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THE DETECTION OF SALMONELLAE FROM THE BIG SIOUX RIVER USING  
FLUORESCENT ANTIBODY TECHNIQUE AND IMPROVED CULTURAL METHODS

BY

RALPH LAMAR PIERCE JR.

A thesis submitted  
in partial fulfillment of the requirements for the  
degree Master of Science, Department of  
Bacteriology, South Dakota State  
University

1970

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RLP

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## INTRODUCTION

The Water Quality Act of 1965 was passed with the intention of enhancing the quality and value of water resources and establishing a national policy for the prevention, control, and abatement of water pollution.

The Federal Water Pollution Control Administration has issued guidelines to be followed by the States in developing standards for interstate waters. As an interstate stream, the Big Sioux River must conform to the Federal requirements.

Pollution in South Dakota is derived principally from agricultural, municipal and industrial wastes. Dairy, meat processing and mining industries are the most important sources of industrial wastes (65).

Bacterial pollution is the contamination of the water by bacteria, most significantly the pathogenic bacteria such as salmonellae. Coliform densities have been traditionally used as an indication of pathogenic bacteria in the water.

Only a limited number of workers (7, 60, and 64) have done studies showing the presence of salmonellae in surface waters but none have done studies in South Dakota. This is due to the reluctance of working with a pathogenic organism, the low numbers present in the water and the inadequate methods of isolation.

The purpose of this study was to show the presence of salmonellae in the Big Sioux River, improve the methods for their isolation and develop a method for their quantitation.

## LITERATURE REVIEW

Salmonella typhi was first recognized in 1856 by William Budd to be infectious and to be transmitted by sewage-contaminated water. It was not until 1885 that Salmon and Smith isolated Salmonella choleraesuis from a case of hog cholera.

There has been much work done on the methods of salmonellae isolation from foods and feeds but only a limited number of workers have studied its isolation from water (7, 60, and 64).

Salmonellae in Water

Moore (51) isolated paratyphoid organisms from sewage using gauze swabs submerged in the water.

Spino (64) used the modified Moore swab to concentrate organisms present in the river. He isolated salmonellae from 15 of the 16 sampling sites along the Red River of the North below Fargo, North Dakota. Some of the serotypes recovered were Salmonella typhimurium, S. kentucky, S. saintpaul, S. heidelberg, S. infantis, and S. enteritidis.

Gallagher (19) reported that salmonellae were isolated at total coliform densities of 862 organisms/100 ml and fecal coliform densities of 35 organisms/100 ml in Las Vegas Wash, Nevada. Further down the wash, below a point of chlorination, salmonellae were not isolated but the total and fecal coliform concentrations were 6860 organisms/100 ml and 1280 organisms/100 ml, respectively. Three different serotypes were isolated, these were Salmonella montevideo,

S. manhattan, and S. nottingham.

West (77) showed the presence of salmonellae on the Chattahoochee River at the Columbus, Georgia water supply intake. The total coliform density was 800 organisms/100 ml and the fecal coliform density was 20 organisms/100 ml. Salmonella montevideo and S. muenchen were isolated during this study.

Miner et al. (48) recovered 10 isolates of Salmonella infantis from runoff and fecal samples taken at two feedlots near Manhattan, Kansas.

Slanetz et al. (59 and 60) isolated salmonellae from seawater. Some of the serotypes recovered were Salmonella thompson, S. tennessee, and S. braenderup.

Brezenski and Russomanno (7) isolated numerous salmonellae from polluted tidal estuaries. Among the serotypes recovered were Salmonella saintpaul, S. montevideo, S. typhimurium, S. derby, S. oranienberg, S. newington, and S. heidelberg.

#### Survival Studies

Gallagher et al. (21) reported that on a survey of the Red River of the North salmonellae were isolated 62 miles and 3 days travel downstream from the waste source. The total and fecal coliform densities immediately below the source of wastes were 432,000 organisms/100 ml and 155,000 organisms/100 ml, respectively while those at the point of isolation of salmonellae were 6,630 organisms/100 ml and 1,610 organisms/100 ml. This showed the isolation of salmonellae at a point where there was a 99 percent

decrease in both the total and fecal coliform densities.

Spino (64) isolated salmonellae 73 river miles and 4 days time from Fargo-Moorehead along the Red River of the North (Minnesota-North Dakota). The total and fecal coliform densities 7 miles below the source of wastes were 162,000/100 ml and 61,000/100 ml, respectively while those at the point of isolation of salmonellae were 6,140/100 ml and 2950/100 ml.

Gallagher and Spino (20) studied salmonella survival in sugar beet-waste water that had been sterilized by filtration. After 14 days there was only 95 percent die-off at 10 C. Salmonella typhimurium was shown to be much more persistent than fecal coliforms at low temperatures.

#### Media and Methodology

Bacterial Concentration The isolation of salmonellae from water has been difficult because of the low numbers present as compared to the large numbers present when foods or feces are studied. For this reason the organisms in the water sample have to be concentrated into a workable volume.

As mentioned earlier, Moore (51) and Spino (64) both used gauze swabs submerged in the water for several days to collect organisms present in the water. The Moore swab procedure will only show the presence or absence of salmonellae without knowledge of the sample size.

Kenne et al. (40) concentrated the water samples by passing them through membrane filters to remove the organisms that were in

the sample. This method has the advantage over the Moore swab because of the known sample size.

Slanetz (59) concentrated water samples using two methods. First, samples of up to 4 liters were filtered through cloth coated with celite. He also used Moore swabs that were kept submerged for 3-7 days. In 1968, Slanetz used membrane filters coated with celite to concentrate salmonellae in the water samples.

Spino (64) and Miner (48) isolated salmonellae using modified Moore swabs that had been submerged for a period of several days.

Brexenski and Russomanno (7) used gauze pads that were tied to barbecue racks and submerged for two days.

Pre-enrichments and Enrichments Pre-enrichment and enrichment media are used for the propagation of the salmonellae. Pre-enrichment media contain no inhibitors and they allow the growth of most organisms present. Enrichment media contain inhibitors and are selective for salmonellae.

Pre-enrichment techniques were used by North (54) which gave higher most probable numbers of salmonellae. He used lactose broth for the pre-enrichment of salmonellae in egg products. North's reasoning was that a mixed flora would produce acid conditions which are inhibitory for many organisms but not for salmonellae. Montford and Thatcher (49) used a lactose broth pre-enrichment when they isolated salmonellae from cake mixes and dried eggs. Reamer et al. (57) used this same broth for pre-enrichment of salmonellae in non-fat dry milk. Sperber and Deibel (63) used lactose broth for

salmonellae detection in dried foods and feeds. Fantasia (17) also used this broth for isolation of salmonellae from raw chicken and hamburger.

