

SALMONELLAE IN MARKETED FOODS:  
ISOLATION FROM PRE-PREPARED AND PACKAGED SAMPLES  
AT THE CONSUMER LEVEL

by

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## INTRODUCTION

Salmonellosis has always been one of the significant international public health problems. A variety of Salmonella serotypes could be pathogenic to human beings (Ratna and Dolman, 1947; Edwards et al., 1948; Bynoe et al., 1953; Edwards, 1958; and Galton and Arnstein, 1960).

Contaminated foods have often been considered as potential sources in initiating and perpetuating the chain of salmonellosis infection. It has been estimated that in the United States alone nearly 2,000 people are affected per month. Intensive field investigation in many recent outbreaks has uncovered a specific contaminated food as the origin (Friedman, 1962).

Increased awareness of these facts has naturally resulted in research workers focusing their attention on the detection of salmonellae from marketed foods intended for human consumption. Attempts to improve the available laboratory techniques for isolation of these pathogens have yielded tangible results.

The object of this study was to attempt isolation of salmonellae from foods sold at the consumer level. Incidentally, this work has given a general idea of the prevalence of bacterial contamination of foods particularly by members of the family Enterobacteriaceae. Also the efficacy of two pre-enrichment media which recently have received much attention in isolating salmonellae from food samples could be compared.

## REVIEW OF LITERATURE

Enrichment Procedures and Media: Research in the field of enteric bacteriology during the past four decades has definitely established the necessity for enrichment procedures for increasing the efficiency of isolation

of pathogens. For the isolation of salmonellae, tetrathionate broth of Muller (1923) and selenite 'F' broth of Leifson (1936) have been used extensively. However, it soon became apparent (Daack, 1955) that the presence of large amounts of organic matter in food products markedly affected the selectivity of the enrichment media. Also the growth of other organisms like Proteus, Pseudomonas, etc., had to be suppressed for getting better results.

To obviate these difficulties, modifications of these selective enrichment media have been suggested by various workers from time to time. Newer enrichment media and pre-enrichment procedures have also been evolved. Kauffman (1930, 1935, 1954) improved the tetrathionate broth of Muller (1923) by the addition of brilliant green and bile salts which he called the "combined enrichment medium." Galton et al. (1952), while isolating salmonellae from canine fecal swabs, found the addition of 0.125 mg of sodium sulfathiazole per 100 ml of tetrathionate broth prevented excessive growth of Proteus species. Jamason (1961) reported that the addition of sodium lauryl sulfate and bismuth sulfite solutions to tetrathionate broth favored active multiplication of salmonellae from sewer swabs, while selectively suppressing the growth of Proteus species.

North (1950), while attempting the isolation of salmonellae from dried and frozen eggs, observed that the reduction of phosphate content from 1.0 percent to 0.25 percent increased both the selectivity and productivity of selenite broth. Banwart and Ayres (1953) studied the effect of various enrichment broths upon the growth of several species of salmonellae. Evaluating the growth curve they found that nutrient broth was the best. Tetrathionate was found to be less inhibitory than selenite 'F', though the former was definitely inhibitory to Salmonella paratyphi and destructive to S. anatis. Hurley and Ayres (1953) compared the efficacy of various enrichment media,

for isolating S. pullorum from egg products. When Escherichia coli, Proteus morgani, Pseudomonas aeruginosa and Alcaligenes faecalis were grown in the enrichment broths with egg products added, the selectivity of the enrichment media was found to be reduced. North and Bartram (1953) evaluated the efficiency of selenite broths of different composition and called attention to the possibility of false negative results from the use of a medium customarily regarded as satisfactory. Certain batches of peptone affected the productivity of medium. Incorporation of a small amount of yeast extract corrected this unsatisfactory condition. Also addition of cystine in a concentration of 10 ug per ml effected a marked improvement. Jensen (1955) suggested the use of both tetrathionate and selenite broths for enrichment to obtain better recovery, when salmonellae were present in small numbers in the specimens. Stokes and Osborne (1955) described a brilliant green medium, consisting of peptone, yeast extract, sodium selenite, sodium taurocholate, brilliant green and phosphate buffer. This supported luxuriant growth of salmonellae from very small inocula and at the same time markedly suppressed the growth of Proteus, Escherichia, Pseudomonas, Alcaligenes and Streptococcus species. Though Aerobacter was significantly reduced in comparison to the conventional selenite 'F' broth, it was not totally inhibited. The amount of phosphate in the medium was a critical factor. If phosphates were omitted, no growth or salmonellae occurred. Mannitol was chosen as a fermentable substrate to facilitate the multiplication of salmonellae. Osborne and Stokes (1955), working further on the selenite brilliant green medium, felt that it was not entirely satisfactory for use with egg products. The addition of 0.05 percent of sodium sulfapyridine nullified the untoward effect and restored the sensitivity and selectivity of the enrichment broth. Wells et al. (1958) confirmed the value of this brilliant green sulfapyridine selenite broth for

screening egg products. Sillicker and Taylor (1958) noted that the presence of gelatin and albumen in food materials markedly diminished the performance of enrichment broths. The use of centrifugation for separating the bacteria from soluble food materials improved the function of enrichment media. They observed that the preponderance of coliforms did not affect the selectivity of either selenite or tetrathionate broths. Taylor et al. (1958) studied the factors affecting the choice of media for the detection and enumeration of salmonellae in naturally contaminated dried egg albumen which contained both salmonellae and coliforms. They compared the cystine selenite 'F' broth of conventional formula with one they had modified by substituting mannitol and dulcitol in the place of lactose. Selenite brilliant green sulfapyridine medium also was used simultaneously. Their modification failed to give any better result. Likewise, in their hands, selenite brilliant green sulfapyridine medium was also not quite productive.

Improvement of procedures and problems associated with recovery of salmonellae from prepared foods were discussed in a symposium during 1955. Slocum (1955) stressed the importance of pre-enrichment to initially boost the number of salmonellae prior to transfer to selective media. According to him the selective enrichment media commonly employed were primarily designed for fecal or clinical material. The selectivity of these media was adversely affected by large amounts of organic matter present in foods. Earlier Schneider (1946) recognized that direct isolation on differential media was difficult, so the egg powder was suspended in distilled water and a large sample of this reconstituted material was added to double strength selenite 'F' medium. Smith (1952) found that direct plating of fecal material was superior to selective enrichment for isolating S. cholerae-suis and S. abortus-ovis. Tetrathionate and selenite enrichment media were highly inhibitory to

these serotypes. Byrne et al. (1955) initially soaked egg powder in distilled water and later agitated it to have a uniform dispersion of material prior to transfer to replicate tubes of cystine selenite 'F' broth. Taylor et al. (1958) used tricarboxylic acid intermediary metabolites for pretreatment of known positive foods, before transferring these to cystine selenite 'F' broth. This was done with the object of rejuvenating the cells physiologically damaged while under manufacture. However, this failed to enhance the isolation rate. Sugiyama et al. (1960) found preliminary incubation of foods in lauryl tryptose broth with polyvalent Salmonella 'H' antisera, centrifugation and subsequent transfer of sediment to cystine selenite broth was superior to direct inoculation. Galton (1961) reviewed the findings of earlier workers and discussed the various facets of the problems involved in laboratory procedures. The use of tetrathionate enrichment broth with brilliant green was recommended for isolation of salmonellae from packaged foods. Incorporation of Tergitol No. 7(R) in pork sausage and dog meal specimens facilitated better dispersion and thus enhanced the growth. Tween 80 (R) was also useful serving the same purpose. Excessive dumping of material was detrimental and decreased the efficiency of isolation. In view of the inhibitory action of tetrathionate medium on certain salmonellae it was suggested that nutrient broth with Tergitol could be used. North (1961), employing lactose broth for pre-enrichment, confirmed the superiority of this procedure to direct transfer of material in selective media. It was stated that plain broth could be equally effective with pure cultures. However, in the presence of a mixed flora, fermentation of lactose could lower the pH sufficiently enough to inhibit other microorganisms, with no apparent ill effect on salmonellae. Taylor and Sillicker (1961) established that reconstitution of albumen in distilled water prior to inoculation into enrichment media was better than direct transfer.



Lactose broth pre-enrichment was superior to reconstitution in distilled water. However, they found that the type of carbohydrate added to the pre-enrichment broth was not significant. Dulcitol, mannitol and lactose gave comparable results. Edwards and Ewing (1962) briefly summarized the contributions of workers in this field. They pointed out the necessity of rehydration of dried products before transfer into selective enrichment broths.

Differential Plating Media: Plating media which are both differential and highly selective have been used for the isolation of enteric pathogens for quite some time. They have been marketed in a dehydrated form by various reliable commercial concerns. MacConkey Agar (MAC), Desoxycholate Citrate Agar (DCA), Eosin Methylene Blue Agar (EMB), Salmonella-Shigella Agar (SS), Brilliant Green Agar (BGA) and Bismuth Sulfite Agar (BSA) have gained wide recognition. The Difco Manual (1953) has details of their composition, modes of preparation and indicates the relative merits. While pathogens are facilitated to grow luxuriantly the majority of non-pathogenic contaminants are prevented from excessive multiplication. Non-lactose-fermenters are differentiated from lactose-fermenting ones by the characteristic appearance of the colonies and the changes the latter produce on these plating media. However, the experiences gained through the years have indicated that the advantages are not absolute. As necessitated by circumstances, modifications of these plating media have also come in vogue.

Barnwart and Ayres (1953) found that BGA gave luxuriant growth of S. paratyphi, S. typhi-murium, S. oranienburg, S. pullorum, S. anatis, and S. worthington. BSA, DCA and SS were highly inhibitory for these organisms. Galton et al. (1954) found that the addition of 8-16 mg of sodium sulfadiazine per 100 ml of BGA markedly inhibited the growth of Pseudomonas. Later, Galton (1961) recommended this medium for the isolation of salmonellae from

foods and pointed out the possible altered appearance of Salmonella colonies in the presence of excessive coliform contamination. Byrne et al. (1955) used BSA and BGA in their studies with S. pullorum. BGA was inhibitory to this organism while BSA was not. They felt that no single plating medium could suffice. The employment of many plating media was a necessity to increase the chances of better recovery. Taylor et al. (1958) established that BGA was superior to SS and BSA. On BGA, distinct differentiation could be made easily between coliforms and non-coliforms in about 24 hours of incubation. Salmonellae grew unrestricted in this medium. However, they advocated the desirability of using many plating media for getting a higher number of isolations. Wells et al. (1958) had comparatively good results on BGA, BSA and Brilliant Green Sulfa Agar, though BGA was slightly superior to the others. Edwards and Ewing (1962) commented favorably on the use of BGA for the isolation of salmonellae other than S. typhi. According to them, BSA was another efficient medium. Though colonial appearance was a reasonably good index for isolation it was not absolute because of atypical forms which might preponderate and could confuse even the experienced worker.

