Preparation and Testing of Polyvalent Conjugates for Fluorescent-Antibody Detection of Salmonellae

BERENICE M. THOMASON AND JOY G. WELLS
Center for Disease Control, Atlanta, Georgia 30333

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A polyvalent OH conjugate for Salmonella O groups A through I, K, L, and O was prepared and tested against pure cultures of salmonellae, nonsalmonellae, and a variety of food, fecal, and environmental specimens. Examination of pure cultures revealed that the conjugate gave negligible staining with representative strains of Shigella, Proteus, Providence, Serratia, and Pseudomonas. However, it stained 12% of the Escherichia coli and Citrobacter freundii strains and 36% of the Arizona strains. Over 1,200 specimens of various types were examined by both fluorescent-antibody (FA) and cultural procedures. Results indicate that, when used with discretion, FA screening can be a useful tool for rapid presumptive indication of the presence of salmonellae. The need for careful selection of strains used for preparing antisera and the importance of adequate evaluation of Salmonella FA reagents are discussed.

Fluorescent-antibody (FA) techniques for detecting salmonellae have become popular in the last few years partly because of the increased interest of the food industry in a rapid, economical screening method for detecting Salmonella-contaminated products.

Most investigators have used antisera prepared for agglutination tests as their primary reagent in the indirect FA test (7, 9, 12) or for preparing a direct FA conjugate (4). The complete antigenic coverage of these reagents is difficult to ascertain from the publications. The inadequate description of Salmonella polyvalent H reagents and the claims of greater specificity obtained with them have led some investigators to assume that the flagellar antibodies are responsible for the better performance in FA tests (4). The typical cell wall staining obtained with Salmonella H antisera has been shown to be due to the O antibodies in the reagent (6, 14), but FA reagents produced with formalinized whole cell antigens have also given higher staining titers than those produced with heated antigens (2, 13).

Developing an FA reagent for salmonellae is a time-consuming and complicated task. The multiplicity of antigens within the group and their similarity to antigens of nonsalmonellae necessitate a compromise between the sensitivity and specificity of the reagent. Any FA reagent devised for detecting Salmonella must be characterized as to its antigenic coverage and cross-reactivity with other members of the Enterobacteriaceae. The preparation and testing of a polyvalent conjugate designed to detect all O and H antigens of Salmonella O groups A through I, K, L, and O are described in this report.

MATERIALS AND METHODS

Strains. In this study, we used 133 Salmonella serotypes representing O groups A through 64. In addition, the following strains were tested: 142 Escherichia coli representing O groups 1 through 145; 35 Shigella representing O groups A through D; 33 Citrobacter freundii representing O groups 1 through 31; 36 Arizona representing O groups 1 through 34; 73 Klebsiella representing K types 1 through 72; 15 Serratia representing O groups 1 through 15; 13 Providence representing O groups 1 through 29; five strains each of Proteus morganii, P. vulgaris, P. rettgeri, and P. mirabilis; five strains of Pseudomonas fluorescens; two strains each of P. diminuta, P. maltophilia, P. aeruginosa, P. putida, and P. stutzeri; and one strain each of P. alcaligenes and P. dinitriificans.

Antiserum. The first antiserum used was the Salmonella polyvalent H antiserum produced by the Biological Reagents Section, Center for Disease Control (CDC), Atlanta, Ga. This reagent was produced by injecting, into rabbits, pools of formalinized broth antigens of the salmonellae listed in Table 1. The resulting antiserum were combined into one polyvalent reagent and preserved by adding 50% glycerin. To increase the O antigen coverage of this antiserum, an additional pool of antibodies was prepared by combining Trypticase soy-Tryptose broth cultures of actively motile strains of Salmonella new-brunswie (3, 15:1, v:1, 7), S. carrau (6, 14, 24:y:1, 7), S. gaminara (16:d), S. minnesota (21:b:e,
TABLE 1. Composition of the CDC Salmonella polyvalent H antisera

