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# DETERMINATION OF *SALMONELLA* SPECIES IN THE BIG SIOUX RIVER AND DEVELOPMENT OF A QUANTITATIVE MOST PROBABLE NUMBER METHOD

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor Date

Head, Department of Bacteriology Date

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MHK

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# INTRODUCTION

In recent years the pollution problems in rivers and lakes have forced the Federal Water Pollution Control Administration to issue requirements for minimum water standards for each State with an interstate stream. Since the Big Sioux River is an interstate river, studies are needed to determine its quality. According to the South Dakota Committee on Water Pollution (39), water pollution in South Dakota originates from agricultural, municipal, and industrial wastes. For the Big Sioux River, the municipal and meat-processing wastes are the important sources of pollution.

Little is known of the sanitary bacteriological quality of rivers and lakes in South Dakota. Standard methods are available (42) for determining the sanitary indicator bacteria in water (coliforms and fecal streptococci), but there is not a standard method available for the determination of pathogens such as the Salmonella spp.

Having understood that the Big Sioux River is an interstate stream, Pierce (32) examined the river by bacteriological methods to demonstrate the presence of salmonellae in the river and developed a qualitative method for isolating salmonellae. He showed the presence of salmonellae in the Big Sioux River by isolating Salmonella enteritidis serotypes Anatum, Bredeney, Derby, Infantis, Javiana, Montevideo, and Oranienburg. He also improved the isolation method by using brain heart infusion (BHI) broth as a pre-enrichment medium.

Since the Salmonella spp. present in water are likely to have low viability, they need to be made more viable before being placed into highly selective media. Following this line of reasoning, he used BHI broth as a pre-enrichment step and he proved it by the experimental comparison between lactose broth and BHI broth.

Although Pierce developed an improved qualitative method using BHI pre-enrichment and membrane filtration for isolations of the salmonellae, he was not able to quantitatively study the distribution of number and kind of the salmonellae in the Big Sioux or James rivers. Thus, there are two objectives in this thesis study: one, the determination of the distribution of Salmonella spp.; two, the development of a method for Salmonella quantitation.

# LITERATURE REVIEW

Bacteriological methods for determining the presence of salmonellae in water were examined by the following researchers; Moore (28) from sewage samples; Spino (40) from the Red River of North; Gallagher and Spino (10) from the Las Vagas Wash; West (46) from the Chattahoochee River; Miner et al. (26) from feedlot runoff near Manhattan, Kansas; Slanetz et al. (36 and 37) from sea water; and Brenzenski and Russomanno (3) from polluted tidal estuaries.

However, only one worker, Pierce (32) had studied the isolation of salmonellae in South Dakota. Pierce determined the presence of salmonellae in the Big Sioux River near Sioux Falls, South Dakota. He confirmed the presence of Salmonella spp. in the river by isolating Salmonella enteritidis serotypes Anatum, Bredeney, Cubana, Derby, Infantis, Javiana, Montevideo, and Oranienburg.

## Isolation of Salmonella

Samples The detection of salmonellae from water is much more difficult than isolating them from clinical specimens or from food samples because of their low density and low viability in water and inadequate techniques for their detection. Therefore, researchers tried to develop methods of concentrating the water samples for the isolation of salmonellae.

Three different methods can be used for concentrating the samples. First, the Moore gauze swab method (28) is done by submerging the gauze in water for a few days to collect some of the organisms present. These swabs are then placed into the enrichment media. This method of

concentrating does not indicate the size of sample, therefore one cannot estimate the quantitative count of bacteria. Second, the diatomaceous earth method (3 and 42) was used to concentrate a relatively large proportion of the microorganisms present in a sample. The filtration was done by placing an absorbent pad on a funnel, then adding sufficient diatomaceous earth to pack the funnel neck loosely. Two or more liters of sample water were poured slowly through the diatomaceous earth filter. After this was done, the funnel was disassembled and the resulting "plug" of diatomaceous earth divided with a sterile spatula into portions which were added to suitable enrichment media (3 and 42). Again, with this method of concentrating the sample, the actual quantitation of salmonellae in a small volume of sample is not accurate because such a large volume of sample must be used and there is no assurance that all the bacteria are collected. Thirdly, the membrane filtration method, which was developed by Kenner et al. (15), is favored over the other two concentration methods for sampling water because the bacteria that are present in a sample can be quantitatively removed and the size of sample can be regulated. Thus the membrane filtration method was used for this study.

