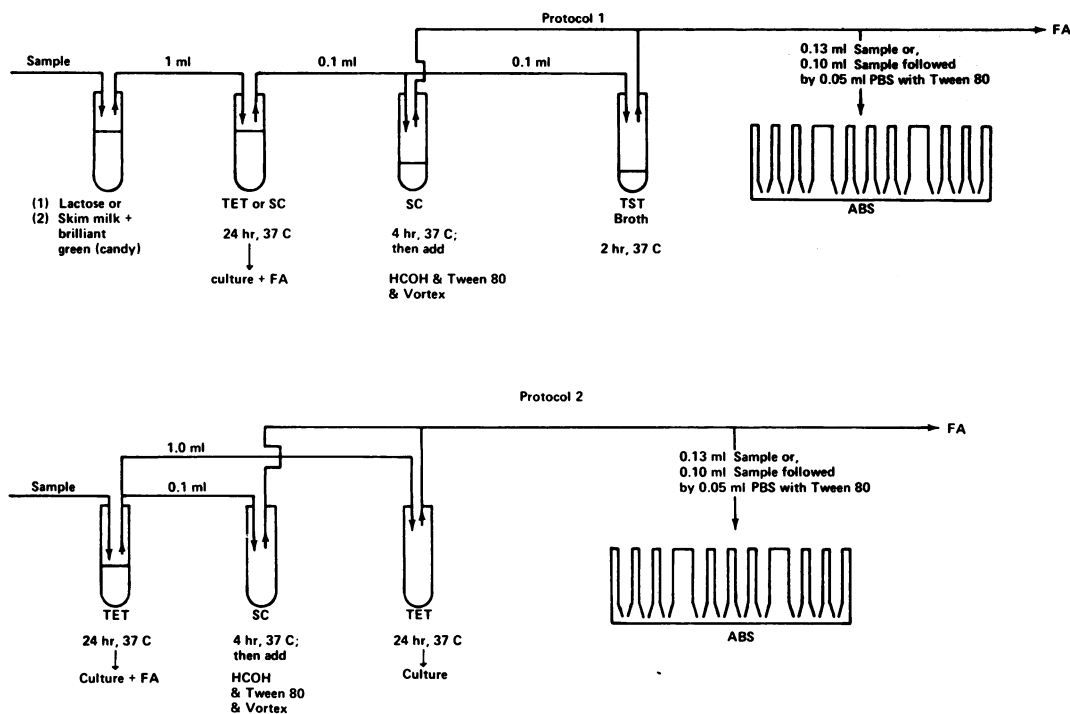


FIG. 4. Flow diagrams of processing of foods for detection of *Salmonella* by ABS, manual FA, and by culture.

TABLE 1. Relative sensitivity of the ABS and manual FA tests

Dilution of stock <i>Salmonella</i> culture ^a	No. of stained organisms/field 1000×	ABS readings ^b	Avg ABS readings
1:2	TNTC ^c	200, 200, 200, 116	179
1:4	TNTC ^c	200, 184, 140, 179	176
1:8	40	200, 107, 102, 126	134
1:16	19	174, 127, 94, 94	122
1:32	7	84, 130, 98, 62	94
1:64	1	73, 64, 65, 67	67
1:128	<1	69, 68, 48, 54	60
1:256	<1	52, 58, 44, 44	49
1:512	<1	42, 40, 42, 44	42
PBS blank		26, 22, 27, 18, 22	23
Broth blank		28, 22, 34, 34, 27	29

^a Approximately 10⁶/ml.

^b Readings on replicate slides.

^c TNTC, Too numerous to count.

(6.6%) samples negative by culture (Fig. 5). The manual FA test showed fluorescent organisms in 17 samples; by culture 9 of them were positive and 8 were negative. There were no false-negative results by either ABS or manual FA as judged by the cultural results (Table 2).

Meatmeal. A total of 100 samples of meatmeal were examined. The ABS gave positive readings on 9 samples (Fig. 5). Of these, 8 were

confirmed by culture and were also positive by manual FA. One false-negative result was obtained by the ABS test on a sample that was positive by both manual FA and culture (Fig. 5). The manual FA and cultural results agreed completely (Fig. 5; Table 2).

