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One-Day Fluorescent-Antibody Procedure for Detecting Salmonellae in Frozen and Dried Foods[1]

J. M. GOEPFERT, M. E. MANN, AND R. HICKS

Food Research Institute and Department of Microbiology, University of Wisconsin, Madison, Wisconsin 53706

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The indirect fluorescent-antibody technique was used to examine 422 food samples for the presence of salmonellae. A cultural phase involving a 16-hr preenrichment in buffered nutrient broth-milk medium followed by a 4- to 5-hr subculture into fresh medium of the same composition was evaluated. This procedure yielded a sufficient population of salmonellae so that no false-negative results were obtained. Of the 31 false-positives obtained, 12 samples yielded positive cultural results upon extensive subculture of the original enrichment broths. Yeast cells and both vegetative and spore forms of bacilli were observed to fluoresce when stained with anti-*Salmonella* serum. Efforts to ascertain the cause of these cross-reactions and several alternate explanations are discussed.

In the past several years, efforts to develop procedures enabling rapid detection of salmonellae in foods have intensified. Various alternate methods to the present multistep cultural procedure that requires 3 to 7 days have been proposed. Silliker et al. (18) described a rapid serological method for determining whether suspect colonies that appeared on selective plating media were salmonellae. Banwart (2) and Fung and Kraft (6) have evolved procedures employing selective motility and simultaneous biochemical determinations as presumptive tests for the presence of salmonellae in various food products. A method involving flagellar agglutination tests on mixed cultures was recently described by Sperber and Deibel (20).

The fluorescent-antibody (FA) method for the rapid detection of salmonellae in foods has been studied by many investigators. The major advantages of the FA technique are its sensitivity and rapidity. The specificity of the procedure is limited only by the antiserum which is employed, and salmonellae can be detected in mixed cultures even in the presence of large numbers of other microorganisms. Application of this immunofluorescence technique to the examination of foods was first suggested by Arkhangel'skii and Kartoshova (1) in 1962. Two years later, Georgala and Boothroyd (7) employed the FA technique to examine raw meats for the presence of *Salmonella*. Their analyses, which were completed within 18 to 24 hr after taking the sample, indicated the potential of this method. In a subsequent publication, the same authors (8) reported that the centrifugation of 18- to 24-hr enrichment broths to concentrate the organisms before staining increased the sensitivity of the test. Silliker et al. (19) reported favorable results with the FA technique for detecting *Salmonella* in various egg products. Insalata, Schulte, and Berman (12) employed centrifugation of enrichment broths and staining by the direct procedure to detect salmonellae in various foods. A similar approach for the examination of animal feeds and feed ingredients was described by Laramore and Moritz (14). Ellis and Harrington (4) described a method involving extraction with organic solvents and gradient centrifugation in which the elapsed time from sampling until microscopic examination was reduced to 9 to 12 hr. In 1969, Insalata and Sunga (Bacteriol. Proc., p. 2, 1969) reported that the preserological cultural phase could be reduced from 36 to 24 hr by agitating the cultures in a shaker-water bath during incubation.

There is need for a method of detecting salmonellae in foods that is both rapid and simple and which has sensitivity and specificity equal to or greater than the cultural method currently employed.

MATERIALS AND METHODS

Organisms. Single strains of *Salmonella* representing somatic groups B–I were employed to test the reactivity of the Spicer-Edwards antiserum in

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certain absorption experiments. Strains of *S. typhimurium*, *S. newport*, *S. rostock*, *S. london*, *S. illinois*, *S. simsbury*, *S. rubislaw*, *S. carrau*, and *S. woodstock* were obtained from N. F. Insalata, General Foods Corp. *S. gaminara* was obtained from E. Christenson, Wisconsin State Laboratory of Hygiene. *S. tennessee*, *S. montevideo*, *S. javiana*, *S. cubana*, and *S. ohio* were isolated from foods used in this study. *S. anatum*, *S. typhimurium*, *S. kentucky*, *S. manhattan*, *S. newington*, *S. illinois*, *S. rubislaw*, and *S. carrau* were from the Food Research Institute collection. *Bacillus* sp. utilized in the absorption and cross-reaction experiments were isolated from food products examined in this study.

Food samples. Naturally contaminated foods examined in these experiments were generously provided by various government agencies and food processors.