| Organism                        | Strain | Group | O antigens | H antigens |
|---------------------------------|--------|-------|------------|------------|
| S. paratyphi A                  | 1015   | A     | 1, 2, 12   | a          |
| S. paratyphi B                  | Java   | B     | 1, 4, 5, 12| b          |
| S. cholerae-suis                | Minn. 179 | C₄ | 6, 7       | c          |
| S. typhi                         | H901   | D     | 9, 12      | d          |
| S. reading                      | 24682  | B     | 4, 5, 12   | e, h       |
| S. salinatis (230)              | 216    | B     | 4, 12      | e, n, z15  |
| S. worthington                  | Ill. 61 | G     | 1, 13, 23, 37 | 1, w |
| S. westerstede                  | 607    | E₄    | 1, 3, 19   | 1, z13     |
| S. chittagong, ph. 2            |        |       |            | z35        |
| S. derby                         | 31     | B     | 1, 4, 5, 12| f, g       |
| S. enteritidis                   | 1891   | D     | 1, 9, 12   | g, m       |
| S. senftenberg                  | 3007   | Eᵢ    | 1, 3, 19   | g, s, t    |
| S. oranienberg                  | NTCC 6709 | C₄ | 6, 7       | m, t       |
| S. typhimurium                  | 190a   | B     | 1, 4, 5, 12| i          |
| S. rubislaw                      | Ont. 401 | F   | 11         | r          |
| S. thompson                     | 152    |        |            | k          |
| Salmonella 28-y                  |        |       |            |            |
| Salmonella 13, 23, 36:2,-       | 433    | G     | 13, 23, 36 | z          |
| S. cerro                        | 415    | K     | 18         | z₄, z23    |
| S. diesseldorf                   | 4908   | C₄    | 6, 8       | z₄, z24    |
| S. tallahassee                   | Fla. C750 | C₄ | 6, 8       | z₄, z32    |
| S. illinois, ph. 1              | Minn. 2594 | Eᵢ | (3), (15), 34 | z10 |
| S. simsbury                      | 11077  | Eᵢ    | 1, 3, 19   | z27        |
| S. tennessee                    | KI     | C₄    | 6, 7       | z29        |
| S. weslaco                      | Watt 155 | T   | 42         | z36        |
| S. fresno                       | 2332-53 | D    | (9) 46     | z38        |
| S. quimbamba                    |        | X     | 47         | z39        |
| S. karamoja                     |        | R     | 40         | z41        |
| S. wichita, ph. 2               | 6093-53 | G    | 1, 13, 23  | z37        |
| S. bunick                       |        | U     | 43         | z42        |
| S. senftenberg                  | 2170-58 | Eᵢ    | 1, 3, 19   | z43        |
| S. quinhon                      | 660    | X     | 47         | z44        |
| S. senftenberg                  | 2282-58 | Eᵢ    | 1, 3, 19   | z45        |
| S. senftenberg                  | 840-59 | Eᵢ    | 1, 3, 19   | z46        |
| S. mikawashima, ph. 3           | 2547-60 | C₄    | 6, 7       | z47        |
| S. paratyphi B, ph. 2           | N25 3  | B     | 1, 4, 5, 12| 1, 2       |
| S. anatum, ph. 2                | FDA 3  | Eᵢ    | 3, 10      | 1, 6       |
| Salmonella 4, 5, 12:z6         | 371    | B     | 4, 5, 12   | z6         |
| S. thompson var. berlin         |        | C₄    | 6, 7       | 1, 5       |

n, x), and S. alachua (35:z4:z23). The pooled formalized antigens were injected into rabbits. The other polyvalent antisera were made by injecting rabbits with pools of formalized broth cultures of selected colonies of the salmonellae shown in Table 2. The colonies were selected for smoothness and for agglutinability with O-factor antisera. All antisera were produced in rabbits by methods recommended by Edwards and Ewing (3).

Preparation of labeled reagents. The globulin fractions of all antisera were obtained by three or more precipitations in the presence of 50% ammonium sulfate. The CDC poly H antisera contained 50% glycine. The glycine was removed before the antisera were fractionated by dialyzing first against running tap water for several hours and then against 0.85% sodium chloride overnight. The ammonium sulfate was removed by dialysis against 0.85% sodium chloride; protein concentrations were determined by the biuret method. The readings of the protein concentrations were made at 560 nm λ.

The globulins were labeled with high-quality fluorescein isothiocyanate by the method of McKinney as described by Cherry (1).
TABLE 2. Composition of the CDC polyvalent 
*OH II* conjugate

| Organism                | O antigens | O group | H antigens |
|-------------------------|------------|---------|------------|
| *Salmonella paratyphi A*| A          | B:1, 2  | a          |
| *S. typhimurium*        | B          | B:1, 4, 5, 12 | i:1, 2 |
| *S. schwarzengrund*     | B          | B:1, 4, 12, 27 | d:1, 7 |
| *S. thomson*            | C          | 6, 7    | k:1, 5     |
| *S. newport*            | C          | 6, 8    | e: h:1, 2  |
| *S. kentucky*           | C          | (8), 20 | i:z:6      |
| *S. gallinarum*         | D          | 9, 12   |             |
| *S. gatehead*           | D          | (9), 46 | s, s, t    |
| *S. anatum*             | E          | 3, 10   | e, h:1, 6  |
| *S. newington*          | E          | 3, 15   | e, h:1, 6  |
| *S. illinois*           | E          | 3, 15, 34 | z:10, 1, 5 |
| *S. senftenberg*        | E          | 1, 3, 10 | s, s, t    |
| *S. rubislaw*           | F          | 11      | r:e, n, x  |
| *S. poona*              | G          | 13, 22, 36 | z:1, 6 |
| *S. worthington*        | G          | 13, 23, 37 | z:1, w |
| *S. carrau*             | H          | 6, 14, 24 | y:1, 7 |
| *S. florida*            | H          | 6, 14, 25 | d:1, 7 |
| *S. gaminara*           | I          | 16      | d:1, 7     |
| *S. cerro*              | K          | 18      | z:4:z:23   |
| *S. minnesota*          | L          | 21      | b:e, n, x  |
| *S. alachua*            | O          | 35      | z:4:z:23   |