Utility of Genus Specific "O-1" Phage: Cherry et al. (1954) reported on the utility of the genus specific "O-1" phage for differentiating salmonellae from other gram-negative organisms. Phage susceptibility was tested on 427 strains of Salmonella, 195 strains of Shigella, 9 strains of Proteus, 3 strains of Serratia, 93 strains of Escherichia, 7 strains of Aerobacter, 195 strains of Bethesda, 162 strains of monophasic Arizona, 29 strains of diphasic Arizona and 95 strains of miscellaneous organisms. With rare exceptions (one strain of Escherichia, one strain of Shigella and one strain from the miscellaneous group), only Salmonella and diphasic Arizona strains were lysed by this phage. They recommended the use of BGA for routine tests because this

medium permitted easy recognition of lactose fermenters as well as provided a good contrast in background for reading lysis results. Occasional strains of S. derby, S. anatum, S. newington, S. pullorum, S. tennessee and S. senftenberg were fairly resistant to phage lysis.

Isolations Reported from Prepared Foods and Food Ingredients: Gibbons and Moore (1944) reported the occurrence of salmonellae in Canadian egg powder. Ratna and Dolman (1947) noted a high incidence of S. thompson in human beings in Western Canada which was isolated from dried egg powder produced in the same locality. Thomson (1955) isolated S. paratyphi B from wheat flour. Samples of the specimen in nutrient broth were positive for the organism for nearly a year. Polak (1960) isolated salmonellae from hens' egg products in Holland. Taylor (1960) quoted figures of isolation from imported egg products while discussing the problem of diarrheal diseases in England. Daniels-Bosman and Huisman (1961) found that at Amsterdam imported desiccated coconuts contained salmonellae. Harvey et al. (1961) traced an outbreak of Salmonella food poisoning to contaminated baker's confectionery. They isolated S. thompson and S. typhi-murium from the remnants of trifles eaten at a party. Thatcher and Montford (1962) isolated a variety of salmonellae from prepared food products such as frozen whole eggs, cake mixes with eggs and cake mixes without eggs. Butler and Josephson (1962) found cake mixes were highly contaminated with salmonellae in Newfoundland. In the United States, Shotts et al. (1961) isolated S. tennessee, S. infantis and S. oranienburg from 3 out of 22 cake mixes and bread mixes containing egg products. According to Yurack (1962), S. thompson, S. heidelberg and S. tennessee were commonly encountered in cake mixes and in egg products. S. bareilly and S. muenchen were infrequent. Recently S. worthington and S. pullorum had been isolated

from commercial egg white. S. thompson was noted in coconut and frozen ready-to-eat cakes.

#### MATERIALS AND METHODS

Purchase of Samples: Undamaged, intact packages of prepared foods available for sale at Manhattan (Kansas) were purchased, taking care that they belonged to different batches of manufacture. The printed wrappers furnished details of contents. Within two hours of purchase these were all stored in the refrigerator at 5 to 8 C where they were retained until the work was completed. Cultural examination was carried out on every sample within two months of purchase. Excepting baby foods, cookie doughs and dinner rolls, the others were dry. Of a total of 247 specimens processed, 116 contained some egg product as an ingredient, 87 contained some animal product, while the remainder (44) were free of any animal products.

Handling: The packages were handled with sterile precautions to avoid extraneous contamination. The cartons were opened with a hot, flamed spatula. In the case of cookie doughs the surface of the wrapper was wiped twice at an interval of five minutes with a sterile cotton swab moistened in 70 percent alcohol. When dry, a square incision of two inches was made in the center with a flamed scalpel. The metal caps of containers of baby foods were likewise treated with 70 percent alcohol and flamed before opening.

Wooden tongue depressors wrapped in Kraft paper and sterilized in the hot-air oven were used to transfer specimens to pre-enrichment media. In all cases surface materials were scooped and discarded. Sufficient quantities were obtained from the mid portion of these samples for culturing.

Pre-enrichment: Two pre-enrichment media, viz. (1) Nutrient broth and (2) Marmitol purple broth were used in this study. The nutrient broth used

had the following composition:

Proteose peptone No. 3 "Difco"	10 grams
Beef extract "Difco"	3 grams
Yeast extract "Difco"	3 grams
Sodium chloride	5 grams
Distilled water	1 liter

The ingredients were heated to dissolve completely and the medium was adjusted to pH 7.6 by Normal sodium hydroxide solution. Mannitol purple broth was prepared by adding 0.5 percent of mannitol to purple broth base "Difco" dissolved in distilled water. After preparation the media were measured in 90 ml quantities into screw-capped, flat-bottomed bottles (4" x 2") of 250 ml capacity and were sterilized in the autoclave at 121 C for 15 min. When cooled, the bottles were incubated at 37 C for 48 hr and left at room temperature until used. Only bottles with media showing no evidence of contamination were used.

In the experiments the bottles were individually weighed on a balance. Approximately 10 grams of material from each of the different samples were aseptically added. The pabulum was stirred with the tongue depressor, previously used for transfer of the material, to form a homogeneous suspension. The pre-enrichment media containing the suspended sample were incubated at 37 C for 12 to 15 hr. During incubation the bottles were gently agitated every 2 to 3 hr to resuspend the sediment. At the end of this preliminary incubation period 1 ml of material from each of the bottles was aseptically transferred to 15 ml of appropriate selective enrichment media in sterile metal-capped test tubes.

Selective Enrichment: Both the tetrathionate broth "Difco" and Selenite 'F' broth "Difco" conventionally used for isolation of enteric pathogens and prepared according to directions of the manufacturer were employed for

selective enrichment. For transfer of nutrient broth pre-enrichment cultures these media were used as such without the addition of any other chemical. However, for the transfer of mannitol purple broth pre-enrichment cultures, selenite broth was enriched with 100 mg of cystine and 500 mg of sulfa-pyridine per liter of the medium. Brilliant green was incorporated in tetrathionate broth to give a final concentration of 1:100,000. These selective enrichment media containing transfers from pre-enrichment broths were incubated at 37 C for 24 hr. Two loopfuls of the selective enrichment cultures were surface streaked on the surface of each of the various differential plating media.

Differential Plating Media: The following differential plating media of "Difco" manufacture prepared according to the manufacturer's specifications were used.

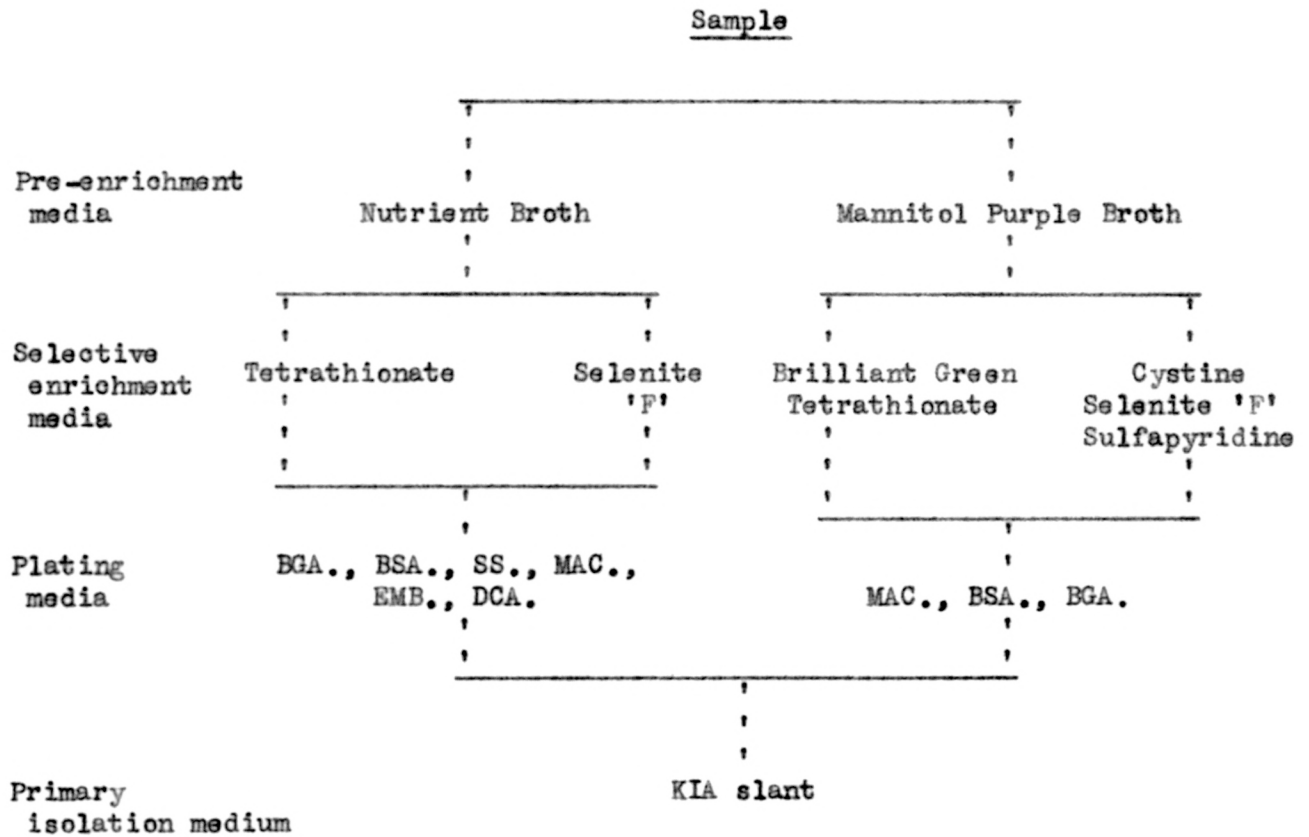
- (1) MacConkey Agar (MAC)
- (2) Brilliant Green Agar (BGA)
- (3) Eosin Methylene Blue Agar (EMB)
- (4) Bismuth Sulfite Agar (BSA)
- (5) Desoxycholate Citrate Agar (DCA)
- (6) Salmonella-Shigella Agar (SS)

The former three media were sterilized in the autoclave at 121 C for 15 min in 120 ml quantities in prescription bottles for storage and were remelted in the Arnold steam sterilizer, for pouring into sterile petri-dishes. The latter three media were immediately poured into plates and after solidifying were stored in the refrigerator. Before use the plates containing these media were incubated at 37 C for 24 hr and left at room temperature for an additional 24 hr in order to check sterility and also have a perfectly dry surface for streaking. The plates were all used within 10 days after preparation.