Enrichment media are used to selectively inhibit as many organisms as possible while allowing the salmonellae to grow relatively unrestricted. The two enrichments most commonly used are Mueller's (52) tetrathionate broth as modified by Kauffman (37) and Leifson's (45) Selenite broth. Selenite broth had been shown to be superior to tetrathionate broth for detecting salmonellae in feces (33, 61, and 55). North and Bartram (55) reported that the incorporation of L-cystine into selenite broth gave higher salmonellae recovery. Other enrichments that have been used are GN broth (Difco) for the selection of Gram negative organisms and Raj's (56) dulcitol sodium selenite enrichment that is reported to have the capacity to recover from 2 to 7 salmonellae cells from a mixed flora of  $10^4$  to  $10^6$  organisms.

Isolation Isolation of salmonellae on solid plating media follows the enrichment step. The enrichment media are streaked for isolation onto the highly selective media in order to obtain isolated colonies of salmonellae. The most commonly used plating media are brilliant green agar (42) and bismuth sulfite agar (79 and 80). Other plating media which have been used to a lesser degree are desoxycholate citrate agar (61), Salmonella-Shigella agar (62), MacConkey agar (47), XLD agar Taylor (69) and Hektoen agar (41).

Identification and Confirmation Selective enrichment and the use of inhibitory plating media decreases the number of organisms that are allowed to grow on the solid media. Further classification can be made by testing only those colonies that show typical morphology. Those that are typical are tested for their biochemical reactions.

The most common medium used for biochemical testing is triple sugar iron (TSI) agar which is a modified Russell's (58) medium. It was modified in 1917 by Krumwiede and Kohn (43) with the addition of sucrose. Later Sulkin and Willett (68) improved the medium with the addition of a hydrogen sulfide indicator.

Edwards et al. (14) developed a lysine iron agar (LIA) to show the production of lysine decarboxylase. There are only three genera of enteric organisms species of Edwardsiella, Salmonella and Arizona that produce both hydrogen sulfide and lysine decarboxylase. Some workers do more biochemical tests in various combinations. Isolates giving positive reactions for the biochemical tests are further classified using serology. Both somatic and flagellar are usually tested for according to the Kauffmann-White schema (13, 38 and 39).

The final confirmation is normally done by having the isolates typed by the National Salmonella Typing Laboratory, National Communicable Disease Center, Atlanta, Georgia.

Optimum Temperature Different incubation temperatures have been studied to find the one that will decrease the generation time of salmonellae to the greatest degree. Harvey and Thompson (31) increased the incubation temperature to 44 C to isolate shigellae

from feces. While at this elevated temperature it was salmonellae, not shigellae that developed in the selenite enrichment. Further study indicated that 43 C was the optimum temperature for a 24 hr salmonellae enrichment.

The London Water Board (46) ran duplicate plates, one at 37 C and the second at 43 C. The 43 C temperature was found to be too high because very little growth was observed on the plates.

Salmonella paratyphi B and S. typhimurium were isolated at 42 C from bismuth sulfite plates in large numbers with few extraneous organisms. The tetrathionate enrichment did not produce any growth at either 42 C or 43 C. The selenite enrichment that was plated did produce growth at 37 C, 42 C, and 44 C. There was a tendency for more suspected salmonellae colonies to occur at the higher temperature.

Jameson (35) did studies to find the optimum temperature for salmonellae growth. He compared three temperatures: 37 C, 41 C, and 44 C. At 37 C and 44 C the lag phase was more than 45 minutes. At 41 C the lag phase was approximately 30 minutes. The fastest growth rate occurred at 41 C while it was slowest at 44 C. The small differences in the growth rate (generation time) produced an eight fold increase of viable counts after 6 hr.

Spino (64) incubated duplicate plates, one at 37 C and the second at 41.5 C. Although the elevated temperature decreased the coliforms on the plates by 58 percent, it increased the salmonellae by 58 percent. The percent of salmonellae confirmed was increased



from 0 to 100.

Motility Flasks Special apparatus has been developed to isolate salmonellae more rapidly from feces and foods. Stuart and Pivnick (67) used a modified "U" tube in which one side had a much larger bore than the other. They used semi-solid enrichment media utilizing the rapid motility exhibited by salmonellae for selection. The side with the large bore was inoculated with the fecal sample and the growth was observed to migrate to the side with the small bore. When the growth reached the surface of the small tube it was plated on solid media for confirmation and found many times to be a pure culture of Salmonella.

Banwart (5) used a flask containing three "U" tubes fused through the side of the flask. The flask contained lactose broth while the "U" tubes contained semisolid SIM agar, mannitol agar, and dulcitol agar, respectively. Brain Heart Infusion (BHI) broth was placed above the semisolid agars on the outside portion of the "U" tubes. Egg products were inoculated into the lactose and the reactions in the side arms were recorded. If the reactions were positive then the BHI broth was tested serologically with Salmonella-H polyvalent antiserum.

Abrahamsson et al. (1) isolated salmonellae from foods using a flask with one side arm from the bottom of the flask. The medium used was Raj's dulcitol sodium selenite enrichment. A pH drop from 6.9 to 6.0 was considered positive for salmonellae and was confirmed using fluorescent antibody technique.

The motility flask techniques seem promising but they are not yet universally accepted.

### Fluorescent Antibody Detection of Salmonellae

Development of Methods Coons (11) developed fluorescent antibody techniques (FAT) and utilized them to locate various antigens in tissues. Weller and Coons (76) used the techniques by indirectly labeling antigens. Coons et al. (12) also used these methods to detect the presence of antibody.

Application for Detection Moody (50) used fluorescent antibody techniques to detect and identify group A streptococci in throat swabs. Moody's results were comparable to that produced by cultural isolation. Winter and Moody (81) used the technique to detect Pasteurella pestis in mice infections. These experiments showed the value of quicker results.

Whitaker et al. (78) were the first to apply fluorescent antibody techniques to the detection of Enterobacteriaceae. Thomason et al. (72) used this method to detect salmonellae in pure culture. The presence of salmonellae in dried eggs and egg products was demonstrated by Haglund et al. (28). Georgala and Boothroyd (22) showed the presence of salmonellae in raw meat. Both showed a considerable saving in time over conventional methods. Isolations have also been made from meat (23), foods and feeds (15, 34 and 44), dry milk (57), eggs (4 and 28) and fecal material (6, 9, 70 and 71).

Specificity The main argument against the fluorescent antibody technique is that it has not been highly specific for the organism in question. Early tests using fluorescent antibody to detect pneumococcal antigens in tissues were shown to be specific (11). This technique has been shown to be specific for the detection of other microorganisms in clinical materials (16, 24, 25, 26, 36 and 39). Thomason et al. (71) showed numerous cross reactions with normal intestinal flora when fluorescent antibody techniques were used to detect salmonellae in feces. The cross reactions were explained by the fact that salmonellae were serologically related to the enteric organisms, the presence of "normal antibodies" against enteric organisms in the serum of the rabbits and the use of conjugates of lower titer and poor quality. Even ten years later Cherry and Thomason (10) stated that "Commercial fluorescent antibody reagents for salmonellae may not be a good investment because we do not yet know what types of reagents or what ancillary methods will prove most advantageous." Fluorescent antibody detection of microorganisms causing food poisoning or infections was demonstrated to be specific (22, 28, 74 and 75). Thomason and McWhorter (73) proved that fluorescent antibody technique was at least equal in sensitivity and specificity to the cultural examination for detecting typhoid bacteria in fecal specimens.

