Detection of salmonellae in whole egg

Barbara Jean Pincomb

The University of Montana

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DETECTION OF SALMONELLAE IN WHOLE EGG

by

Barbara Pincomb

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Approved by:

[Signature]
Chairman, Board of Examiners

[Signature]
Dean, Graduate School

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A direct fluorescent antibody (FA) procedure was experimentally developed for the rapid detection of salmonellae in frozen whole egg. Currently accepted detection methods are time-consuming (5-7 days), contain many laborious procedural stages, and often produce ambiguous results. Based on reactions characteristic of the salmonellae, the conventional procedural sequence is capable of detecting as few as one Salmonella organism per 25-g sample of egg. Significant increases in sensitivity and specificity of salmonellae recovery were observed by increasing the whole egg sample size, concentrating the selective enrichment, and incorporating a suitable FA procedure in the experimental system.

Whole egg samples (100 g), tested negative by the standard cultural method, were inoculated with as few as two salmonellae and blended. The contaminated samples were then blended and enriched in 100 ml double-strength Selenite-F. After incubation for 7 hours at 37°C, smears were heat-fixed, incubated with Bacto-FA Salmonella Poly A-S (Difco), washed in 0.1M potassium phosphate buffer, and blotted dry. Upon fluorescent microscopic examination, fluorescent Salmonella cells were readily seen.

Two alternative procedures were employed as control features. Samples from the same selectively-enriched cultures were (1) plated on Millipore filters on brilliant green agar, and (2) suspended in Selenite-F. After appropriate incubation periods, the presence of salmonellae was confirmed.

Thus, a screening procedure recommended for detection of Salmonella contamination in frozen whole egg proved to be at least as sensitive as standard cultural methods and reduced to 8 hours the minimum time necessary to complete the assay.
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Most of all, I thank my husband, Art, for keeping me happy.
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CHAPTER I
INTRODUCTION

Description of the research medium

According to the Food and Drug Administration (FDA), frozen pasteurized whole egg is defined as a semi-uniform mixture of "eggs from domesticated fowl intended for use or for sale for human consumption" (33). Processing the egg product includes large-volume delivery of shell eggs to an industrial breaking plant. Here eggs are broken and the contents are collected and pasteurized. This process involves heating the liquid egg to $140^\circ$ F for 3.5 minutes to destroy pathogenic microorganisms. After pasteurization, the liquid whole egg is immediately frozen and stored at $0^\circ$ F in 30-pound metal or plastic containers. Commercial institutions such as bakeries, ice cream manufacturers, and confectioneries are the primary purchasers of these eggs (26). Prior to delivery to and utilization by these institutions, the frozen whole egg must be inspected by government regulatory agencies and/or approved quality control laboratories for the presence of Salmonella.

Frozen whole egg has been shown to contain salmonellae
with sufficient frequency to be considered a significant source of the organisms (3, 9, 13, 14, 28). Thus, it is identified as a potential source of salmonellosis outbreaks. These eggs and the formulated foods in which they are used as ingredients can present a potential contamination hazard to the health of the consumer.

Contamination of the whole egg may occur via many routes. These include: (a) contact with wet and dirty surfaces; (b) improper storage temperature; (c) improper handling in channels of commerce; (d) unsanitary breaking plant practices; and (e) inadequate pasteurization. Most *Salmonella* infections in the whole egg come from reintroduction of the organisms into the egg after pasteurization by human beings (1). The pathogenicity of the microorganisms points to the necessity of preventing eggs containing *Salmonella* from reaching the consumer. Accordingly, it should be the function of the laboratory to use the most efficient microbiological method for the detection and isolation of salmonellae if they are present in the whole egg.

Methods employed for the detection of pathogenic *Salmonella* species in whole eggs have been continuously revised during the past two decades to improve the effectiveness of quality control surveillance. The prevailing screening procedure, accepted by the FDA, used to determine that the egg does not contain detectable levels of salmonellae
is designated as the standard cultural method. An outline of this method, as adapted from the *Bacteriological Analytical Manual* (23), is summarized in the Appendix, part A. It essentially embodies the following series of procedural steps: an initial non-selective pre-enrichment, followed in turn by, selective enrichment, plating on one or more differential isolation agars, one or more biochemical determinations, and serological confirmations. Pre-enrichment is designed to revive the microorganisms which may have survived adverse environmental conditions such as freezing, heating, or storage and would otherwise go undetected without this initial step. Selective enrichment media and agars are designed to favor the multiplication of salmonellae while at the same time restrict the growth of competing microorganisms by utilizing various concentrations of selectively inhibitory chemicals. The selective media also contain indicators which impart color changes to the surrounding medium or colonies of bacteria. Biochemical determinations are established through fermentation reactions with various sugars. Serological confirmations rely on antigenic features of the bacteria. Based on reactions characteristic of the salmonellae, this procedural sequence is capable of detecting as few as one *Salmonella* organism per 25-gram sample of the egg. Negative samples may be identified after 3 days. For suspicious or presumptive positive cultures, the minimum time necessary to complete all of these steps for positive and exclusive re-
covery of any Salmonella is 5 to 7 days.

In addition, Sperber and Deibel (29) offered an alternative accelerated procedure for use as an acceptable quality control measure termed "enrichment serology" (ES). The ES method is based on flagellar agglutination after an added elective enrichment. This method has apparently proved equally as sensitive as the standard cultural method in some laboratories and offers the added advantage of reducing the minimum amount of time for screening to 50 hours (8, 22). Hilker and Solberg (17) noted, however, that nonmotile Salmonella species are not detected by the ES method. When used, it is primarily in conjunction with dried egg products. The technique is outlined in the Appendix, part B. Recently, Boothroyd and Baird-Parker (4), in evaluating the recovery of Salmonella from various food raw materials, reported a substantial increase in numbers of Salmonella isolations if the incubation time for the food sample in "M" broth (elective enrichment) is extended from 6 to 24 hours. The percent increase in recovery was from 69% to 95% recovery, using the standard cultural method as the reference.

Thus, even though fallible, these two procedures are currently recognized as the conventional means for detection of the Salmonella in the whole egg. Disadvantages to the methods focus largely on: (1) tedious, time-consuming, and laborious stages in analysis; (2) ambiguity in interpretation
of reactions assumed to be unique to *Salmonella*; (4) lack of detection of some non-motile *Salmonella* species; (5) inconvenient waiting period for positive or negative results; and (6) the large volume and variety of costly laboratory media, reagents, and utensils necessary for testing.

Realizing these inherent problems, investigators have proposed modifications to simplify and shorten these detection procedures. Variations suggested in the cultural methods have included the use of special glassware, motility media, or additions of diversified inhibitory chemicals to certain media (2, 4, 10, 24). However, the essential problems of the accepted detection methods were not completely eliminated.

**Review of Literature**

A fast and simple modification which with further research may finally prove to be the most satisfactory for isolation and detection of *Salmonella* is the fluorescent antibody (FA) technique. The technique has already proved to be a valuable diagnostic aid in the hospital laboratory clinic. Its application to the food industry is a topic currently under investigation. Beginning with its inception by Coons *et al.* (5) in 1942, various modifications have been made for screening different food products, including meats (11, 20), frozen and dried foods (7, 12, 15, 19, 22, 24, 25, 27), food byproducts (17), and animal feeds and feed
ingredients (6, 7, 16, 18, 19, 21). Indications are that the technique not only is rapid, but has sensitivity and specificity equal to or greater than established cultural methods.