Cultural procedures. For pure cultures, working cultures of the salmonellae and bacilli were routinely grown for 24 hr at 37 C in a buffered nutrient broth-milk medium (nutrient broth, 8 g; nonfat dry milk, 10 g; KH_2PO_4, 7 g; K_2HPO_4, 11 g; $CaCO_3$, 4 g; distilled water, 1 liter; *p*H 7.4 to 7.6 after autoclaving). Stock cultures were maintained on nutrient agar slants at room temperature.

Cultural examination of food products. Nonfat dry milk, dried buttermilk, and powdered eggnog were tested by rehydrating 100 g in 1 liter of sterile distilled water containing Brilliant Green dye (1:20,000). After suspension of the powdered material, filter-sterilized trypsin was added to a final concentration of 1:10,000 (18). Frozen spinach-egg souffle, dried egg noodles, and fermented sausage were preenriched by suspending 100 g in 1 liter of the buffered nutrient broth-milk medium described above. After 16 hr of incubation at 37 C, 1 ml was transferred to 9 ml of fresh buffered nutrient broth-milk that had been prewarmed to 37 C. These subcultures were incubated for 4 to 5 hr at 37 C. Subsequently 1 ml was transferred to 9 ml selenite-cysteine broth and tetrathionate broth. The enrichment cultures were incubated at 37 C for 18 to 24 hr. A loopful of each of the enrichment cultures was streaked on a Salmonella-Shigella agar plate and a Brilliant Green-agar plate. The plates were incubated at 37 C for 18 to 24 hr. Suspicious colonies were picked and inoculated into lysine-iron-agar and Brain Heart Infusion broth. Those cultures evidencing the typical salmonellae reaction in lysine-iron-agar were confirmed by testing for O group antigens by slide agglutination and testing for H antigens by tube agglutination. Serotyping was done by E. Christenson, Wisconsin State Laboratory of Hygiene. *Bacillus* sp. were isolated from nutrient agar plates streaked with a loopful of preenrichment subculture.

Quantitation of salmonellae in foods. The most-probable-number (MPN) procedure employing triplicate samples of 100, 10, 1, and 0.1 g was used to enumerate viable salmonellae in the food products. Each sample was analyzed separately by the cultural procedure described above.

FA examination of food products. Smears for FA staining were made from the 4- to 5-hr subculture of the preenrichment material. A loopful of the subculture was spread within the etched circle of a precoated FA slide (Clay Adams, Parsippany, N.J.). These slides were precoated with 0.2% agar by placing two drops of the melted agar, cooled to 50 C, on the surface of the slide and spreading the agar over the entire surface of the slide with the side of a pipette. The slides were allowed to drain dry. A 1:10 dilution of the subcultured material was made in 0.1% peptone, and a loopful of this material was placed in the second etched circle of the slide. These smears were allowed to air-dry and then were fixed by immersion in modified Kirkpatrick's fixative (ethyl alcohol-chloroform-Formalin, 110:60:30) for 3 min and subsequent rinsing in 95% ethyl alcohol for 1 min. The slides were allowed to air-dry before staining.

Staining procedures and reagent. The indirect staining procedure was used throughout this study. A 0.02-ml amount of Spicer-Edwards pooled antisera (1:500) or the appropriate dilution of a fraction of the pooled antisera was placed on each smear, and the slides were incubated for 15 min in a moist chamber at room temperature. The slides were then briefly rinsed in 0.1 M potassium phosphate buffer (*p*H 8.0) followed by soaking for 5 min in fresh 0.1 M phosphate buffer (*p*H 8.0). After air-drying the smears, 0.02 ml of fluorescein-conjugated 7*S* goat-antirabbit globulin (Cappel Laboratories, Downington, Pa.), at the highest working dilution determined by preliminary titration, was placed on each smear and the slides were again incubated for 15 min at room temperature in a moist chamber. After this the slides were briefly rinsed in 0.1 M phosphate buffer (*p*H 8.0) and given a 5-min soak in fresh buffer. After air-drying the stained smears, a drop of mounting fluid (glycerol-0.1 M phosphate buffer, 9:1, *p*H 8.0) was placed on the smear, and a no. 1 cover slip was placed over the mounting fluid. Counterstaining, when necessary, was performed by adding one drop of an Evans Blue-rhodamine mixture [10 ml of 0.05% aqueous Evans Blue dye plus 0.1 ml of rhodamine (Difco)] to the drop of conjugate on the smear about 15 to 30 sec before the conjugate was washed from the slide in the buffer solution.