When streaking material from selective enrichment media, all six differential plating media listed above were used for cultures originating from nutrient broth pre-enrichment. But in the case of cultures from mannitol purple broth pre-enrichment, MAC, BSA and BGA plates only were used.

Isolation of Cultures: The plates of the various differential media with the inocula were incubated at 37 C for 48 hr. At the end of the first 24 hr they were screened to check the distribution and types of colonies. Two colonies of each type from each of the plates were isolated. The plates were returned for further incubation. The center of each colony was gently touched with the tip of a sterile inoculating needle which was first stabbed into the butt and later stroked on the slant of Kligler's Iron Agar (KIA). Cultures on these KIA slants were incubated at 37 C for 12 hr and later stored in an air-conditioned room at 25 C until identified. Before the plates of differential media were discarded on the third day they were checked and additional isolations were made if necessary. The cultures were suitably indexed with a serial number, date of isolation, their enrichment origin and the specimen number. All the cultures isolated from the first 136 specimens were typed biochemically. However, excepting cultures suggestive of salmonellae, others originating from the rest of 111 specimens were not typed biochemically. A schematic representation of the methods employed is presented in Fig. 1.

Identification of Cultures: Cultures on KIA slants were examined for changes that had occurred in the medium. Hydrogen sulfide positive cultures were separated into one group, and H<sub>2</sub>S negative strains were arranged into two lots. Those showing evidence of pigment production were kept separate while the non-pigmented ones formed another lot.



Key: BGA = Brilliant Green Agar  
 BSA = Bismuth Sulfite Agar  
 SS = Salmonella-Shigella Agar  
 MAC = MacConkey Agar  
 EMB = Eosin Methylene Blue Agar  
 DCA = Desoxycholate Citrate Agar  
 KIA = Kligler's Iron Agar

Fig. 1. Flow diagram showing details of media employed for pre-enrichment, selective enrichment and isolation of salmonellae from samples.



Purification of Cultures: The cultures from KIA were emulsified in about 5 ml of sterile nutrient broth and immediately surface streaked on a MAC plate. After 24 hr of incubation, isolated colonies were picked and transferred from these plates again to KIA. The growth characteristics were recorded. The plates were discarded after a check which indicated that the re-isolated cultures produced changes on the KIA identical to the original tubes.

The scheme presented in Fig. 2 was adopted for establishing the identity of the cultures.

Phage lysis: The genus specific Salmonella "O-1" phage, so useful in separating salmonellae from other enteric organisms, was obtained from Dr. Cherry of the Communicable Disease Center, Atlanta, Georgia. A loopful of each KIA culture was emulsified in about 5 ml of sterile nutrient broth. The tubes were incubated for 3 to 4 hr, when there would be evidence of slight turbidity. A loopful of these young, actively growing cultures was carefully spread on an area of about 1 square centimeter in appropriate sections marked on the back of BGA plates suitably indexed with numbers. The propagating strain (S. paratyphi B<sub>1</sub>) was used as the positive control and a known E. coli stock strain was used as the negative control. When the plates were dry in each area previously spread with a culture, a drop of the phage was carefully delivered from the tip of a finely drawn sterile Pasteur pipette, without touching the surface of the medium. The plates were incubated at 37 C, results read at the end of 6 hr and later confirmed at the end of 24 hr. If the substrate strain was sensitive to the phage an area of confluent lysis developed simulating the appearance of a moon while in other cases there was luxuriant growth in the entire area of inoculation. All the strains isolated

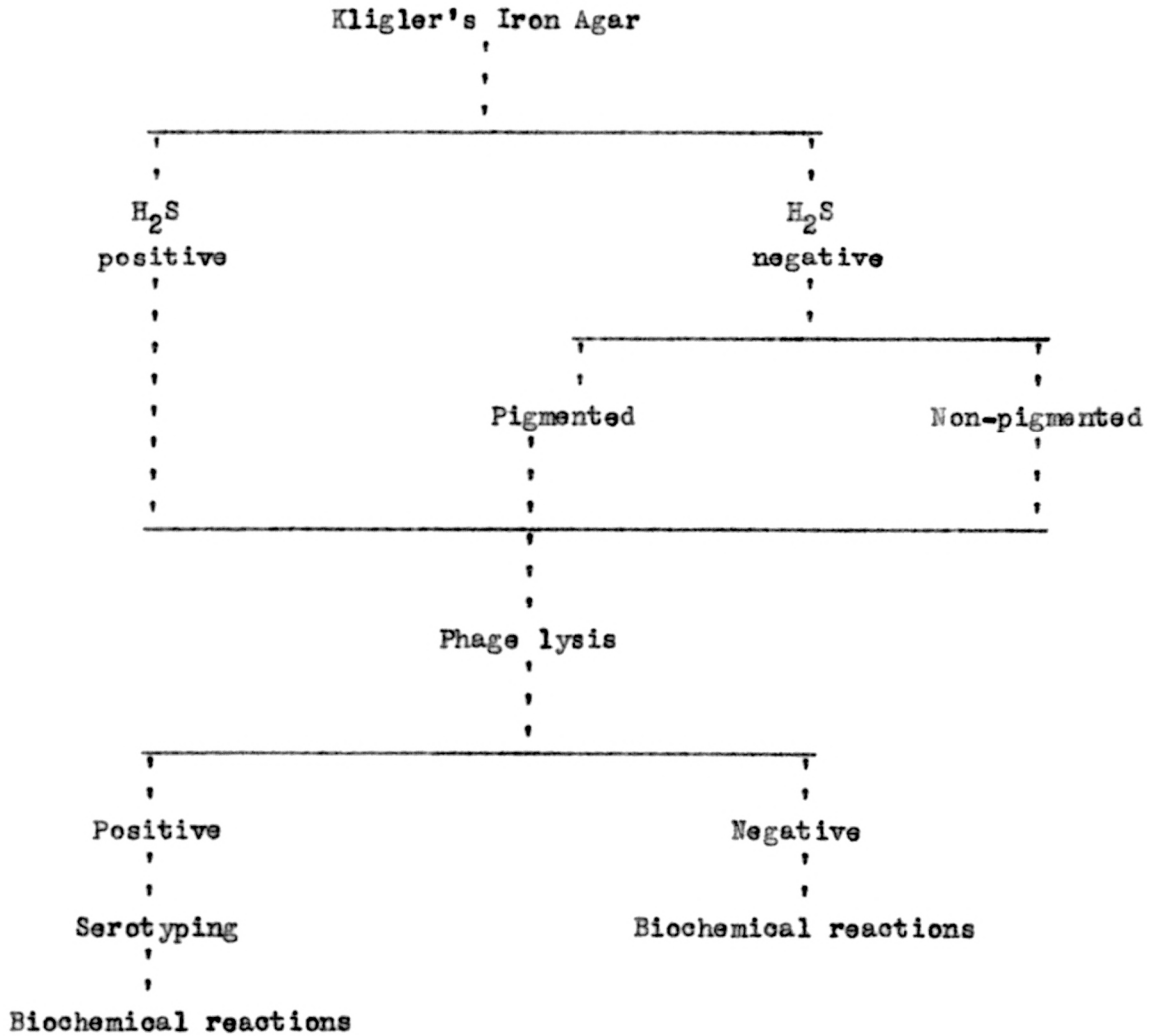


Fig. 2. Scheme for identification of isolated cultures.

in this work were subjected to phage lysis. Only the salmonellae were acted upon by the phage.

Serological Identification: Cultures lysed by the Salmonella genus specific "O-1" phage were subjected to antigenic analysis to the extent possible. "Difco" commercial 'H' and 'O' Salmonella typing sera were employed. In order to conserve the amount of sera used, slight modification of the procedures was adopted.

Determination of Somatic Antigen: Each of these KIA stock strains was seeded on the entire surface of a proteose peptone agar slant. After 18 hr incubation, smears prepared from each culture, stained with Gram's procedure were examined under the microscope to ascertain purity. Employing a sterile loop and repeatedly scooping, a sufficient amount of the growth was removed and suspended in 1 ml of 95 percent ethanol in small test tubes just before use. All suspensions were carefully indexed. These were heated in a water bath at 60 C for 1 hour and later centrifuged at 1800 RPM in an International centrifuge for 10 min. By careful manipulation the supernatants were discarded, retaining the sediments. The tubes were left inverted for drying for 4 hr in a test tube rack, the bottom of which was provided with a filter paper. When dry, the sediment in each tube was reconstituted with 0.5 ml sterile normal saline. The mixture was aspirated in the pipette and dislodged a sufficient number of times to get a uniform stable suspension.

Slide agglutination was carried out in determining the 'O' group. Clean microscope slides were marked with a glass marking pencil into two equal halves. Using a clean inoculating loop approximately 0.05 ml of the saline culture suspension was placed on each side. To one of the spots 0.05 ml of the antiserum was added, leaving the other blank to serve as control. The mixture was quickly mixed with a clean toothpick. The slide was gently warmed over a

flame and carefully rocked to hasten the reaction, and results were read with the aid of transmitted light from a table lamp. Commencing with poly A-I the other groups, viz., A, B, C<sub>1</sub>, C<sub>2</sub>, D, E<sub>1</sub>, E<sub>2</sub>, E<sub>4</sub>, F, G, H, I and Vi, were successively used one at a time. A positive reaction developed in the homologous mixture within 10 sec and was complete within 30 sec. Clumps were distributed in good-sized masses leaving clear, watery areas in the field. The controls remained unaltered and uniform. The cultures were agglutinated by the poly 'O' and only one of the other group sera employed without cross reaction with others.