Pre-enrichment To promote the growth of the Salmonella spp. present in water, pre-enrichment media were used by a few researchers. The pre-enrichment media were only for the purpose of enriching the growth, and usually did not have added inhibitors. Thus researchers, North (31), Montford and Thatcher (27), Reamer et al. (34), Sperber and Deibel (41), and Fantasia et al. (9) used lactose broth as a pre-enrichment medium for

the detection of salmonellae in foods. This was because a mixed flora produced acidic conditions which were inhibitory for many organisms but not for the Salmonella spp. However, Pierce (32) showed that using brain heart infusion (BHI) broth improved the growth and recovery of salmonellae compared to the commonly used lactose broth. The Salmonella spp. that were present in water had a tendency to have low viability. Therefore, it was necessary to have rich media. The incubation time in BHI broth was limited to a six hour period.

Enrichment Previous workers used the enrichment medium to promote the growth of salmonellae and to inhibit the growth of other enteric bacteria. There were at least four different enrichment broths, such as, tetrathionate (8 and 14), selenite cystine (2, 22, and 30), GN broth (Difco), and Raj's dulcitol sodium selenite (33) broth. Commonly, the two enrichment broths tetrathionate and selenite cystine were used for studies of river water.

Screening Procedure After the enrichment step, the cultures were transferred to highly selective solid agar media to differentiate the typical colonies of salmonellae from those of other enteric bacteria. There were seven commonly used selective media for the differentiation of enteric bacteria, such as, Brilliant green agar (BG) (20), Bismuth sulfide agar (BS) (47 and 48), Deoxycholate citrate agar (38), Salmonella-Shigella agar, MacConkey agar (24), XLD agar (45), and Hektoen agar (16 and 17). Among these selective media, BG agar and BS agar were most commonly used for the isolation of salmonellae.

Biochemical Tests The biochemical tests were performed to differentiate the Salmonella spp. from the other enteric bacteria. Typical Salmonella colonies were picked from the selective solid agar medium, and transferred to biochemical test media. The triple sugar iron agar (TSI) slant (35, 7, 21, and 43), and lysine iron agar (LIA) (5) were commonly used as biochemical test media.

Serological Identification The results from the biochemical tests were confirmed by serological methods. For rapid serological screening, growth from the TSI slant was used to determine the Salmonella spp. somatic antigen by using Salmonella O Antiserum Poly A-I. The individual somatic antigenic group of salmonellae was characterized by Kauffmann-White schema (6 and 13). The H antigen of salmonellae was identified by the Spicer-Edwards method (6).

## Enumeration of Salmonella

The quantitation of salmonellae in river water samples was a greater problem than the qualitative detection of salmonellae because of the low number of salmonellae in the river water and the inadequate methods of enumeration.

One method of enumerating Salmonella sp. from foods was a modified most probable number (MPN) method which involves the enrichment of bacteria from a known quantity of sample and subsequent isolation of salmonellae from a selective medium.

Byrne et al. (4) used a five tube, three decimal dilution MPN method to estimate the number of salmonellae in dried egg and other

food products. He used five tubes of selenite broth for each dilution as an enrichment step, then transferred a loopful of culture from selenite broth and streaked it on BG agar and BS agar to confirm the presence of salmonellae. Taylor et al. (44) used a three tube MPN method with selenite cystine broth substituting dulcitol or mannitol for lactose. They attempted to improve the enrichment medium so that the enumeration of salmonellae could be determined. However, he could not make any improvement by substituting a different carbohydrate for lactose. He concluded that the medium combination of selenite cystine broth and BG agar was more valuable than the other combinations of media for the quantitation of salmonellae.

North (31) tried to improve the MPN method for salmonellae by using lactose broth as a pre-enrichment before the food samples were placed into enrichment media. By the use of lactose broth, he tried to prevent the "skip" in the MPN method.

As one can see, the present technique of enumeration needs much improvement. Since the enrichment media do not demonstrate the presence of salmonellae, and the selective solid media may indicate the presence of salmonellae by showing typical Salmonella colonies, the colonies cannot be assumed to be Salmonella until they are confirmed by biochemical tests and serological tests. Also, the time factor involved in the experiment must be considered because a person may waste his time carrying on the test when the sample does not have Salmonella spp.

