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Detection of *Salmonella* in Eggs and Egg Products With Fluorescent Antibody[1]

JOHN R. HAGLUND, JOHN C. AYRES, ALAN M. PATON[2], ALLEN A. KRAFT, AND LOYD Y. QUINN

Departments of Bacteriology and Dairy and Food Industry, Iowa State University, Ames, Iowa

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ABSTRACT

HAGLUND, JOHN R. (Iowa State University, Ames), JOHN C. AYRES, ALAN M. PATON, AND ALLEN A. KRAFT. Detection of *Salmonella* in eggs and egg products with fluorescent antibody. Appl. Microbiol. **12**:447-450. 1964.—Organisms of the genus *Salmonella* are detected in eggs and egg products within 24 hr in the presence of Pseudomonadaceae and other Enterobacteriaceae by combining selective cultural methods with fluorescent-antibody techniques. These techniques are specific for *Salmonella* when H antibodies are used. Absorption techniques are necessary before the O antibodies give specific reactions for *Salmonella*. No cross-reactions appear when H antiserum is used. Absorption and interference techniques indicate the test is specific for *Salmonella*.

Salmonellosis is one of the important food-borne infections in the world today; the United States alone reported almost 19,000 cases in 1963 (U.S. Department of Health, Education, and Welfare, 1964). Conventional methods for the isolation and identification of *Salmonella* are tedious and time-consuming. The development of a rapid and accurate test would be an invaluable aid toward controlling these organisms in dried eggs and egg products.

Coons et al. (1942) introduced the fluorescent-antibody (FA) technique and made possible a new and rapid serological method of detecting microorganisms. In principle, this technique is an immunological staining procedure which uses homologous antibody conjugated with suitable fluorochromes.

FA technique is now a common tool in microbiology and has generally been proved satisfactory. Thomason, Cherry, and Moody (1957) applied this method for the detection of several serotypes of *Salmonella* in pure culture. Later, Thomason, Cherry, and Edwards (1959) found that the value of the method for the rapid detection of *Salmonella* in feces was impaired by nonspecific staining.

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[2] Present address: Bacteriology Department, University of Aberdeen, Aberdeen, Scotland.

The purpose of the present study was to determine the usefulness of the FA technique for rapid *Salmonella* detection in dried egg products. The procedure employed combines the respective advantages of selective enrichment and the FA method. By reducing the proportion of other organisms, the incidence of undesirable cross-reactions is diminished.

MATERIALS AND METHODS

Slides were prepared by placing a drop of culture on a clean glass slide, drying, and then fixing the film in acetone for 10 min at room temperature. The slides were stained by the indirect FA technique as described by Weller and Coons (1954). The effects with either somatic O or flagellar H antibodies were determined. H antibodies were preferred because they showed no cross-staining reactions. The γ-globulins were precipitated twice with $(NH_4)_2SO_4$, redissolved in an equivalent volume of sterile physiological saline, and dialyzed for 24 hr at 2 C to remove the ammonium salt. The preparation was preserved by adding sodium ethyl mercuric thiosalicylic acid (1:10,000). To eliminate cross-staining, the globulin preparation was absorbed with four strains of *Escherichia coli* according to the method described by Nairn (1962). The antiserum, prior to globulin precipitation and absorption, was composed of group antisera in proportions designed to give a suitable titer of each. The antiserum, identified as "multiserum" to avoid confusion with polyvalent antiserum, contained antibodies against *Salmonella* antigens 1 to 10, 12, 13, 15, 19, 22, 36, 46, and V_i. The component antisera were obtained from the Communicable Disease Center, U.S. Public Health Service, Atlanta, Ga. Some commercially available sera, although useful for agglutination purposes, did not provide the desired intensity of staining reaction. The FA Goat Anti-Rabbit Globulin (GAR; Difco) was conjugated with fluorescein.

Pure cultures of known *Salmonella* were stained by the FA technique to ascertain the uniformity of results. Twenty *Salmonella* serotypes which are all common agents of salmonellosis were selected for this purpose. The positive reaction showed cells clearly defined with bright-green fluorescence. The specificity of the staining reaction was determined by absorbing the O and H antibodies and observing the reduction of fluorescence on stained slides. Films were viewed with a Reichert Zetopan research

microscope (Wm. J. Hacker Co., Inc., West Caldwell, N.J.) equipped with an Osram HBO 200 high-pressure mercury lamp for fluorescence and dark-field microscopy.