Candy. A total of 101 samples of chocolate candy were pre-enriched in skim milk with a 1:50,000 concentration of brilliant green dye (4) for 24 h; then 1 ml of each sample was transferred to 10 ml of TET broth. Because the initial processing was done in another laboratory and we received the TET broths several days later, they were subcultured to a fresh tube of TET and incubated at 35 C for 24 h. One-tenth milliliter of subculture was transferred to 2 ml of TST broth for incubation in a 37 C waterbath for 2 h. The TST broth cultures were killed by the addition of 0.05 ml of formaldehyde and were processed by ABS and manual FA. Of the 101 samples, 6 were positive by cultural tests (Fig. 5). All of these were positive by both the ABS and manual FA tests. The ABS test gave positive readings on five culturally negative samples, three of which were also positive by manual FA for a false-positive rate of 5.3% (Table 2). The manual FA test gave positive results on four additional samples that were negative by both ABS and cultural tests for a

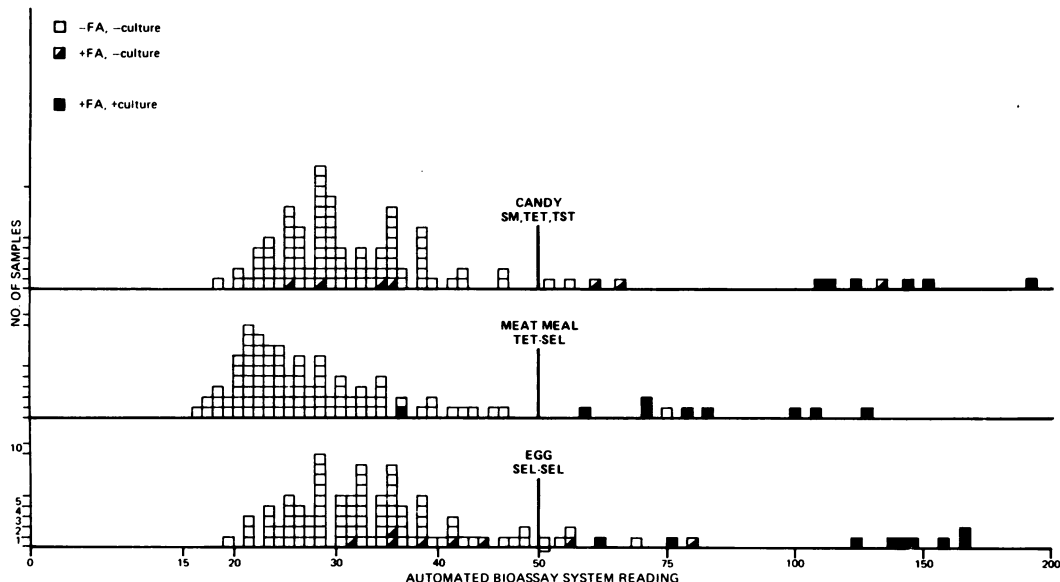


FIG. 5. Frequency distribution of ABS (Protocol 1) readings of three 100-sample groups of dry processed foods. Abbreviations: SM, skim milk with brilliant green; TET, tetrathionate broth; TST, Trypticase soy and tryptose broth in equal proportions; SEL, selenite-cystine broth.

TABLE 2. ABS and manual FA false-positive and false-negative results on processed food samples obtained with Protocol 1

Sample	No. examined	Test	No. of	
			False positive ^a	False negative ^b
Eggs	100	FA	8.8%	0
		ABS	6.6%	0
Meatmeal	100	FA	0	0
		ABS	1.1%	11.1%
Candy	101	FA	7.4%	0
		ABS	5.3%	0

^a Based on total of culture-negative samples.

^b Based on total of culture-positive samples.

7.4% false-positive rate (Table 2; Fig. 5). Although both manual FA and ABS gave small percentages of false-positive results when compared to culture, neither gave false-negative results (Table 2).

Inspection of the frequency distribution histogram (Fig. 5) of ABS readings obtained with powdered eggs, meatmeal, and candy samples shows the difficulty of deciding on an arbitrary instrument value by which to divide ABS-positive from ABS-negative samples. At the ABS value of 50, only 1 of 24 culturally positive samples would be called negative. On the other hand, 7 of 258 samples which were negative by both manual FA and culture would be positive

by ABS readings. Also, 5 of 15 FA positive but culturally negative samples gave ABS readings higher than 50.