A neutral red lysine iron cystine (NRLIC) broth was developed by Hargrove, McDonugh, and Reamer (11) for the qualitative detection of salmonellae in dairy products. This NRLIC broth gave a very distinct color reaction in response to growth of Salmonella compared to color reaction of the other enteric bacteria. After an 18 hour incubation period in NRLIC broth Salmonella changes its color from red to yellow and the production of iron sulfide causes a blackening of the medium.

# MATERIALS AND METHODS

## Source of Cultures

The *Salmonella* cultures, used in this study included a culture of *Salmonella enteritidis* serotype Typhimurium (CDC 3-93-20) which was obtained from Center for Disease Control (CDC), Atlanta, Georgia, and the *Salmonella* spp. that were isolated from the Big Sioux River by Pierce (32). The identity of these cultures has been confirmed by the CDC and include *Salmonella enteritidis* serotypes:

| | |
|---|---|
| Anatum | Bredeney |
| Cubana | Derby |
| Infantis | Javiana |
| Montevideo | Oranienburg |

(Nomenclature of *Salmonella* according to Ewing (8))

## Water Samples

*Sampling Site* Water samples were taken from the Big Sioux River at the center of the Cliff Avenue Bridge located in Sioux Falls, South Dakota (Figure 1). This site is ideal because of its location which is downstream from the Sioux Falls Municipal Sewage Disposal Plant, and the Morrell Meat Packing Plant. According to the previous studies (32), this site has been highly contaminated with salmonellae. In addition to these samples, two samples were obtained from the Huron Municipal Sewage Disposal Plant and from the Armour Meat Packing Plant.