Cherry et al. (8) stated that careful adsorption of globulins to remove antibody reacting with organisms other than salmonellae may remedy the problem of cross reactivity.

Bissett et al. (6) compared fluorescent antibody detection of Salmonella typhi with conventional isolation procedures. The fluorescent antibody technique was done according to Thomason and McWhorter (73). An enrichment using Selenite broth was used along with the direct fluorescent antibody technique. Enrichment cultures proved more sensitive yielding positive fluorescent antibody results from 40 of 41 patients positive by culture. There were 9 positive and 2 questionable fluorescent antibody results on specimens that were negative by cultures.

Arkhangel'skii and Kartashova (3) were able to detect Salmonella dublin in artificially contaminated milk using fluorescent antibody methods.

The fluorescent antibody technique for the identification of salmonellae is easiest and most rapid method if the specificity can be depended upon.

## MATERIALS AND METHODS

### Source of Cultures

The organism used in this study was a culture of Salmonella typhimurium obtained from the National Communicable Disease Center at Atlanta, Georgia. The culture was given a reference number of 3-93-20 then checked biochemically and serologically.

All stock cultures and isolates were maintained in screw-cap tubes of Trypticase Soy Agar (BBL).

The purpose of this study is to rapidly isolate and identify salmonellae from water and if possible, to quantitate their numbers.

### Sampling Techniques

Water samples were obtained along the Big Sioux River at selected points below Volga, S. D., and Sioux Falls, S. D. The Volga site is a point where highway 14 passes over the Big Sioux River approximately one mile east of Volga, S. D. The Sioux Falls site is a point where interstate 229 passes over the Big Sioux River just east of Sioux Falls, S. D. Samples were taken just under the surface using aseptic techniques. The Volga site in winter was covered with as much as 18 inches of ice. The river at the Sioux Falls site did not freeze even though the temperature fell as low as -37 F.

### Bacterial Concentration

The organisms present in the water samples were removed or concentrated by filtering 200 ml of sample through membrane filters (Millipore) with a pore size of 0.45  $\mu$ . A second method used to concentrate the organisms in the water was the use of the modified Moore swab (64). The swab was left just below the water level for 2 to 7 days. This method has been used commonly but it has the disadvantage that it is not comparable to any set volume of water. Another technique that was used was to filter water through a buchner funnel containing 1.0 grams of sterile Celite (Johns-Manville) between two #1 Whatman filter discs (7).

### Preliminary Studies using Pure Culture

Temperature Studies The optimum temperature for growth of salmonellae was studied so isolation could be made as rapidly as possible.

A pure culture of Salmonella typhimurium was used for this study. The temperatures studied were 35 C, 37 C, 39 C, 41 C and 43 C. Growth at these temperatures were compared turbidimetrically using a Bausch and Lomb Spectronic 20 at a wavelength of 440 nm. The medium used was brain heart infusion (BHI) broth which was dispensed in 100 ml aliquots into spectrophotometric flasks (Bellco No. 300). Each flask of medium was inoculated with 1.0 ml of a  $10^{-4}$  dilution of an overnight culture of S. typhimurium in BHI. The adsorbance was read at selected intervals up to 10 hr.

Survival Studies River water from the Volga sampling site was sterilized by autoclaving. Aliquots of 200 ml of sterile river water were each inoculated with 1.0 ml of an 18 hr BHI culture of Salmonella typhimurium. The flasks were held static at 10 C, 20 C, and 30 C. The most probable number technique (MPN) was used to measure the bacterial densities which ranged initially from  $10^8$  to  $10^9$  organisms/100 ml. The MPN's were done daily over a period of 40 days. The MPN's were done using BHI broth.

Pre-enrichment and Enrichment Studies The enrichment media studied were GN broth, Selenite cystine broth, brain heart infusion broth, and lactose broth as described in Difco literature. Raj's (56) dulcitol sodium selenite enrichment was also used.

The enrichments were compared turbidimetrically in duplicates using a Spectronic 20 at a wavelength of 440 nm. The enrichments were dispersed in 100 ml aliquots into photometric flasks (Belco No. 300). All the media were sterilized by autoclaving except selenite cystine and the dulcitol sodium selenite enrichments. The selenite cystine enrichment was prepared using freshly autoclaved distilled water. The dulcitol sodium selenite enrichment was steamed for 30 minutes. Both were prepared just prior to inoculation.

The first experiment was carried out in a shaker water bath at 41.5 C. The inoculum used was 0.1 ml of a BHI culture of Salmonella typhimurium that had been frozen at -70 C for several weeks to

"weaken" the organisms. The absorbance was read at selected intervals up to 12.5 hr. The lactose broth and the dulcitol sodium selenite enrichment were reinoculated after 24 hr because no growth had occurred.

The second experiment was carried out on a shaker in an incubator at 41.5 C. An inoculum of  $5.4 \times 10^3$  organisms according to MPN's were inoculated into each flask. The absorbance was read on the same Bausch and Lomb Spectronic 20 (440 nm) at selected intervals up to 22.5 hr.

The third experiment was done by running MPN's using each of the different media. Appropriate dilutions of an overnight culture of Salmonella typhimurium in nutrient broth were used to measure which medium would support the lowest number of salmonellae.

#### Initial Isolation of Salmonellae

The initial isolation of salmonellae was done to qualitatively show the presence of salmonellae in the river water. Later studies were run in an attempt for quantitation of salmonellae in the water. The Volga site was used as the source of water for this initial isolation.

The modified Moore swab (51) was used as the means of concentration of the organisms present in the river. The swab was kept submerged in the river for seven days. Strips of the swab were placed into 100 ml aliquots of tetrathionate broth and selenite cystine broth for 24 hr. All the commercial media used were Difco



and the incubation temperature used was 41.5 C unless stated otherwise. After incubation the enrichments were streaked for isolation on brilliant green agar plates and bismuth sulfite agar plates, which were incubated for 24 and 48 hr, respectively. Typical salmonellae colonies were inoculated into triple sugar iron agar and lysine iron agar which were then incubated for 24 hr at 37 C. Those cultures that gave typical reactions for salmonellae were tested serologically. Both somatic and flagellar antigen were determined using Difco antisera. Salmonella O Antiserum Poly A-I was used to show the presence of somatic antigens and the Salmonella H Antisera Spicer-Edwards Set was used to show the presence of specific flagellar antigens. The somatic antigen determination was done using growth directly from the triple sugar iron slant. For the flagellar determination the isolate was grown up for 18 hr in veal infusion broth then inactivated using formalized saline solution. The reactions were carried out in 1.0 ml durham tubes at 50 C for 1 hr. A positive test was shown by the presence of a flocculent precipitate. Upon termination of these tests the cultures were sent to the Public Health Department at Pierre, S. D., and then to the National Communicable Disease Center at Atlanta, Georgia, for confirmation.

