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Immunofluorescent Staining of *Salmonella* Species with Flagellar Sera[1]

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Salmonellae stained with flagellar sera by either the direct or indirect fluorescent-antibody technique fluoresced at the cell surface. Investigations showed that fimbriae, flagellar material, and capsule antigens were not the participating antigens in this reaction. Cell surface staining was inhibited by mannose but was unaffected by glucose, galactose, lactose, maltose, and sucrose. Specific absorption of the flagellar sera by intact cells and purified somatic antigen showed that O antigens were the site of the surface staining. The advantages of using flagellar sera in fluorescent-antibody screening of materials for the presence of salmonellae are discussed.

The increased attention that has been focused on the problem of *Salmonella* in foods and feeds has prompted intensified research directed at the establishment of simpler and more rapid methods for the detection of these organisms. These efforts have resulted in the development of several procedures that appear to hold promise for earlier detection of salmonella organisms (11, 18, 20, 21). Although the preliminary manipulations, i.e., preenrichment and enrichment vary according to the author, each of these techniques has in common the use of serology as the definitive criterion for the presence or absence of *Salmonella* in the sample. A report in 1950 by Hajna and Damon (10) that outlined the use of a polyvalent flagellar serum as a screening tool forms the basis of most of today's suggested accelerated procedures.

In 1941 Coons et al. (2) introduced the principle of fluorescent tagging of specific antibody and the use of such antibody to detect untagged antigen. This technique has long found widespread application in the clinical laboratory. Georgala and Boothroyd (7, 8) and Haglund et al. (9) described the use of the fluorescent-antibody (FA) technique as a screening method to detect salmonellae in certain food materials. Silliker et al. (19) reported excellent correlation between the indirect FA technique and standard cultural procedures. The use of the direct technique has been described by Insalata et al. (11) and Schulte et al. (17).

Several authors (11, 19) have referred to fluorescing cells when the organisms were stained with flagellar sera. These observations were confirmed in our laboratory and led to an investigation of the site of fluorescence on the cell surface. The results of the study are reported in this paper.

MATERIALS AND METHODS

Organisms. The strains of *Salmonella typhimurium*, *S. infantis*, *S. thompson*, *S. anatum*, *S. montevideo*, *S. tennessee*, and *S. gallinarum* were obtained from the Food Research Institute collection. *S. typhimurium* 1287 fim$^+$ and fim$^-$ were obtained from J. P. Duguid, Queen's College, Dundee, Scotland.

Cultural procedures. The salmonellae were routinely grown in nutrient broth at 37 C. The working cultures were transferred daily, whereas the stock cultures were maintained on nutrient agar slants stored at room temperature and transferred at 6-month intervals. Anaerobiosis was achieved by the use of Gaspak (BBL) anaerobic generators and jars.

Antisera. The Spicer-Edwards, poly H, and poly A, B, C, D, E, and F antisera were obtained from Difco Laboratories, Detroit, Mich. The seven Spicer-Edwards sera were pooled to form a polyvalent serum that was used at a 1:500 dilution throughout this study. Similarly the poly A, B, C, D, E, and F sera were pooled and diluted 1:500. This pool is referred to as poly A-F. The poly H serum was diluted to 1:100 as directed by the manufacturer. The fluorescein-conjugated goat antirabbit globulin was obtained from Difco and diluted 1:35 for use. Fluorescein-conjugated *Salmonella* O (groups A to G) and H (antibodies to i, 1, 2, 5, 6, and 7) sera were obtained from Sylvana Co., Milburn, N.J. These sera were used at a working dilution of 1:2.

Fixation procedures. A loopful of culture was smeared within an etched circle on a precleaned Trident-fluoro slide (Aloe Scientific Co., St. Louis, Mo.). New slides were cleaned by applying a thick suspension of Bon Ami soap, allowing the soap to dry, and then

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removing it with a cloth. The smears were then fixed by one of the following procedures. (i) For Kirkpatrick's method (15), slides were immersed in an ethyl alcohol chloroform-Formalin (60:30:10) solution for 3 min; slides were washed without agitation for 4 min in ethyl alcohol (95%) and rinsed thoroughly in water. (ii) For fixation with absolute methanol, slides were immersed in absolute methanol for 5 min and air-dried before staining (16). (iii) With acetone, slides were immersed in acetone at 20 C for 1 hr and air-dried (13), and cells were formalized in a tube prior to making the smear. The smears were fixed in acetone for 5 min and air-dried (19). (iv) Slides were placed in formalized saline (9 parts saline to 1 part formalin) for 20 min. The slides were then rinsed in phosphate-buffered saline (*p*H 7.2) and washed twice in 95% ethyl alcohol, once in xylene —95% ethyl alcohol (1:1), and finally in a xylene bath. The slides were air-dried before staining (11).