Creek samples. A total of 35 samples consisting of Moore swabs, algae, vegetation, and soil were collected from a nearly polluted stream. The samples were placed directly into TET broth and incubated for 24 h at 41.5 ± 1.0 C. An aliquot (0.1 ml) of TET broth was transferred to selenite-cystine broth (2 ml) and the cultures were incubated for 4 h at 37 C. Of the 35 samples, 22 were positive by culture (Table 3). Of these, 14 were positive by manual FA tests but only 4 gave a positive response by ABS tests. Eight of the 18 culturally positive but ABS negative samples also were negative by the manual FA tests. The manual FA test was positive on one sample that was negative by culture.

Poultry products. A total of 54 samples of poultry consisting of unprocessed turkey and chicken meat and chicken liver and gizzards were examined. Twenty-two samples were positive by culture and by manual FA tests (Table 4). The ABS test results were positive on 11 of these 22 samples and on one additional sample that was negative by manual FA and culture. The manual FA gave positive results on an additional 21 samples that were negative by culture.

Sausage. Twenty samples of bulk pork sausage were examined. The cultural tests yielded

isolates from 13 of the 20 samples (Table 4). Of these, 12 were positive by manual FA. The ABS results were positive on 7 of the 12 and one additional sample that was negative by both manual FA and cultural tests. One culturally positive sample was negative by both FA tests.

ABS-FA Protocol 2. Because of the poor results obtained when Protocol 1 was used on unprocessed samples (poultry, sausage, and creek samples), the procedure was modified as follows. At the time the transfer was made from the 24-h TET broth to the selenite-cystine broth for a 4-h postenrichment period, a second TET broth was inoculated with 1 ml of the first TET broth. After this second TET broth was incubated for 24 h, a 2-ml portion was withdrawn and examined by manual FA, ABS, and culture. Both Protocol 1 and Protocol 2 (Fig. 4) were used to examine 20 additional samples each of chicken parts, pork sausage, and creek samples. The number of false-positive and false-negative results obtained when the two protocols were used are compared in Table 5. With Protocol 1, the ABS gave no false-positive

results on 11 of the culturally negative samples of chicken. Two of nine culturally positive samples of chicken were negative by ABS giving a false-negative rate of 22.2%. The manual FA test was positive on 6 or 54.5% of the culturally negative samples. There were no false-negative results by the manual FA test with Protocol 1. Using Protocol 2 the ABS gave a false-positive rate of 71.4% but no false-negative results. The manual FA test gave false-positive results on 28.6% of the culturally negative samples and false-negative results on 7.7% of the culturally positive samples.

When Protocol 1 was used, 13 of the 20 sausage samples were positive by culture (Table 5). The ABS gave a false-positive rate of 14.3% and a false-negative rate of 46.2%. There were no false-positive results by the manual FA, but 7.7% of the culturally positive samples were negative by manual FA. With Protocol 2, the false-positive rate by ABS increased to 25% but there were no false-negative results. The manual FA gave a 12.5% false-positive rate and no false-negative results.

TABLE 3. ABS, manual FA, and cultural results on creek samples obtained with Protocol 1^a

Sample	No.	No. of					
		ABS + culture+/total culture+	FA + culture+/total culture+	ABS + culture-/total culture-	FA + culture-/total culture-	ABS - culture+/total culture+	FA - culture+/total culture+
Creek	35	4/22 (18.2%)	14/22 (63.6%)	0/13	1/13 (7.7%)	18/22 (81.8%)	8/22 (36.4%)

^a+, Positive; -, negative.

TABLE 4. ABS, manual FA, and cultural results on nonprocessed food samples obtained with Protocol 1^a

Sample	No.	No. of					
		ABS + culture+/total culture+	FA + culture+/total culture+	ABS + culture-/total culture-	FA + culture-/total culture-	ABS - culture+/total culture+	FA - culture+/total culture+
Poultry	54	11/22 (50%)	22/22 (100%)	1/32 (3.1%)	21/32 (65.6%)	11/22 (50%)	0/22
Sausage	20	7/13 (53.8%)	12/13 (92.3%)	1/7 (14.3%)	0/7	6/13 (46.2%)	1/13 (7.7%)

^a+, Positive; -, negative.