Georgala and Boothroyd (11) concluded the FA procedure lacked precision, but could be used effectively as a rapid presumptive _Salmonella_ test. Goepfert and Insalata (13) published an excellent description, historical review, and evaluation of the advances in and problems with the use of both direct and indirect FA technique up to 1969. The majority of subsequent research with the fluorescent antibody technique has dealt with variations in staining procedures and emphasized improvements in sensitivity using the direct staining procedure (17, 18, 20, 22, 30). Basically, both the direct and indirect procedures utilize enrichment broth of one kind or another and proceed to the detection and recognition of _Salmonella_ with FA.

Immunofluorescence may prove to be a feasible system for detection of _Salmonella_ in whole egg. Haglund et al. (15) first demonstrated the potential use of the FA procedure for screening large numbers of egg samples to obtain presumptive results within 24 hours. Using the indirect method, they reported extensive cross reactions between _Salmonella_ somatic O-antisera and a variety of bacterial isolates from the egg. No cross reactions were observed later using polyvalent flagellar antisera. In addition,
the major problem encountered was non-specific fluorescein-staining debris from the egg material which prevented concise interpretation of the prepared slide. Suggestions then proposed to improve serum specificity and elimination of interfering egg particles were, respectively, absorption with antigenically related genera of the Enterobacteriaceae, and centrifugation.

Following this work, Silliker and co-workers (27) employed the indirect method to examine whole egg samples incubated 24 hours and stained with polyvalent H-antisera. They noted far greater sensitivity with their FA procedure in that egg products with as high as 2,300 (MPN determination) salmonellae per gram were negative by cultural methods. Also, most probable number determinations estimated the sensitivity of FA to vary from less than 0.3 to 30 salmonellae per gram of egg. Probably due to numerous transfer dilutions in Silliker's procedure, no non-specific fluorescence by yolk particles was reported (13).

In the investigations of Insalata et al. (19), good correlation between the direct FA and cultural method was shown though the former gave positive presumptive results 48 hours earlier than cultural methods alone. Significant data included the identification of Salmonella on a smear of egg culture artificially inoculated with less than one cell per 25 grams of liquid egg. Although this procedure incorporated centrifugation of the enrichment broth to eliminate
unwanted background fluorescence, occasional non-specific fluorescence and sample debris were again reported. Using commercially-supplied polyvalent O-(Groups A-E and H) and H (I complex)-conjugated antisera, minimal problems arose with false negative results. In spite of this, results also indicated the presence of salmonellae in samples which were culturally negative.

The direct immunofluorescence screening technique of Insalata et al. was then altered by Schulte, Witzeman and Hall (25). Not only was the time required to obtain negative results reduced to 50 hours, but, a modified polyvalent O-fluorescein-conjugated antiserum (which included Salmonella Group G antibodies) was employed to improve coverage and provide a more effective FA screen for Salmonella detection. Because the process of centrifugation broke flagellar antigens, anti-H serum was not used.

Continuing work with the 50 hour direct FA method, Fantasia (7) also found Salmonella recovery to be comparable to conventional procedures for inspecting frozen whole egg. Like Schulte et al. (25), only commercial conjugated O-antiserum (Groups A-G) was used, with greater specificity accounted for by absorption with strains of Escherichia coli and Citrobacter freundii. Even though small particles of culture sediment were carried over to the smears, he reported no problem with readability. His FA method included pre-enrich-
merit, and selective enrichment prior to staining, but eliminated many of the washing and smear treatments presented in earlier procedures. Finally, it was stated that a conjugate with broader coverage of *Salmonella* somatic groups would increase the usefulness of the method as would a decrease in pre-enrichment or enrichment time or both.

In 1971 Thomason (32) tested a polyvalent somatic and flagellar conjugate suitable for the direct FA procedure. With centrifugation discarded, Insalata et al. (18) reported 90.8% agreement with control methods when using a new commercially-supplied (Difco) polyvalent O- (Groups A-S) and H-conjugated antiserum to detect salmonellae in 18 different naturally contaminated foods, none of which were whole eggs. The Sylvana commercial somatic conjugate used by Fantasia (7) gave only 86.2% agreement with cultural methods.

In 1974, Mohr and co-workers (22) reported the use of a 54-hour direct fluorescent antibody procedure, now termed "conventional FA," which employed Difco FA *Salmonella* Poly antiserum. Although frozen whole egg was not examined, whole egg powder was. Their FA procedure was compared with ES and cultural methods for detection of *Salmonella* in food prototypes. Conventional FA identified all culturally positive samples but due to "excessive false positive" results, the ES procedure was their method of choice.
Thus, a suitable direct immunofluorescence test has not been developed for routine surveillance of frozen whole egg. The FA technique is fast and simple. However, in applying this method to whole egg, certain criteria must be taken into consideration. First, salmonellae have a very inconsistent distribution in whole egg. Since only a few organisms in the egg can multiply to thousands under certain circumstances and thereby produce a potential health hazard, the method of analysis must be specific for and sensitive to that small nonuniform population. The sensitivity, i.e. the smallest number of salmonellae capable of being detected, is directly expressed in terms of the size of the egg sample taken. The opportunity of detecting salmonellae would increase with an increase in the quantity of material being examined.

Second, should the small contaminating Salmonella population survive the heating and freezing process, they may be somewhat debilitated and incapable of growth without sufficient metabolites for proliferation (30). Incubation in an appropriate enrichment broth is a necessity in procedure. Since salmonellae are commonly present in egg in association with other members of the Enterobacteriaceae, the enrichment should be selective in nature to inhibit the growth and influence of these unwanted microorganisms which may mask the exclusive recovery and identification of the salmonellae.
Next, profitable use of the FA technique is possible only if the demonstrable results are reliable, reproducible, and rapid. Rapid test results for egg samples, representing greater quantities of the produce, would prove advantageous to both producer and consumer by reducing the time and cost of storing the product. Early test results would also mean that a large number of egg samples could be tested during a normal 40-hour work schedule.

Finally, results of previous research show that revision in FA slide preparation and treatment is needed. Non-specific fluorescing egg particles interfere with interpretation. In addition, the specificity and availability of the conjugated antisera applied are very critical features of the test.

**Statement of the problem**

The goal of this study was to determine if a direct fluorescent antibody procedure could be developed to improve quality control analyses for *Salmonella* infection in frozen whole egg. Previous investigators demonstrated the direct FA technique to be a method which improved sensitivity and specificity of detection of the bacteria in various foods. Application of the technique to the examination of frozen whole egg necessitated proposing modifications in existing methods for preparation and culture of the samples, and in treatment of the smears for examination by FA.
Consequently, the objectives of this present research were divided into three phases. The first phase involved the establishment of the cultural isolation media to be employed for optimal recovery of salmonellae from the artificially inoculated egg. It was also of prime interest to determine the usefulness of Millipore filters in the recovery procedures.

The second phase was designed to develop a method capable of improving current levels of detection of salmonellae in whole egg. The influence of variations in egg sample size, size of inoculum, and incubation times was correlated with recovery incorporating a direct FA procedure.