Microscopic examinations. Stained smears were examined with a Leitz Laborlux microscope by using an oil immersion dark-field condenser for all examinations. The light source was an Osram HBO 200 mercury arc lamp. A heat filter, 2-mm UGl and 4-mm BG38 exciter filters, and a K 460 barrier filter were used for all examinations. A 54× objective was used for scanning slides and a 100× objective was used in some instances to verify the presence or absence of flagella when they were not readily visible with the 54× objective. Leitz fluorescence-free immersion oil was used for all examinations.

Preparation of the globulin fraction of Spicer-Edwards antisera. The globulin fraction of the pooled sera was removed by precipitation with ammonium sulfate. One volume of Spicer-Edwards antisera was diluted with 2 volumes of 0.85% saline. Ammonium sulfate was added with constant mixing to 50% saturation at 0 C. This was allowed to stand for 30 to 60 min at 0 C, and the precipitate was removed by

centrifugation at 10,000 × *g* for 15 min in a refrigerated centrifuge. The precipitate was resuspended to the original volume in saline, diluted with 1 volume of saline, and reprecipitated by the addition of ammonium sulfate to 50% saturation. This latter procedure was repeated twice. The precipitated globulins were then resuspended in saline to the original volume (10.5 ml). The globulin component and the supernatant fluid (~12 ml) from the initial ammonium sulfate treatment were dialyzed at 4 C against three 1 liter changes of saline.

Fractionation of Spicer-Edwards globulins. Separation of 7*S* and 19*S* globulins was accomplished by column chromatography by using Sephadex G-200 (3). A 5-ml amount of globulins (10 to 15 mg of protein per ml) was applied to a column (73 by 2.5 cm) of Sephadex G-200 and eluted with 0.85% saline solution containing 0.02% sodium azide as a bacteriostatic agent. Column flow rate was approximately 30 ml per hr. Portions (5 ml) of the eluant were collected with a fraction collector.

Absorption of sera. Absorption of Spicer-Edwards antisera with cross-reacting organisms was accomplished by resuspending a dense suspension of washed cells and spores of cross-reacting organisms in 5 ml of pooled, untreated antiserum, incubating for 1 hr at 37 C, and centrifuging at 10,000 × *g* for 15 min. Absorption was repeated until the cross-reacting organisms could no longer be stained with the absorbed antisera.

Protein determinations. Protein concentrations of untreated and fractionated antisera were determined by measuring the absorption at 260 and 280 nm in a Beckman spectrophotometer.

RESULTS AND DISCUSSION

The purpose of these studies was to develop a sensitive and specific method for the detection of salmonellae in dried and frozen food products that would be completed within 24 hr of the initiation of the analyses. It was decided to employ reagents and media that were commercially available, since evaluation and application of this technique by industrial and commercial laboratories could be possible only if this were the case.

Preliminary trials with several "naturally contaminated" foods indicated that a 16-hr preenrichment period alone was sufficient to permit detection of salmonellae in these foods. However, certain problems were encountered that suggested that a single 16-hr preenrichment phase was somewhat less than optimum. First, many smears that were judged positive contained only one or very few fluorescing cells in all of the fields examined. Consequently, it was feared that eventually some smears would be counted as negative because the minimum number of salmonellae necessary for microscopic detection might not be generated in the 16-hr period. A greater problem however was the examination of the smears made directly from the preenrichment broth of certain food products. The most troublesome foods were the dairy products. Smears made from the 16-hr preenrichment were too thick, and location and interpretation of fluorescing bacteria were not possible. Usually, in these cases, the application of a counterstain offered little or no benefit. Thus, it was necessary to dilute the preenrichment before making the smear. However, dilution only enhanced the possibility of missing low numbers of salmonellae present in the preenrichment. Because of this, it was deemed desirable to transfer a sample from the initial preenrichment broth to a second medium. This step would not only effect a dilution of the interfering food material but, also upon incubation, would allow further proliferation and antigen synthesis by the salmonellae. Several choices of media and incubation periods were considered. The medium selected was the buffered nutrient broth-milk used for the initial preenrichment phase. This choice of this medium was governed largely by the coverage of the serological reagents employed in this study. Previously, Goepfert and Hicks (10) demonstrated that Spicer-Edwards sera contained antibodies against both the O and H antigens of salmonellae, and the advantages of using an OH serum were discussed at that time. To take advantage of the benefits of OH staining, it is essential that the cells evidence good flagellar development. It is generally accepted that flagellar synthesis by *Salmonella* in selenite and tetrathionate broths is less than optimum and in some cases does not occur at all. Consequently, use of these media would negate the advantages of the Spicer-Edwards sera. The use of M broth (20) was also considered. Although flagellar synthesis is stimulated in this medium, the mannose could adversely affect somatic staining (10). On the other hand, the buffered nutrient broth-milk medium stimulated flagellar synthesis and maintained the *p*H within a range favorable for this synthesis (R. L. Hicks, M.S. Thesis, Univ. of Wisconsin, 1969).

