Rapid Detection of *Salmonella* Microcolonies by Fluorescent Antibody

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A microcolony fluorescent-antibody (FA) procedure for detecting *Salmonella* was compared to the usual direct FA procedure on 304 environmental, food, and feed samples. The microcolony FA test detected all of the specimens found positive by culture, whereas the direct FA missed 3.1% of them. Both FA tests revealed stained organisms in some of the culturally negative specimens. The microcolony FA test has several advantages over the direct FA test: ease of examining the smears, elimination of the fluorescent background material, and increased sensitivity.

In the past few years, the need for more rapid methods of detecting *Salmonella* in food, feeds, and their raw products has stimulated a number of investigators to use fluorescent-antibody (FA) procedures for this purpose (3, 4, 6-8, 10-14). Some investigators have found it necessary to prepare smears from centrifuged sediments of enrichment broth cultures. Centrifugation is time-consuming, and smears prepared from these sediments may contain too many organisms. When this occurs, the fluorescence of the *Salmonella* is often faint and uneven. The presence of organic material and heterologous bacteria in the stained preparations frequently makes interpretation of fluorescence difficult.

A method is needed which increases the numbers of *Salmonella* in the stained preparation without prior centrifugation and which lessens the amount of background fluorescence. The microcolony FA method developed by Chadwick and Abbott (1) for the detection of small numbers of enteropathogenic *Escherichia coli* seemed to offer a possible solution to this problem. This report describes the adaptation of their method for use with food and feed specimens.

**MATERIALS AND METHODS**

**FA reagent.** The FA reagent used in this study was a pool of five polyvalent OH conjugates covering *Salmonella* O groups A through I, 18, 21, and 35. A 1:8 dilution of the conjugate in phosphate-buffered saline (PBS), pH 7.6, was used to stain all smears. The preparation and testing of this reagent was described in a previous report (15).

**Specimens.** Three categories of specimens were examined. The environmental specimens consisted of surface swabs and both fresh and salt water samples. The food specimens were fresh fish and pork. The feed specimens were fishmeal, bone meal, and pelleted pet foods. All specimens were examined by both FA and cultural procedures. The cultural methods used were those recommended by Galton, Morris, and Martin (5).

**Preparation of multiple-well slides for FA tests.** Microscope slides (1 by 3 inches (2.54 by 7.62 cm)) were cleaned with 95% ethanol and scrubbed dry with a lintless towel. The cleaned slides were spotted with glycerol by using a syringe fitted with an 18-gauge needle which had the head cut off (Fig. 1). The slides were sprayed with Fluoro-Glide, Film-Bonding Grade (Chemplast, Inc.), allowed to dry for a few minutes, and then rinsed well in running tap water to remove all traces of glycerol. They were blotted gently to remove most of the water and allowed to air-dry. Each slide was flame well before using. This method of preparing coated slides for FA tests was first described by Goldman (9).

**Preparation and staining of smears for the direct FA test.** The direct FA test was performed on smears prepared from 24-hr tetrathionate with Brilliant Green (TET) enrichment broth cultures. A 2-mm loopful of each TET broth was placed in a well of the coated slides. After air-drying, the smears were fixed for 2 min in a fixative containing 60 parts of absolute ethanol, 30 parts of chloroform, and 10 parts of formaldehyde solution. The fixed slides were rinsed briefly in 95% ethanol and allowed to drain dry. A small drop of conjugate was placed on each smear, and the slides were covered with a large petri dish fitted with a moist piece of filter paper for 30 min. Excess conjugate was drained off on a paper towel. The slides were rinsed briefly in a bath of PBS and placed in fresh PBS for 10 min. Then they were dipped in distilled water to remove the salt, and cover glasses were mounted with buffered glycerol (pH 9). Smears were examined under the 95× oil immersion objective of a Leitz Ortholux fluorescence assembly. A 3-mm BG 12 exciter filter was used in combination with a 1-mm blue-absorbing ocular filter. The light source was the Philips CS 150 mercury arc lamp.