Flagellar Antigen: When the 'O' antigenic analysis was completed, flagellar agglutination was carried out. Stock cultures were inoculated in 10 ml of sterile nutrient broth and incubated for 18 hr at 37 C. These were inactivated by the addition of an equal volume of 0.6 percent formalinized saline. Employing these suspensions, first slide agglutination (as already described) was carried out to determine phase 1 and phase 2. At this stage undiluted "Difco" Bacto Salmonella 'H' antisera of fractions a, b, c, d, eh; G complex; i, k, L complex; r, y, s, z<sub>4</sub>; l complex; z<sub>10</sub>, z<sub>29</sub> and en complex were employed. Agglutination was clear cut and complete in about 20 sec. No cross reactions were observed.

This was further confirmed by the tube test. For this, 0.1 ml of anti-serum was diluted with 3.3 ml of sterile normal saline. The various diluted flagellar antisera were measured in 0.5 ml quantities in separate agglutination racks. The formalinized cultures were added serially in each set in 0.5 ml quantities. Immediately after the addition the tubes were rocked vigorously. The racks were set in a water bath at 50 C for half an hour. Every 5 min the rack was lifted gently and checked for evidence of

agglutination. In positive cases a fluffy, woolly mass developed which later settled to the bottom in a coarse, nondescript mass.

The results were compared with the Kauffman-White schema furnished by Edwards and Ewing (1962) and the approximate identity was elucidated. This identification was only approximate and not taken as absolute in view of the inherent incompleteness of a total antigen study of this nature.

Biochemical Tests: All the isolated cultures were subjected to biochemical typing. Eighteen-hr-old proteose peptone agar slant cultures emulsified in sterile distilled water were used for inoculation in appropriate media. The 'IMViC' pattern, fermentation reactions, nitrate reduction, urease activity, gelatinase production, citrate utilization and motility were ascertained first. Later, production of phenylalanine deaminase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, malonate utilization, and growth in phenol-red tartrate agar, Stern's glycerol fuchsin broth and mucate broth were studied. Composition of the media, reagents, methods of procedure and interpretation were according to the recommendations of Edwards and Ewing (1962), with the following exceptions - Simmon's citrate agar, phenol red tartrate agar, malonate medium, mucate broth and decarboxylase media which were of "Difco" manufacture. These latter media were prepared and interpreted as per directions in their brochure. Results were arranged on a record chart shown in Fig. 3.

All the strains of salmonellae isolated from 17 different samples and a random of 80 of the remainder of the cultures which were not salmonellae were forwarded to Dr. Charles A. Hunter, Public Health Laboratory, Topeka, Kansas, for final identification.

Viability Trials: Fifteen positive samples were retained in the refrigerator. Cultural examination was carried out on these at intervals of 3, 5, 8, and 12 months from the date of initial isolation.

Culture No.SourceMorphologyStainingColonies on MacConkey's AgarAgar Stroke

Form

Amount of growth

Elevation

Form

Surface

Density

Margin

Chromogenesis

Density

Change of medium if any

Chromogenesis

Fermentation testsDays

	: 1	: 2	: 4	: 7	: 14	: 21	: 28	: 35	: 42	: 50
Glucose	:	:	:	:	:	:	:	:	:	:
Lactose	:	:	:	:	:	:	:	:	:	:
Sucrose	:	:	:	:	:	:	:	:	:	:
Mannitol	:	:	:	:	:	:	:	:	:	:
Dulcitol	:	:	:	:	:	:	:	:	:	:
Salicin	:	:	:	:	:	:	:	:	:	:
Adonitol	:	:	:	:	:	:	:	:	:	:
Inositol	:	:	:	:	:	:	:	:	:	:
Sorbitol	:	:	:	:	:	:	:	:	:	:
Arabinose	:	:	:	:	:	:	:	:	:	:
Raffinose	:	:	:	:	:	:	:	:	:	:
Rhamnose	:	:	:	:	:	:	:	:	:	:

Fig. 3. Chart employed to record the various cultural, biochemical and serological characters of organisms isolated.

Fig. 3 (concl.).

Growth on SS

Growth on BGA.

Growth on BSA.

Motility

<u>Kligler's iron agar:</u>	Slant
	Butt
	H <sub>2</sub> S

Urease

Gelatinase

Indol

M.R.

V.P.

Simmon's citrate

Nitrate test

KCN

Growth on Phenol red tartrate agar (stab)

Phenyl-alanine deaminase

Lysine decarboxylase

Arginine dihydrolase

Ornithine decarboxylase

Malonate utilization

Stern's glycerol fuchsin broth

Mucate broth

Phage lysis

Serology

Culture identified as

## RESULTS

A total of 247 specimens of 23 different categories was subjected to bacteriological examination. Seventeen of these were positive for salmonellae. The nature of the sample and the number from which salmonellae were isolated in each item are furnished in Table 1.

Table 1. The type and number of specimens examined and the number which yielded salmonellae.

Type of specimen	Number examined	Number containing salmonellae
Cake mixes	57	5
Canned baby foods	46	0
Cookie doughs	41	5
Dinner rolls	25	4
Corn bread mixes	15	1
Noodles (dry)	15	0
Frosting mixes	11	0
Soup mixes	8	0
Corn muffin mixes	6	1
Biscuits (bakein type)	5	0
Drinks	3	0
Bakein cookies	2	0
Wheat germ	2	0
Dried milk	2	0
Dog meal	1	0
Malt mix	1	0
Date bread mix	1	0
Bone ash	1	0
Corn meal	1	0
Nuts	1	0
Sunflower seeds	1	0
Pizza dough	1	1
Yeast powder	1	0
Total	247	17

Fifty-seven cake mixes were examined. Of these, 31 contained some egg product. From these, 2 contained salmonellae. The serotypes isolated were one each of S. infantis and S. tennessee. The remaining 26 had no egg product



in them but had some animal product like gelatin, buttermilk or dried milk. Of these, 3 yielded salmonellae, the serotypes being two cultures of S. tennessee and one of S. infantis. All 41 cookie doughs examined contained some egg product. Of these, 5 gave positive isolations, the serotypes being two of S. oranienburg, two of S. tennessee and one of S. infantis. Of the 25 dinner rolls processed, 17 contained egg material, one did not have any egg product and 7 were free of any animal product. Of the 17 containing egg material, 3 contained salmonellae, the serotypes being one each of S. litchfield, S. miami and S. tennessee. The second group which did not contain any egg product failed to yield salmonellae. Of the 7 free of any animal product, one sample yielded S. litchfield. One of the 15 corn bread mixes examined contained S. tennessee. Incidentally, this one specimen was free of any animal product. Six corn muffin mixes which were free of any animal product were examined. One yielded S. muenchen. Of 103 miscellaneous samples, one pizza dough contained S. newington. Table 2 summarizes details of these results. It can be seen from the table that 10 of 116 specimens containing egg products, 4 of 87 samples containing no egg products and 3 of 44 free of any animal products harbored salmonellae.

Colonial Appearance of Different Organisms on Differential Plating Media:

The features of colonial morphology of the different types of organisms met with in this study are summarized in Fig. 4. Although in the majority of instances these characteristics were salutary there were deviations. On BGA, in the presence of excess coliforms, salmonellae colonies were pinkish white with yellow zone because of lactose fermentation by the coliforms (see Plate I). On this medium, Citrobacter cultures simulated Salmonella cultures to a marked extent. Both had a typical butyrous consistency. Also in crowded zones it was difficult to differentiate Pseudomonas cultures from these two. Generally

Table 2. Types of specimens and composition, the number of samples yielding salmonellae, and Salmonella serotypes isolated from the different types of specimens.

Type of specimen	Number of specimens containing egg products	Number containing salmonellae and organism isolated	Number of specimens with no egg product, but some animal product	Number containing salmonellae and organism isolated	Number that contained neither egg nor animal product	Number containing salmonellae and organism isolated
Cake mixes	31	(2) (1) <u>S. tennessee</u> (1) <u>S. infantis</u>	26	(3) (2) <u>S. infantis</u> (1) <u>S. tennessee</u>	Nil	Nil
Cookie doughs	41	(5) (2) <u>S. oranienburg</u> (2) <u>S. tennessee</u> (1) <u>S. infantis</u>	Nil	Nil	Nil	Nil
Dimmer rolls	17	(3) (1) <u>S. tennessee</u> (1) <u>S. litchfield</u> (1) <u>S. miami</u>	1	Nil	7	(1) <u>S. litchfield</u>
Corn bread mixes	6	Nil	1	Nil	8	(1) <u>S. tennessee</u>
Corn muffin mixes	Nil	Nil	Nil	Nil	6	(1) <u>S. muenchen</u>
Others	21	Nil	59	(1) <u>S. newington</u>	23	Nil
<b>Total</b>	<b>116</b>	<b>10</b>	<b>87</b>	<b>4</b>	<b>44</b>	<b>3</b>