The poly OH I reagent was prepared by combining four parts of the CDC poly H conjugate with one part of the supplemental conjugate. CDC poly OH II was prepared by combining equal quantities of the OH pools 1 through 5. All conjugates were tested against their homologous antigens, and the staining titers were determined. The highest dilution that gave 4+ fluorescence intensity with the homologous antigens was taken as the staining titer. The routine test dilution (RTD) was half the staining titer, e.g., staining titer, 1:32; RTD, 1:16.

Preparation and staining of smears with fluorescent antibodies. Smears of pure cultures of *Salmonella* or other organisms were made either from saline suspensions or directly from nutrient broth cultures. All specimens were grown first in either selenite or tetrathionate-Brilliant Green enrichment broth. The smears were made on Teflon-coated slides prepared by the method of Goldman (8). After drying, the slides were immersed for 2 min in a fixative solution containing 60 parts of absolute ethanol, 30 parts of chloroform, and 10 parts of formaldehyde, then rinsed briefly in 95% ethanol, and allowed to air dry.

Fixed smears were stained with the labeled conjugates for 30 min at room temperature in a moist chamber. The conjugate was then drained off, and the slides were rinsed briefly with agitation in a bath of phosphate-buffered saline (PBS) at pH 7.6 and put in a bath of fresh PBS for 10 min, after which they were dipped in a bath of distilled water and allowed to drain dry. A cover glass was mounted on each slide with buffered glycerol (pH 9), and the smears were examined for fluorescing organisms.

Fluorescence equipment. A Leitz Ortholux microscope fitted with an oil immersion condenser and illuminated by either a Philips CS-150 or an Osram HBO-200 mercury vapor lamp was used in this study. The primary filter was the Schott BG-12 (3 mm), and the ocular filter was either an OG-1 or a Leitz blue-absorbing filter. The smears were examined under the 95X oil immersion fluorite objective.

Culture procedures. Specimens of foods, feeds, water, etc., were cultured by methods recommended by Galton et al. (5). Environmental swabs and water samples were inoculated directly into tetrathionate-Brilliant Green broth in field studies and transported back to the laboratory. If more than 48 hr. elapsed before they reached the laboratory, a subculture was made in which 1 ml of the initial tetrathionate broth was transferred to 9 ml of fresh tetrathionate. The latter was incubated for 24 hr before being streaked to plating media. Fecal specimens were cultured by methods recommended by Edwards and Ewing (3).

## RESULTS AND DISCUSSION

The fluorescein to protein ratio of the CDC poly I conjugate was 34 and of the CDC poly II conjugate, 36. The cellulose acetate strip electrophoresis (CASE) patterns of the two conjugates showed that the poly OH I conjugate had 69% gamma globulin, whereas the poly II had 70%. However, conjugates with F/P ratios of 25 or above show erroneously high per cent gamma by CASE.

The CDC poly OH I conjugate was titrated against *Salmonella* serotypes representing *Salmonella* O groups A through I, K, L, and O. The results given in Table 3 show that the staining with various strains varies from a 2+ intensity at the 1:2 dilution to a 4+ intensity at the 1:64 dilution.

In carefully evaluating the immunizing strains, we noted that the pooled antigen was unequal in the number of serotypes with particular O factors. This inequality is reflected in the staining titer with certain strains. For example six strains of *Salmonella* had 1, 4, 5, 12 antigen factors and none had 4, 12, 27 antigen factors in the immunizing antigen pools. The staining titer of the conjugate for *S. typhimurium* (1, 4, 5, 12) was 1:32, whereas the titer for *S. schwarzengrund* (1, 4, 12, 27) was only 1:4.