TABLE 5. ABS and FA results obtained with Protocols 1 and 2 for three classes of samples

Test	Chicken (20)		Sausage (20)		Creek (20)	
	False + ^a	False - ^b	False + ^a	False - ^b	False + ^a	False - ^b
Protocol 1						
ABS	0/11	2/9 (22.2%)	1/7 (14.3%)	6/13 (46.2%)	0/7	10/13 (76.9%)
Manual FA	6/11 (54.5%)	0/9	0/7	1/13 (7.7%)	0/7	7/13 (53.8%)
Protocol 2						
ABS	5/7 (71.4%)	0/13	2/8 (25%)	0/12	3/4 (75%)	3/16 (18.7%)
Manual FA	2/7 (28.6%)	1/13 (7.7%)	1/8 (12.5%)	0/12	3/4 (75%)	1/16 (6.2)

^a False + (positive) determined by ABS + or FA +; culture - samples/total culture -.

^b False - (negative) = ABS - or FA -; culture + samples/total culture +.

Twenty additional samples consisting of Moore swabs and of vegetation and soil from a creek were examined by both Protocols (Table 5). The primary TET broth (Protocol 1) yielded isolates from 13 samples. There were no false-positive results by either ABS or manual FA but the false-negative rates were 76.9% by ABS and 53.8% by manual FA. With Protocol 2, salmonellae were isolated from 16 of the TET broths. Both ABS and manual FA gave a false-positive rate of 75% based on cultural results. The false-negative rates were 18.7% by ABS and 6.2% by manual FA.

DISCUSSION

When results obtained with both FA methods are compared to those obtained by culture, it is obvious that there are serious deficiencies in each FA test procedure. The only products examined that yielded completely satisfactory results by the ABS system were powdered eggs and candy, although only one false-positive and one false-negative sample of meatmeal was found (Table 2). The better performance on the first two food groups may have been due to the fact that the candy samples had two enrichments in TET broth and the FA tests were performed on 2-h TST broths instead of the usual 4-h selenite-cystine broth.

The false-negative rates obtained with the ABS in Protocol 1 (Tables 3 and 4) were quite high on samples of creek water (81.8%), poultry (50%), and sausage (46.2%).

The false-negative rate of the manual FA test versus cultural examination was 7.7% on the sausage (Table 4) and 36.4% on the creek samples (Table 3). The latter figure is much higher than that found in earlier studies on creek samples (2). This indicates the inadequacy of the procedure followed in this study (Protocol 1) for samples that contain small numbers of salmonellae and which are not pre-enriched. When the protocol was modified by subculturing the original enrichment to a second TET broth for 24 h and performing both manual and ABS-FA tests, the results were considerably improved (Table 5).

The results obtained on 20 samples each of chicken parts, sausage, and creek samples with Protocols 1 and 2 (Table 5) indicate that modification of procedures can severely affect the results obtained. For example, the ABS false-negative rates obtained with Protocol 1 decreased for all sample classes in Protocol 2, but the false-positive rate increased concomitantly. Also, the manual FA false-positive rate on chicken parts obtained with Protocol 1 decreased when Protocol 2 was used, but the false-

negative rate increased. The reverse occurred when sausage and creek samples were examined. (Table 5).

We isolated salmonellae from 41 samples using Protocol 2 and from only 35 using Protocol 1. One cultural failure occurred with Protocol 2 when salmonellae that were present in the primary TET broth were not isolated from the second TET broth even though both types of FA tests indicated its presence. Cultural failures occur, and they explain some positive FA results that cannot be confirmed by culture (3, 5, 6). Under the conditions used in our evaluation, the manual FA tests are approximately twice as sensitive as the ABS tests. In addition to requiring a minimum concentration of 10^6 organisms per milliliter of test sample and a 0.1-ml volume of sample for a significant response, the ABS test also requires some background fluorescence. This observation was derived from microscope examination of test slides on which the membrane filters (Millipore Corp.) gave low or negative electronic responses in the reader, although they were prepared from specimens that were positive by both manual FA and by culture. The Leitz Ortholux microscope fitted with the Ploem incident illuminator was used for examining these membranes. Numerous fluorescent organisms were seen on the filters, but the background was very dark. Conversely, a bright background was always seen on filters which gave positive ABS responses. However, the brightness of the background was not always positively correlated with the number of stained bacteria which were present. The filter slides from specimens that were negative by manual FA and culture but which gave a positive response by ABS revealed a bright background with amorphous fluorescent deposits but no discernible bacteria.