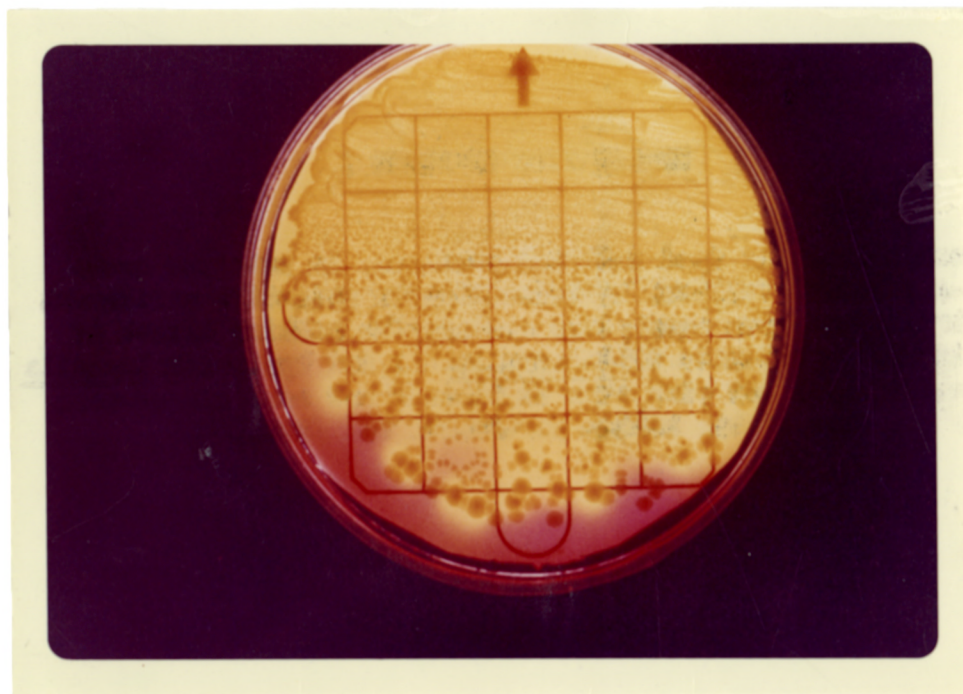
Genus of organism	Brilliant green agar	Bismuth sulfite agar	MacConkey agar	Salmonella-Shigella agar	Desoxycholate citrate agar	Eosin methylene blue agar
<u>Salmonella</u>	Small, circular, umbonate, smooth, entire with an intense red zone, butyrous.	Small, circular, umbonate or umbonate, smooth, entire, dark brown or black with a metallic sheen, butyrous.	Small, circular, umbonate, smooth, entire, colorless, with a clear zone, butyrous.	Small, circular, umbonate, smooth, entire with a clear zone, butyrous.	Small, circular, umbonate, smooth, entire, opaque, clears the zone of precipitated material around, butyrous.	Small, circular, umbonate, smooth, entire, translucent, no change on the medium, butyrous.
<u>Aerobacter</u>	Medium, circular, raised, smooth, entire, yellow with a yellow zone, unctuous.	Medium, circular, raised, smooth, entire, brownish with no change on the medium, unctuous.	Medium, circular, raised, smooth, entire, dirty pinkish center with a clear zone, unctuous.	Medium, circular, raised, smooth, entire with brownish red center with a clear zone, unctuous.	Medium, circular, raised, smooth, entire, pale red center, clears the zone of precipitated material around, unctuous.	Medium, circular, raised, smooth, entire, dark reddish center, opaque, no change on the medium, unctuous.
<u>Klebsiella</u>	As above but large, convex and mucoid.	As above but large, convex and mucoid.	As above but large, convex and mucoid.	As above but large, convex and mucoid.	As above but large, convex and mucoid.	As above but large, convex and mucoid.
<u>Citrobacter</u>	Small, circular, umbonate, smooth, entire, pink with an intense red zone, butyrous.	Small, circular, umbonate, smooth, entire, greenish brown, no change on medium, butyrous.	Small, circular, umbonate, smooth, entire, colorless with a clear zone, butyrous.	Small, circular, umbonate, smooth, entire, dark center and clear margin, butyrous.	Small, circular, umbonate, entire, smooth, opaque, clears the zone of precipitated material around, butyrous.	Small, circular, umbonate, smooth, entire, translucent, no change on the medium, butyrous.
<u>Serratia</u>	Large, circular, convex, smooth, entire, intensely red with a light pink zone, butyrous.	Large, circular, convex, smooth, entire, intensely red. No change on medium, butyrous.	Large, circular, convex, smooth, entire, intensely red, no change on medium, butyrous.	Medium, circular, convex, smooth, entire, light red, no change on medium, butyrous.	Small, circular, convex, smooth, entire, pale pink, clearing of medium, butyrous.	Large, circular, convex, smooth, entire, bluish red with light center and dark periphery, butyrous.
<u>Pseudomonas</u>	Medium, circular, flat, rough, irregular, greenish pink with a pink zone, butyrous.	Medium, circular, flat, rough, wrinkled, crenate greenish brown with or without a metallic sheen, butyrous.	Medium, circular, flat, rough, irregular, green, no change on medium, butyrous.	Medium, circular, flat, rough, irregular, green, no change on the medium, butyrous.	Medium, circular, flat, rough, irregular, green, no change on the medium, butyrous.	Medium, circular, flat, rough, irregular, greenish blue with dark center, no change on the medium, butyrous.

Fig. 4. Chart indicating the colony characters of the different organisms on the various differential plating media.

#### EXPLANATION OF PLATE I

Streak culture of food material processed in nutrient broth pre-enrichment followed by tetrathionate selective enrichment on brilliant green agar plate showing the masking effect of coliforms on colonies of Salmonella. A few typical Salmonella colonies which are pink with an intense red zone can be seen near the edge of the bottom portion of the plate.

## PLATE I



Serratia, Klebsiella and Pseudomonas isolations grew more luxuriantly than others. Serratia and Pseudomonas were identified by their characteristic pigment production, while Klebsiella colonies were prominent in their mucoid consistency.

The distribution of Salmonella colonies was better on BGA than on any other medium. Also, Salmonella colonies were comparatively easy to recognize and differentiate from the rest of the organisms. BSA was another good medium. On this, Citrobacter could easily be differentiated. However, Pseudomonas cultures and colonies of Salmonella which did not produce a metallic sheen were difficult to differentiate. On SS and DCA, distribution was poor and these media were markedly inhibitory for Salmonella which grew as stunted colonies. MAC produced abundant growth of all genera isolated. However, the distribution of Salmonella was poor and Citrobacter colonies confused the picture while selecting colonies for isolation. On EMB, Salmonella colonies were fewer in number because of excessive growth of coliforms. Klebsiella colonies flowed on the entire surface of the medium, submerging all other colonies.

Results of occurrence and distribution of the various contaminants apart from Salmonella in the first 136 specimens are furnished in Table 3. Aerobacter was the predominant contaminant. Pseudomonas was frequent. Klebsiella was less frequent. Citrobacter and Serratia were occasionally found. Specimens yielding salmonellae invariably contained two or more of the contaminants.

Phage lysis: Three hundred twenty-nine strains of different organisms (99 Salmonella, 107 Aerobacter, 59 Pseudomonas, 30 Citrobacter, 24 Klebsiella and 10 Serratia), identified from the first 136 samples were tested for phage lysis. All Salmonella strains were completely lysed by the phage while none of

Table 3. Results of bacteriological examination of 136 samples of food showing the distribution of various organisms.

Type of specimen	Number examined	Number of specimens from which genus of organisms was isolated					
		<u>Salmonella</u>	<u>Aerobacter</u>	<u>Klebsiella</u>	<u>Citrobacter</u>	<u>Serratia</u>	<u>Pseudomonas</u>
Cake mixes	36	2	13	4	3	1	12
Noodles	15	Nil	8	1	Nil	2	4
Cookie doughs	14	2	6	2	1	1	7
Frosting mixes	11	Nil	1	3	Nil	Nil	1
Soup mixes	8	Nil	6	Nil	2	Nil	3
Baby foods	46	Nil	Nil	Nil	Nil	Nil	1
Corn bread mixes	6	1	3	1	Nil	1	1
<b>Total</b>	<b>136</b>	<b>5</b>	<b>37</b>	<b>11</b>	<b>6</b>	<b>5</b>	<b>29</b>

the others was acted upon by the phage. Since some doubt existed about the identity of Citrobacter strains, two of these cultures were referred to Dr. Cherry, for study. They were identified as Citrobacter in his laboratory.

Biochemical Characters and Serology: The biochemical characters and antigenic structure of Salmonella serotypes and the rest of the organisms are furnished in the Appendix. Though 'O' antigenic structure of the Salmonella strains could be identified in this laboratory, it was difficult to elucidate 'H' antigenic configuration. Since fractionated flagellar factor sera were unavailable we could not perform "forced induction" to differentiate Phase 1 and Phase 2. Hence we failed to identify S. muenchen, S. newington and S. miami specifically. All strains of Salmonella were identified by the Kansas State Board of Health Salmonella Laboratory.

Comparison of Enrichment Procedures and Media: All 17 positive samples yielded Salmonella cultures both in nutrient broth as well as mannitol purple broth pre-enrichments. Insofar as the isolations were concerned both gave identical results and neither was superior to the other. Isolations were from the same specimens. In other words, mannitol purple broth did not detect anything new that had not been recovered from nutrient broth and vice versa. However, there was a very interesting picture of colony distribution on the differential media, because of different selective enrichments through which the pre-enriched cultures were processed prior to plating.

It may be recalled that nutrient broth cultures were transferred to unmodified tetrathionate and selenite 'F' media. All six differential plating media were used for seeding the selective enrichment culture material. In nutrient broth-tetrathionate combination, distribution of Salmonella colonies was better than the nutrient broth-selenite 'F' combination. However, in both combinations contaminants predominated to a great extent as evidenced by their



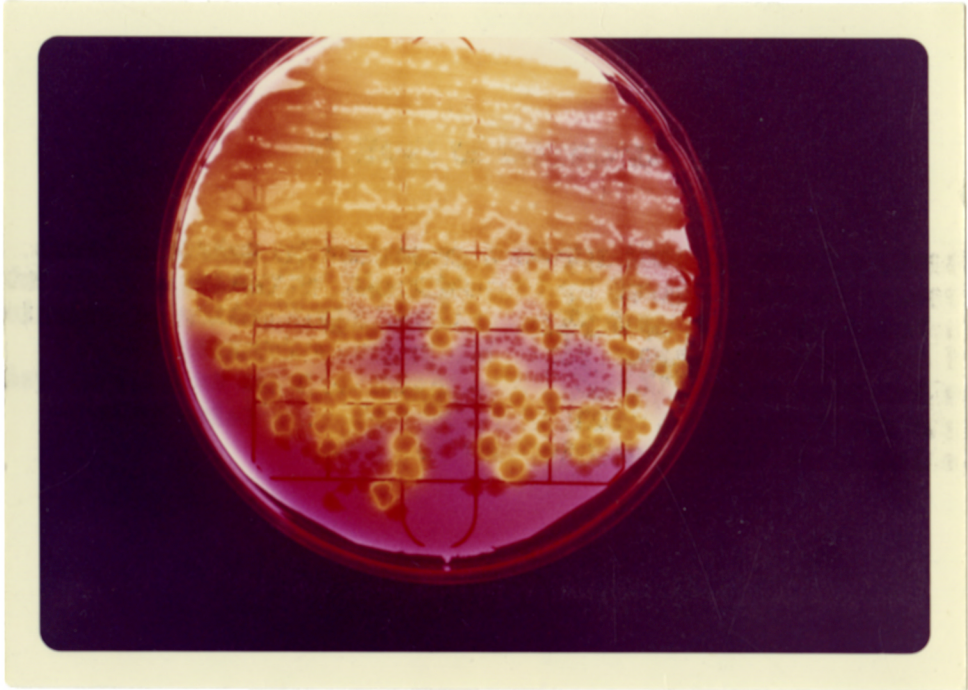
growth on non-inhibitory media like MAC and EMB. BSA was the best in suppressing the contaminants while BGA, though giving a better distribution of Salmonella colonies, failed to suppress the growth of the contaminants to any extent (see Plate II). The most common contaminants were Aerobacter, Klebsiella, Citrobacter and Pseudomonas. In the case of nutrient broth-selenite 'F' combination the contaminants were slightly reduced in number. However, it was felt that there was an apparent inhibitory effect on Salmonella as well, as evidenced by the distribution of colonies on the plates of differential media (see Plate III). Since the contaminants were less in number, Salmonella colonies could be recognized easily. Thus, though tetrathionate and selenite 'F' media gave identical results on the basis of positive isolations, selenite 'F' apparently appears to be a better choice if one were to consider the relative ease with which one could fish out Salmonella colonies in a contaminated environment.