#### Improved Methods for Isolation

The isolation of salmonellae from water has normally been done by placing the concentrated sample directly into enrichment (7, 60 and 64). A few workers have used a pre-enrichment step of proteose

peptone broth (48 and 53).

Pre-enrichment steps have been used extensively when isolation has been attempted from foods and feeds. The reason given for the pre-enrichment was to allow the "weak" salmonellae to adapt and grow before being subjected to an inhibitive enrichment (54). Salmonellae present in river water are very likely to be "weak" also. A 6 hr pre-enrichment in brain heart infusion (BHI) broth was used allowing all organisms to grow. A longer period of time was not used for fear that other organisms could produce products that might destroy salmonellae. After the pre-enrichment step the inhibitors present in the enrichments are less likely to restrict growth of salmonellae (2 and 27).

The flow diagram shown in Figure 1 was used for the improved method of isolation of salmonellae. The water sample (A) was taken from the Sioux Falls site and filtered through membrane filters (Millipore) with a pore size of 0.45  $\mu$ . A sample size of 200 ml was chosen and many times required as many as four filters because of the large amount of organic material in the water. These filters were placed in 100 ml of Brain Heart Infusion broth (B) for 6 hr as the pre-enrichment step. At the end of this time 1.0 ml of the broth was inoculated into tubes containing 9 ml of enrichment, both tetrathionate broth and selenite cystine broth were employed (C). These enrichments were allowed to incubate for 24 hr at which time the selenite cystine broth had turned a deep orange color while the tetrathionate broth had remained unchanged. From each enrichment 3

Water Sample (A)

BHI broth

Tetrathionate broth                      Selenite-cystine broth (C)

Brilliant green agar	Bismuth sulfite agar	Brilliant green agar	Bismuth sulfite agar	(D)
----------------------------	----------------------------	----------------------------	----------------------------	-----

Typical colonies inoculated into triple sugar iron agar (E)

Typical reactions tested serologically. (F)

Positive isolates confirmed by other laboratories. (G)

Fig. 1. Procedure for improved isolation of salmonellae from river water.

loops of broth were streaked for isolation onto bismuth sulfite agar and brilliant green agar (D). These plates were incubated for 48 hr and 24 hr, respectively, at which time typical colonies were observed. Typical salmonellae colonies on bismuth sulfite agar appeared as gray to black colonies surrounded by a metallic halo. These organisms on brilliant green agar plates appeared as pink colonies surrounded by red discoloration of the medium. These colonies were inoculated into triple sugar iron agar and lysine iron agar (E). Typical salmonellae reactions in triple sugar iron agar showed an alkaline slant and an acid butt with the demonstration of hydrogen sulfide and abundant gas. Arizona sp. which are very closely related to salmonellae show the same reactions except the slant is also acid. The typical salmonellae reactions in lysine iron agar show an alkaline slant and an alkaline butt with varied amounts of hydrogen sulfide. Gas is usually not produced in this medium. The serology (F) has already been fully covered in the initial isolation section along with the confirmation (G) procedure.

#### Quantitation using Pure Culture

A culture of S. typhimurium was grown up at 41.5 C for 13 hours. The number of organisms present was shown by the most probable number method using BHI. Five dilutions ranging from  $10^{-6}$  to  $10^{-10}$  were inoculated into lactose broth and BHI broth. After incubation for 6 hr 1.0 ml of BHI broth was inoculated into tetrathionate broth and selenite cystine enrichment. After 12 hr 1.0 ml of BHI broth

and lactose broth were inoculated into the enrichments. After 24 hours 1.0 ml of the lactose broth culture was inoculated into the enrichment media. The enrichment media were allowed to incubate for 24 hours when they were streaked on brilliant green agar and bismuth sulfite agar plates for isolation. The plates were observed after 24 and 48 hours respectively, for the presence of typical salmonellae colonies.

#### Quantitation of Salmonellae in River Water

The quantitative determination of Salmonella in river water was carried out using a modification of the five tube most probable number technique.

Five flasks containing 100 ml of BHI broth were used, each receiving the same inoculum. Set quantities of river water were filtered and the filters were placed in the flasks.

The first set of five flasks received membrane filters which had filtered 50 ml of water. Each set of flasks after that received filters that had filtered 50 ml more than the last set of flasks. The five dilutions were: 50 ml, 100 ml, 150 ml, 200 ml, and 250 ml. After incubation for 6 hours 1.0 ml from each flask was inoculated into selenite cystine enrichment medium and tetrathionate enrichment medium. The enrichments were incubated for 24 hours then each was streaked for isolation on brilliant green agar and bismuth sulfite agar plates. After 24 and 48 hours respectively the plates were observed for typical colonies of salmonellae. Those found were

inoculated into TSI and LIA. Those cultures that gave typical salmonellae reactions were tested serologically.

#### Fluorescent Antibody Techniques

The Salmonella conjugate was provided by the General Bacteriology Unit, Diagnostic Services, National Animal Diseases Laboratory. The procedures used to prepare the conjugate and information on its specificity of their conjugate in detail various controls were run to check the application to our studies. Numerous cultures of Salmonella spp., Proteus spp., E. coli variants, Shigella spp., Enterobacter spp., Arizona sp., and Citrobacter were used as controls. The only organisms found to give good 4+ fluorescence were the Salmonella spp. and Arizona sp.

Samples of river water in 200 ml aliquots were taken using aseptic techniques and filtered through membrane filters (0.45 u, Millipore). The filters were placed in GN broth for 24 hr when smears were made on nonfluorescing slides. After air-drying the smear was fixed with gentle heating. The staining was done according to the methods of Ellis and Harrington (15 and 30). A counterstain of flazo orange (National Biochemicals Corp.) was used (29).

Cells were observed after staining and arbitrarily given values of +4, +3, +2, and +1 based on the degree of cell fluorescence. All preparations were observed using an American Optical Fluorolume illuminator (Model 645) with an Osram HBO 200 W maximal-pressure mercury-vapor arc with a UGI (Corning no. 5840) as the exciter filter and a Schott GG-99 as the barrier filter.

## RESULTS AND DISCUSSION

Preliminary Studies with Known Cultures

Temperature The temperature that will give the fastest generation time for salmonellae was of importance to make the isolation procedure as rapid as possible. Of the five temperatures that were tested, 41 C gave the fastest rate of growth followed by 39 C, 43 C, 37 C and 35 C, in that order. These results support the finding of Spino (64) and Jameson (35) that 41.5 C or 41 C is the optimum temperature for growth of salmonellae. Again, it should be said that the growth curve was done turbidimetrically in a BHI medium using a known culture of Salmonella typhimurium.