FA procedures. For the direct method, one drop of conjugated O or H serum was applied to the fixed smears. The slides were incubated in a moist chamber at room temperature for 20 min, briefly rinsed with distilled water, and then washed for 5 min with agitation in 0.2 M phosphate buffer at *p*H 8.0. The slides were air-dried at 37 C prior to mounting.

Except where otherwise noted, the indirect procedure was used throughout this study. One drop of the appropriate H serum was placed on the fixed smear. The slides were incubated in a moist chamber for 30 min at room temperature. The slides were rinsed with water and washed for 5 min with agitation in 0.2 M phosphate buffer (*p*H 8.0). Excess moisture was gently blotted from the slide prior to applying a drop of the fluorescein-conjugated antirabbit globulin (goat) to the smear. The slides were incubated at 37 C for 15 min in a moist chamber, briefly rinsed with water, and washed again with the 0.2 M phosphate buffer. Excess moisture was removed by careful blotting. The slides were air-dried at 37 C.

All stained smears received a drop of Difco FA mounting fluid, a cover slip, and a drop of Leitz fluorescence-free immersion oil.

Microscopic examinations. FA stained smears were viewed through a Leitz Laborlux microscope equipped with an Osram HBO 200 mercury arc lamp, an immersion dark-field condenser with numerical aperture 1.20 and a 54 × fluorite objective. The filter system consisted of 2 exciter filters (a 2-mm UG 1 and a 4-mm BG 38) and a barrier filter (Leitz K 460). All fluorescent work was performed by using dark-field microscopy.

Preparation of purified O antigen. Salmonellae from a 24-hr APT broth culture were harvested by centrifugation and washed once with 0.85% saline. The cells were then extracted and the degraded polysaccharide was obtained as described by Luderitz et al. (14).

Absorption procedures. Absorption by purified O antigen was accomplished by adding concentrated O antigen to 30 ml each of the pooled Spicer-Edwards, poly H, and poly A-F sera and incubating at 37 C for 3 hr. The mixtures were centrifuged at 16,000 × *g* for 60 min, and the supernatant fractions were collected. Each serum was then reabsorbed five successive times with concentrated antigen. The incubation conditions for the latter five absorptions were changed to 60 min each at 32 C with agitation.

Absorptions by whole cells were performed by adding a heavy suspension of washed cells to 5-ml portions of the sera to be absorbed. The mixture was incubated at 37 C for 60 min. The cells were removed by centrifugation at 10,000 × *g* for 15 min. This procedure was repeated until the sera would no longer permit the fluorescent staining by the indirect method of the organism used to absorb the sera (usually 4 to 6 times).

RESULTS

Early in this investigation some difficulty was encountered in achieving suitable fixation of broth-grown cells to the slides for FA staining. This difficulty resulted either in complete loss of the smear or a severe reduction in the number of cells remaining on the slide after the staining and washing procedures. Five different fixation methods were evaluated with regard to efficiency of retention of cells during the staining and washing manipulations. To quantitatively measure the efficiency of the fixation procedures, a series of slides prepared in duplicate was smeared with broth-grown salmonellae and air-dried. One set of slides was heat-fixed and stained with crystal violet and served as a control. The second set of slides was fixed by the methods described above, and, after fluorescent staining, each slide was compared with the control. A quantitative estimation of the fixation efficiency was determined by counting the cells in a series of fields and comparing the counts of the cells on fluorescent-stained slides with the counts made on control set (Table 1). The method of Kirkpatrick (15) was found to yield the fewest losses and was used throughout the remainder of this work.

TABLE 1. *Efficiency of various fixation techniques for broth-grown salmonellae*

Fixation procedure	Percentage of cells retained after fluorescent staining by the indirect technique[a]
1) Ethyl alcohol-$CHCl_3$-Formalin (60:30:10), 3 min	96
2) Methanol (absolute), 5 min	23
3) Acetone, 1 hr	36
4) Acetone, 5 min[b]	8
5) Formalin-saline (9:1), 20 min	43

[a] Control was heat-fixed, crystal violet-stained cells; percentages represent the average of ten trials.

[b] Cells formalized prior to making the smear.