In the case of mannitol purple broth pre-enrichment cultures, brilliant green tetrathionate and cystine selenite 'F' sulfapyridine were used for selective enrichment. Only MAC, BGA and BSA were used for plating. Both selective enrichment media inhibited contaminants to a remarkable extent. However, a few Aerobacter, Klebsiella and Pseudomonas still persisted in brilliant tetrathionate selective enrichment (see Plate IV). Cystine selenite 'F' sulfapyridine selective enrichment cultures presented a remarkable picture on plating media. Pseudomonas was totally absent. Klebsiella and Aerobacter developed occasionally in small numbers (see plate V). In both cases BGA proved itself to be the medium of choice. Though salmonellae were isolated in both instances with ease, cystine selenite 'F' sulfapyridine medium appeared superior in suppressing the growth of contaminants, thus facilitating the isolation of salmonellae in almost pure cultures.

#### EXPLANATION OF PLATE II

Streak culture of food material processed in nutrient broth pre-enrichment followed by tetrathionate selective enrichment on brilliant green agar plate showing distribution of colonies of coliforms and Salmonella. The yellow colonies with a yellow zone are coliforms. Pink colonies with an intense red zone are Salmonella. Note the Klebsiella colonies have coalesced at the top portion of the plate.

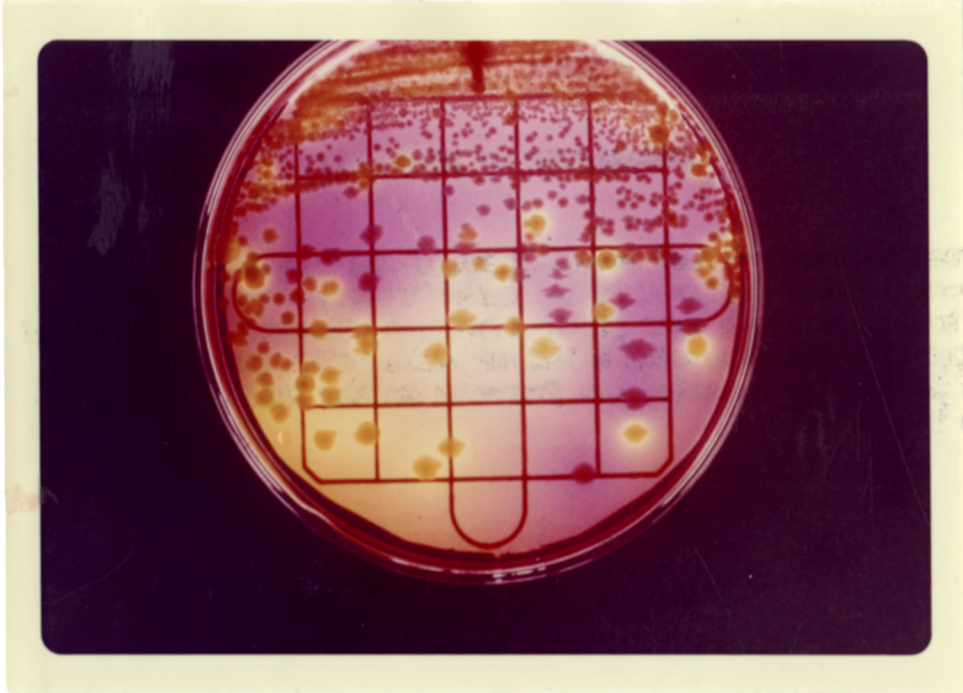
PLATE II



### EXPLANATION OF PLATE III

Streak culture of food material processed in nutrient broth pre-enrichment followed by selenite 'F' selective enrichment on brilliant green agar plate showing a better distribution of Salmonella colonies with a lesser number of coliforms. Note the Salmonella colonies are fewer in number indicating apparent inhibition.

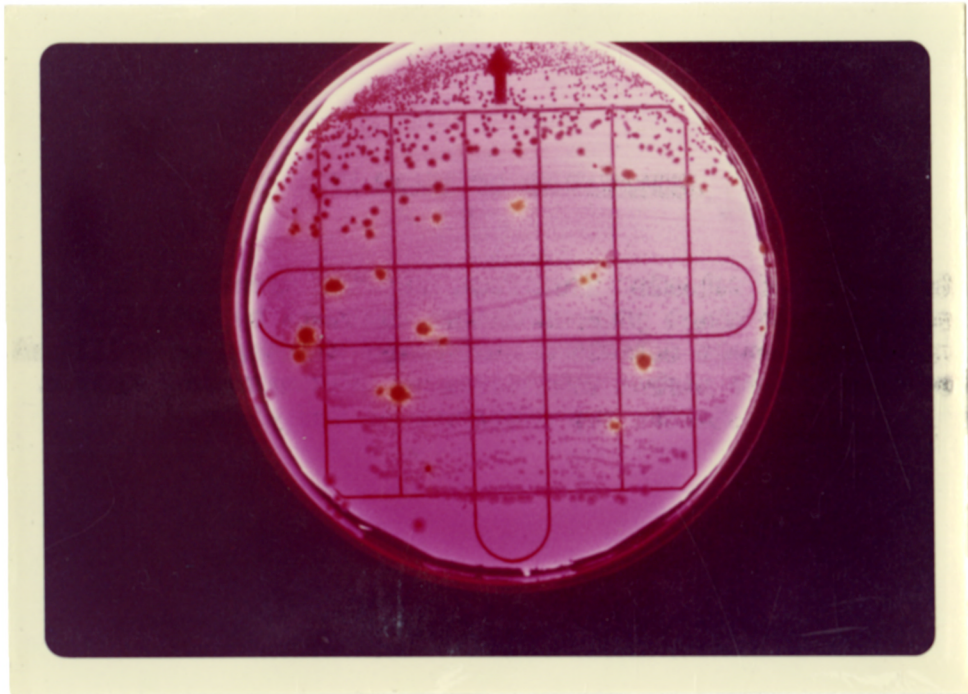
PLATE III



#### EXPLANATION OF PLATE IV

Distribution of colonies from food material processed in mannitol purple broth pre-enrichment followed by brilliant green tetrathionate selective enrichment streaked on brilliant green agar. Note marked inhibition of contaminants. Only a few Klebsiella colonies are dispersed in the plate.

PLATE IV

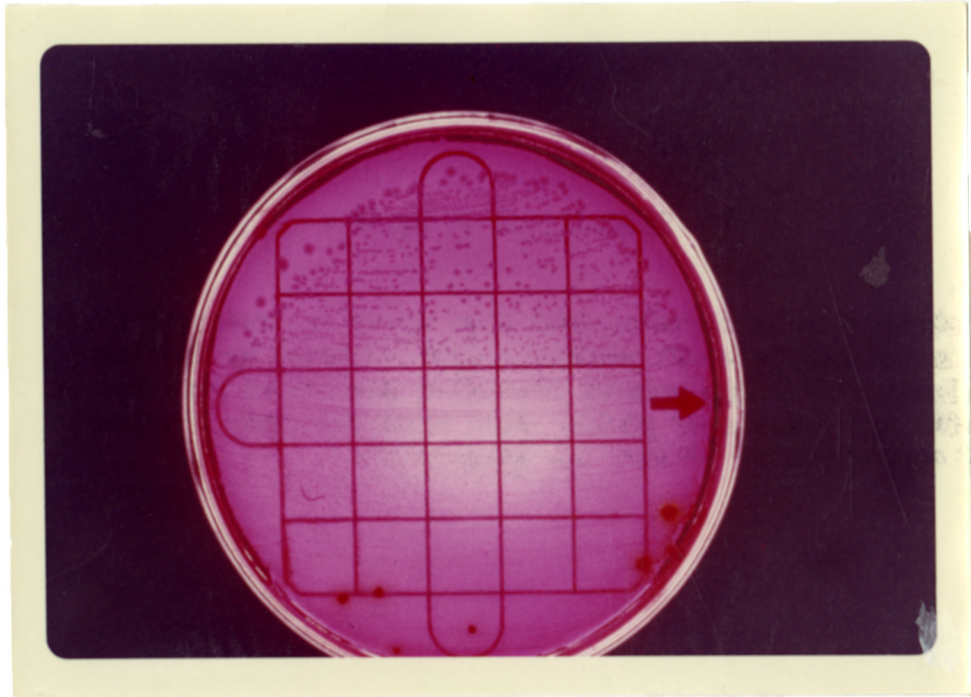


## EXPLANATION OF PLATE V

Distribution of colonies from food material processed in mannitol purple broth pre-enrichment followed by cystine selenite 'F' sulfapyridine selective enrichment streaked on brilliant green agar plate. Note there are only 6 colonies of contaminants. Others are Salmonella.



PLATE V



In summary, both nutrient broth and mannitol purple broth pre-enrichment gave identical results with regard to isolations. In selective enrichment of nutrient broth pre-enrichment cultures, tetrathionate failed to inhibit contaminants to any desirable extent. Selenite 'F' was slightly more advantageous in this respect, but for some unexplainable reason this latter medium had a tendency to suppress the growth of salmonellae as well. In the mannitol purple broth pre-enrichment procedure both brilliant green tetrathionate and cystine selenite 'F' sulfapyridine media proved quite effective in markedly reducing the number of contaminants. Of the two, cystine selenite 'F' sulfapyridine was better than brilliant green tetrathionate medium.