Survival Studies The survival studies done in this experiment were carried out using a pure culture of S. typhimurium and are not necessarily the same survival rates as those occurring in river water. There were many factors not considered in this experiment that exist in river water such as the fluctuation in temperature and aeration, presence of other organisms, degree of sunlight, etc. A measurement of the percentage of salmonellae surviving in river water at different temperatures was one objective of this study.

The graph of this study as shown in Figure 2 shows a greater percentage of survival as the temperature was decreased from 30 C to 10 C. A summary of this graph is shown in Table 1. The initial density was estimated to be approximately  $10^7$  cells of S. typhimurium/100 ml using MPN's (66).

Table 1. Effect of Temperature on the Survival of Salmonella Typhimurium in Sterile River Water

Day	Percent surviving		
	10 C	20 C	30 C
0	100	100	100
5	80	80	90
10	70	60	60
15	60	40	20
20	40	20	5
25	30	10	0.4
30	20	8	0.03
35	9	6	0.004
40	7	4	0.0008

Pre-enrichment and Enrichment Studies It was of importance to find whether a pre-enrichment step would improve the recovery of salmonellae from the river samples. It was also of importance to know which enrichment broth gave the best propagation of salmonellae.

The most significant proof that a pre-enrichment was beneficial was the fact that Salmonella spp. were isolated from the river more often using the improved methods than when the initial isolations



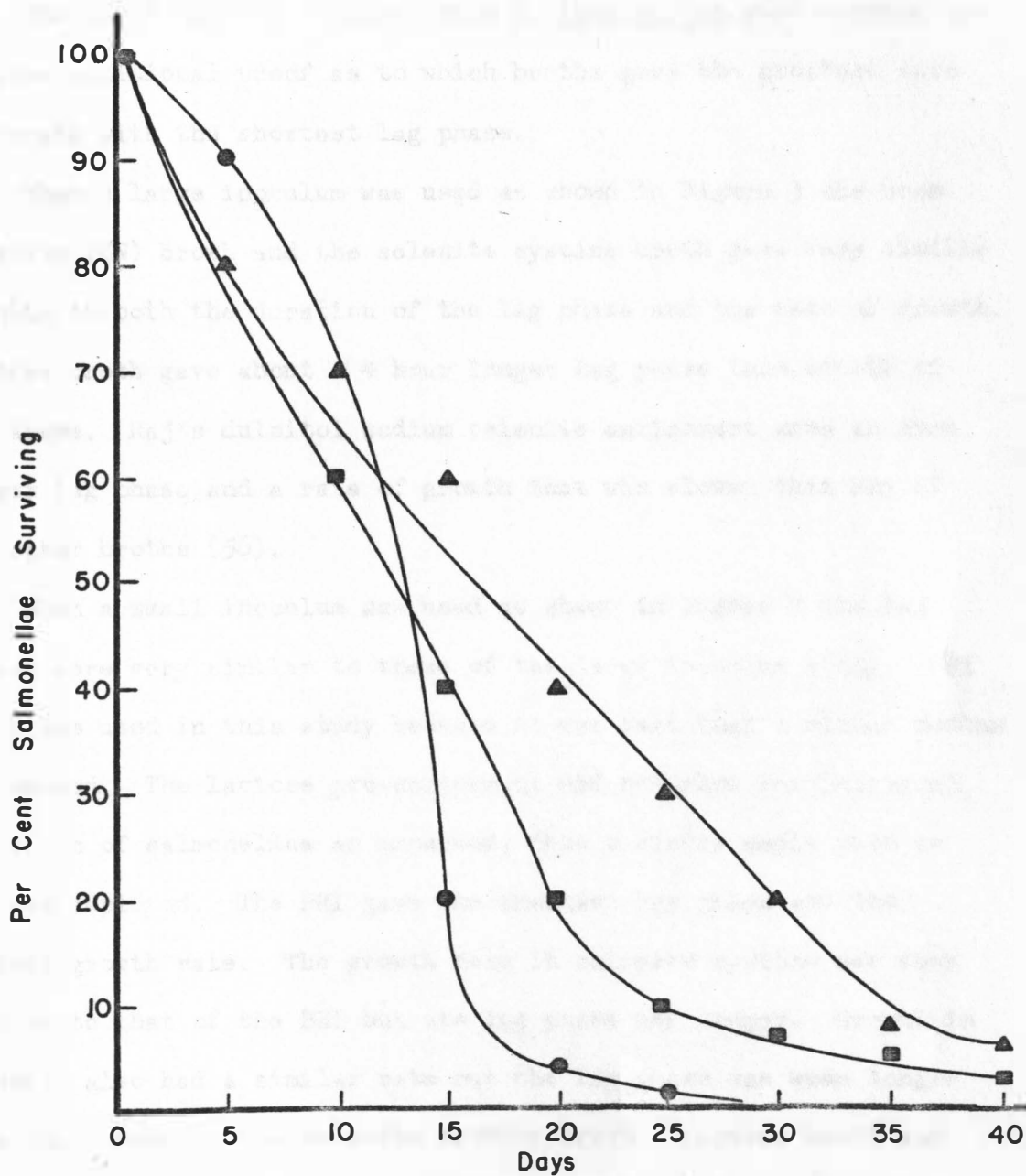


Fig. 2. The effect of temperature on the survival of *Salmonella typhimurium*. Symbols: ▲, 10 C; ■, 20 C; and ●, 30 C.

were run. It was also shown that the Brain Heart Infusion (BHI) broth was a better pre-enrichment than the lactose broth because more isolations were made using the BHI broth.

The turbidimetric studies using S. typhimurium were carried out to give additional proof as to which broths gave the greatest rate of growth with the shortest lag phase.

When a large inoculum was used as shown in Figure 3 the Gram Negative (GN) broth and the selenite cystine broth gave very similar results in both the duration of the lag phase and the rate of growth. Lactose broth gave about a 4 hour longer lag phase than either of the above. Raj's dulcitol sodium selenite enrichment gave an even longer lag phase and a rate of growth that was slower than any of the other broths (56).

When a small inoculum was used as shown in Figure 4 the lag phases were very similar to those of the large inoculum study. BHI broth was used in this study because it was felt that a richer medium was needed. The lactose pre-enrichment did not show the increased isolation of salmonellae as expected, thus a richer media such as BHI was employed. The BHI gave the shortest lag phase and the highest growth rate. The growth rate in selenite cystine was very similar to that of the BHI but its lag phase was longer. Growth in GN broth also had a similar rate but the lag phase was even longer than that shown in the selenite cystine broth. Lactose broth and the dulcitol sodium selenite enrichments had much longer lag phases and smaller growth rates.