**Preparation and staining of microcolony smears.** At
the time the direct FA smears were made, a loopful of each TET broth was placed on the surface of a Brilliant Green agar (BG) plate (9 cm square) to develop microcolonies in the manner shown in Fig. 2. Nine specimens were inoculated onto each BG plate. After incubation for 3 hr at 35°C, impressions of the inoculated areas (three impressions on each 1- by 3-inch slide) were made on alcohol-cleaned microscope slides. The impressions were made by firmly pressing the slide onto the agar and pushing it over the surface for a few millimeters before lifting it with forceps. The impression smears were rinsed with a diamond marking pen, and each slide was labeled appropriately. They were fixed and stained as described above. The smears were scanned with the 10X objective, and suspect areas were further examined with a 45X oil immersion objective.

**Sensitivity tests.** *Salmonella thompson*, *S. illinois*, *serfienberg*, *S. cerro*, and *S. kentucky* were grown in Trypticase soy broth (BBL) for 18 hr. Serial dilutions were prepared in PBS, and plate counts were made on Trypticase soy agar plates. The counts ranged from $6.3 \times 10^6$ to $1.6 \times 10^8$ organisms per ml of the original broth culture. A 0.1-ml amount of each dilution through $10^{-4}$ was added to 0.9 ml of a 24-hr TET broth culture of bonemeal sample that was negative for *Salmonella* by both FA and culture. Smears for direct FA examinations were made of each dilution, and BG plates were inoculated for microcolony detection.

Two fishmeal specimens previously found positive for *Salmonella* were grown for 24 hr in TET broth. The broth cultures were diluted in sterile TET broth in successive 10-fold dilutions. Smears for FA examination were prepared from each dilution, and BG plates were inoculated for microcolony development.

**RESULTS**

A total of 304 specimens were examined. The results are shown in Table 1.

*Salmonella* cells were isolated by culture tests from 86 of the 134 environmental samples. The direct FA test detected 85 of these. Three specimens that were negative by culture were positive by direct FA. The microcolony FA test detected all 86 culturally positive specimens; one specimen was positive by this test but negative by culture. Thirty of the 80 food samples were positive by cultural methods. The direct FA test detected 27 of these. Six specimens that were negative by culture were positive by direct FA. The microcolony FA test detected all of the culturally

**Table 1. Comparison of results obtained by direct FA and microcolony FA with cultural tests for Salmonella**

| Specimen     | No. examined | Test          | Results                              |
|--------------|--------------|---------------|--------------------------------------|
|              |              | Direct FA     | FA positive/culture positive         |
| Environmental| 134          | Microcolony FA| 85/86                                |
|              |              | Direct FA     | 86/86                                |
|              |              | Microcolony FA| 27/30                                |
|              |              | Direct FA     | 30/30                                |
|              |              | Microcolony FA| 43/44                                |
|              |              | Direct FA     | 44/44                                |
| Food         | 80           | Microcolony FA| 86/86                                |
| Food         | 80           | Direct FA     | 27/30                                |
| Feed         | 90           | Microcolony FA| 30/30                                |
| Food         | 90           | Direct FA     | 43/44                                |
| Feed         | 90           | Microcolony FA| 44/44                                |
| Totals       | 304          | Direct FA     | 155/160 (96.9)                       |
| Totals       | 304          | Microcolony FA| 160/160 (100.0)                      |

* Values in parentheses are expressed as percentages.
positive specimens, and 13 specimens that were negative by culture were positive by the microcolony FA test.

Of the 90 feed samples, 44 were culturally positive for Salmonella. The direct FA test detected 43 of these and the microcolony FA detected 44. Three samples were positive by both the direct and microcolony FA tests but were negative by culture.

The salmonellae isolated in this study are listed in Table 2.

For comparison of the appearance of the direct FA smears with that of the microcolony FA smears, see Fig. 3 through 6. Figure 3 shows the appearance of a direct FA smear under the 95X oil immersion objective. Note the amount of background fluorescence and the lack of contrast between some of the stained organisms and the debris. Figure 4 shows the microcolony im-

| Specimen       | Serotype                      |
|---------------|-------------------------------|
| Environmental water | S. bareilly, S. eimsbuettel, S. montevideo, S. norwich, S. oranienburg, S. blockley, S. kentucky, S. binza, S. newbrunswick, S. illinois, S. thomasville, S. sentenber, S. cerro |
| Food          | B, S. derby, S. heidelberg, S. saintpaul, S. typhimurium, S. bareilly, S. eimsbuettel, S. kentucky, S. anatum, S. muenster, S. newbrunswick, S. thomasville |
| Feed          | B, S. bredeney, S. kentucky, S. tennessee, S. drypool, S. newbrunswick, S. sentenber, S. cubana, S. cerro, S. sieburg, S. matopeni |