Viability Trials: Five positive specimens from the first lot of 136 samples processed during June, 1962, continued to retain viable salmonellae at the end of one year. Since insufficient material was collected from two specimens of dinner rolls for storage, only 6 of the 8 positive samples from the second lot of 68 examined during December, 1962, were available for viability experiments. They all yielded salmonellae after 6 months from the initial date of isolation. Each of the 4 positive samples from the third lot handled during April, 1963, yielded salmonellae at the end of 3 months. This proved that no matter whether the specimens were dry or moist, salmonellae could prevail in a viable state for a long time when stored in the refrigerator.

#### DISCUSSION

The choice of proper laboratory procedures is an essential prerequisite for the isolation of any pathogenic organism. Employment of appropriate media and techniques coupled with good judgment on the part of the research worker will yield the best result. Recognition of biological variations is equally

important so as not to miss aberrant forms. In dealing with the problem of isolation of salmonellae from foods there are certain inherent difficulties. While processing such materials one has to take cognizance of the advantages and disadvantages of the procedures and media employed in order to strike a balance and thus surmount some of the difficulties, if not all. Admittedly procedures that have been hitherto used or adapted have largely come from the background of information gathered from those primarily employed for screening fecal specimens. Workers engaged in isolating salmonellae from foods have concentrated their efforts during the past few years to study the suitability of various procedures, formulated or modified the composition of media, determined their defects and thus constantly endeavored to improve upon these procedural details. In processing food materials pre-enrichment and selective enrichment have been found to be a necessity. Since the number of salmonellae in prepared foods is small, it is essential to build this population before transferring to selective media. The organisms must be stimulated to grow from a state of reduced viability and physiological inactivity suffered during the process of manufacture. Some kind of pre-enrichment is necessary to accomplish this. Uniform dispersion of materials in pre-enrichment media facilitated better recovery. Some of the enrichment media were either inhibitory to certain strains of salmonellae or they favored the growth of contaminants equally well. Further it has been realized that the presence of large amounts of organic matter exerts a deleterious effect on the performance of selective enrichment media. People trying to understand the mechanics of this have not come to any definite conclusion. Even the differential plating media presented problems. Some of these media were too inhibitory. Others failed to suppress the growth of contaminants to any marked extent so much so that it was difficult to differentiate colonies of salmonellae from others. For all intents and purposes it has to be conceded that there has been much progress

in the methodology. However, despite the better efficiency claimed by various workers in this field there still remains room for improvement. The primary object of this study was to isolate salmonellae from prepared foods as available for sale in the open market. The methods adapted were mainly focused to achieve this, bearing in mind some of the drawbacks as discussed.

The choice of pre-enrichment media used in this study was made after due consideration of available information from the experiences of previous workers. Hurley and Ayres (1953), Stokes and Osborne (1958), Sillicker and Taylor (1958) and Galton (1961) had recognized the untoward effect of large amounts of organic matter in the performance of selective enrichment media. Daek (1955), Slooem (1955) and Edwards and Ewing (1962) had expressed the desirability of pre-treatment of materials prior to transfer to selective media. Schneider (1946) and Byrne et al. (1955) had favorable results with reconstitution of materials in distilled water. Banwart and Ayres (1953) found that nutrient broth supported luxuriant growth of salmonellae. North (1961) and Galton (1961) advocated the feasibility of using nutrient broth for pre-enrichment. North (1961) had favorable results with lactose broth. Taylor and Sillicker (1961) found pre-enrichment broths gave better results than reconstitution of materials in distilled water. Lactose, mannitol and dulcitol incorporated in pre-enrichment broths gave comparative results. North and Bartram (1953) found that the quality of peptone affected the productivity of selenite 'F' medium. Incorporation of a small amount of yeast extract remedied the defect. It was thus decided to use two noninhibitory pre-enrichment media, nutrient broth and mannitol purple broth, in this work. The former was prepared fresh in the laboratory using basic ingredients. Proteose peptone No. 3 "Difco" was found to be an excellent variety of commercial peptone for use in this work. In addition, yeast extract and beef extract were incorporated to provide essential growth factors. Mannitol purple broth was made by reconstituting

purple broth base "Difco" and adding mannitol. It was felt that the presence of a fermentable substrate usually utilized by salmonellae could be an added advantage over nutrient broth. This could facilitate active multiplication of salmonellae when present even in small numbers. A 10 percent suspension of the various food stuffs in both these media provided optimal ratio of bulk volumes for the equitable distribution of materials. Only a small portion of these were later transferred to selective enrichment media after incubation. Thus the possible ill effects on the growth of salmonellae due to the presence of excess amounts of extraneous organic matter could be avoided. Transfers of these pre-enrichment cultures were made at the end of a preliminary incubation period of 12 to 15 hr when one could expect a large and steady population.

For selective enrichment of nutrient broth pre-enrichment cultures both tetrathionate and selenite 'F' media were chosen because even if some strains were affected by one medium, the other medium could possibly detect the organism. Also the efficiency between tetrathionate and selenite 'F' in reducing the contaminant population could be elucidated. It may be recalled that Banwart and Ayres (1953) reported tetrathionate was inhibitory to S. paratyphi and S. anatis. According to Smith (1952) both tetrathionate and selenite 'F' were inhibitory to S. cholerae-suis and S. abortus-ovis. For selective enrichment from mannitol purple broth pre-enrichment cultures, tetrathionate medium was modified by the addition of brilliant green, while selenite 'F' medium was modified by addition of both cystine and sulfapyridine. The effectiveness of brilliant green in limiting contaminants has been reported by Stokes and Osborne (1955) and Osborne and Stokes (1955). Brilliant green was one of the constituents of the medium they had formulated. The brilliant green tetrathionate medium gave excellent results in the hands of experienced workers like Galton (1961) and Edwards and Ewing (1962) in isolating salmonellae from varied sources. Cystine and sulfapyridine were incorporated

into selenite 'F' because of good results obtained by North and Bartram (1953) and Osborne and Stokes (1955). It was felt that a choice combination of both the pre-enrichment and selective enrichment media could supplement and complement each other and thus result in maximum recovery. Our results indicated that both nutrient broth and mannitol purple broth pre-enrichments were identical, and neither was superior to the other. However, the distribution of colonies of salmonellae in comparison to the other contaminating organisms was by far superior in mannitol purple broth pre-enrichment. This could be explained by considering 3 factors: (1) the ratio of salmonellae to the contaminants in the initial population at the time of transfer to selective enrichment media; (2) the function of the selective enrichment media; and (3) the value of differential plating media in inhibiting the contaminants. Probably in nutrient broth, salmonellae as well as the contaminants grew equally well. The utilization of mannitol could have helped in building a rapid population of salmonellae by allowing them to outgrow the contaminants in the case of mannitol purple broth. Once sufficient numbers of salmonellae were present, the acidity developed as a result of mannitol fermentation could have checked the excessive multiplication of the remainder of the organisms. A similar concept was expressed by North (1961) for lactose broth pre-enrichment. Since the inhibition of contaminants was largely the function of selective media, addition of a suitable selective bacteriostatic substance could have evidently improved their efficiency. In general, judging from the observation of distribution of colonies on the plates of differential media which reflected the population transferred to them, tetrathionate and selenite 'F' could not be considered as satisfactory. The selective differential plating media though capable of dealing with small numbers of contaminants were unable to check the development of contaminant colonies, when present in

excessive numbers. Evidently nutrient broth facilitated the luxuriant growth of salmonellae along with the contaminants. Both brilliant green tetrathionate and cystine selenite 'F' sulfapyridine media successfully inhibited the contaminants to a remarkable extent. Mannitol purple broth could have had a preponderance of salmonellae with a lesser number of non-mannitol fermenters which were inhibited with facility in the selective enrichment media. This could be attributed to the probable selective bacteriostatic propensity of brilliant green and sulfapyridine because these were the additional ingredients incorporated into the commercial base of tetrathionate and selenite 'F' media, respectively.

The inhibitory effect of certain plating media on some strains of salmonellae had been recognized by various workers. Banwart and Ayres (1953) had good results with BGA but found DCA, BSA and SS inhibitory for a number of serotypes. Byrne et al. (1953) noted that BGA was not a suitable medium for S. pullorum. Others used BGA with advantage notably Taylor et al. (1958) and Wells et al. (1958). Edwards and Ewing (1962) recommended BGA and BSA for general use. Even though we could isolate all the serotypes met with in this study on each of the six differential plating media used, BGA proved more advantageous than the others. However, Citrobacter and Pseudomonas were difficult to differentiate from Salmonella on this medium. BSA was good, while DCA and SS were highly inhibitory. MAC and EMB were the least selective.

A variety of prepared foods, as listed in Table 1, contained salmonellae. Cake mixes, cookie doughs and dinner rolls constituted the main source, while occasional specimens of corn bread mixes and corn muffin mixes were positive. Cookie doughs and dinner rolls were moist while the rest were dry. As indicated in Table 2 not only foods which had some egg product but also the ones devoid of egg matter yielded salmonellae. It was interesting to note that in

cake mixes, the same serotypes were found in samples that had egg products as well as those that did not contain any egg material. If one were to consider the overall situation, apparently, foods that contain egg products yielded the maximum number of positive isolations, while specimens free of any animal product were least productive. The samples having some animal products contained a moderate number of positives. However, it must be pointed out that the number of specimens used in this study that did not contain any animal or egg product was not large. The frequency of occurrence of the various serotypes in the 17 positive samples were: S. tennessee (6), S. infantis (4), S. oranienburg (2), S. litchfield (2), S. miami (1), S. muenchen (1) and S. newington (1).