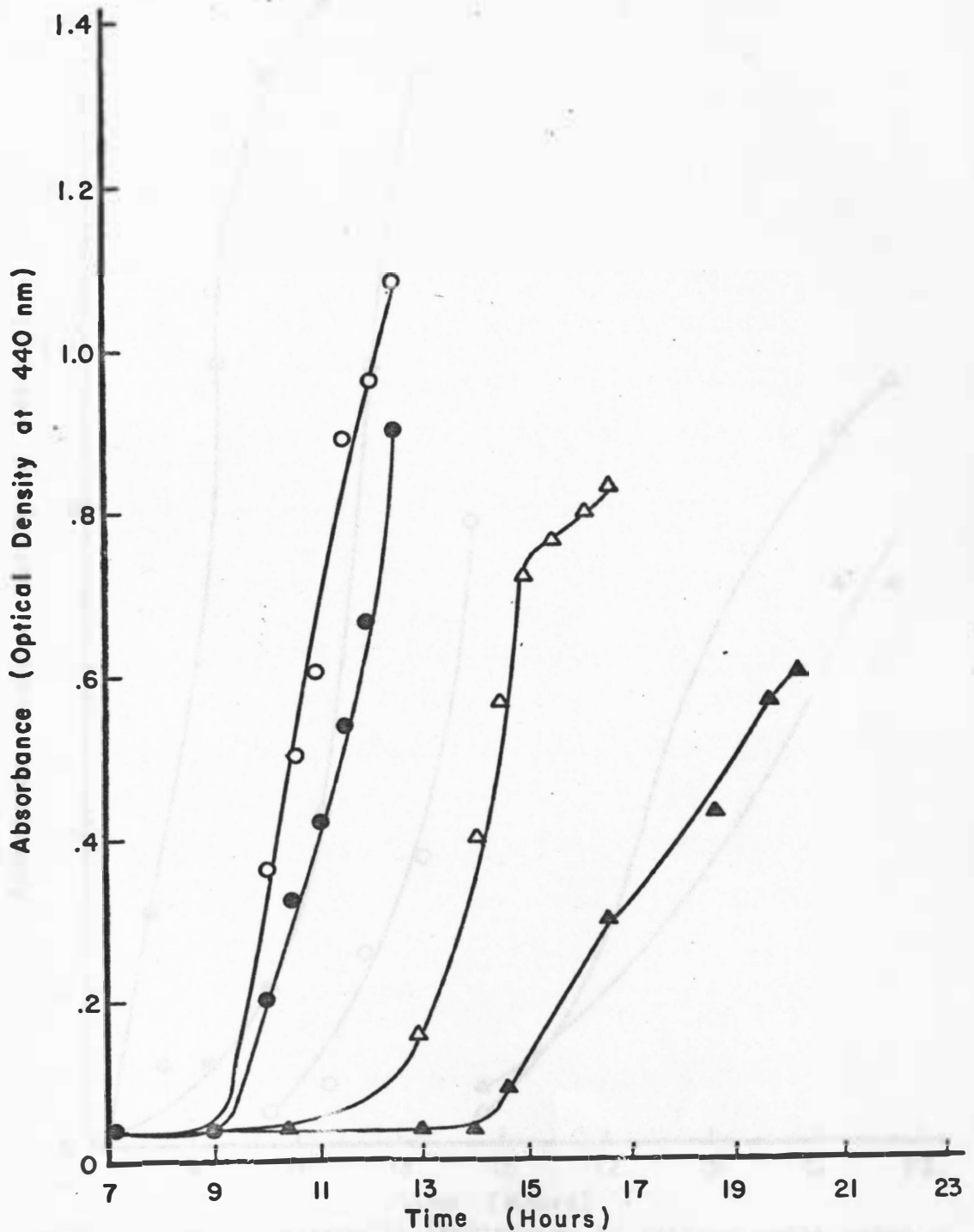


Fig. 3. Growth curves of *Salmonella typhimurium* in various media using a large inoculum (approx.  $10^9/100$  ml). Symbols: ○, GN broth; ●, selenite-cystine broth; △, lactose broth; and ▲, dulcitol sodium selenite enrichment.