The third phase was an analysis of methods to enhance and facilitate FA smear interpretation.

In conclusion, this study was undertaken to devise an improved salmonellae detection method that could be suitable for an 8-hour work day schedule.
CHAPTER II
MATERIALS AND METHODS

General Materials and Methods

Organisms employed

*Salmonella typhimurium*, type T2, was used throughout this investigation. Several studies also included *Salmonella enteritidis*, *Escherichia coli*, *Shigella flexneri*, *Proteus vulgaris*, *P. mirabilis*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Arizona hinshawii*. These organisms were obtained from Dr. George Card, Dr. Jon Rudbach, and Dr. John J. Taylor, Microbiology Department, University of Montana. All cultures were originally grown in trypticase soy broth and subsequently transferred to trypticase soy agar slants. The cultures were stored at 4°C and periodically were tested for cultural purity.

Frozen whole egg material

Frozen whole eggs were generously supplied by the University of Montana Food Service, Bakery Department. Collection of the egg samples was accomplished by opening a 30-pound can of frozen egg, taking precautions to prevent dust or extraneous matter from entering. As the frozen material
was removed in small slivers from the top layers of the can by means of a sterilized chisel, it was transferred to a sterile, wide-mouthed, plastic screw-capped jar. The egg was kept solidly frozen at all times at -20°C until needed for use in the investigation.

Media and Reagents Employed

All media used were commercially available from Difco, Detroit, Michigan, and Baltimore Biological Laboratories.

0.85% physiological saline, was prepared by adding 8.5 g reagent grade sodium chloride to 1000 ml distilled water, adjusting the pH to 6.8 with 1 N HCl or 1 N NaOH, and then autoclaving for 15 minutes at 15 pounds pressure (121°C).

0.1M Potassium Phosphate Buffer, pH 8.0. 5.3 ml of monobasic K$_2$HPO$_4$ (0.2M) and 94.7 ml of dibasic KH$_2$PO$_4$ (0.2M) stock solutions were diluted to 200 ml volume in distilled water. The buffer was stored at 4°C.

Normal rabbit serum was supplied by Mrs. SuRang Tantivanich, Department of Microbiology, University of Montana.
Salmonella (O) group antiserum pools A, B\textsubscript{4-5}, C\textsubscript{1}, C\textsubscript{2}, D, E\textsubscript{12}, 34, F, G, H, and L, supplied by Mr. Milton Hazelhurst Brown, Montana State Department of Health and Environmental Sciences, Helena, MT.

Difco Bacto FA Salmonella Poly antiserum representing antibodies to somatic groups A-S, factors 1-25, 27, 28, 30, 34 through 41, 45, and Vi. Also the conjugate included flagellar spectrum a through i, k through n, p, r through u, w through z, z\textsubscript{4}, z6, z10, z13, z15, z23, z24, z27, z28, z29, z32, z35, z38, z42, 1, 2, 5, 6, and 7. The antiserum was prepared for use by rehydrating with distilled water and was stored at -20° C.

Control Procedures for whole egg material

The standard cultural method for detection of salmonellae as outlined in the Appendix, part A, was employed as the control procedure for screening the frozen whole egg material. Thus, egg material producing negative results by the cultural method was considered appropriate for use in this investigation.

In addition, standard plate counts were performed on a 10-g sample representative of each 30-pound can from which the whole egg was taken. The sample was diluted 1:10.
After being vigorously shaken, ten ml of this suspension were pipetted into a second 90-ml dilution bottle. Four more serial decimal 1:10 dilutions were performed in this manner. One ml of the 1:10,000 dilution was transferred to a Petri plate, and plate count agar, tempered to 50°C was poured over the 1 ml aliquot. This pour plate process was repeated for the 1:100,000 and 1:1,000,000 egg dilutions. Each dilution was plated in triplicate. The plates were incubated for 48 hours at 37°C. After incubation, the number of colonies on each plate was counted and multiplied by the reciprocal of the corresponding dilution factor in order to obtain cell counts. According to the Food and Drug Administration, if the standard plate count is less than 10,000 per 10-gram sample the whole egg is considered "clean."

Detailed Methods and Materials

The Standard inoculum

For each experiment, a population of Salmonella cells was grown to contaminate artificially the whole egg. A 2 mm loopful of S. typhimurium from the stock culture slant was transferred to 100-ml trypticase soy broth. The freshly inoculated broth was incubated 24 hours at 37°C. After incubation, the cell suspension was serially diluted in sterile physiological saline. The dilution tubes were shaken with a Vari-Whirl mixer to distribute the inoculum evenly. One-tenth ml of each dilution was pipetted into Petri dishes
which were then poured with total plate count agar. Plates were prepared in triplicate. Preparatory experiments indicated those dilutions which were the most suitable for plating to produce reproducible colony counts. The plates were incubated at 37°C for 48 hours after which colonies on each plate were counted, with the aid of a darkfield Quebec colony counter. The number of viable colony forming units in the *Salmonella* culture was determined by multiplying the number of colonies by the reciprocal of the dilution factor. An average of the counts was taken and designated as the *Salmonella* population in the standard inoculum for each experiment performed.

**Preparation, inoculation, and homogenization of the whole egg**

The frozen whole egg was thawed as rapidly as possible by immersing the plastic storage jar which contained the egg in 55°C water. The jar was vigorously shaken at intervals to hasten the process. In preliminary investigations, only 10-g volumes of the liquid egg were inoculated with *Salmonella* cells and then subjected to "homogenization" using the Vari-Whirl mixer. In succeeding studies, 100-g volumes of egg were inoculated. Determinations were then made on the best method to use to homogenize this larger volume. Ultimately, the thawed egg material was aseptically pipetted into a glass Waring blender jar, a suspension of *S. typhimurium* was then introduced into the jar, and the mixture was blended for one minute at low speed.
A later experiment included artificially contaminating 100 g of whole egg and storing it for 24 hours in a freezer (-20°C) and in a refrigerator (4°C) prior to enumeration of the S. typhimurium culture.

Selection of isolation media

One-tenth ml of a 24-hour culture of S. typhimurium was added to 10 g of thawed whole egg in a test tube. The inoculated egg was then serially diluted in sterile physiological saline. After each dilution was made, the tubes were shaken with the Vari-Whirl mixer. One-tenth ml aliquots of each dilution were then plated, in triplicate, on bismuth sulfite, brilliant green, MacConkey and SS agars. The inoculum on each plate was uniformly spread by means of a bent glass rod, sterilized by immersion in 70% alcohol. The plates were then incubated at 37°C for 24 hours after which they were examined for typical Salmonella colonies. The entire process was then repeated with the same differential isolation media using pour plate method.

Modified cultural and Millipore filter methods for recovery of salmonellae

One-hundred gram samples of thawed whole egg were inoculated with selected concentrations of the standard inoculum. One-hundred ml of double-strength Selenite-F broth, pre-warmed to 37°C, was added to the inoculated egg mixture. The 200-ml culture was blended for one minute and
immediately placed in the 37° C water bath. After varied incubation periods, the mixture was removed from the water bath and blended for one minute. The contents were allowed to settle. Separate tubes containing 9.9 ml Selenite-F enrichment broth, tempered to 37° C prior to use, were inoculated with one-tenth ml of the incubated sample. The minimum incubation time at 37° C necessary for the color change to occur which indicated growth of salmonellae was recorded. That change in color was from pale yellow to red-orange and was accompanied by heavy turbidity. This procedure was designated as the "modified cultural (MC) method" for salmonellae detection.