This study presented substantial evidence that foods purchased by the consumer (no matter whether dry or moist, whether they contain some animal product or are free of any ingredient of animal origin) could harbor a potential pathogen. Viability trials indicated that salmonellae could survive for a long time under refrigerated condition. Edwards et al. (1948) have furnished interesting information regarding the occurrence of many of the serotypes listed above, from divergent sources. S. oranienburg had been isolated from veal salad, a food handler and from a patient who had eaten the salad. S. miami was isolated from pickles and persons who had consumed this delicacy. S. oranienburg and S. newington were isolated from chocolate-covered ice cream suspected of having been the cause of salmonellosis in Kentucky. S. muenchen and S. tennessee have been found to occur in reptiles, birds, animals and men. Galton (1960), in a review for epidemiologists, suggested the possibility of fowls and egg products playing a major role in human infections. Many workers in different parts of the globe have isolated salmonellae from raw ingredients that go into the making of prepared foods. The frequent occurrence of



salmonellae in egg products has been noted by Gibbons and Moore (1944), Ratna and Dolman (1947), Polak (1960) and Yuraok (1962). In Canada various brands of cake mixes screened recently have yielded many serotypes. Shotts et al. (1961) have isolated the same serotypes met with in this study from cake mixes. The isolation of salmonellae from wheat flour (Thomson, 1955) and from dried coconuts (Daniëls-Bosman and Huisman, 1960) have substantiated the possibility of the prevalence of salmonellae in foods with no animal matter.

The genus specific salmonella "O-1" phage was very useful in rapid, early identification of salmonellae cultures from the other organisms isolated. The phage was highly specific and did not lyse any other organism excepting the salmonellae strains. For an epidemiologist or a research worker looking for salmonellae amidst large number of cultures this could be an invaluable adjunct in routine laboratory procedures. Apart from saving a lot of time, money, and effort it could lead to rapid identification of the strain or strains directly by serological procedures conveniently bypassing the tedious biochemical examination. However, as pointed out by Cherry et al. (1954), occasional strains might not be sensitive to the phage, in which case one can always fall back upon biochemical methods to discern their identity. The old dictum that no biological test is 100 percent true still holds good.

#### SUMMARY

Bacteriological examination to determine the occurrence of salmonellae was carried out on 23 different kinds of pre-prepared and packaged food samples purchased from various grocery stores at Manhattan, Kansas. Seventeen of 247 samples examined were found to harbor salmonellae. Each of the samples yielding salmonellae contained only one of the following seven serotypes: S. tennessee, S. infantis, S. oranienburg, S. litchfield, S. muenchen, S. miami

and S. newington. Samples yielding salmonellae were found to contain two or more of the following contaminants: Aerobacter, Pseudomonas, Klebsiella, Citrobacter and Serratia.

Two pre-enrichment media (nutrient broth and mannitol purple broth) were used to compare their efficiency in isolating salmonellae. Nutrient broth pre-enrichment cultures were transferred to each of unmodified tetrathionate and selenite 'F' media for selective enrichment prior to plating. Mannitol purple broth cultures were transferred to each of brilliant green tetrathionate and cystine selenite 'F' sulfapyridine media for selective enrichment. With regard to results of isolation, both nutrient broth and mannitol purple broth yielded salmonellae from the same samples. The unmodified tetrathionate and selenite 'F' media used following nutrient broth pre-enrichment failed to suppress the growth of contaminants. Both brilliant green tetrathionate and cystine selenite 'F' sulfapyridine selective enrichments following mannitol purple broth pre-enrichment effectively inhibited the growth of contaminants. The cystine selenite 'F' sulfapyridine medium was superior to the brilliant green tetrathionate medium. Of the plating media, BGA was better than the others. BSA was satisfactory while DCA and SS were highly inhibitory to salmonellae. MAC and EMB were least selective. It appears that mannitol purple broth pre-enrichment followed by selective enrichment in cystine selenite 'F' sulfapyridine coupled with employment of BGA, BSA and MAC for plating is an efficient procedure for the isolation of salmonellae from foods.

The genus specific salmonella "O-1" phage was found to be highly specific. It was very useful in rapidly identifying salmonellae cultures.

Salmonellae in foods under refrigerated conditions were found viable at the end of one year from the initial date of isolation. The prevalence of

such potential pathogens in human foods coupled with the prolonged viability of these organisms under storage conditions should be regarded as a serious menace to public health.

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APPENDIX

Table 5. Reactions of the different species of bacteria isolated from foods in a study to detect Salmonella species.

Name of organism	Kligler's iron agar			Mor- phology and Gram's stain	Chro- mo- geni- sis	Fermentation reactions												
	Slant	Butt	H <sub>2</sub> S			Glucose	Lactose	Sucrose	Mannitol	Dulcitol	Salicin	Adonitol	Inositol	Sorbitol	Arabinose	Raffinose	Phannose	
<u>Salmonella tennessee</u>	Alk	A	+	Gram negative rods measuring 1-2 x 0.6-0.8	-	Ag	-	-	+	+	-	-	+	+	+	-	+	
<u>Salmonella infantis</u>	Alk	A	+		-	Ag	-	-	+	+	-	-	+	+	+	-	-	+
<u>Salmonella oranienburg</u>	Alk	A	+		-	Ag	-	-	+	+	-	-	+	+	+	-	-	+
<u>Salmonella litchfield</u>	Alk	A	+		-	Ag	-	-	+	+	-	-	+	+	+	-	-	+
<u>Salmonella miami</u>	Alk	A	+		-	Ag	-	-	+	+	-	-	+	+	+	-	-	+
<u>Salmonella muenchen</u>	Alk	A	+		-	Ag	-	-	+	+	-	-	+	+	+	-	-	+
<u>Salmonella newington</u>	Alk	A	+		-	Ag	-	-	+	+	-	-	+	+	+	-	-	+
<u>Citrobacter</u> species	Alk	A	+		-	Ag	Ag	+	+	+	-	-	+	+	+	-	-	+
<u>Klebsiella</u> species	A	A	-		-	Ag	Ag	+	+	+	+	+	+	+	+	+	+	+
<u>Aerobacter</u> species	A	A	-		-	Ag	Ag	+	+	+	+	+	+	+	+	+	+	+
<u>Serratia</u> species	Alk	A	-	-	+	Ag	Ag	-	+	-	+	-	-	+	-	-	-	
<u>Pseudomonas</u> species	Alk	Alk	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	

Alk = Alkaline

A = Acid

Ag = Acid and gas

+ = positive (in the case of fermentation reactions, indicates acid production only)

- = negative

Table 5 (concl.).

Name of organism	Other biochemical reactions																Serology		
	Urease	Gelatinase	Indol	M. R.	V. P.	Motility	Simmon's citrate agar	Nitrate reduction	Phenol red tartrate agar	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Malonate utilization	Stern's glycerol fuchsin broth	Mucate broth	Phage lysis	Somatic	Flagellar, phase I
<u>S. tennessee</u>	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	C <sub>1</sub>	29	-
<u>S. infantis</u>	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	C <sub>1</sub>	r	1,5
<u>S. oranienburg</u>	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	C <sub>1</sub>	m,t	-
<u>S. litchfield</u>	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	C <sub>2</sub>	l,v	1,2
<u>S. miami</u>	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	D	a	1,5
<u>S. muenchen</u>	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	C <sub>2</sub>	d	1,2
<u>S. newington</u>	-	-	-	+	-	+	+	+	-	-	+	+	-	+	+	+	E <sub>2</sub>	e,h	1,6
<u>Citrobacter sp.</u>	-	-	-	+	-	+	+	+	-	-	+	+	Inconclusive	-	+	-	Not determined		
<u>Klebsiella sp.</u>	-	-	-	-	+	-	+	+	-	-	+	+	+	-	+	-	"		
<u>Aerobacter sp.</u>	-	+	-	-	-	+	+	+	+	-	+	+	+	-	+	-	"		
<u>Serratia sp.</u>	-	+	-	-	+	+	+	+	+	-	+	+	-	-	+	-	"		
<u>Pseudomonas sp.</u>	-	+	-	-	-	+	-	+	+	-	+	+	+	-	+	-	"		

SALMONELLAE IN MARKETED FOODS;  
ISOLATION FROM PRE-PREPARED AND PACKAGED SAMPLES  
AT THE CONSUMER LEVEL

by

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Salmonellosis continues to be one of the significant international public health problems. Contaminated foods can be a potential source for the initiation and perpetuation of the chain of infection. In order to find out the extent of prevalence of these pathogens in foods mainly intended for human consumption, bacteriological examination was carried out on various prepared and packaged samples sold at consumer level.

In this study, two pre-enrichment media (nutrient broth and mannitol purple broth) which have gained much popularity during recent years in detecting salmonellae in foods were used in order to assess their efficacy. Cultures originating from nutrient broth pre-enrichment were transferred to each of unmodified tetrathionate and selenite 'F' selective enrichment media, prior to plating on differential media. Likewise, mannitol purple broth pre-enrichment cultures were processed in each of brilliant green tetrathionate and cystine selenite 'F' sulfapyridine media for selective enrichment. Both pre-enrichment procedures gave identical results. However, judging from the distribution of colonies on plates of differential media, the unmodified tetrathionate and selenite 'F' media used for selective enrichment in the case of nutrient broth cultures failed to suppress the growth of contaminants. Both brilliant green tetrathionate and cystine selenite 'F' sulfapyridine media used for selective enrichment in the case of mannitol purple broth pre-enrichment cultures effectively inhibited the multiplication of contaminants. Between the two, cystine selenite 'F' sulfapyridine medium was superior to brilliant green tetrathionate medium. Of the six differential plating media employed, BGA was superior to all others. BSA was satisfactory while SS and DCA were highly inhibitory to both salmonellae as well as the contaminants. MAC and EMB were least selective.

Seventeen of 247 samples examined were found to harbor salmonellae. Five of 57 cake mixes yielded either serotype S. tennessee or S. infantis. From 5 of 41 cookie doughs, one of 3 serotypes (S. oranienburg, S. tennessee and S. infantis) was isolated. Four of 25 dinner rolls processed were found to contain one of 3 serotypes viz., S. tennessee, S. litchfield and S. miami. From one of 15 corn bread mixes examined, S. tennessee was isolated. One of 6 corn muffin mixes was found to contain S. muenchen. One pizza dough from the remainder of 103 miscellaneous specimens yielded S. newington.

The genus specific salmonella 'O-1' phage used in this study was highly specific and facilitated early recognition of salmonellae cultures.

Salmonellae in foods under refrigerated conditions were found viable at the end of one year from the initial date of isolation. The prevalence of such potential pathogens in human foods coupled with the prolonged viability of these organisms should be regarded as a serious menace to public health.