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Immunofluorescence Technique for Detection of Salmonellae in Various Foods

N. F. INSALATA, S. J. SCHULTE, AND J. H. BERMAN[1]
General Foods Technical Center, Tarrytown, New York 10591

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Salmonella species have been detected in nine food varieties by use of fluorescent antibodies without false-positive or false-negative results. Test antisera were specially prepared, commercially available, conjugated polyvalent O globulin absorbed with cultures of *Escherichia coli* and *Citrobacter freundii*, and polyvalent phase II H globulin antibodies. Use of this technique permits a decrease of 24 hr in time normally required for *Salmonella* detection when compared with cultural *Salmonella* recovery methods.

During the past 24 years, salmonellosis has become an increasing problem in the United States. From the 9,680 reported outbreaks in 1962, there were 20,865 reported cases in 1965 (13, 14, 15). The total number of nonhuman *Salmonella* isolations reported has also steadily risen in this period. The foods and feeds portion of these statistics show the largest percentage increase (520 in 1963 to 1,108 in 1965) of salmonellae isolations (14, 15).

The epidemiological significance of *Salmonella* is well known. Industrial organizations testing for this organism now are compelled to use lengthy cultural methods (1, 7-9). There are definite needs for rapid microbiological test methods in *Salmonella* detection.

Coons et al. (3) introduced the fluorescent-antibody technique, facilitating identification of microorganisms. Thomason, Cherry, and Moody (12) used the fluorescent-antibody procedure to detect *Salmonella* in pure culture work. Difficulty in recovering *Salmonella* from feces due to nonspecific staining was reported in 1959 by Thomason et al. (11). Haglund et al. (6) found cross-reactions occurred while using O antibodies. However, these cross-reactions did not occur when H antibodies were used in studies on eggs. Caldwell et al. (2) reported similar results in clinical specimens. Georgala succeeded in recovering *Salmonella* from meat and poultry, but also obtained a relatively high number (14.7%) of false-positive reactions (4, 5). Silliker (10) reported some degree of correlation between the fluorescent-antibody technique and existing cultural methods.

[1] Present address: Eric Sobotkin, Columbus, Ohio 43215.

The purpose of this study was to assess the validity and reliability of the fluorescent-antibody technique in detection of *Salmonella* from various food prototypes by use of absorbed O globulin antibodies and phase II globulin H antibodies. It was felt that the use of these reagents in conjunction with one another would increase the specificity of the fluorescent-antibody technique in *Salmonella* detection.

MATERIALS AND METHODS

Since it is difficult to obtain foods naturally contaminated with *Salmonella*, a variety of food materials were inoculated with salmonellae to test recovery by use of the fluorescent-antibody technique.

Sample materials were inoculated with cultures of *S. senftenberg*, *S. anatum*, *S. typhimurium*, *Escherichia coli*, and *Erwinia carotovora* in several combinations.

Food samples were blended in a Hobart mixer and were concomitantly sprayed with the bacterial culture from an atomizer to insure a homogenous distribution of inoculum in the food. Seven food types were inoculated with *Salmonella* at a level of 10 organisms per g and with *E. coli* and *E. carotovora* at a level of 200 organisms per g. One food type (egg) was inoculated with *S. senftenberg* at a level of less than one organism per 25 g.

Each of the seven food varieties was divided into four samples before inoculation. One portion was inoculated with salmonellae; a second, with salmonellae and *E. coli*; and a third, with *E. coli* and *E. carotovora*. An uninoculated sample served as a negative control.

After inoculation, samples contaminated with *E. coli* and *E. corotovora* were stored for 1 week at 4 C before analysis. Samples containing only the *Salmonella* were stored for 3 weeks at 4 C before the fluorescent-antibody procedure was used. Variation in storage time was designed to afford maximal recovery of inoculated organisms. This was based on an

assumption that coliform organisms, which are less tenacious, would die first.

Samples (25 g) were pre-enriched at 37 C in 225 ml of lactose broth to which 1% Tergitol-7 was added (9). A 24-hr lactose broth culture (10 ml) was then selectively enriched for 24 hr at 37 C in 90 ml of selenite cystine broth (Difco). Cultures from the selenite cystine broth were used for fluorescent-antibody analysis.

Portions (10 ml) were removed from each enriched sample and centrifuged in 15-ml conical tubes at 10,000 $\times$ *g* for 15 min. The supernatant fluid was decanted, and the pellet was resuspended in 0.5 ml of fluorescent-antibody phosphate-buffered saline at *p*H 7.2 (Difco). One loopful of suspension was smeared onto each of two inscribed areas on a nonfluorescent glass slide (Trident-floro slides; Aloe Scientific, St. Louis, Mo.), and allowed to air-dry.

After drying, slides were fixed by immersing in formalized saline (1 part Formalin to 9 parts phosphate-buffered saline) for 20 min (4). After fixation, slides were immersed in phosphate-buffered saline and vigorously shaken, followed by successive washing in two baths containing absolute alcohol, a 50:50 alcohol-xylene bath, and finally a xylene bath. The slides were then air-dried (3, 4).