Examination of several strains of the same serotype revealed marked differences in staining intensity (Table 4). Study of five strains of *S. derby* by indirect FA with absorbed single-factor O sera for the 1, 4, 5, and 12 factors as the primary reagents and an antirabbit immunoglobulin G (IgG) conjugate showed only one
TABLE 3. Staining titers* of CDC polyvalent OH (I) conjugate

| Antigens          | Staining titer at conjugate dilution of |
|-------------------|----------------------------------------|
|                   | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
| Salmonella para-  |     |     |     |      |      |      |
| typhi A           | 4+  | 4+  | 4+  | 4+  | 3+  | 2+  |
| S. typhimurium    | 4+  | 4+  | 4+  | 4+  | 3+  | 3+  |
| S. schwarzengrund.| 4+  | 4+  | 3+  | 2+  | 1+  | -   |
| S. thompson       | 4+  | 4+  | 4+  | 4+  | 3+  | 2+  |
| S. newport        | 4+  | 3+  | 2+  | 1+  | ±   | -   |
| S. kentucky       | 2+  | 1+  | ±   | -   | -   | -   |
| S. gallinarum     | 4+  | 4+  | 3+  | 1+  | -   | -   |
| S. gatesshead     | 4+  | 3+  | 1+  | ±   | -   | -   |
| S. anatum         | 4+  | 4+  | 4+  | 4+  | 3+  | 1+  |
| S. newton         | 4+  | 4+  | 4+  | 4+  | 4+  | 4+  |
| S. illinois       | 4+  | 4+  | 4+  | 4+  | 2+  | -   |
| S. senftenberg    | 4+  | 4+  | 4+  | 4+  | 3+  | 3+  |
| S. rubislaw       | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. poona          | 3-4 | 2+  | 1+  | -   | -   | -   |
| S. worthington    | 4+  | 4+  | 4+  | 4+  | 3+  | 2+  |
| S. carrau         | 4+  | 4+  | 3+  | 2+  | 1+  | -   |
| S. florida        | 4+  | 4+  | 4+  | 4+  | 3+  | 2+  |
| S. gaminara       | 4+  | 4+  | 4+  | 4+  | 3+  | 2+  |
| S. cerrro         | 4+  | 4+  | 4+  | 4+  | 2+  | 1+  |
| S. minnesota      | 4+  | 4+  | 4+  | 4+  | 4+  | 4+  |
| S. alachua        | 4+  | 4+  | 4+  | 4+  | 4+  | 3+  |

* Staining titer is the highest dilution that gives a 4+ fluorescence intensity with the homologous antigens.

TABLE 4. Antigenic analysis of Salmonella by indirect fluorescent-antibody (FA) with absorbed single factor sera and anti-rabbit immunoglobulin G (IgG) conjugate

| Salmonella serotype | Strain | FA reactions with anti-rabbit IgG and single factor sera |
|---------------------|--------|--------------------------------------------------------|
|                     |        | 01 | 04 | 05 | 012a | 012a |
| S. derby             | 3935   | 4+ | 4+ | 1-3+| 4+  | 4+  |
|                     | 3940   |     | 4+ | 2+ | -   | -   |
|                     | 3660   | 1-2+| 2-3+| -  | -   | -   |
|                     | 4508   |     | 2-3+| -  | -   | -   |
|                     | 3897   | ±1+| 1-2+| -  | -   | -   |
| S. typhimurium      | 4830   | 4+ | 4+ | 4+ | -   | 1+  |
|                     | 5123   | 3+ | 4+ | 4+ | ±   | 1-2+|
|                     | 4399   | 4+ | 4+ | 4+ | 4+  | 2-4+|
|                     | 4956   | 2+ | 2-3+| 4+| ±   | 1-2+|
|                     | 4836   | 3+ | 4+ | 4+ | ±   | -   |

TABLE 5. Fluorescent-antibody (FA) staining titers* of CDC polyvalent OH II conjugate

| Antigens          | FA titers at conjugate dilutions of |
|-------------------|------------------------------------|
|                   | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
| Salmonella paratyphi A          | 4+  | 4+  | 4+  | 4+  | 3+  | 2+  |
| S. typhimurium                | 4+  | 4+  | 4+  | 3+  | 2+  | 1+  |
| S. schwarzengrund             | 4+  | 4+  | 3+  | 2+  | 1+  | -   |
| S. thompson                   | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. newport                    | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. kentucky                   | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. gallinarum                 | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. gatesshead                 | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. anatum                     | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. newton                     | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. illinois                   | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. senftenberg                | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. rubislaw                   | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. poona                      | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. worthington                | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. carrau                     | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. florida                    | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. gaminara                   | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. cerro                      | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. minnesota                  | 4+  | 4+  | 4+  | 3+  | 2+  | -   |
| S. alachua                    | 4+  | 4+  | 4+  | 3+  | 2+  | -   |

* Staining titer is the highest dilution that gives a 4+ fluorescence intensity with the homologous antigens.
strains showed flagellar fluorescence with the FA reagent. Arizona. Arizona strains are biochemically and serologically related to members of the Salmonella group, so FA staining was expected to occur with the Salmonella polyvalent reagent.