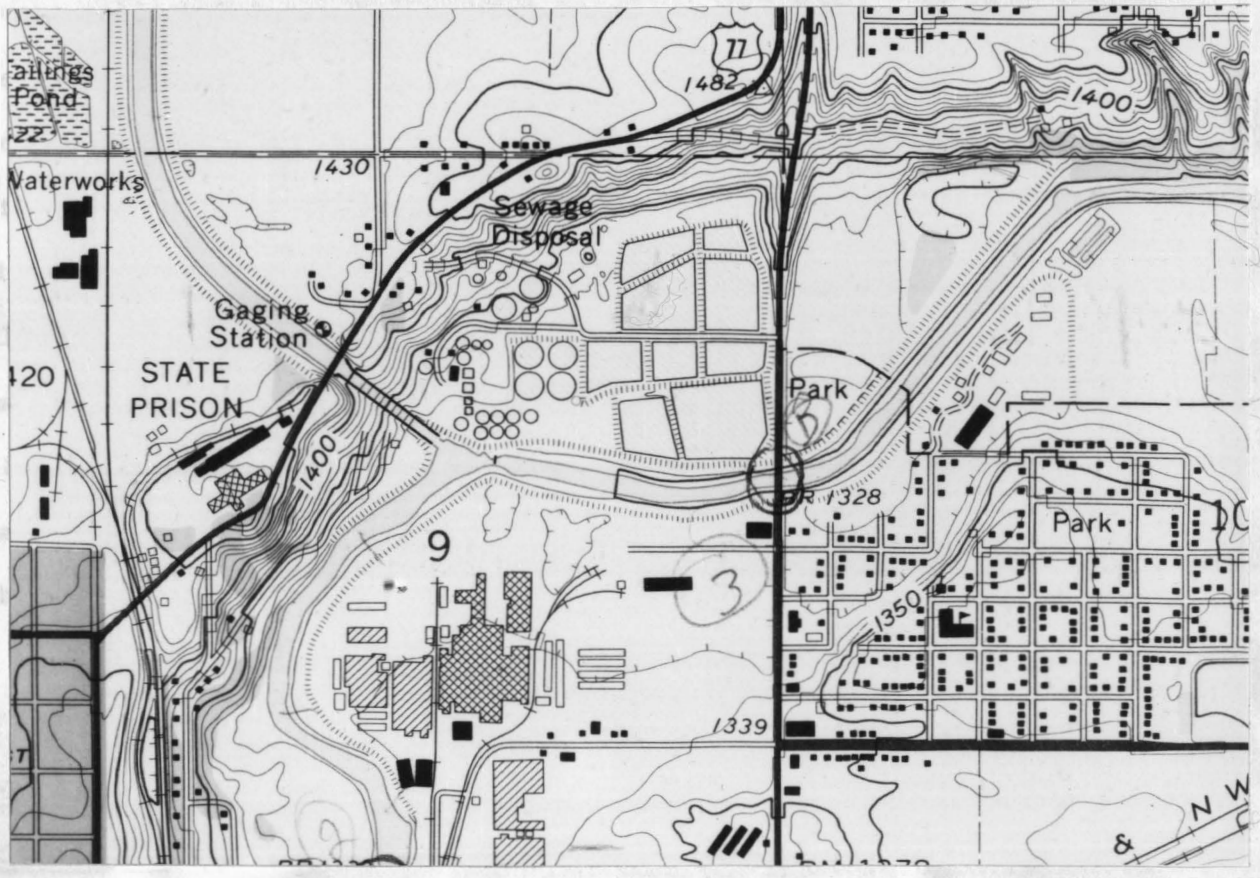


Figure 1. The Big Sioux River water sampling site at Cliff Avenue Bridge, Sioux Falls, South Dakota.

Sampling Techniques The Big Sioux River water samples were collected two inches below the water surface. This sampling was done using the 800 ml bottle sampler developed by Koupal (18). The sedimentation samples were collected from the top two inches (5 cm) of the river bed. These samples were collected by an Ekman dredge which had spring-operated jaws on the bottom. At the top of the dredge are two hinged overlapping lids that are held partially open during the descent. These lids are shut by a descending weight or messenger when the dredge reaches a silt layer. The collected silt from the sedimentation layer was poured over a window-screen to collect only the finely textured silt with the water (42). The collected samples were mixed and aliquotes of 10 ml, 1 ml, and 0.1 ml were placed into the neutral red lysine iron cystine (NRLIC) broth tubes.

## Isolation of Salmonella

Concentration of River Water Samples and Pre-enrichment Procedure

Salmonella spp. in Big Sioux River water samples were isolated by a membrane filtration method (32). The pore size of the membrane filter was 0.45 μ (Milipore type HAWG). Water sample volumes of 100, 10, 1, and 0.1 ml were used. After appropriate filtration of the water samples the membrane filters were placed into the pre-enrichment (Figure 2, I) medium Brain Heart Infusion broth (BHI) for six hours (32) at 41.5 C (39).

Enrichment One ml of each culture that had been pre-enriched in BHI broth was transferred to tetrathionate broth (TT) (Figure 2, IIa), and to selenite-cystine broth (SC) (Figure 2, IIb). The cultures were