Cultures of Pseudomonadaceae and Enterobacteriaceae were examined to detect possible cross-staining reactions. Eighty-two miscellaneous bacterial cultures isolated from dried eggs were similarly checked. This procedure screened bacteria both likely and unlikely to produce cross-reactions. When cross-staining was encountered, the O antiglobulin was absorbed to eliminate this reaction. It was not found necessary to absorb the H antiglobulin.

The specificity of the staining reaction was further determined by examining mixed cultures containing various numbers of microorganisms, by replacing immune with nonimmune sera and by using sera absorbed with homologous organisms. The numbers of fluorescent organisms observed increased within the period of incubation.

Two media were used for primary enrichment of salmonellae. The first was a modification of the one described by Hurley (*unpublished data*; Table 1). Portions (80 ml) of the broth mixture were dispensed in 200-ml prescription bottles and steamed for 15 min. After cooling, 2 ml of the iodine solution were added to each portion. The second enrichment medium, prepared by dissolving 23 g of Selenite cystine broth (Difco) in 800 ml of distilled water, was dispensed in 80-ml portions and steamed for 10 min.

Portions (10 g) of dried egg were made into a paste with 20 ml of sterile distilled water containing 0.3 g of yeast extract to counteract inhibitory properties of egg albumen (Ayres and Stewart, 1947), and were used to inoculate the two enrichment media. The samples were then incubated for 8 to 16 hr at 37 C.

After incubation, three 0.1-ml portions of each culture were transferred to three 10-ml portions of H broth (Hajna and Damon 1950), modified by substituting mannitol for glucose. Mannitol was substituted after observing that FA slides prepared from cultures grown with mannitol produced a brighter fluorescence than in the presence of glucose or lactose.

The Institute of American Poultry Industries (IAPI) method (1960) and the procedure preferred by Montford and Thatcher (1961) were used as controls. The FA technique gave comparable results. Positive FA smears were confirmed by streaking enrichment cultures on Brilliant Green (BG) Agar (Difco). Resulting isolates were subjected to fermentation tests on Triple Sugar Iron (TSI) Agar (Difco) and typed with specific agglutinating sera. (A mimeographed sheet providing exact details for a step-by-step procedure is available upon request.)

TABLE 1. *Composition of tetrathionate mannitol medium*

Component	Amt
Broth mixture	
Tetrathionate Broth Base (Difco)	46.0 g
Mannitol	5.0 g
Phenol red	0.2 g
Distilled water	800 ml
Iodine solution	
Iodine	7.6 g
Potassium iodide	5.6 g
Distilled water	20.0 ml

RESULTS AND DISCUSSION

Proof of specificity of the FA test. The use of either absorbed or nonimmune serum prevented the staining of the cells (Table 2). Staining reactions occurred only when the appropriate homologous *Salmonella* antisera were used, and no effect was observed with heterologous antisera, including the conjugated GAR. In all cases where staining was not evident, cells could be detected when viewed with dark-field and tungsten light. The results indicated that the staining reaction was specific.

Cross-staining reactions. A comprehensive selection of isolates derived from samples of dried egg yolk and albumen by the IAPI method and capable of growing on BG agar was screened for cross-staining reactions (Table 3). Five *E. coli* cultures obtained from the Iowa State University Department of Bacteriology were included. Some cross-staining was experienced with somatic O antibodies but not with flagellar H antibodies. When the absorption

TABLE 2. *Fluorescent-antibody staining specificity*

Salmonella group*	Type of staining			Serum absorbed with group				
	Normal procedure	GAR only†	Nonimmune sera	B	C_1	D	E_1	Combined
B	+	−	−	−	+	+	+	−
C_1	+	−	−	+	−	+	+	−
D	+	−	−	+	+	−	+	−
E_1	+	−	−	+	+	+	−	−

* Group according to Kauffman White Schema (Kauffmann, 1961).

† GAR = FA Goat Anti-Rabbit Globulin (Difco).