Thirty-six Arizona strains representing O antigens 1 through 34 were tested. The Arizona strains exhibiting 2+ or greater fluorescence are listed in Table 6. In addition to the 13 strains listed, five showed spotty fluorescence and 25 had well stained flagellae.

**Table 6. Heterologous staining of Arizona, Escherichia, and Citrobacter with CDC polyvalent OH II conjugate**

| Heterologous organisms | No. tested | O groups staining 2+ to 4+ with 1:8 dilution of CDC poly OH II conjugate |
|------------------------|------------|---------------------------------------------------------------------|
| Arizona O groups 1-34  | 36         | 01, 3; 07ab; 07ac; 012; 013; 017; 018; 020; 022; 025; 027; 028; 032 |
| E. coli O groups 1-145  | 142        | 03; 011; 017; 018ac; 044; 062; 066; 068; 070; 073; 075; 077; 085; 0106; 0111; 0127; 0135 |
| C. freundii O groups 1-31 | 33         | 021ab; 022; 023; 028 |

**Table 7. Agglutination titers with Salmonella antisera of all Escherichia coli serogroups stained with CDC polyvalent OH II conjugate**

| E. coli | Salmonella sera | Titer | E. coli | Salmonella sera | Titer |
|--------|-----------------|-------|--------|-----------------|-------|
| 03     | 1, 4, 5, 12     | 400   | 44     | 1, 2, 12        | 200   |
|        | 8, 20           | 400   |        | 6, 7            | 400   |
|        | 6, 7            | 100   |        | 6, 14, 24       | 800   |
|        | 6, 14, 24       | 400   |        | 6, 14, 25       | 800   |
|        | 6, 14, 25       | 100   |        | 18              | 100   |
|        | 3, 15, 34       | 400   |        | 13, 22          | 200   |
|        | 13, 22          | 400   | 62     | 6, 7            | 100   |
|        | 3, 10           | 400   | 21     | 400             |       |
|        | 16              | 3,200 | 66     | 6, 7            | 200   |
| 011    | 1, 2, 12        | 800   |        | 6, 14, 24       | 100   |
|        | 1, 4, 5, 12     | 400   |        | 6, 14, 25       | 400   |
|        | (8), 20         | 100   |        | 18              | 1,600 |
|        | 6, 7            | 200   | 68     | (8), 20         | 100   |
|        | 6, 14, 24       | 3,200 | 21     | 100             |       |
|        | 6, 14, 25       | 3,200 | 70     | 28              | 1,600 |
|        | 18              | 1,600 | 39     | 200             |       |
|        | 1, 3, 19        | 800   | 73     | 1, 2, 12        | 400   |
|        | 13, 22          | 400   | 3, 19  | 1, 4, 5, 12     | 200   |
|        | 18              | 100   | (8), 20| 200             |       |
|        | 18              | 100   |        | 6, 7            | 800   |
|        | 16              | 100   |        | 6, 7            | 800   |
| 73     | 6, 14, 24       | 3,200 | 77     | 8, 20           | 100   |
|        | 6, 14, 25       | 1,600 | 6, 14, 24| 3,200 |
|        | 1, 3, 19        | 200   | 6, 14, 24| 3,200 |
|        | 13, 22          | 400   | 6, 14, 25| 3,200 |
|        | 13, 23          | 400   | 18     | 1,600           |       |
|        | 35              | 200   | 85     | 17              | 1,600 |
|        | 75              | 200   | 106    | 1,600           |       |
|        | 6, 7            | 200   |        | 6, 14, 24       | 800   |
|        | 6, 14, 25       | 100   |        | 6, 14, 25       | 1,600 |
|        | 3, 10           | 800   |        | 1, 2, 12        | 200   |
|        | 3, 15           | 400   | 111    | 35              | 1,600 |
|        | 3, 15, 34       | 400   | 127    | 13, 22          | 1,600 |
|        | 1, 3, 19        | 400   | 13, 23 | 1,600           |       |
| 11     | >6,400          | 135   | 1, 2, 12| 200   |
| 16     | 400             | 400   | 1, 4, 5, 12| 200 |

* Source of data: reference 11.
Citrobacter. Of the 33 strains of *C. freundii* tested, only 4 stained 2+ or better.

Providence, Serratia, Pseudomonas, Shigella, and Proteus. All strains tested stained no more than 1+ with the conjugate.

*E. coli*. A total of 142 strains of *E. coli*, representing O groups 1 through 145, were tested. Of these, 17 were stained 2 to 4+ by the conjugate. These results are shown in Table 6. Several investigators have studied the antigenic relationships between *E. coli* and *Salmonella* extensively. Table 7 shows the titers Kampelmacher (11) obtained when he performed tube agglutination tests with *Salmonella* antibodies and *E. coli* antigens of the same O groups stained by FA in this study. Note that *E. coli* 073 reacts with 10 different *Salmonella* O sera, and *E. coli* 017 reacts with nine *Salmonella* O sera with titers ranging from 1:200 to 1:3,200.