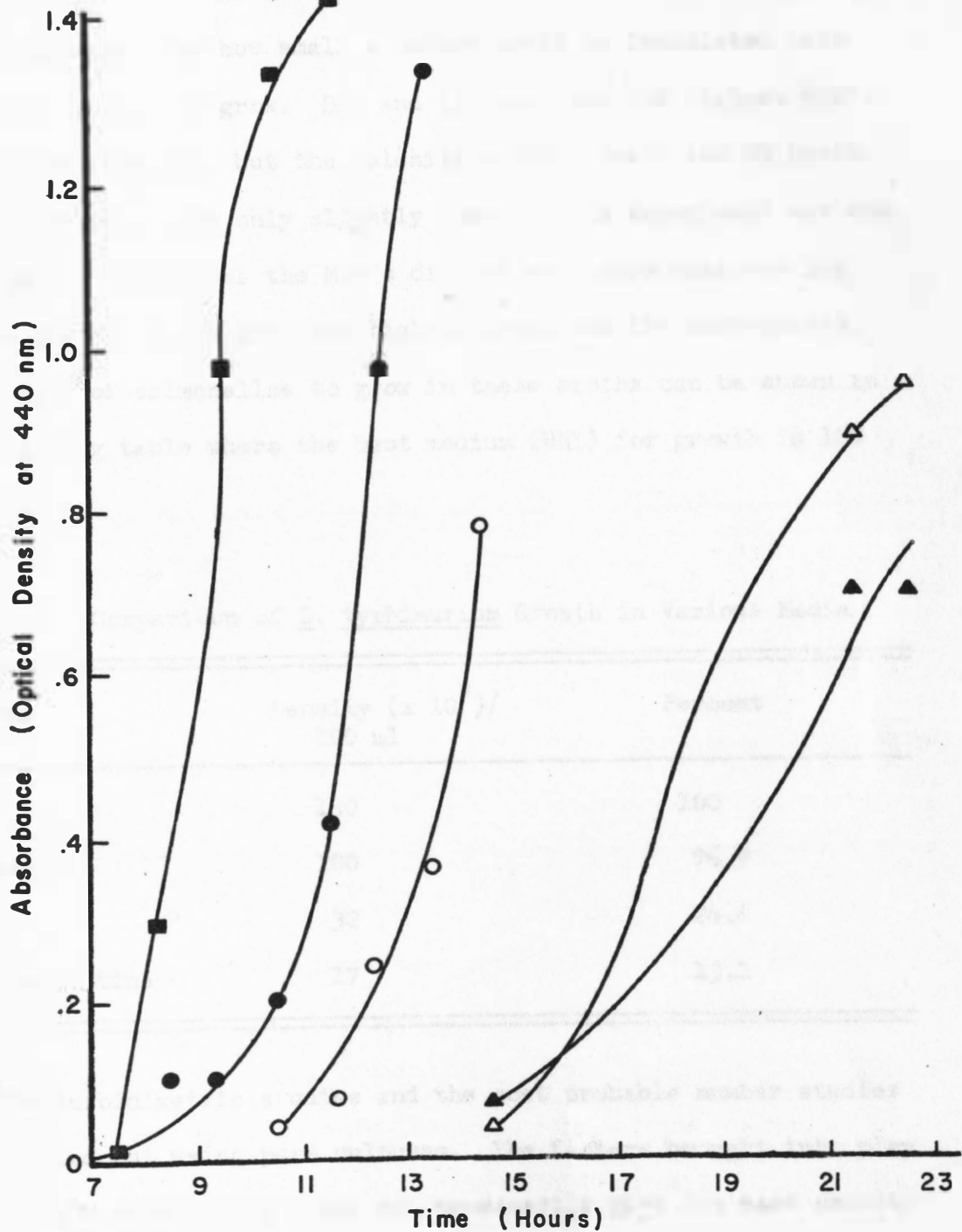


Fig. 4. Growth curve of *Salmonella typhimurium* in various media using a small inoculum (approx.  $5 \times 10^3/100$  ml). Symbols: ■, BHI broth; ●, selenite cystine broth; ○, GN broth; ▲, lactose broth; and △, ducitol sodium selenite enrichment.

The most probable number technique was used to estimate the smallest number of salmonellae that the enrichments would support. This would also show how small a number could be inoculated into the broths and still grow. BHI and lactose gave the highest MPN's as would be expected, but the selenite cystine broth and GN broth gave counts that were only slightly lower. This experiment was run twice and in both cases the MPN's did not vary more than one log between the BHI which gave the highest count and the enrichments. The ability of salmonellae to grow in these broths can be shown in the following table where the best medium (BHI) for growth is 100 percent.

Table 2. Comparison of S. typhimurium Growth in Various Media

Medium	Density ( $\times 10^7$ )/ 100 ml	Percent
BHI	130	100
Lactose	100	76.9
GN	32	24.6
Selenite cystine	17	13.1

The turbidimetric studies and the most probable number studies were carried out using pure cultures. The factors brought into play when using a mixed flora might not necessarily give the same results as this study.

Initial Isolation of Salmonellae The initial isolation of salmonellae from the river was done as a qualitative determination only. There was no consideration given for quantitation or speed of recovery.

The Volga sampling site was examined utilizing the modified Moore swab technique as described in the Concentration section of the Materials and Methods. This was done without a pre-enrichment step. This procedure was employed five times. Following enrichment and plating all isolated colonies were tested using triple sugar iron (TSI) agar and lysine iron (LIA) agar. Only one of the five swabs tested was found to contain salmonellae. Sixteen isolates were found to be positive for presumptive (TSI and LIA) and completed (Poly O and Spicer-Edwards Serology) tests. Twelve of these were confirmed by the State Health Laboratory and the National Salmonella Typing Laboratory, National Communicable Disease Center, Atlanta, Georgia. Both laboratories identified the 12 isolates as Salmonella typhimurium by biochemical and serological testing.

From these efforts salmonellae were shown to exist in the Big Sioux River at the Volga sampling site. The isolation was shown not to be quantitative and not very productive. This initial isolation without the use of a pre-enrichment step yielded 12 isolates out of 400 colonies that were picked from the solid media. This is a recovery of less than 4 percent. This study indicates a need for improved methods and a quantitative procedure for enumerating salmonellae.

### Improved Methods for Salmonellae Isolation

Once the presence of salmonellae had been confirmed from the Volga site it became my goal in this study to improve the methods for isolation and to shorten the time for identification.

The procedure for the improved method involved the use of a pre-enrichment step and the complete removal of all organisms from the water sample using membrane filters. By using the membrane filters it could be said that salmonellae were or were not present in the water sample. This has the advantage over the Moore swab of showing the presence of salmonellae in a measured volume, eg. 200 ml, instead of just the presence of salmonellae in an unknown volume of water. This makes the procedure more highly specific. The pre-enrichment step was employed in order to give the "weakened" salmonellae a better chance of survival before they were placed into a medium containing inhibitors. A lactose pre-enrichment step is used in the isolation of salmonellae from foods and feeds, again because of the "weakened" state of the organisms present. For this reason lactose broth was used for the pre-enrichment step. This procedure was used for 11 water samples taken at the Volga sampling site and all gave no salmonellae isolation. The sampling site was changed to a point below Sioux Falls because of the fact that the bacterial densities have been shown to be much higher than normal by Foley, (18) and Herreid, (32). Still, no salmonellae isolations were made so a richer pre-enrichment broth was utilized. Brain Heart Infusion broth (BHI) was employed as the richer pre-enrichment

but lactose broth was run in parallel as a control to show that the difference was due to the richer pre-enrichment and not due to the sampling differences. Nine samples were obtained from the Sioux Falls site using lactose broth pre-enrichment and three were found to contain salmonellae. From the same site 6 out of 6 samples taken were positive for salmonellae when the BHI broth pre-enrichment was used. Five of the 6 samples were done in parallel with the lactose broth pre-enrichment. Beside the fact that BHI broth is richer, lactose is not fermented by the salmonellae.

This method gives better recovery of salmonellae but it is still qualitative. A method which would quantitate salmonellae in the water was needed for this study.

#### Quantitation using Pure Culture

Quantitation of salmonellae was achieved by inoculating several flasks of BHI broth with varying sized samples then each BHI was tested using the improved method. This method was tested using a pure culture of S. typhimurium. The culture was grown in nutrient broth, then dilutions of  $10^{-6}$  through  $10^{-10}$  were inoculated into BHI broth. The estimated density of the nutrient broth culture was found to be approximately  $1.4 \times 10^8$  organisms per ml by MPN. These same dilutions were inoculated into the lactose broth controls. Both the 6 hr and 12 hr BHI pre-enrichment gave recovery through the  $10^{-8}$  dilution. Each dilution was also inoculated into GN broth and tested using the fluorescent antibody technique (FAT). This procedure



showed salmonellae were present in smears through the  $10^{-9}$  dilution. In this experiment the FAT and BHI procedure gave comparable results. The lactose broth pre-enrichment procedure showed a sensitivity that was 1 log less than either the BHI pre-enrichment procedure or the fluorescent antibody technique. These results are described below.

Table 3. Detection of S. typhimurium by Cultural and FAT

Dilution	Lactose	BHI	FAT
$10^{-6}$	+	+	+
$10^{-7}$	+	+	+
$10^{-8}$	+	+	+
$10^{-9}$	-	+	+
$10^{-10}$	-	-	-

#### Quantitation of Salmonella in river water

The actual quantitation of salmonellae from the river was done using the 6 hr BHI pre-enrichment procedure. Samples ranging from 50 ml through 250 ml were used to find the smallest volume which contains salmonellae. Typical salmonellae colonies were found on most of the plates but only those on the 50 ml plates were checked. These isolates which were positive for both presumptive and completed tests were sent to the State and National laboratories where they were confirmed to be S. typhimurium, S. infantis, S. oranienburg, S.

anatum, S. montevideo and S. javiana. The best recovery was noted as coming from the brilliant green plates rather than the bismuth sulfite plates.