It was experimentally determined that 2 hr of incubation at 37 C in the fresh buffered medium was necessary to restore the salmonellae to the original population level that existed in the initial preenrichment broth before making the transfer. Since one of the objectives of the study was the development of a 24-hr procedure, the upper limit on incubation in the second preenrichment was set at 7 hr. Further study showed that in some cases a diminution in number of viable salmonellae occurred at 6 to 7 hr of incubation. Although dead salmonellae would be detected, they may not remain flagellated, and a compromise incuba-

tion period of 4 to 5 hr was found to be satisfactory and was used throughout this study. Although it is conceivable that nonsalmonellae may proliferate to the exclusion of salmonellae in the second preenrichment, the absence of false-negatives in this study indicated that either this did not occur or that there were sufficient salmonellae in the initial preenrichment to be detected after the 1:10 dilution.

One of the most important aspects of the FA procedure is the establishment of the determinative criteria to be employed. This is governed by the reagents being used as well as prior experience with pure cultures and given food materials. Since many of the investigators in the field have used different reagents and staining procedures, it was not considered prudent to employ their criteria. Our experience with pure cultures had taught us that all of the salmonellae in a given population did not necessarily fluoresce to the same degree of brightness. This was true for both somatic and flagellar staining. Based on these observations, it was decided that the presence of a single organism (of proper morphology) fluorescing at a 3 to 4+ level was sufficient to interpret the smear as positive for salmonellae. Even though the following situation was not encountered, it is conceivable that a smear having numerous cells fluorescing 2+ (but no 3 to 4+ cells) could be visualized. In such a case, continued cultural analysis would have to be employed to make a final determination with any degree of certainty. We did not encounter any smears that contained 1+ cells only that were positive in cultural examination. Although most (~70%) of the smears containing salmonellae had flagellated cells, some smears did not contain flagella-bearing cells. Thus, the necessity for fluorescing cells to have attached fluorescing flagella to be considered salmonellae is too rigorous a criterion to be implemented at present. The presence of flagellated cells lends considerable confidence to the judgment of positivity, and in one instance in this study the benefit of using an OH serum was underscored. In that instance, a smear that contained flagellated cells whose flagella, but not the cell wall, were fluorescing was observed. Cultural analysis yielded a rough strain of *Salmonella*. Thus, the use of the OH serum resulted in the detection of a positive sample that would have been considered negative if the serum contained antibodies against only the somatic antigens.

In our experience and in that of others (D. L. Georgala, *personal communication*), most flagellated cells are observed in the perimeter of the smear. Therefore, it is necessary to include several sections of this area of the smear in the microscopic examination.

The results of the cultural and FA examination of 422 food samples are shown in Table 1. There was exact agreement on 403 of the 422 samples. In no case was a sample that was positive by the cultural procedure found negative by the FA procedure. However, 31 false-positive, i.e., FA-positive–cultural-negative samples were encountered. In each case, the enrichment media were saved and additional efforts (i.e., subculture to fresh enrichment media for incubation at 37 and 43 C before restreaking) were made to isolate salmonellae. These efforts were successful in 12 of the 31

TABLE 1. *Results of cultural and fluorescent-antibody (FA) determinations of salmonellae in selected food products*