Experiments by Kampelmacher (11) showed that absorption of *Salmonella* O serum 6, 14, 24 by *E. coli* 017, 066, 073, or 077 reduced the homologous agglutination titers from 8- to 32-fold. *Salmonella* 1, 6, 14, 25 O serum absorbed by *E. coli* 017, 077, or 0106 was reduced in titer from four- to eightfold. *Salmonella* 011 and *E. coli* 075 were found to be almost identical in antigen composition as were *Salmonella* 035 and *E. coli* 0111. These studies point up the futility of attempting to render *Salmonella* sera free of *E. coli* agglutinins by absorption procedures.

In 1969, Julian et al. (10) demonstrated that IgG is the major immunoglobulin reactive with *Treponema pallidum* in all fluorescent treponemal antibody tests. To determine the degree of cross-reactivity of the major immunoglobulins present in the *Salmonella* polyvalent conjugate, a 6-ml portion was separated by zonal centrifugation into IgG and IgM fractions. The fractions of each immunoglobulin class were pooled, and the protein was precipitated with 50% ammonium sulfate. The precipitates were collected on membrane filters (Millipore Corp.), washed off in PBS (pH 7.6), and dialyzed in PBS to remove the sulfate. The final volume of each immunoglobulin was adjusted to that of the conjugate before separation. Both the IgG and the IgM were tested against pure cultures of *Salmonella*. The IgG fraction was found to have the same staining titer (1:8) with the homologous *Salmonella*, but only 11 of the 21 *Salmonella* were stained as much as 2+ with the 1:8 dilution of the IgM. The cross-reacting *E. coli* and *Citrobacter* strains were tested with both reagents. The results are shown in Table 8. When the IgG fraction was used to stain *E. coli*, the number of cross-reactions obtained with the whole conjugate was reduced from 17 to 7. All cross-reacting *Citrobacter* strains were negative with the IgG fraction. The reactions obtained with the IgM fraction are difficult to assess because the antibody activity of this fraction rapidly deteriorates. Further studies with a stabilized solution of IgM are needed to assess the reactivity of this immunoglobulin. The IgG fraction has remained stable for several months with no loss of staining titer. As further evidence of the cross-staining activity of the IgM fraction, the whole conjugate was diluted 1:8 in 0.2 N mercaptoethanol in PBS (pH 7.6), and incubated for 1.5 to 2.0 hr in a 37 C water bath. When tested against pure cultures of *Salmonella* and the cross-reacting *E. coli* and *Citrobacter*, the fraction gave the same reduction in the cross-staining as the IgG fraction obtained by zonal centrifugation, and no loss in staining titer was noted. The mercaptoethanol treatment is a simpler process than zonal centrifugation, and the IgG obtained in this way appears to give staining activity comparable to that of the pure IgG fraction. Existing FA reagents can thus be made more specific without sophisticated separation procedures.

**Table 8. Escherichia coli and Citrobacter freundii stained with immunoglobulin G (IgG) and immunoglobulin M (IgM) fractions of CDC polyvalent OH II conjugate**

| Antigens | CDC OH II 1:8 | IgG 1:8 | IgM 1:8 |
|----------|---------------|---------|---------|
| *E. coli* | 03 | 2+ | - | ± |
| 011 | 4+ | 2+ | 2+ |
| 017 | 3+ | - | 2+ |
| 018ac | 3-4+ | - | ± |
| 044 | 4+ | - | 1+ |
| 062 | 4+ | - | ± |
| 066 | 3+ | - | ± |
| 068 | 4+ | - | 1+ |
| 070 | 3+ | - | 1+ |
| 073 | 4+ | ±-1+ | 2-3+ |
| 075 | 4+ | 3+ | 2+ |
| 077 | 4+ | 2-3+ | 1+ |
| 085 | 4+ | 2+ | - |
| 0106 | 4+ | 2+ | - |
| 0111 | 4+ | 4+ | ±-1+ |
| 0127 | 3-4+ | - | ± |
| 0135 | 3+ | - | ± |
| *C. freundii* 021ab | 2-3+ | - | ±-1+ |
| 022 | 2-3+ | - | 1+ |
| 023 | 4+ | - | 2+ |
| 028 | 2+ | - | ± |

FA and cultural examination of specimens. A total of 1,221 specimens of various types were examined by both FA and culture techniques. The results are shown in Table 9. The number of FA-positive specimens that were shown to con-
procedures ranged from feeding FA-positive specimens, confirmed by culture. The salmonellae isolated are shown in Table 10. In most cases, only one attempt was made to isolate salmonellae from the FA-positive specimens. If more stringent isolation had been attempted, additional salmonellae probably would have been found. Goepfert et al. (7) found salmonellae in 12 of 31 samples reported negative by culture when they recultured the enrichment media. Their finding proves that cultural procedures, as routinely performed, are not sensitive enough to be used for evaluating the specificity of the FA reactions. Only by repeated culturing of FA-positive, culture-negative specimens can the true sensitivity of the FA technique be determined.