For the Millipore filter (MF) method, the double-strength Selenite-F culture was sampled after the same varied incubation periods. A Millipore membrane was aseptically secured on the Millipore filter holder and moistened with sterile physiological saline. One-tenth ml of the blended 200-ml culture was withdrawn and pipetted over the membrane. In addition, the sides of the filter were washed with saline in order to prevent loss of the egg sample. The filter apparatus was sealed and evacuated thereby concentrating the bacterial contaminants of the egg on the membrane. Finally, the membrane was transferred to a brilliant green agar plate and incubated 18 hours at 37° C or until typical Salmonella colonies appeared. The number of Salmonella colonies present
on the membranes was counted and recorded. This sampling procedure was performed in triplicate for each 200-ml culture incubated. Both the MC and MF methods were considered reference standards for subsequent development of the modified fluorescent antibody (MFA) technique.

**Modified fluorescent antibody technique for detection of salmonellae**

Further analyses of the double-strength Selenite-F cultures were accomplished by employing the MFA technique. This procedure was also repeated at varied incubation times. A 2 mm loopful of culture was smeared within three etched circles, about 8 mm in diameter, on a clean glass slide. The smears were heat fixed. A 1:5 dilution of Difco FA Salmonella Poly antiserum was prepared with double-distilled sterile water. Two drops of the conjugate were applied to each smear. The slides were then incubated at room temperature for 30 minutes in a Petri dish with moistened filter paper to prevent desiccation of the antiserum. Following incubation, the slides were soaked for one minute in two changes of chilled 0.1M potassium phosphate buffer, pH 8.0. After washing, the slides were gently blotted dry with "Kim-Wipe" papers. No mounting fluid or coverslips were used. Slides were examined with the aid of a Zeiss fluorescence microscope and suitable activating and barrier filters. An oil immersion objective providing a total magnification of 1000 x was used to detect the bright yellow-green fluorescence
of the Salmonella cells. A slide was judged positive if at least one fluorescing rod-shaped bacterial cell was seen in each of the three etched circles.

The diluted conjugate was screened for possible cross reactions with other enteric bacterial organisms known to occur in frozen whole egg. Separate test tubes containing 10-ml trypticase soy broth were inoculated from slant cultures of E. coli, C. freundii, S. flexneri, K. pneumoniae A. hinshawii, P. mirabilis, and P. vulgaris. After 24 hours at 37°C, a loopful of each culture was smeared, stained with the conjugate, and examined as described above.

Also, in order to verify the specificity of the conjugate, two types of control smears were prepared. Loopfuls of double-strength 15-hour Selenite-F cultures were spread on duplicate slides. For one control, two drops of normal rabbit serum were placed on the smears on one side. For the second control, two drops of unconjugated Salmonella-specific serum were applied to the smears on the remaining slide. The slides were incubated in the moist chamber for 30 minutes, then rinsed twice for one minute in buffer and allowed to air dry. Following application of the conjugate to both slides, they were incubated, rinsed, blotted, and examined as outlined above.
Several alternative procedures for direct preparation and staining of FA smears were investigated. These included the methods outlined by Insalata et al. (19), Geopfert et al. (12), and Schulte et al. (25).

Precision and accuracy of the MC, MF, and MFA techniques

The influence of competing organisms on the sensitivity of the MC, MF, and MFA techniques was studied by inoculating frozen whole egg with both *S. typhimurium* and *E. coli*. The standard inoculum of the former was made. *E. coli* cells were grown by inoculating 100 ml of trypticase soy broth and incubating for 24 hr. at 37°C. Standard plate count was determined for the culture. For purposes of this study, the concentration of *E. coli* cells inoculated into the egg was greater than that of the salmonellae. Following 7, 8, 9, and 15 hours of incubation in double-strength Selenite-F, enriched cultures were smeared for FA examination. Also, at each sampling time, the MC and MF procedures were initiated. A control culture of only *S. typhimurium* and 100 g of whole egg was included for comparison of recovery results.

Inasmuch as *Salmonella* species other than *S. typhimurium* may contaminate frozen whole egg, the reliability and sensitivity of MC, MF, and MFA techniques for detecting other salmonellae were investigated by artificially inoculating the egg with *S. enteritidis*. 
Finally, the precision of results obtained with the MC, MF, and MFA procedures was determined by initially inoculating 1000 g of thawed whole egg with 20 *S. typhimurium* cells. The inocula was then distributed uniformly by means of a magnetic stirring bar. One-thousand ml of double-strength Selenite-F broth was added to the egg and thoroughly mixed. This 2000 ml culture was distributed into ten 200 ml aliquots. The detection procedures outlined above were executed as specified and results recorded.
CHAPTER III
RESULTS

Throughout this investigation, control whole egg samples from the University of Montana Bakery Department gave negative results when screened by the standard cultural method for the presence of salmonellae. Likewise, standard plate counts consistently indicated less than 10,000 organisms per 10 grams of egg sample. Accordingly, the egg material was acceptable for further test purposes.

In the earliest experiments, brilliant green agar proved the most suitable of selected differential isolation media for use in this study. The selection was based on ease of identification of typical, complete Salmonella reactions. The pour plate method was superior to the streak-plate method in recovery of salmonellae following inoculation of the species in whole egg. Difficulty was experienced with the latter method, as the inoculum adhered to the rod and the resultant growth overcrowded and prevented identification of colonies. Inoculation of egg and subsequent passage into Selenite-F broth gave results similar to those obtained when only the contaminated egg was sampled by the pour plate method.
A cell density determination of the standard inoculum was needed for quantitative recovery studies. The number of colony forming units (CFU) ranged from 19-31 x 10^8 per millimeter of Salmonella culture. Approximately 32% of the culture contained 24 x 10^8 CFU per ml. With this basic background information, it was possible to calculate cell densities for inocula.

It was assumed that blending whole egg inoculated with salmonellae would produce a uniform mixture. Table 1 reveals that blended whole egg-Salmonella samples were, in fact, well-mixed suspensions. In four separate determinations, plate counts of the blended egg samples corresponded to those of the respective saline samples with either a high or low concentration of Salmonella cells in the inoculum. Also, neither the whole egg nor the blending process was significantly inhibitory to cell recovery.

An investigation was made into the effect of storing inoculated whole egg for 24 hours at 4°C and -20°C. After enumeration it was found that at both storage temperatures, the surviving populations of S. typhimurium remained relatively unchanged from those of the original inocula.