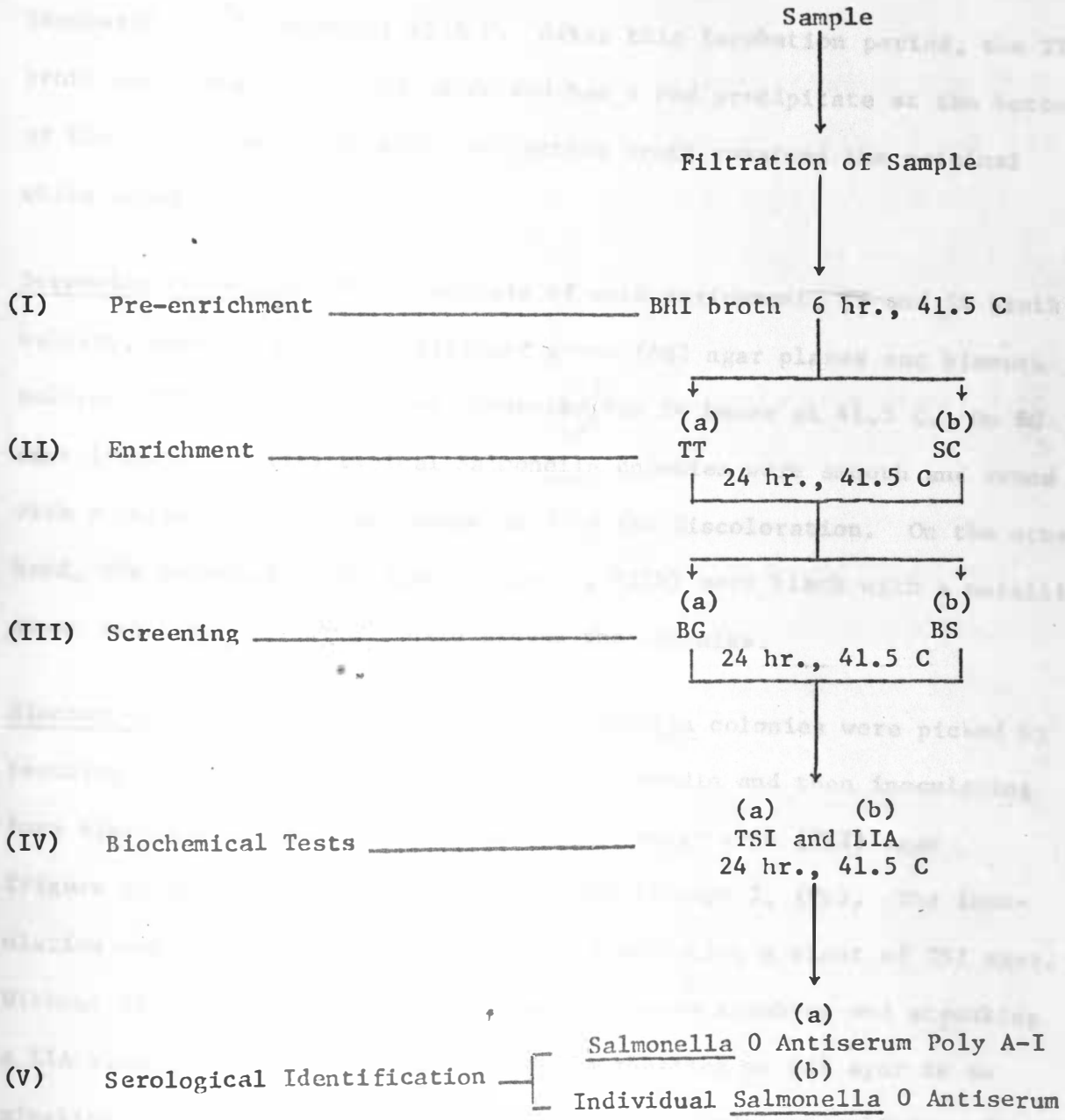


Figure 2. Isolation of Salmonella from water samples.

incubated for 24 hours at 41.5 C. After this incubation period, the TT broth was changed to a red color and had a red precipitate at the bottom of the test tubes. The selenite cystine broth remained the original white color.

Screening Procedure Three loopfuls of each enrichment, TT and SC broth culture, were streaked on brilliant green (BG) agar plates and bismuth sulfide (BS) agar plates, and incubated for 24 hours at 41.5 C. On BG agar (Figure 2, IIIa) typical Salmonella colonies were smooth and round with a white to pink color surrounded by red discoloration. On the other hand, the colonies on BS agar (Figure 2, IIIb) were black with a metallic sheen and sometimes with a halo around the colonies.

Biochemical Tests Typical, isolated Salmonella colonies were picked by touching the center of the colonies with a needle and then inoculating into biochemical test media such as triple sugar iron (TSI) agar (Figure 2, IVa) and lysine iron agar (LIA) (Figure 2, IVb). The inoculation was done by stabbing the butt and streaking a slant of TSI agar. Without reinoculation this was followed by twice stabbing and streaking a LIA slant. A characteristic Salmonella reaction on TSI agar is an alkaline slant, an acid butt, and production of hydrogen sulfide. Gas may or may not be produced. A Salmonella culture on LIA shows an alkaline slant, alkaline butt, and with various amounts of hydrogen sulfide production. It is very rare to have gas production in LIA medium.

## Serological Identification

All the cultures which showed Salmonella reactions on biochemical test media were tentatively considered to be Salmonella spp. These were confirmed by a rapid slide agglutination test with Salmonella O Antiserum (Difco) Poly A-I (Figure 2, Va) and by individual Salmonella O group antisera (Difco) (Figure 2, Vb).

Preparation of Antigen The antigens of the Salmonella were prepared by several different methods:

1. Direct Method

   Cells from a TSI agar slant culture were directly mixed with a drop of Salmonella O Antiserum Polyvalent A-I on a slide, or mixed with a drop of individual group antiserum on a slide.

2. Saline Method

   A dense suspension of organism was prepared by suspending the growth from an 18 hour heart infusion agar (HIA) slant in 0.5 ml of 0.85 percent sodium chloride solution. This produced a dense homogeneous suspension approximating 50 times that of Bacto-McFarland Barium Sulfate Standard number three (Difco).

3. White's Method

   The growth from an agar slant (HIA) culture was suspended in one ml of 95 percent alcohol, and heated at 60 C for one hour. The organisms were sedimented by centrifugation, the alcohol decanted, and the cells resuspended in 0.5 ml of phenolized saline (6). When preparing Salmonella antigen by White's

method, heavy growth from an entire slant should be used. For best results the loss of antigen during preparation must be considered.