Four (0.8%) of the culture-positive specimens were reported as FA negative. This failure of the FA test was due to the presence of Salmonella cells that were not covered by the reagent but to inadequate sampling or technical error. Two of the misses occurred when the enrichment broth of swab samples taken from chicken carcasses were examined. The scarcity of organisms in these broths could account for failure to detect some salmonellae by either FA or culture.

The FA tests on most of these specimens were made directly from the primary selective enrichment broth. Smears made from specimens that were grown first in the selective enrichment broth without prior preenrichment sometimes contained part of the original specimen. Thus, interpretation was difficult because of background fluorescence. Specimens of ground meat required subculturing to a second selective enrichment to minimize nonspecific or background fluorescence. When we examined the drain cleaners which contained several enzymes, we could not demonstrate any staining of the salmonellae even though they were present in large numbers in the primary selective enrichment broths and were easily isolated. After a subculture to a second selective or tetrazolium broth, the salmonellae stained 4+ with the conjugate.

The reason for this failure is not clear, but these

### Table 9. Fluorescent-antibody and cultural results on a variety of specimens

| Specimens          | No. examined | FA+ culture+ per total no. FA+ | FA+ culture- per total FA+ | FA- culture+ per total culture+ | FA- culture- |
|---------------------|--------------|--------------------------------|---------------------------|---------------------------------|-------------|
| Human feces         | 426          | 156/180 (86.7)                 | 24/180 (13.3)             | 0/156 (0)                       | 246         |
| Environmental swabs | 225          | 125/132 (94.7)                 | 7/132 (5.3)               | 0/125 (0)                       | 93          |
| Chicken carcass swabs | 168     | 34/48 (70.8)                   | 14/48 (29.2)              | 2/36 (5.6)                      | 118         |
| Chicken feeds       | 118          | 27/44 (61.4)                   | 17/44 (38.6)              | 0/27 (0)                        | 74          |
| Animal feeds        | 56           | 39/52 (75.0)                   | 13/52 (25.0)              | 0/39 (0)                        | 4           |
| Raw meat            | 52           | 17/28 (60.7)                   | 11/28 (39.3)              | 1/18 (5.6)                      | 24          |
| Drain cleaner       | 36           | 34/36 (94.4)                   | 2/36 (5.6)                | 0/34 (0)                        | 0           |
| Eggs                | 31           | 10/12 (83.3)                   | 2/12 (16.7)               | 0/10 (0)                        | 19          |
| Bone meal           | 30           | 16/19 (84.2)                   | 3/19 (15.8)               | 1/17 (5.9)                      | 10          |
| Candy               | 22           | 3/4 (75.0)                     | 1/4 (25.0)                | 0/3 (0)                         | 18          |
| Fish meal           | 20           | 14/15 (93.3)                   | 1/15 (6.7)                | 0/14 (0)                        | 5           |
| Frog legs           | 12           | 8/12 (66.7)                    | 4/12 (33.3)               | 0/8 (0)                         | 0           |
| Milk                | 12           | 10/12 (83.3)                   | 2/12 (16.7)               | 0/10 (0)                        | 0           |
| Cheese              | 8            | 2/2 (100.0)                    | 0/2 (0)                   | 0/2 (0)                         | 6           |
| Grain               | 2            | 0                             | 0/0 (0)                   | 0/0 (0)                         | 2           |
| Chicken litter      | 2            | 1/2 (50.0)                     | 1/2 (50.0)                | 0/1 (0)                         | 0           |
| Totals              | 1,221        | 496/598 (82.9)                 | 102/598 (17.1)            | 4/500 (0.8)                     | 619         |