As stated previously, brilliant green agar (BGA) gave satisfactory results when used for the recovery of Salmonella from whole egg. The additional use of the Millipore membrane incubated on BGA proved even more advantageous. Table 2 shows
## TABLE I

Recovery of *S. typhimurium* from blended whole egg and saline suspensions.*

<table>
<thead>
<tr>
<th>S. <em>typhimurium</em> number added to egg and saline determinations (cells/ml)</th>
<th>S. <em>typhimurium</em> recovered/ml from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole egg</td>
</tr>
<tr>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>210</td>
<td>169-180</td>
</tr>
<tr>
<td>24</td>
<td>15-24</td>
</tr>
</tbody>
</table>

* Determined by plate counts of appropriate decimal dilutions.
**TABLE 2**

Sensitivity of Millipore and pour plate methods for recovery of *S. typhimurium* from whole egg

<table>
<thead>
<tr>
<th><em>S. typhimurium</em> added to egg (cells/ml)</th>
<th>Number of Determinations</th>
<th>Recovered with Millipore (cells/ml)</th>
<th>Recovered with pour plate (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Average</td>
</tr>
<tr>
<td>24</td>
<td>4</td>
<td>18-24</td>
<td>23</td>
</tr>
<tr>
<td>26</td>
<td>6</td>
<td>23-27</td>
<td>25</td>
</tr>
</tbody>
</table>
that the sensitivity of the MF-BGA combination was much
greater than that of the traditional pour plate method. In
comparing both methods, 95-96% of the original inoculum was
detected by the MF-BGA procedure, whereas much lower recovery
rates resulted with poured plate counts. Moreover, the
variance in these recovery rates (70% and 11%) was not ex­
perienced with the use of the membrane. The range in the
number of colonies counted per plate was very narrow, in­
dicating consistency in technical steps. It was decided,
therefore, to make further use of the technique in detection
procedures.

Table 3 summarizes the data from the first in a series
of investigations on the sensitivity of the MF procedure.
Varied numbers of salmonellae were inoculated into 100-gram
samples of whole egg and incubated in double-strength
Selenite-F for the periods indicated. As anticipated, with
an increase in incubation time, an increase in the number of
CFU per plate was seen with each different concentration of
organisms. At the two greatest inoculum levels, 190-250
and 95-125 Salmonella cells per ml, significant numbers of
salmonellae were detected in egg-Selenite-F cultures incubated
5 hours. After 6-7 hours, the lowest levels of inocula,
10-13 Salmonella cells per ml, were detected by the MF pro­
cedure. The range in CFU values in some cases appeared very
wide. This was probably a direct result of the range in
TABLE 3

Detection of *S. typhimurium* in 100-g samples of whole egg by the MF method

<table>
<thead>
<tr>
<th>Incubation time of egg-Selenite-F mixture (hr)</th>
<th>Number of CFU of <em>S. typhimurium</em> per ml egg-Selenite-F mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-13*</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>3-8</td>
</tr>
<tr>
<td>7</td>
<td>20-22</td>
</tr>
<tr>
<td>8</td>
<td>36&gt;250</td>
</tr>
<tr>
<td>10</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

*Number of *S. typhimurium* added per 100 g whole egg
inoculum concentrations. If more than 250 CFU were visible on the Millipore membrane, accurate colony counts could not be made because of overcrowding. In summary, the MF procedure detected salmonellae in each 100-g egg sample that was inoculated with at least 10 *S. typhimurium* organisms and incubated for a minimum of 6 hours in double-strength Selenite-F broth.

Results for the detection of salmonellae in 100 g of whole egg by the modified cultural (MC) method are presented in Table 4. The time required for the characteristic Selenite-F color change was a function of the initial number of *S. typhimurium* organisms inoculated in the egg, the incubation period in double-strength Selenite-F. For example, the samples with the greatest inoculum size of salmonellae (190-250 cells per ml) incubated for the longest period of time (8-10 hours) showed the typical color reaction after only 12 hours, whereas samples with the smallest inoculum size (10-13 cells per ml) incubated for the shortest time periods (4 hours), reacted after 26 hours. Further subcultivation of the Selenite-F broth verified the growth in the turbid, red-orange suspensions to be salmonellae. Uninoculated control samples of whole egg gave negative results with both MC and the MF procedures. Providing adequate incubation periods were allowed, the MC method was capable of detecting salmonellae in every 100-g whole egg sample.
### TABLE 4

Detection of *S. typhimurium* in 100-g samples of whole egg by the MC method

<table>
<thead>
<tr>
<th>Incubation time of egg-Selenite-F mixture (hr)</th>
<th>Minimum additional time required for a presumptive positive reaction (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-13*</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
</tr>
</tbody>
</table>

*Number of *S. typhimurium* added per 100 g whole egg
The next phase of investigations involved development of a direct FA procedure which would improve current salmonellae detection methods for whole egg. Studies included testing the commercially-supplied fluorescein conjugate for specificity and possible cross reactions. Examination of control smears revealed Difco FA Salmonella Poly antiserum to be specific for Salmonella species. Fluorescence was not prevented by application of normal rabbit serum, but was inhibited by Salmonella O-group antiserum. Moreover, no cross reactions in fluorescence occurred following the staining of E. coli, C. freundii, S. flexneri, K. pneumoniae, A. hinshawii, P. mirabilis, and P. vulgaris smears.

The direct FA procedure employed in this research was designed as "modified." Preparation and treatment of smears as outlined by Insalata et al. (19), Goepfert et al. (12), and Schulte et al. (25) gave unfavorable results in this laboratory. Non-specific background fluorescence from egg particles, buffer crystals, and smear fixatives made interpretation impossible. Smears of egg cultures were easily interpreted when prepared and treated by the method presented in Chapter II.

Encouraging results for the detection of S. typhimurium in whole egg were obtained with the modified fluorescent antibody (MFA) technique (Table 5). After 7 hours of incubation of whole egg in Selenite-F, S. typhimurium could be
detected with MFA, i.e. positive results. Preliminary studies with this system demonstrated that the temperature of the enrichment broth was a critical factor in determination of the minimum incubation period required to obtain presumptive results. It was important that the broth be tempered to 37°C before inoculation, particularly when the level of Salmonella organisms in the inoculum was low (less than 20 cells per ml).

In Table 6 it is seen that the MFA technique gave positive results with an initial inoculum level of only 2 salmonellae in 100 g of whole egg. The Selenite-F-egg cultures required a 7-hour incubation period for this low concentration of organisms to be detected by the immunofluorescence procedure. Both the MC and MF procedures verified the presence of the organisms. Each sample of the enriched cultures produced at least 21-28 CFU on the Millipore membranes. As enrichment time increased, the number of CFU increased also. Finally, Selenite-F broth became red-orange 24 hours after the cultures were sampled.

The precision and sensitivity of these results were further established. In a later investigation, each of ten 200-g egg-Selenite-F test samples of whole egg inoculated with equivalent low numbers of salmonellae gave positive results. Thus, the MC, MF, and MFA procedures were capable of detecting as few as approximately 2 salmonellae per 100-g whole egg samples.
**TABLE 5**

Detection of *S. typhimurium* in 100 g samples of whole egg by the MFA technique*

<table>
<thead>
<tr>
<th>Incubation time of egg-Selenite-F mixture (hr)</th>
<th>Fluorescent salmonellae detected (+) or not detected (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-13**</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
</tr>
</tbody>
</table>

*Data based on four determinations within each range of cell densities listed.