Food material	No. of samples	MPN[a] range per g	FA-positive/cultural-positive	FA-negative/cultural-negative	FA-postive/cultural-negative	FA-negative/cultural-positive	*Salmonella* species isolated
Spinach-egg souffle	36	2.3–21	22	6	8	0	*S. montevideo*
Nonfat dry milk	154	0.003–0.023	32	114	8	0	*S. cubana, S. tennessee, S. bredeney, Salmonella* sp. (rough)
Nonfat dry milk	33	ND[b]	18	13	2	0	*S. bredeney, S. minnesota, S. tennessee, S. montevideo*
Dried buttermilk	27	ND	11	16	0	0	*S. tennessee*
Dried eggnog	6		0	6	0	0	
Egg noodles	94	0.093–240	61	32	1	0	*S. ohio* and group C_2 *Salmonella*
Thuringer sausage	72	0.93–4.6	42	30	0	0	*S. typhimurium*
Totals	422		186	217	19	0	

[a] Most probable number.
[b] Not determined.

samples. It is impossible to say whether more rigorous treatment of these enrichment broths would have yielded additional cultural positives, but these data indicate that the FA procedure is more sensitive than the standard cultural procedure. This observation has also been made by other investigators (5, 11).

The absence of false-negatives was not only encouraging but fortuitous. False-negative results would be expected were any of the food samples contaminated with salmonellae of somatic or flagellar groups not covered by the sera. These data support the contention that 20 to 21 hr of incubation in a noninhibitory medium is sufficient to generate enough cells to be detected microscopically. Naturally, these observations need further substantiation, but the implications are obvious.

Frequently during the course of the examination of the stained food preenrichment smears, various organisms other than salmonellae were observed to be fluorescing. Although, in most cases, the morphology of these organisms precluded their being identified as salmonellae, their presence was often distracting and caused some inconvenience in interpreting the slides. The organisms observed to be fluorescing were most often yeast cells and both vegetative cells and spores of the genus *Bacillus*. Most commonly the yeast cells were observed to fluoresce at a 2 to 3+ level. Vegetative forms of *Bacillus* sp. fluoresced dimly in some cases, but it was not rare to encounter some cells fluorescing at a 4+ level. Spores fluoresced from 1 to 4+, and the morphology of these forms often resembled the typical short-rod appearance of salmonellae. Differentiation was accomplished by examining the field under dark-field for the characteristic refractility of the spore.

There are several possible explanations for the nonspecific staining of yeast and bacilli. First, it is conceivable that the rabbits in which the anti-*Salmonella* serum was prepared were exposed to these nonsalmonellae and had developed antibodies against them. This seems unlikely, however, since several lots of sera were employed in this study and in each case the staining titer for the cross-reacting organisms was always equal to the titer for salmonellae (e.g., $\geq 1:500$). It is also conceivable that the yeast and bacilli possess a surface antigen identical or closely related to the somatic or flagellar antigens of salmonellae. Recently, Gettner and Rezai (9) reported that *Candida albicans* agglutinated in serum prepared against group C_1 salmonellae. When group C_1 serum was absorbed with *C. albicans*, part of the reactivity for salmonellae was removed. When group C_1 salmonellae were used to absorb serum prepared against *C. albicans*, the absorbed serum no longer agglutinated the yeast cells. Another consideration was that the nonspecific serological reaction involved a component of the Spicer-Edwards serum other than the globulins. A fourth possibility was that these cells were reacting nonspecifically with a globulin (antibody to *Salmonella*) in the Spicer-Edwards serum. Nickerson et al. (15) recently reported this type of interaction between the protein A of *Staphylococcus aureus* and F_c portion of 7*S* globulins of human myeloma or hyperimmune rabbit sera.

Because a better understanding of the reactivity of the reagents would bring about more efficient utilization of the procedure, an investigation into the basis of the nonspecific staining was undertaken.

Pooled Spicer-Edwards serum was fractionated by $(NH_4)_2SO_4$ precipitation into globulin-containing and globulin-free components. After removal of the excess $(NH_4)_2SO_4$, each component was tested for its ability to participate in the fluorescent staining of *Bacillus* sp. isolated from the food products examined in this study. Fluorescent staining was obtained only when the globulin fraction was employed.

Having established that the cross-reactions were attributed to the antibody-containing fraction of the serum, a series of experiments involving absorption of Spicer-Edwards serum with a mixture of spores and vegetative forms of the *Bacillus* sp. was undertaken. After absorption (*see above*) the serum was tested for its ability to stain salmonellae of somatic groups B to I. Absorption reduced the intensity of fluorescence of *Salmonella* somatic groups C_2, E_1, F, and G.