TABLE 3. *Staining reactions of selected organisms*

No. of cultures	Description	Fluorescent antibody stain		Absorbed* O serum
		O	H	
20	*Salmonella* sp.	20+	20+	20+
5	*Escherichia coli*	4+	−	−
23	Gram-negative rods, *Escherichia* intermediates	8+	−	−
2	*Aerobacter aerogenes*	−	−	−
3	Gram-negative rods, *Aerobacter* sp.	1+	−	−
6	*Proteus* sp.	−	−	−
4	*Pseudomonas* sp.	−	−	−
31	Gram-positive aerobic rods, *Bacillus* sp.	−	−	−
15	Miscellaneous gram-positive rods	2+	−	−

* Absorbed against four reacting *E. coli* strains.

technique described by Nairn (1962) was used, cross-staining reactions were eliminated by twice absorbing the O antibody with a mixture of *E. coli* strains and then removing the cells by centrifugation at 25,000 × *g* for 15 min. Since this absorption was also found to eliminate the cross-reactions with the other observed contaminants, it is anticipated that, if additional cross-reactions are encountered when O serum is used, these may be removed by an extension of the absorption procedure.

Detection of Salmonella in the presence of contaminants. Enterobacteriaceae and Pseudomonadaceae are common contaminants of eggs and egg products (Florian and Trussel, 1957). When varying levels of likely contaminants were mixed with *S. thompson*, it was shown (Table 4) that these cultures did not interfere with *Salmonella* detection. For this reason, isolation of pure cultures was not considered necessary for detection of these organisms.

TABLE 4. *Effect of contaminants on detection of Salmonella*

Test	C_1 (*S. thompson*)	*Pseudomonas*	*Proteus*	*Escherichia coli*	FA results	
					O	H
1	0*	29	50	17	−	−
2	4.1	29	50	17	+	+
3	0	29	0	0	−	−
4	0	0	50	0	−	−
5	0	0	0	17	−	−
6	4.1	0	0	0	+	+

* Figures indicate number of contaminants per gram of albumen (× 100).

TABLE 5. *Recovery of Salmonella from dried egg products*

Sample	IAPI* (MPN/g)	Method of Montford and Thatcher (1961)	Fluorescent antibody†	
			O	H
Yolk 1	0.03	−	+	+
2	<0.03	−	+	+
3	<0.03	−	−	−
4	<0.03	−	−	−
5	0.091	+	+	+
6	0.061	+	+	+
7	<0.03	−	−	−
8	<0.03	−	−	−
Albumen 1	<0.03	−	−	−
2	<0.03	−	−	−
3	<0.03	−	−	−
4	<0.03	−	−	−
5	<0.03	−	−	−
6	0.036	+	+	+
7	<0.03	−	−	−
8	2.4	+	+	+
9	0.072	+	+	+
10	0.15	+	+	+
11	0.03	+	+	+
12	0.061	+	+	+

* Institute of American Poultry Industries 1960 method. Results shown as most probable number per gram.

† Results confirmed by streaking culture on Brilliant Green Agar, picking colonies to Triple Sugar Iron Agar for biochemical confirmation, and checking cultures by serological methods.

Tests on egg products. Preliminary tests on naturally contaminated egg products indicate this method will detect presence of *Salmonella* within 24 hr. One limitation of this procedure is that a viable pure culture is not available, at this stage, for further work. However, the FA tests may be readily confirmed by streaking on BG agar from the enrichment culture. Such results are in agreement with other standard tests (Table 5).

A difficulty encountered in preparing slides from broth cultures containing egg yolk was the interpretation of fluorescence on yolk particles. The specificity of this staining effect is uncertain but may be due to *Salmonella* growth within the debris. The degree of fluorescence increases with incubation time, as do the numbers of particles showing fluorescence. Large fluorescent particles may interfere with observation of bacterial cells; it is suggested, therefore, that they be removed by filtration through coarse filter paper and that slides be prepared from the filtrate. The criterion of a positive smear is the presence of stained bacterial cells; other stained particles or amorphous debris should be treated with suspicion. In all instances, positive results obtained by FA technique were confirmed by streaking the culture on BG agar and isolating typical *Salmonella* colonies. Biochemical confirmation was obtained with TSI agar and serological confirmation, with diagnostic sera.

The application of this test will depend on the general availability of absorbed sera suitably prepared for the FA technique. It is essential to know the animal source of the antisalmonella serum to ensure that the appropriate fluorescent globulin is used. With each series of tests and, in particular, when a new batch of serum is used, it is advisable to check the reactions of known *Salmonella* and also a selection of possible interfering organisms.

ADDENDUM IN PROOF

Since completion of the present study, a communication has been received from D. L. Georgala, Unilever Research Laboratory, Bedford, England, which indicates that *Salmonella* in meats can be detected with fluorescent antibody. A report of his investigation has been accepted for publication in the *Journal of Applied Bacteriology*.

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