**S. typhimurium** cells added per 100 g whole egg.
<table>
<thead>
<tr>
<th>Incubation time of egg-Selenite-F mixture (hr)</th>
<th>MC</th>
<th>MF</th>
<th>MFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum additional incubation time required for a presumptive positive (hr)</td>
<td>24</td>
<td>21</td>
<td>&gt;28</td>
</tr>
<tr>
<td>Number of CFU/ml**</td>
<td>21-28</td>
<td>59-73</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Fluorescent salmonellae observed</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Whole egg inoculated with *S. typhimurium* to produce a calculated 2 cells/100 g sample

**Each range represents four determinations
Table 7 summarizes the studies made on the effect of *E. coli* on salmonellae detection by the MC, MF, and MFA procedures. In the test samples salmonellae were present in small numbers compared with *E. coli*. Control samples were inoculated with only the same low level of *S. typhimurium*. The presence of *E. coli* did not alter the sensitivity of the MFA procedure. In samples analyzed by the MF method following 7-9 hours incubation in double-strength Selenite-F, *E. coli* caused a decrease in the number of Salmonella CFU per plate. Likewise, for the same samples a longer incubation period was necessary for Selenite-F cultures containing the mixed inoculum to change color.

The reliability and sensitivity of the MC, MF, and MFA techniques for detecting *S. enteritidis* are illustrated in Table 8. The inocula of *S. enteritidis* and *S. typhimurium* are similar. The MFA procedure gave positive results for all cultures after the indicated time periods. The MF and MC procedures confirmed the presence of the species. Also, as seen with these results, it appeared that *S. enteritidis* and *S. typhimurium* had similar growth characteristics in enrichment cultures.

In Table 9 the minimum total time necessary to complete the MC, MF, and MFA assays is summarized. The number of hours listed for completion of each procedure is based on results obtained with the indicated inocula and also includes
### TABLE 7

Influence of *E. coli* on *S. typhimurium* detection by the MC, MF, and MFA procedures

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>Minimum additional incubation time required for a presumptive positive (hr)</th>
<th>Number of CPU S. typhimurium per ml</th>
<th>Fluorescent S. salmonellae observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC</td>
<td>MF</td>
<td>MFA</td>
</tr>
<tr>
<td></td>
<td>Mixed Inoculum*</td>
<td>Control**</td>
<td>Mixed Inoculum Control</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>24</td>
<td>8-18</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>21</td>
<td>19-31</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>20</td>
<td>129&gt;250</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>17</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

*48 *E. coli* and 2 *S. typhimurium* cells per 100 g whole egg

**2 *S. typhimurium* cells per 100 g whole egg
TABLE 8

Detection of \textit{S. enteritidis} and \textit{S. typhimurium} by the MC, MF, and MFA procedures

<table>
<thead>
<tr>
<th>Incubation time of egg-Selenite mixture (hr)</th>
<th>Minimum additional incubation time required for a \textit{S. typhimurium} presumptive positive (hr)</th>
<th>Number of CPU per ml</th>
<th>Fluorescent</th>
<th>S. typhimurium salmonellae observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC</td>
<td>MF</td>
<td>MFA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*2 \textit{S. enteritidis} cells per 100 g whole egg

**2 \textit{S. typhimurium} cells per 100 g whole egg
TABLE 9

Comparison of minimum total times required to complete MC, MF, and MFA detection assays

<table>
<thead>
<tr>
<th>Salmonella cells per 100 g whole egg</th>
<th>Total hours required to complete test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MC</td>
</tr>
<tr>
<td>2-5</td>
<td>29</td>
</tr>
<tr>
<td>10-13</td>
<td>28</td>
</tr>
<tr>
<td>20-25</td>
<td>28</td>
</tr>
<tr>
<td>95-125</td>
<td>24</td>
</tr>
<tr>
<td>190-250</td>
<td>23</td>
</tr>
</tbody>
</table>
preparation and incubation of culture media. The MFA procedure requires significantly less total time to detect salmonellae in whole egg.

In summary, the total time required for presumptive salmonellae results with these three procedures is a maximum of 29 hours by the MC method, 26 by the MF procedure and 8 hours by the MFA technique. These time periods are sharply in contrast to the 3 day period necessary to obtain negative salmonellae results with either the standard cultural method or the ES procedure. Furthermore, presumptive positive results can not be obtained until 5-7 days after initiating the former conventional detection method.
The presence of salmonellae in frozen whole egg is a cause of concern among commercial egg producers, commercial egg consumers such as bakeries, restaurants, and confectioneries, and public health officials. It has been established that improvements are needed in present Salmonella detection methods. Conventional screening procedures are very time-consuming (5-7 days), contain many laborious stages of analysis, and often produce ambiguous results. The number of egg samples that can be screened by the quality control laboratory is markedly limited by the large amount of media and equipment consumed in the process. It has been suggested that application of an immunofluorescence technique to detection methods would provide more rapid, simple, and reliable means of detecting the organisms.

Much attention has been given to applying the fluorescent antibody (FA) technique to the food industry. A review of the literature revealed that several attempts to use FA to detect Salmonella in frozen whole egg have been reported, namely those of Haglund et al. (15), Silliker et al. (27),
Insalata et al. (19), Schulte et al. (25), and Fantasia (7). Since the publication of those studies, revisions and new developments have been made in the direct FA methodology and reagents. It seemed reasonable that application of the updated techniques to the examination of whole egg for salmonellae merited further investigation.

In designing an experimental procedure for Salmonella detection, it was felt that the system should include both cultural and FA phases. The cultural phase would be needed to augment growth of salmonellae and the FA phase would provide a fast and accurate means of detecting the growth. Considerable attention was given to determining maximal recovery and sensitivity values of the system. For this reason, the whole egg used throughout the study was actually termed a "diluent" for various concentrations of Salmonella inocula. Important factors evaluated for their effects on optimal recovery in the cultural phase of the system included: 1) the enrichment and isolation media; 2) the sizes of the inocula and of the egg samples; and, 3) the possible use of the Millipore membrane.

To artificially contaminate test samples of frozen whole egg with Salmonella and evaluate a detection procedure, several criteria were considered. First, the Salmonella population in naturally infected whole egg has been subjected to freezing temperatures for an extended period of time.
The process of freezing may have denatured the bacterial proteins (30). Thus, the choice of isolation media is very important. Although Straka and Stokes (30) report that when salmonellae were stored in foods at subzero temperatures for months, the bacterial species became less able to grow on selective agar media as compared to non-selective, highly nutritious media, brilliant green agar (BGA) was chosen for the present investigation due to the large microbial population in whole egg. Taylor (31) suggests that whole egg might afford protection to microorganisms from the damaging effects of freezing. Frequently, even though subjected to the same low temperatures, coliforms, Pseudomonas, and Proteus species are present in greater numbers than the salmonellae. Thus, if the egg samples tested had been plated on a non-selective media, the presence of Salmonella could probably not have been detected as easily as on a media which selects for salmonellae. Salmonellae were readily distinguished from the natural microflora of whole egg in artificially inoculated test samples.

Incubation of the Millipore membrane on BGA made a significant difference in percent recovery of the salmonellae from test samples when compared to either a BGA pour plate or streak-plate procedure for recovery. This probably was a result of the inocula being quantitatively recovered on the membrane. Whole egg diluted $1 \times 10^3$ passed easily through
the pores of the membrane. It is possible that greater recovery with the membrane would have been experienced had the filter been incubated in a non-selective broth prior to addition of the test sample. The poor recovery noted with the other procedures could have been due to loss of organisms during pipetting, plating, or stirring.