It should be noted that the problem encountered in fixing broth cultures of cells was probably a result of the relative solubility in the protein precipitants (fixatives) of the peptone fragments of the medium. This problem would be circumvented when proteinaceous food materials are added to broth for the preenrichment phase of a food analysis. Foods low or devoid of protein could be handled by (i) incorporating a small amount of protein, e.g. milk, in the preenrichment broth or (ii) by using a fixation procedure that efficiently fixes broth-grown salmonellae.

When smears from cultures of *Salmonella* grown in nutrient broth were stained by either the indirect technique employing pooled Spicer-Edwards sera, poly H, or pooled poly A-F sera, or by the direct technique using conjugated H serum, microscopic observation revealed that the surface of cells fluoresced in addition to or in the absence of fluorescing flagella.

Effect of mannose on fluorescence. The incorporation of mannose in the growth medium for salmonellae had several pronounced effects. The percentage of cells that could be stained with fluorescent antibody was considerably reduced. Moreover, the cells that were stained showed a greatly diminished degree of brightness (i.e., 1 to 2+). This same effect was noted whether the cells were grown in mannose broth or when mannose was incorporated just prior to making the smear. This indicated that the monosaccharide and not a metabolic by-product of the sugar was the responsible agent. The effect of mannose was concentration dependent and increased in effectiveness (of blocking fluorescent staining) with increasing concentrations from 0.01 to 0.5%. There was no effect at 0.01% or less. The age of the culture had a definitive effect on its sensitivity to mannose. Maximal sensitivity of most strains to the "mannose-effect" was shown by 6-hr cultures. The 6-hr cultures were 2 to 4 times more sensitive to the mannose effect than younger (4 hr) and older (12 to 24 hr) cultures as measured by the percentage of brightly fluorescing cells in the smears (Table 2). Under the conditions employed, cultures would show maximal fimbriae formation after 6 hr of incubation. Cells that were grown in the presence of mannose or to which mannose was added prior to making the smear could be rendered fully sensitive to fluorescent staining by washing. A single washing of the "mannose-treated" cells in phosphate-buffered saline (*p*H 7.2) was sufficient to restore the cells to receptiveness to surface staining. This was interpreted as indicating a rather loose association of the mannose with the cell surface. This inhibition was associated only with mannose, and the addition of sucrose, lactose, glucose, galactose, or maltose was without effect on the fluorescent staining of salmonellae.

TABLE 2. *Effect of age of the culture on the sensitivity of Salmonella infantis to inhibition of fluorescent staining by mannose*

Age of culture (hr)	Percentage of cells staining 3 to 4+ in presence of mannose (0.5%)[a]
0	50–60
4	90–100
6	25–35
12	50–60
24	50–60

[a] Mannose added just prior to making the smear.

Effect of anaerobiosis and growth on solid media. Duguid et al. (3) reported that fimbriae formation by salmonellae was greatly diminished or even abolished by serial transfer of the organisms on a solid medium or by anaerobic culture. Each of the salmonella strains was cultured on nutrient agar under anaerobic conditions for four successive transfers. The resulting growth was resuspended in nutrient broth and smears were made for fluorescent staining. Organisms grown in this manner fluoresced as brightly at the cell surface as did cells grown aerobically in nutrient broth. Moreover, the addition of mannose to the resuspended, anaerobically grown cultures did not affect the fluorescence of the cell surface. Electron microscopic examination confirmed the absence of fimbriae on the anaerobically grown cells and the presence of fimbriae on the cells that were grown aerobically.

Examination of fimbriaeless mutants. To positively rule out fimbriae as the substrate of fluorescent staining at the cell surface, two strains of *S. typhimurium* 1287 were obtained from J. P. Duguid. These organisms were identical except that one culture was genotypically fimbriae negative. These cultures were grown in nutrient broth under conditions that resulted in fimbriae formation by the culture possessing the genotypic capability of synthesizing fimbriae. Microcsopic examination of the two cultures grown in this manner and stained by the indirect FA method disclosed that there was no difference in the degree of fluorescence between the two cultures. The addition of mannose reduced the fluorescence of the fimbriae-bearing cells but did not alter the fluorescence of the fimbriaeless strain.