Chadwick and Abbott (1) reported that the microcolony technique was approximately 100 times as sensitive as the direct smear method when fecal suspensions were examined for enteropathogenic E. coli. The results of the sensitivity tests run in this study are shown in Table 3. With two naturally contaminated fishmeal samples and five artificially contaminated bovine samples, we found the microcolony test approximately 10 times as sensitive as the direct FA test. The microcolony FA test was 100 times as sensitive as the direct FA test in only one of the five artificially contaminated specimens. The difference between these results and those reported by Chadwick and Abbott may result from the treatment of the specimens examined. The Salmonella specimens were incubated in a selective enrichment broth before the direct smears were prepared, whereas the fecal specimens in Chadwick and Abbott’s study were examined directly.

DISCUSSION

The direct FA test is very sensitive, although failures do occur as evidenced by negative results for salmonellae in 3.1% of the specimens found positive by culture. These failures must be considered technical failures, since all of the salmonellae isolated were stained by the FA reagent.

The sensitivity of the FA tests on food specimens appeared to be greater than that on either the environmental or feed samples (Table 1). This may be due to the nature of the material examined. Included in the food samples were 47 fresh pork sausage specimens. All six of the specimens positive by direct FA but negative by culture were sausage samples. Also, 12 of the 13 specimens positive by the microcolony FA test but negative by culture were sausage samples. Only one attempt was made to isolate salmonellae in this study, and many of the BG plates inoculated with TET enrichment broths of the sausage were overgrown with Proteus. It has been shown by many workers that repeated culture will confirm the majority of FA results. Whether FA results that are not confirmed by culture should be considered as “false-positive” was discussed in a previous paper (2). However, since the FA reagents for Salmonella are not genus-specific, the possibility exists that cross-reactions will occur, and this must be considered in evaluating FA results on grossly contaminated products. The sensitivity of the FA test should encourage more stringent isolation procedures since food products
FIG. 3. Smear of tetrathionate broth culture of Salmonella-positive fishmeal sample stained with Salmonella polyvalent OH conjugate. × 1,187.

FIG. 4. Impression smear of 3-hr microcolonies stained with Salmonella polyvalent OH conjugate. × 125. Specimen is the same as in Fig. 3.

subject to regulation by the Food and Drug Administration are required to be free from viable Salmonella microorganisms.

The sensitivity of the microcolony FA test is at least 10 times that of the direct FA test. The apparent disadvantage of the microcolony test, the three additional hours of incubation required, is outweighed by the unequivocal results ob-
Fig. 5. Same as Fig. 4, except photomicrograph taken at 563× with dry dark-field condenser on microscope and 45× dry objective.

Fig. 6. Same as Fig. 5 showing large microcolony of Salmonella.

tained. Impression smears of the microcolonies could be read as either distinctly positive or distinctly negative; the doubt encountered in interpreting some of the direct smears was almost totally eliminated.

The specificity of the FA test is the same regardless of whether the direct or microcolony technique is used. When microcolonies of a cross-reacting organism develop on the plates, false-positive results will be obtained. However, the salmonellae develop more rapidly on the selective BG plates than do most other gram-negative
organisms. Only the stained microcolonies were apparent in many of the impression smears, even when they were viewed under tungsten light.

The microcolony smears permit the use of low-power objective for scanning, thereby greatly accelerating the examination of specimens. Also, if dry objectives are used, a dry darkfield condenser may be substituted for the standard oil immersion condenser.

The development of automated FA tests has been hampered, because it is difficult for a machine to distinguish between clumps of amorphous debris and clumps of organisms. The microcolony technique, by the virtual elimination of background material, may help solve this problem. Automation of the FA procedures will make the technique more attractive to many laboratories.

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| Samples | Contaminated with Salmonella | No. examined | Test | Highest dilution giving positive results |
|---------|-----------------------------|--------------|------|---------------------------------------|
| Fishmeal| Naturally                    | 2            | Direct FA Microcolony FA | 10^{-1} | 10^{-2} |
| Bone-meal| Artificially              | 4            | Direct FA Microcolony FA | 10^{-3} | 10^{-4} |
| Bone-meal| Artificially              | 1            | Direct FA Microcolony FA | 10^{-3} | 10^{-5} |

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