Also considered was the distribution of the inocula in the egg samples. Although naturally infected whole eggs have a nonuniform population of microorganisms, in order to evaluate experimental recovery procedures, the use of the Waring blender was necessary. It was proposed that blending might help to produce a test sample more representative of the whole egg microflora.

Development of an experimental procedure continued with the addition of an enrichment phase in Selenite-F broth. It is known that if a contaminating population of salmonellae in whole egg is either small in number or has survived adverse environmental conditions, sufficient incubation in an enrichment broth allows proliferation and revival of the organisms. Selenite-F broth, along with tetrathionate broth, are presently accepted as the two best enrichment media for frozen whole egg samples. The choice of Selenite-F for use in this experimental system was based on two parameters. First, it has been reported that tetrathionate quenches fluorescence (6, 7). Second, it is conceivable
that most of the viable organisms introduced into the egg samples were metabolizing optimally and that the contaminating salmonellae in naturally-infected whole egg may not be in the same physiological state. Selenite-F, being selective in nature, would enhance the salmonellae growth but inhibit that of coliform organisms which are known to occur in whole egg. As later demonstrated, samples of egg that were originally inoculated with very few Salmonella and subsequently enriched in Selenite-F gave positive results with the various detection procedures.

The volume of the egg material samples was a critical factor in proposing the experimental plan. Presently, the FDA requires that a 25-g unit of whole egg be sampled for conventional analyses. Samples which produce "negative" results are assumed to contain less than one viable Salmonella in 25 g. This limit of sensitivity is determined arbitrarily and is based on the sensitivity of the standard cultural method. As a practical matter, it seemed prudent to develop a procedure that would be more sensitive and reliable than those used by the regulatory agencies. Essentially, it was believed that if the quantity of material examined were increased so would be the chance of detecting small numbers of contaminating salmonellae. In this study, the sample was increased from 25 g to 100 g of frozen whole egg.
Further investigations of the MF method were conducted to determine how few cells could be recovered from the 100-g sample of egg. The outcome of these studies established not only the necessity for an appropriate incubation period, but, also the importance of pre-tempering the enrichment media to $37^\circ$ C. In this system, inoculation of as few as 2 cells per egg sample gave positive results. There seems to be no mention in the literature of similar attempts to use the Millipore filter to screen whole egg for *Salmonella* with which to compare these findings.

The modified cultural (MC) method was designed as an added control feature and/or an alternative detection procedure. Results with this system mirrored those with the MF method. The amount of time which elapsed between initiation and completion of analysis was dependent on the number of *Salmonella* in the sample. After inoculation of as few as 2 cells per 100 g of egg, positive results were obtained within 24 to 29 hours by the MF and MC methods, respectively. These results revealed the ability of the experimental system, at this stage of development, to allow for proliferation and eventual detection of an initially low number of salmonellae in 100 g of sample.

Immunofluorescence procedures for the detection of salmonellae in various foods, in general, have been demonstrated to be improvements over established cultural methods (7,
12, 15, 17, 19, 22, 24, 25, 27). In proposing that a direct FA technique be included in the experimental system, it was hoped that sensitivity and specificity could further be increased.

The data collected were encouraging. It was experimentally possible to detect as few as two salmonellae cells per 100-g sample of egg following 7 hours of selective enrichment. Indications were that the procedure could be completed within an 8-hour industrial working day. It was strongly felt that the main factors responsible for the results in recovery were the specificity and sensitivity of the fluorescein-conjugate employed.

Difco FA Salmonella Poly A-S antiserum has only recently been reported in the literature (18, 22). In those investigations cited, however, it was not employed in connection with examination of whole egg. Previous application of other commercially-available conjugates to the examination of whole egg has resulted in difficulties in smear interpretation arising from cross reactions with non-salmonellae and non-specific background fluorescence. In this study no cross reactions appeared if the proper dilutions of antisera were used. Similar results were reported by Silliker et al (27). Problems were encountered, however, with non-specific fluorescence. It was found that in the use of the direct slide preparation procedures of Insalata et al. (19),
Schulte et al. (25), and Mohr et al. (22), fluorescent egg particles and buffer crystals made interpretation impossible. Revisions in the slide treatment were made and, fortunately, easier interpretation resulted. This was probably due to elimination of the need for many of the fixing and washing procedures in the other methods, by sampling the blended selenite culture after it had been allowed to settle, and by blotting the slides dry.

Whole eggs generally contain large numbers of coliform bacteria, and these compete with the salmonellae in the enrichment media. Results with the MFA procedure confirmed the specificity of the Difco conjugate. Inoculation of a large population of *E. coli* into the egg material did not affect detection of the small *Salmonella* population. Furthermore, these observations focus on an important advantage of the FA system in contrast to the cultural method. Coliform populations in the egg often mask recovery of salmonellae on isolation media because of overcrowding.

Reports of false positive results with FA procedures are found throughout the literature (7, 11, 13, 17, 19, 27), pointing to the lack of cultural confirmation in recovery studies. In this investigation, the MC and MF experimental procedures were used as the reference standards with which to compare the MFA results. In each culture analyzed, the sensitivity achieved with MFA was confirmed by the additional but delayed results with the other two methods.
The most probable source of error in this experimental system was the determination of the Salmonella cell density of the inocula. Quantitative determinations necessitated relying on dilutions and plate counts of the pure cultures for the results. An alternative would have been to employ the most probable number procedure for enumeration, which is more time-consuming, requires that greater numbers of dilutions be performed, and is no more accurate than the plate count method. Therefore, as in all studies of this kind, the possible inaccuracy of this determination was taken into consideration.

The experimental FA studies for detection of salmonellae in frozen whole egg were performed with only two species, S. typhimurium and S. enteritidis. Obviously, there may be other species that could have been characterized. Other species may not grow in Selenite-F at the same rate, and therefore at the time of sampling may give false negative results. In a practical application of the system, it is suggested that negative FA cultures be sampled again after 24 hours incubation.

The experimental procedures thus described are schematically illustrated in the following flow sheet, p. 49. An important advantage of the analytical procedure (MFA method) developed is its greater sensitivity than that of traditional cultural methods. Although the detection procedure
Analystic scheme for MC, MF, and MFA procedures

A. Thaw frozen egg
B. Weigh 100 grams into Waring blender
   Blend 1.0 minute
C. Add 100 ml pre-tempered double-strength Selenite-F
   Blend 1.0 minute
D. Incubate at 37°C in water bath for 7 hours

MF
1. Pass 0.1 ml through Millipore membrane
2. Place filter on Brilliant Green agar plate
3. Incubate 18 hr. at 37°C
4. Record presence or absence of Salmonella

MFA
1. Heat fix smear
2. Add Difco conjugate
3. Incubate 30 minutes in moist chamber
4. 2 rinses for 1 minute in 0.1 M potassium phosphate buffer, pH 8.0
5. Blot dry
6. Observe under fluorescent microscope
7. Record positive or negative results

MC
1. Add 0.1 ml to 9.9 ml Selenite
2. Observe color change to orange
3. Streak loopful on Brilliant Green agar
required selective enrichment, no streaking on isolation or pre-enrichment media were necessary. This decreases the number of stages in the procedural sequence necessary to obtain presumptive salmonellae results. If it were needed, samples of the selenite cultures could be retained for serological and biochemical determinations.