In addition to the lack of agreement between ABS responses and cultural results, several design or operational problems were encountered with the slide processor which required constant monitoring by the operator. Since our study, however, modifications have been made which should alleviate the filtration and flooding problems that we experienced.

The conjugate pump of the processor was inconvenient to load and prime and required a large volume of conjugate because of the in-line final filter.

The reader appeared to faithfully record the total field fluorescence but, of course, could not distinguish between specifically stained bacteria and nonspecifically stained background material or autofluorescent particles. In addition, some problems relating to transport of the slides through the reader were encountered,

but these appear to be minor design difficulties.

In regard to rapidity of processing, we observed that when a bacteriologist experienced in doing manual FA examinations, started to prepare slides at the same time that the machine operator started to fill the sample blocks, the manual FA results were usually completed before they were available from the ABS system. However, this observation was based on the processing of only 50 samples per work day and does not discount the fatigue factor inherent in routine visual reading of large numbers of samples.

This first attempt at automation of the FA technique for detection of salmonellae in food-stuffs is promising. The success of the ABS approach may prove to depend more upon the solution of the biological problems than upon the resolution of the engineering and design difficulties. More research is needed to improve enrichment procedures for samples to be processed by the system. This need is especially critical for the examination of unprocessed food and environmental specimens by either FA or cultural procedures.

In summary, the ABS Protocol 1 performed satisfactorily on the processed foods for which it was developed but was unsatisfactory for unprocessed food and environmental samples. We tried a modified procedure, Protocol 2, which decreased the number of false-negative results obtained with Protocol 1 on unprocessed foods and environmental samples. Further refinements will be required, however, to decrease the incidence of false-positive results.

ACKNOWLEDGMENTS

We thank Aerojet Medical and Biological Systems (now Organon Diagnostics) for the opportunity to evaluate the Automated Bioassay System. We are particularly indebted to W. H. Hartung, Manager, Advanced Programs, and to John J. O'Connor for installing the instruments and training us to use them, and for providing operational protocols and the required reagents. We also thank Eli Mishuck, President of AMB and Albert Balows, Director of the Bacteriology Division, CDC, for their valuable assistance on administrative and logistical aspects of the evaluation effort.

LITERATURE CITED

1. Binnings, G. F., M. J. Riley, M. E. Roberts, R. Barnes, and T. C. Pringle. 1969. Automated instrument for the fluorescent treponemal antibody-absorption test and other immunofluorescence tests. *Appl. Microbiol.* 18:861-868.
2. Cherry, W. B., J. B. Hanks, B. M. Thomason, A. M. Murlin, J. W. Biddle, and J. M. Croom. 1972. Salmonellae as an index of pollution of surface waters. *Appl. Microbiol.* 24:334-340.
3. Fantasia, Leonard D. 1969. Accelerated immunofluorescence procedure for the detection of *Salmonella* in foods and animal by-products. *Appl. Microbiol.* 18:708-713.
4. Galton, M. M., G. K. Morris, and W. J. Martin. 1968. Salmonellae in foods and feeds. A review of isolation methods and recommended procedures. National Communicable Disease Center, Atlanta, Ga.
5. Goepfert, J. M., M. E. Mann, and R. Hicks. 1970. One-day fluorescent antibody procedure for detecting salmonellae in frozen and dried foods. *Appl. Microbiol.* 20:977-983.
6. Laramore, C. R., and C. W. Moritz. 1969. Fluorescent antibody technique in detection of salmonellae in animal feed and feed ingredients. *Appl. Microbiol.* 17:352-354.
7. Thomason, B. M., and G. A. Hebert. 1974. Evaluation of commercial conjugates for fluorescent antibody detection of salmonellae. *Appl. Microbiol.* 27:862-869.