A second quantitation experiment was done using 1.0, 10.0 ml, 100 ml, and 200 ml samples. Both BHI and lactose broths were used for pre-enrichment. Only two isolates were found to be positive for salmonellae. Both were from the procedure where BHI was used and both were from the 100 ml sample. The extreme difference between the first and second quantitation experiment can be attributed to heavy rains that fell the night before the second sample was taken. The river had risen drastically and the bacterial counts showed a dilution of approximately 10 times the normal densities. If this is correct then where salmonellae was found in 100 ml samples, they would normally be found in as small a volume as 10 ml.

#### Comparison of FAT with Cultural

The Volga site as shown in Table 4 demonstrated 6 positive FAT smears from the 11 samples tested. The cultural procedures employed lactose broth as the pre-enrichment. None of these gave positive salmonellae identification. The highest total coliform count recorded was 230,000 organisms per ml.

The Sioux Falls site as shown in Table 5 demonstrated 7 positive FAT smears from the 9 samples tested. The cultural procedures employed BHI broth and lactose broth gave salmonellae recovery. Six of the 6 samples tested using the BHI broth gave positive identification for salmonellae.

The FAT and culture technique using BHI pre-enrichment showed good correlation except for sample number 8.

The highest total coliform count recorded was 9.3 million as compared to the highest fecal coliform of 1.3 million and fecal streptococci of 4.3 million.

The FAT is by far the best method of detecting Salmonella species if a high degree of specificity can be maintained. The sensitivity is about the same but the time involved is much shorter.

#### Total Coliform Densities in River Water

The greatest allowable coliform density (MPN) for any water in South Dakota is 20,000 organisms/100 ml (65).

The water at the Volga site as shown in Table 4 had total coliform densities which ranged from 790 to 230,000 organisms/100 ml. Only one of the 11 samples tested were shown to exceed the 20,000/100 ml limit.

The water at the Sioux Falls site as shown in Table 5 had total coliform densities ranging from 330,000 to 9,300,000 organisms/100 ml. Each water sample tested was found to exceed the water quality standards.

There appears to be no correlation between total coliform densities and the presence of salmonellae (20 and 21) but the presence of salmonellae would seem more likely when high bacterial densities are present. From the studies done in this paper one could state that the Big Sioux River below Sioux Falls is a highly polluted stream.

Table 4. Summary of Data from Volga Sampling Site

Sample number	FAT	Lactose	BHI	IC	FC	FS
1	+	-				
2	-	-				
3	+	-				
4	+	-		790	330	20
5	+	-		790	330	230
6	-	-		3,300	3,300	500
7	-	-		9,300	4,300	400
8	+	-		4,300	2,300	400
9	-	-		4,300		
10	+	-		4,300	900	
11	-	-		230,000	7,900	3,300

Table 5. Summary of Data from Sioux Falls Sampling Site

Sample number	FAT	Lactose	BHI	IC	FC	FS
1	+	-		9,300,000	430,000	2,300
2	+	-		1,100,000	430,000	4,300,000
3	+	-	+	930,000	430,000	230,000
4	+	-		7,900,000	1,300,000	490,000
5	-	-		9,300,000	3,000	930,000
6	+	-	+	2,400,000	3,000	430,000
7	+	+	+			
8	-	+	+			
9			+			
10	+	+	+	330,000	110,000	130,000

### Comparison of Fluorescent Antibody Technique with Cultural Methods

The fluorescent antibody technique was used as being the most rapid method for detection of salmonellae in water.

Attempts were made to label Salmonella Spicer-Edwards sera with fluorescein isothiocyanate (FITC) but the conjugate was nonspecific. This nonspecificity was thought to be due to the sera having been freeze-dried.

A Salmonella polyvalent H conjugate was obtained from Ellis and Harrington (15). This conjugate was found to be highly specific in their studies and when controls were tested in this laboratory. The same methods and procedures were used in this study as those used by Ellis and Harrington including the flazo orange counterstain described by Hall (29).

Of the 11 water samples from the Volga site that were tested, 6 were found to be positive using FAT while none were positive using cultural techniques. Seven of the 9 samples that were tested from the Sioux Falls site were found to be FAT positive. Six of the 10 samples that were tested culturally were found to be positive also. Whenever salmonellae were isolated culturally they were also detected using FAT except for sample 8.

It was of significance that salmonellae were detected with FAT when total coliform densities were as low as 790 organisms/100 ml.

The fluorescent antibody technique and the cultural technique using the BHI pre-enrichment was shown to be equally sensitive. The advantage of the FAT is its rapidity whereas with the cultural technique the organism can be isolated and serotyped.

## CONCLUSIONS

1. The optimum temperature for growth of Salmonella typhimurium is 41.5 C.
2. A pre-enrichment step using lactose broth was shown to be superior for the isolation of salmonellae from water compared to the direct inoculation into an enrichment medium.
3. A brain heart infusion pre-enrichment step was found to give a greater recovery of salmonellae from water compared to the lactose pre-enrichment step.
4. The tetrathionate enrichment medium and the selenite cystine enrichment medium are equivalent in their ability to select for salmonellae since they gave the same number of isolation.
5. Brilliant green agar gave more isolations of salmonellae than bismuth sulfite agar.
6. The enrichment medium-plating medium combination that showed the highest recovery of salmonellae was the tetrathionate enrichment when used in conjunction with the brilliant green agar.
7. The survival of S. typhimurium in sterile river water was shown to be increased as the temperature was decreased from 30 C to 10 C. Survival for 40 days at 10 C was 7 percent while at 20 C it was 4 percent. One percent of the S. typhimurium survived for a period of 22 days at 30 C.

8. The turbidimetric studies of the pre-enrichments and enrichments showed growth in brain heart infusion broth to have the shortest lag phase and the greatest growth rate of the broths tested. Selenite cystine broth and GN broth were the most productive enrichments with growth rates like that of BHI but with longer lag phases.
9. Little difference was seen in the ability of Salmonella typhimurium to survive in the noninhibitive pre-enrichment media compared to the selective enrichment media.
10. The fluorescent antibody technique for detecting salmonellae was much more rapid than cultural methods.
11. The combination of the highly specific Salmonella conjugate and the flazo orange counterstain eliminated nonspecific staining.
12. All samples taken from the Sioux Falls site exceeded the total coliform density allowed of 20,000 coliforms/100 ml by as much as 400 times. The river at this point was found to be extremely polluted.
13. Salmonellae were isolated at the Sioux Falls site from water samples as small as 50 ml. Salmonella typhimurium was the most common isolate although S. infantis, S. montevideo, S. javiana, S. anatum, and S. oranienburg were also isolated.

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