In conclusion, the possibility of developing a screening procedure recommended for detection of Salmonella contamination in frozen whole egg was investigated. By application of a direct FA technique to selectively-enriched cultures of contaminated whole egg, a procedure was developed which is at least as sensitive as standard cultural methods, and reduces to 8 hours the minimum time necessary to complete the assay.
A direct fluorescent antibody (FA) procedure was experimentally developed for the rapid detection of salmonellae in frozen whole egg. Currently accepted detection methods are time-consuming (5-7 days), contain many laborious procedural stages, and often produce ambiguous results. Significant increases in sensitivity and specificity were observed by increasing the whole egg sample size, concentrating the selective enrichment, and incorporating a suitable FA procedure in the experimental system.

Whole egg samples (100 g), tested negative by the standard cultural method, were inoculated with as few as two salmonellae and blended. The contaminated samples were then blended and enriched in 100-ml double-strength Selenite-F. After incubation for 7 hours at 37°C, smears were heat-fixed, incubated with Bacto-FA Salmonella Poly A-S (Difco), washed in 0.1 M potassium phosphate buffer, and blotted dry. Upon fluorescent microscopic examination, fluorescent Salmonella cells were readily seen.

Two alternative procedures were employed as control features. Samples from the same selectively-enriched cultures
were 1) plated on Millipore filters on brilliant green agar and, 2) suspended in Selenite-F. After appropriate incubation periods, the presence of salmonellae was confirmed.
REFERENCES CITED


APPENDIX A
STANDARD CULTURAL METHOD

The procedure outlined for the detection and identification of Salmonella in frozen whole egg is adapted from the Bacteriological Analytical Manual (23). It conforms to the method described in the Official Methods of Analysis of A.O.A.C., pp. 8434, 1970 and revised in J.A.O.A.C., 54; 495, 1971.

1. Procedure for preparation of egg sample
   a. Thaw a suitable portion of egg as rapidly as possible
   b. Aseptically weigh 25 grams of sample into sterile, empty wide-mouth, screw-cap pint jar
   c. Add 225 ml of sterile lactose broth
   d. Cap jar securely and let stand at room temperature for 60 minutes
   e. Mix well by shaking and adjust to pH 6.8
   f. Loosen jar cap about 1/4 turn and incubate 24 ± 2 hours at 35°C.

2. Isolation of Salmonella
   a. Gently shake incubated sample mixture, and transfer 1 ml portions each to 10 ml selenite cystine broth and 10 ml of tetrathionate broth
   b. Incubate 24 ± 2 hours at 35°C
c. Streak 3 mm loopful of each selective enrichment broth on brilliant green agar, Salmonella-Shigella agar, and bismuth sulfite agar

d. Incubate 24 \pm 2 hours at 35^\circ C

e. Note appearance of typical \textit{Salmonella} colonies

f. Pick, with needle, 2 or more typical or suspicious colonies from these agar plates onto triple sugar slants (TSI)

g. Incubate 24 \pm 2 hours at 35^\circ C

h. If typical reactions are not observed, pick additional colonies from BG, SS, and BS plates to TSI.

3. \textbf{Identification of Salmonella}

a. Mixed cultures

   (1) Streak any TSI agar slant culture, which appears to be mixed, on MacConkey's agar or BG

   (2) Incubate for 24 \pm 2 hours at 35^\circ C. If typical colonies appear, transfer to TSI

b. Pure cultures

   (1) Urease test:

      (a) Transfer 2 loopfuls of growth from each presumptive positive TSI agar slant to urea broth tubes. Incubate 24 \pm 2 hours at 35^\circ C

      (b) Retain cultures that give a negative test

c. Serological flagellar (H) screening test

   (1) Transfer one loopful of growth from ureaast negative TSI agar slant to

      (a) Brain heart infusion broth. Incubate 4-6 hours at 35^\circ C

      (b) Trypticase soy broth. Incubate 24 \pm 2 hours at 35^\circ C.
(2) To 5 ml broth culture add 2.5 ml formalized physiological saline soln.

(3) Select 2 formalized broth cultures and test with *Salmonella* flagellar (H) antisera
   (a) Place 0.5 ml polyvalent (H) antiserum in serological test tube
   (b) Add 0.5 ml antigen
   (c) Prepare saline control
   (d) Incubate 1 hour at 50° C in water bath
   (e) Observe positive or negative agglutination

(4) If broth cultures are positive, perform additional tests.

d. Testing urease negative cultures in biochemical reactions

(1) Lysine iron agar
   (a) Streak from TSI slant
   (b) Incubate 48 + 2 hours at 35° C
   (c) Examine for acid or alkaline reaction

(2) Lysine decarboxylase broth
   (a) Inoculate with loopful TSI agar culture
   (b) Incubate 48 + 2 hours at 35° C
   (c) Examine for positive or negative reaction

(3) Purple broth base with dulcitol
   (a) Inoculate with loopful of TSI agar culture
   (b) Incubate 48 + 2 hours at 35° C
   (c) Examine for positive or negative growth
(4) Tryptophane broth

(a) Inoculate broth with loopful of TSI agar culture

(b) Incubate 24 ± 2 hours at 35 C and test with

i) KCN broth

ii) Malonate broth

iii) Indole test

e. Additional biochemical tests

(1) Purple lactose broth

(2) Purple sucrose broth

(3) MR-VP medium

(4) Simons citrate agar

4. Salmonella serological test

a. Polyvalent somatic (O) plate test

(1) Place 1/2 of a loopful of culture from 24-48 hour culture TSI in two cupped portions of agglutination plate

(2) Add 1 drop of saline solution to 1 loopful culture

(3) Emulsify culture in saline solution with clean sterile transfer loop

(4) Add 1 drop polyvalent somatic (O) antiserum to the other loopful of culture

(5) Mix antiserum and culture with clean, sterile transfer loop

(6) Tilt mixtures back and forth one minute and observe any degree of agglutination

b. Polyvalent flagellar (H) test tube method

(1) See 3, C, (1)-(4)

5. Repeat entire process on new egg sample for confirmation
The procedure outlined for the detection of Salmonella in whole egg is taken from Sperber and Deibel (29).

1. A 10% suspension of the food sample is prepared in sterile Lactose Broth and incubated at 37°C for 18 to 24 hours.

2. One milliliter of the pree enrichment culture is transferred to 9 ml of Selenite-Cystine Broth and 1 ml to 9 ml of Tetrathionate Broth. The enrichment media are incubated at 37°C for 24 hours.

3. Ten ml of M-broth is inoculated with one drop of each enrichment culture, shaken to distribute the inoculum tempered, incubated for 6 hours at 37°C and tested by the modified H-agglutination test.

4. The modified H-agglutination test is performed as follows. To one drop of Formalin salt solution in a Kahn tube add 0.85 ml of M-broth culture. To this mixture is added 0.1 ml of pooled antisera. The Kahn tube is gently shaken and incubated at least 1 hour at 50°C before observing for agglutination.

5. The pooled antiserum is prepared by adding 0.5 ml of each properly rehydrated Difco H antiserum (Spicer-Edwards sera 1, 2, 3, 4, L complex, en complex; and H antisera z6, poly D, and poly F) to 11.5 ml of 0.85% sodium chloride.