In certain cases, intensity of flagellar fluorescence was also reduced. Attempts to stain *Bacillus* sp. with *Salmonella* O grouping sera corresponding to those O groups whose titer was reduced by absorption have not been successful.

Several investigator (A. L. Markovits, *personal communication;* B. Thomason, *personal communication*) have reported that the 19*S* component of anti-*Salmonella* globulin is responsible for nonspecifically staining enteric organisms other than salmonellae. To determine whether the macroglobulin fraction is involved in staining bacilli, samples of the serum were heated to 65 C for 60 min (16) or treated with mercaptoethanol (0.1 M, 1 hr, 37 C) to destroy the 19*S* material before testing its ability to stain the bacilli. In all cases, each treated fraction was able to stain the bacilli, but the intensity of staining was reduced in comparison to the untreated control sera. Finally, the 7*S* and 19*S* globulins were separated by column chromatography on Sephadex G-200 (Fig. 1). Tubes 16 to 18 were taken to represent 19*S* material and 24 to 26 were used as 7*S* globu-

lins. Each fraction was tested for staining of *Bacillus* sp. and a *Salmonella* control culture. Brightest staining of bacilli and salmonellae was observed when the 7*S* fraction was employed. However, certain strains of *Bacillus* sp. were stained by the 19*S* material. The *Salmonella* culture fluoresced 4+ when the 7*S* component was used but only 2+ when the 19*S* fraction was employed. These data also point out that the 7*S* goat-antirabbit conjugate is capable of reacting not only with the 7*S* component of rabbit globulin (for which it is advertised to be specific) but also with 19*S* globulins.

It is not possible from these data to determine whether antigens common to salmonellae also occur in *Bacillus* sp. or whether a phenomenon similar to that described by Nickerson et al. (15) is responsible for the fluorescence of bacilli when anti-*Salmonella* serum is employed. Further investigation into this problem is in progress.

Since we cannot explain this phenomenon at present, investigations into the possibility of precluding the *Bacillus* sp. problem by means other than treatment of the serological reagents have been initiated. In 1959, Jeffries (13) reported on the use of novobiocin in the enrichment medium to suppress the growth of undesirable enteric organisms. Novobiocin is also known to be active against various species of *Bacillus*. In a very limited number of trials to date, we have found that the addition of novobiocin (generously supplied by Dr. E. Jensen, Upjohn Co., Kalamazoo, Mich.) at 10 μg/ml to the initial preenrichment broth is very effective in reducing the proliferation of bacilli, with the result that the smears became easier to scan and interpret. We have not noted any adverse effect on growth or flagellar synthesis by salmonellae in the presence of novobiocin. Additional work is necessary before definitive statements can be made about the value of this antibiotic in salmonella methodology.

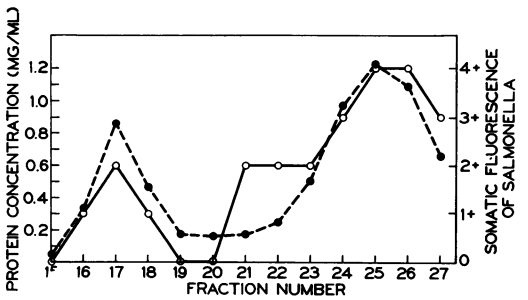

FIG. 1. *Separation of 7S and 19S components of the globulin fraction of Spicer-Edwards serum on a Sephadex G-200 column (73 by 2.5 cm). Symbols: ●, protein concentration; ○, somatic staining of salmonellae. Column flow rate was approximately 30 ml/hr. Five-milliliter fractions were collected.*

The data accumulated in this study strongly indicate that a procedure capable of detecting the presence of low levels of *Salmonella* sp. in food products within a 24-hr period is possible. It certainly would not be scientifically sound to recommend widespread adoption of this or any method solely on the basis of 422 samples examined in one laboratory. However, the indications are that certain manipulations such as agitated incubation, centrifugation of enrichment cultures, and pretreatment of sera with reducing agents, all of which require either additional equipment or reagents, are not essential. The study also points up the rather shallow understanding of the reagents that are employed and some of the interactions associated with these reagents that we cannot explain currently.

We are hopeful that research will resolve some of the complexities encountered and that other laboratories will undertake to evaluate the cultural and serological approach described above so that the rapidity, sensitivity, and specificity of this approach may be scientifically measured.

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