Effect of heat on fluorescent staining. Cultures of *S. typhimurium*, *S. anatum*, *S. tennessee*, *S. thompson*, *S. montevideo*, and *S. infantis* were grown in nutrient broth; the cultures were heated

TABLE 3. *Effect of somatic group absorptions on the fluorescence of cell surface of five serotypes of Salmonella*

Serotype	Somatic group	Fluorescence of cell surface when H sera were absorbed with whole cells of				
		S. typhimurium	*S. infantis*	*S. thompson*	*S. anatum*	*S. senftenberg* 775W
S. typhimurium	B	0[a]	4+	4+	4+	4+
S. infantis	C_1	4+	0	0	4+	4+
S. thompson	C_1	4+	0	0	4+	4+
S. anatum	E_1	4+	4+	4+	0	4+
S. senftenberg 775W	E_4	4+	4+	4+	4+	0

[a] 0 denotes complete lack of fluorescence

to 100 C for 90 min. Cells treated in this manner did not differ from untreated cells in intensity of fluorescent staining.

Effect of flagellaless culture. *S. gallinarium* was grown in nutrient broth and stained by the indirect method with each of the pooled H sera and the poly H serum. In each instance, the surface of the cells fluoresced brightly.

Fluorescence of a rough form of S. tennessee. During this study, a rough colony appeared on a nutrient agar plate culture of *S. tennessee*. Subsequent serological testing showed that the organism agglutinated in O groups A to I as well as in 0.85% saline. The organism was grown in nutrient broth, and smears were stained by each of the H sera by using the indirect technique. The cell surface of this organism did not fluoresce.

Absorption of sera. Poly H, pooled Spicer-Edwards, and poly A–F were divided into 5-ml portions, and each portion was absorbed with a heavy suspension of salmonellae. Each portion was absorbed only by a single somatic group of *Salmonella*. After these absorptions, the sera were tested for the ability to participate in the fluorescent staining of the absorbing group and heterologous groups (Table 3). The data show that the absorptions were group specific with respect to the somatic antigens. Absorption of the sera with a group C_1 organism removed the antibodies that were responsible for staining of group C_1 organisms but had no effect on the staining of group B, E_1, and E_4 organisms. Similarly, the other organisms were also group specific in their absorptive properties.

The possibility that K antigens were responsible for surface staining remained. If indeed K antigens were involved, the data in Table 3 would indicate that these antigens are also O-group specific. It was decided that the definitive proof would be obtained by absorbing the H sera with purified O antigen. The antigen was prepared, and the absorption was conducted (see above). Each of the H sera was subjected to six successive absorptions with the purified antigen. The initial addition of antigen to the pooled Spicer-Edwards sera resulted in heavy agglutination. This did not occur with the poly H or poly A–F. The results of these absorptions were identical to those obtained with whole cells except that the poly A–F serum was still able to effect a very dim fluorescence of homologous somatic group cells. It is likely that further absorptions would result in removal of the remaining antibodies from the poly A–F serum.

These results confirmed that the site of fluorescent staining on the cell surface by H sera in both the direct and indirect test was the somatic antigen.

DISCUSSION

The existence of O, K, H, and fimbrial antigens on or near the surface of salmonellae meant that each of these had to be considered as the site of the antibody binding that resulted in fluorescent staining of the cell surface by flagellar sera.

Staining of cells grown under conditions that were not conducive to fimbriae formation, e.g., anaerobically on nutrient agar, strongly indicated that these structures were not the antigens participating in the fluorescence. Further and more definitive evidence was obtained when a genotypically fimbriae-negative strain of *S. typhimurium* fluoresced to the same degree as a strain that was fimbriated. Mannose inhibited the fluorescence of only those cells possessing fimbriae. This is similar to the mannose-inhibition of hemagglutination by fimbriated salmonellae reported by Duguid et al. (3). These observations led to the conclusion that the effect of mannose was steric, i.e., it inhibited the fluorescent staining by combining with the fimbriae and thereby physically preventing the union of the antibody with the specific antigen beneath the fimbriae. We were also able to confirm the weak (loose) association between the mannose and fimbriae reported by Duguid et al. (4). A single washing

was sufficient to remove the mannose inhibition. In light of this inhibition, it is suggested that media containing mannose should be avoided when preparing cells or food samples for examination by the FA procedure. Although some salmonellae are able to utilize mannose and would reduce the concentration of this sugar in the medium to noninhibitory levels upon prolonged incubation, this should not be relied upon and the use of media devoid of mannose is the prudent choice.

Heating the cells to boiling for 90 min should have destroyed flagellar antigens. Cells treated in this manner fluoresced as brightly as untreated cells. Moreover, cells of *S. gallinarum* also stained brightly, thereby negating any role of flagellar substance in cell surface staining. Assuming that the heating was sufficient to remove any K antigens that may have been present [an assumption that would be valid for Vi-type antigens (5, 12)], there would be little reason to believe that K antigens took part in the staining reaction. If indeed the K antigens were involved, they would have to be O-group specific. There is no evidence in the literature to substantiate this possibility, and rather the occurrence of Vi antigen in O groups C_1 and D would seem to refute it.