Flourescent stain. One smear on each slide was stained with polyvalent O antiserum, conjugated with fluorescein isothiocyanate (FITC). The antiserum used was a globulin fraction; it was prepared by cold ammonium sulfate fractionation, and absorbed with strains of *E. coli* and *Citrobacter freundii* and included O antigens 1-10, 15, 19, and VI (Sylvana Co., Millburn, N.J.). The second smear on each slide was stained with polyvalent H antisera for antigens 1, 2, 5, 6, and 7 and conjugated with FITC (Sylvana). Upon receipt of the sera, the titer was determined by a stain dilution technique. Working dilutions for the O antiserum and H antiserum were 1:4 and 1:2, respectively. Nonspecific staining was found to decrease at higher dilutions.

Slides were exposed to the conjugated antisera for 30 min in a moist chamber to prevent antisera drying. After completion of the staining period, slides were washed vigorously in phosphate-buffered saline and then passed through the baths previously described, except that the formalized-saline bath was omitted.

Once the slides were removed from the xylene bath they were air-dried and mounted in buffered glycerol, fluorescent-antibody mounting fluid (Difco) with a no. 1 cover slip. Slides were then immediately examined for the presence of *Salmonella*.

Inasmuch as this study was designed to test the validity and reliability of the fluorescent-antibody technique, all samples were tested for *Salmonella* by standard cultural methods (1, 7–9) concomitant with fluorescent staining. These methods included primary streaking on Bismuth Sulfite Agar, Brilliant Green Agar, xylose-lysine agar, and *Salmonella-Shigella* Agar, with biochemical confirmation and serological typing from positive Triple Sugar Iron slants.

Microscopy. In all experiments, dark-field microscopy was used. A Leitz Ortholux microscope, equipped with a mercury arc lamp, Osram HBO 200 high pressure bulb, numerical aperture 1.20 dark-field oil-immersion condenser and fluorite objectives, 4-mm heat-absorbing filter (BG 38), and a blue pass filter (BG 12) were used.

The following criteria were used in assessing smears for salmonellae. We considered the number of organisms fluorescing in any given field to be critical. We felt that if the majority of the cells present in a dark field were fluorescing after staining with O antiserum, the sample should be considered positive. On the other hand, the presence of any H-staining organisms was judged as positive. In addition to the number of fluorescing cells, the degree of fluorescence helped in separating positives from negatives. We considered only cells moderately to brightly fluorescing as positive. Weakly fluorescing cells were considered negative unless stained by H antiserum. If an H stain was present, it was considered to indicate strongly a positive reaction.

RESULTS

Preliminary work was concerned with fluorescent staining of pure cultures. Table 1 shows the results of the first pure culture experiments. In this work, unabsorbed whole polyvalent O antiserum was used rather than the globulin fraction used in later work.

All *Salmonella* cultures were detected (Table 1). In addition, a false positive with *E. coli* was recorded. Because of the false positive, a new antiserum was prepared which was absorbed with *E. coli* and *C. freundii* as fluorescent-positive organisms occasionally occurring in foods.

A second pure culture experiment (Table 2) shows results of tests done with polyvalent O material as well as polyvalent nonspecific H conjugate.

Absorption and globulin fractionation of the polyvalent O antiserum with the *E. coli* antigen seemed to resolve the false-positive problem first encountered with *E. coli*.

TABLE 1. *Fluorescent-antibody detection of Salmonella in pure culture*

Organism	*Salmonella* polyvalent O conjugate	Degree of fluorescence[a]
Citrobacter freundii	Negative	−
Aerobacter aerogenes	Negative	−
Salmonella typhi	Positive	4+
S. anatum	Positive	4+
Proteus vulgaris	Negative	−
S. senftenberg	Positive	3+
S. tennessee	Positive	4+
Serratia marcescens	Negative	−
Escherichia coli	Positive	3+
Shigella dysenteriae	Negative	−

[a] Symbols: 1+ = weak fluorescence; 4+ = strong fluorescence; − = no fluorescence.

Since satisfactory results were obtained with pure culture, investigation continued with the study of food prototypes. The first series of foods analyzed consisted of a chocolate drink mix suspected of contamination with *S. tennessee* at a low level. Ten samples were tested and analyzed by both the fluorescent-antibody technique and culture methods previously described. All samples tested were negative by both methods.

TABLE 2. *Effect of absorbed Salmonella antiserum globulin on cross reactions*

Organism	Absorbed *Salmonella* globulin polyvalent O antiserum[a]	Nonspecific H conjugate
C. freundii	Negative	Negative
Aerobacter aerogenes	Negative	Negative
S. typhi	4+	3+
S. anatum	4+	4+
Proteus vulgaris	Negative	Negative
S. senftenberg	4+	3+
S. tennessee	4+	4+
Serratia marcescens	Negative	Negative
Escherichia coli	Negative	Negative
Shigella dysenteriae	Negative	Negative

[a] This was absorbed with *E. coli* and *C. freundii* antigens.