### Table 10. Salmonella isolated from specimens positive by FA tests

| O group | Serotype     | O group | Serotype     |
|---------|--------------|---------|--------------|
| B       | S. california | S. livingston | S. anatum   |
|         | S. typhimurium | S. holly | S. gri     |
|         | S. derby | S. macon    | S. lexington |
|         | S. schwarnie     | S. melanogaster | S. muenster |
|         | S. balear     | S. saint-paul | S. minnesota |
|         | S. infa      | S. st. paul | S. manila |
|         | S. montevideo  | S. new york | S. binza |
|         | S. oranenburg | S. new york | S. new brunswick |
|         | S. infantis | S. new york | S. new brunswick |
| C1      | S. emilbeitlar | S. new york | S. new brunswick |
|         | S. braentorfor | S. new york | S. new brunswick |
|         | S. tennessse  | S. new york | S. new brunswick |
|         | S. norwich   | S. new york | S. new brunswick |
|         | S. thompson  | S. new york | S. new brunswick |
| C2      | S. blockley | S. new york | S. new brunswick |
|         | S. kentucky  | S. new york | S. new brunswick |
|         | S. litchfield | S. new york | S. new brunswick |
|         | S. newport  | S. new york | S. new brunswick |
| D       | S. typhi     | S. new york | S. new brunswick |

The FA tests on most of these specimens were made directly from the primary selective enrichment broth. Smears made from specimens that were grown first in the selective enrichment broth without prior preenrichment sometimes contained part of the original specimen. Thus, interpretation was difficult because of background fluorescence. Specimens of ground meat required subculturing to a second selective enrichment to minimize nonspecific or background fluorescence. When we examined the drain cleaners which contained several enzymes, we could not demonstrate any staining of the salmonellae even though they were present in large numbers in the primary selective enrichment broths and were easily isolated. After a subculture to a second selective or tetrazolium broth, the salmonellae stained 4+ with the conjugate. The reason for this failure is not clear, but these...
observations point up the need to investigate optimal methods of culturing each type of specimen to obtain maximum sensitivity of FA results. Assuming that one method will prove equally effective with all specimens is unrealistic.

All of the FA-positive results reported in this study were based on the presence of peripherally stained organisms typical of salmonellae regardless of whether the organisms had stained flagellae. The presence of FA-stained flagellae attached to a fluorescing bacterial cell is additional evidence of the presence of salmonellae, but even then a positive report is presumptive since many Arizona strains possess both somatic and flagell antigens in common with salmonellae. Some investigators (4) have placed unwarranted emphasis on the importance of H antibodies because of the specificity of flagellar antibodies as reflected by the tube agglutination tests. Since the H tube agglutination test was not designed for use on mixed cultures, the specificity obtained with pure cultures cannot be compared to the results obtained when polyvalent antibodies are used on mixtures of unknown organisms. Because FA tests are usually done with mixtures of organisms, the reactivity of the reagent must be adequately assessed to evaluate the results obtained. The finding of strands of material resembling flagellae is not uncommon in smears prepared from food, feed specimens, or feces. Whether a positive report should be given on the basis of stained flagellae that are not attached to a stained cell is debatable. Goepfert et al. (7) reported the isolation of a rough Salmonella strain that did not stain with the somatic antibodies in the Spicer-Edwards pool of antibodies but did have stained flagellae. Inasmuch as the Spicer-Edwards pools of antibodies are limited in O coverage of salmonellae, the question arises as to whether the Salmonella strain isolated is in reality a serotype not covered by the somatic antibodies. In our work with E. coli, we were able to determine the O group by FA when the organisms were too rough to group by agglutination tests. Furthermore, when we selected colonies for antigen production of the Salmonella OH reagents, we picked colonies that were extremely rough in slide agglutination tests but fluoresced brilliantly when stained with CDC poly I FA reagent. When reagents, such as the Spicer-Edwards pools, containing antibodies for only 12 O groups (Table 11) are used to screen products for consumer use, results must be interpreted with caution.

Ellis and Harrington (4) have reported extensively on the use of an FA reagent prepared from the same antisera as the CDC poly OH I conjugate. Our conjugate made from this antiserum was tested and judged inadequate for reliable screening of foods and feeds. These authors state that their FA reagent used with a flazo orange counterstain is genus-specific for Salmonella with the exception of the Arizona group. This finding contradicts our experience and that of other workers. It is difficult to understand how a counterstain such as flazo orange could make a conjugate more specific. Counterstains usually depress the intensity of both specific and cross-staining fluorescence.

Since the CDC poly OH II conjugate was prepared, two companies have produced FA reagents for screening foods and feeds for the presence of salmonellae. Two of these are OH conjugates specifically designed for FA purposes; they cover all O and H antigens of Salmonella O groups A through S. A third is a polyvalent reagent designed to detect all salmonellae of O groups A through 64. These reagents have been tested and found to stain all of the homologous antigens. The cross-reactivity of the two OH conjugates is comparable to that obtained with the CDC OH conjugate. The A through 64 conjugate was more reactive with nonsalmonellae, as one would expect. Any of these reagents should be useful in screening, if the user recognizes their limitations. Before the reliability of FA results can be adequately assessed, these reagents need to be further evaluated with smears of various foods, feeds, and environmental samples.

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