The somatic group-specific absorption that took place when whole cells were used to absorb the H sera strongly indicated that the cell wall was the site of fluorescent staining. This view was further supported by our failure to stain the rough form of *S. tennessee*. Most probably this is due to the lack of specific antibody in the H sera against the basal core portion of the cell wall. Although these antibodies (antibasal core) may be present in the sera as taken from the rabbit, they are apparently diluted out in preparation of the working dilutions of these sera.

Final proof for the participation of somatic antigens in the staining reaction was obtained by using purified O antigen to absorb the various sera. This material removed fluorescence for the specific O group and did not eliminate fluorescence of the other groups.

Several possibilities can be considered in attempting to explain the presence of somatic antibodies at a titer of 1:500 to 1:1000 (and possibly higher) in the flagellar sera. The methods of preparing the cells to be used as antigens are different, depending on whether H or O antibody production is desired. In contrast to the heating and acetone-drying treatments of cells that are to be used for stimulation of O antibody formation, cells injected into rabbits for production of flagellar antibody are Formalin-killed. Quite possibly the latter treatment does not reduce the antigenicity of the cells to the extent that acetone drying does, and this is then reflected in production of higher titer somatic antibody. Other considerations such as immunization schedule and dilution by the vendor must also be considered. Additionally, it should be mentioned that the inherent sensitivity of the indirect FA method will result in the detection of extremely low levels of O antibody. It is quite possible that the "blocking" or "incomplete" type of antibody (1) is present in the H sera. Ford and DeFalco (6) reported that the titer of "blocking" antibodies was often greater than the titer of complete antibodies in a given serum. These antibodies would react with specific antigen but would not cause an agglutination to occur. They would however be detected by the use of an antiglobulin which is inherent in the indirect FA technique.

The importance of being completely familiar with the antibody content of sera that are used for diagnostic purposes is obvious. Furthermore, it is essential that the nature of the substrate being stained in FA reactions be identified so that the complete range of antibodies that are required can be incorporated into the sera that are used.

The use of FA for screening food materials for salmonellae is being actively studied in several laboratories. One of the prime considerations is the establishment of criteria defining a positive and negative sample. It is desirable to have a method that is both specific and sensitive. The desire for specificity has led to the use of flagellar sera in the FA technique by several workers (9, 11, 19). Paradoxically, these investigators have then chosen to interpret the results on the basis of cell surface staining in the absence of flagella. The validity of this approach depends entirely on the sera that are employed. Presently, there is only a single commercial source of conjugated salmonella sera. The flagellar antigens that are covered include i and the 1 complex (1, 2, 5, 6, 7). It seems reasonable to assume that, at most, five different serotypes were used in the production of these sera. This would mean that, at best, five somatic groups would be represented. If the criterion was that the cell surface must be fluorescing, these flagellar sera (depending on which somatic groups were represented) would not seem to have the desired range of somatic antibodies upon which to base a decision regarding the absence of *Salmonella* in a given sample. Moreover, the use of these sera in conjunction with the conjugated somatic serum would merely be repetitive, since the latter contains antibodies to somatic groups A through G. One investigator (19) has reported on the use of pooled Spicer-Edwards sera in the indirect test. We found that these sera contain antibodies to somatic groups A through H. Similarly, pooled poly A–F also

covers O groups A through H and further groups 17, 18, 38, 39, 40, 45, 47, 57, and 59 (*personal communication*, A. E. Bunner, Difco). The extended coverage of these latter sera is desirable though the investigator should titer the sera against each of the O groups prior to establishing a working dilution for that lot of serum.

The indirect FA technique that uses flagellar sera (either pooled Spicer-Edwards or poly A–F sera) offers several advantages. The sera may be used in dilutions of 1:500 to 1:1000, thereby reducing the number of undesirable cross-reactions that may accompany the use of more concentrated sera. Additionally, the flagellar sera by virtue of their H and O antibody content make it possible to stain both cell wall and flagellar antigens in the same smear. The presence of fluorescing cells with attached fluorescing flagella is strong presumptive evidence for the presence of *Salmonella* (or *Arizona*) in the sample.

The indirect technique is now being used to screen several food prototypes for *Salmonella* and preliminary indications are that the method is at least as sensitive as classical procedures, and can be performed within 16 to 24 hr of taking the sample.

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