TABLE 3. *Fluorescent-antibody detection of Salmonella recovered from artificially contaminated egg yolk solids vs. standard cultural methods*[a]

Sample	Absorbed polyvalent O globulin conjugate	Polyvalent H globulin conjugate	Triple Sugar Iron[b]
A	Negative	Negative	—
B	Positive	Positive	Positive
C	Positive	Positive	Positive
D	Negative	Negative	Negative
E	Negative	Negative	—
F	Positive	Positive	Positive
G	Negative	Negative	—
H	Positive	Positive	Positive
I	Negative	Negative	—
J	Negative	Negative	—
K	Negative	Negative	—
L	Positive	Positive	Positive
M	Positive	Positive	Positive
N	Positive	Positive	Positive
O	Negative	Negative	—
P	Negative	Negative	—
Q	Negative	Negative	—
R	Positive	Positive	Positive
S	Negative	Negative	—
T	Negative	Negative	—

[a] All presumptive positive cultural isolations were *S. senftenberg*.

[b] Only samples which yielded typical *Salmonella*-like colonies when streaked on selective agar plates were inoculated onto TSI slants.

A second series of samples tested consisted of egg yolk solids contaminated with *S. senftenberg* at a level of less than one organism per 25 *g*. Twenty 25-*g* samples were analyzed by pre-enriching in Lactose-Tergitol Broth and selectively enriching in Selenite-Cystine Broth after which fluorescent-antibody and cultural analyses were done (Table 3).

Two salad dressing mixes, two dessert toppings, a mashed potato mix, pet food, and gelatin dessert mix were inoculated with the organisms mentioned at the previously described levels in the final series of this investigation (Table 4). Uninoculated controls for all food types were negative by both cultural and fluorescent-antibody test methods. The cultural method was a North pre-enrichment method recommended by the U.S. Public Health Service (9).

DISCUSSION

E. coli and *E. carotovora* were chosen as organisms to be inoculated because they do fluoresce, especially *E. coli*. Also, they are organisms which occur in food.

We placed great emphasis on the presence of a good H stain. Since the H antiserum that we used covered only phase II antigens, its absence in the presence of a strong O reaction was ignored because the organism in question could be exhibiting only phase I antigens. Use of selenite cystine broth did not seem to prohibit good H staining results. This is contrary to the findings of Georgala et al., who reported that cultures inoculated in Selenite Broth demonstrated poor H stains (4).

In the egg study as in the chocolate drink study, complete correlation between fluorescent-antibody and cultural methods at its conclusion, was achieved. The fluoresent-antibody procedure produced results 48 hr earlier than those obtained culturally.

In the analysis of our uninoculated control, some yeast cells, occasionally found in the pet food, fluoresced. However, these cells were obvious because of their morphology and presented no diagnostic problem. The fluorescent-antibody technique, as modified and used here, was accurate and reliable in detection of *Salmonella*, even in the presence of concomitant microflora.

The results show a significant increase in the rapidity of *Salmonella* detection methodology with the fluorescent-antibody technique. This method represents a great improvement over

TABLE 4. *Food inoculated with Salmonella and Escherichia coli and Erwinia carotovora (storage for 2 weeks at 4 C)*

Sample	No. tested	No. of coliforms positive	No. of E. coli	No. triple sugar iron positive	No. fluorescent-antibody positive[a]	No. fluorescent-antibody negative	Confirmed[b]
Dressing mix A	4	0	0	4	4	0	*S. senftenberg*
Dressing mix B	4	4	0	4	4	0	*S. senftenberg*
Dessert topping A	4	4	0	4	4	0	*S. senftenberg*
Dessert topping B	4	4	0	4	4	0	*S. anatum*
Mashed potato mix	4	4	0	4	4	0	*S. anatum*
Pet food	4	4	0	4	4	0	*S. anatum*
Gelatin dessert	4	0	0	1	1	3	*S. anatum*

[a] Sample assessment after viewing with both O and H stained smear.
[b] Serological agglutination and biochemical tests.

cultural procedures now used in food microbiology by shortening the time required to obtain positive and negative results for the presence of *Salmonella*. The success that was achieved was accomplished with reagent antisera designed and made available to us through commercial sources. The fact that such sensitive and specific reagents are now available should lead to greater usage of the fluorescent-antibody technique. Within the limits of these data, no difficulty was encountered with false positive or false negative results. We feel that most of the early pitfalls encountered with the fluorescent-antibody technique, and described by others, have been eliminated.

This method affords industrial laboratories a saving of 1 day to arrive at negative results for the presence of *Salmonella* within 48 hr. At the completion of a fluorescent-antibody analysis, all negative samples may be discarded. Only those samples which are fluorescent-antibody positive should be confirmed by standard cultural test methods until sufficient experience is obtained to determine whether cultural methods are necessary. This would add an additional saving of time by discarding a large percentage of fluorescent-antibody negatives otherwise examined further.

Further work is in progress investigating the use of bright-field microscopy as a possible time-saver in viewing slides. In addition, we are studying the effects of shake cultures in shortening enrichment times now required. We are currently investigating naturally contaminated foods in our laboratories, and a report of this work will follow shortly. New serum is in preparation, including some O groups not included at this time.

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