Slide Agglutination Test Procedure

1. Growth from a TSI agar slant was tested by a direct method for Salmonella somatic antigens by using Salmonella O antiserum polyvalent A-I.
2. All the positive isolates were then transferred to heart infusion agar slants.
3. The heavy growth from HIA was transferred to one ml of 95 percent alcohol and mixed well.
4. It was then placed into a 60 C water bath for one hour.
5. The organisms were centrifuged for 15 minutes (660 x G).
6. The alcohol was decanted.
7. One-half ml of phenolized saline was added.
8. A drop of antiserum was thoroughly mixed with one loopful of antigen.
9. The slides were rocked for 30 seconds to one minute.
10. Positive agglutination was completed within one minute. Any delayed positive reaction was considered a negative agglutination test.
11. If agglutination occurred, then the individual Salmonella O antiserum group was tested.

Preparation of Antisera The dehydrated antisera, Difco, were prepared by adding three ml of sterilized 0.85 percent sodium chloride per individual vial and agitated gently to complete solution. These antisera were then stored in the refrigerator at two to eight C. The sera

from Sylvana (Millburn, New Jersey) were prepared by adding two ml of 0.85 percent sodium chloride to each vial.

The following groups of antisera and serotypes of Salmonella were routinely used for serological group identification and group identification positive controls.

| Salmonella O Antiserum Group | Serotypes |
|---|---|
| B | Typhimirium |
| $C_1$ | Oranienburg |
| D | Javiana |
| $E_1$ | Anatum |
| G | Cubana |

Determination of Titer for Individual Serum Each individual Salmonella O antiserum (Difco) was examined for its titer by the tube agglutination test method. This was performed with a positive control, a negative control, and a series of two-fold dilutions of each serum. For positive controls the same five cultures were used. One-half ml of each culture was mixed with 0.25 ml of its individual antiserum. Negative controls consisted of 0.25 ml of 0.85 percent sodium chloride and 0.5 ml of each culture. A 0.5 ml aliquote of each antiserum was diluted by ten series of two-fold dilutions for the test.

Slide Agglutination Tests for Isolates All the slide agglutination tests for Salmonella O antigen had positive controls with each different group,

and a negative control was run using 0.85 percent saline to make sure the serum was functioning properly. In addition to this procedure, all the isolates were restreaked on selective medium BG agar, and biochemically rechecked, and confirmed again with Salmonella O Antiserum Poly A-I. This was done to insure the purity of the isolates before they were tested for their individual group somatic antigen. The positive isolates were sent to the Division of Laboratories, South Dakota State Department of Health, Pierre, South Dakota for the confirmation. From there they were sent to CDC, Atlanta, Georgia.

## Enumeration of Salmonella

The quantitation of salmonellae from river water samples is a more difficult problem then the qualitative detection of salmonellae. Common methods of "enumerating" the Salmonella are by enriching the samples in TT broth or in SC broth with subsequent streaking on BG agar or BS agar plates. Cultures are then transferred from selective solid media to biochemical test media, TSI, and LIA. Until the results from biochemical tests are available, the presence of salmonellae cannot be determined. Also, these procedures take at least three to four days. This method does not quantitate the number of salmonellae in the water sample because the growth of salmonellae in TT broth and SC broth is unpredictable. It is our aim to develop a procedure which will shorten the time for testing and yet be specific enough to demonstrate the presence of salmonellae. In searching for a better method of enumeration of salmonellae in river water samples, I found that neutral red lysine iron cystine broth, developed by Hargrove et al. (11), worked well. This

is a very sensitive qualitative medium. The method was developed on the basis of pH changes and blackening of the medium by hydrogen sulfide production. Inoculation of salmonellae cultures into this medium gave very distinct color reactions compared with the other enteric organisms. Since the reactions of this medium were so different for Salmonella, this medium was used as a presumptive test medium for enumeration of salmonellae by a MPN method; this compares to the lactose broth method (42) which is used in quantitation of coliform bacteria. To support this idea the following experiments have been done: (1) determination of the end point or the least amount of sample which have Salmonella sp., (2) a sensitivity test for NRLIC broth using known mixed cultures, and (3) the actual use of NRLIC broth in enumeration of salmonellae from river water samples.

End Point Determination of Salmonella in River Water To quantitate the presence of salmonellae in a sample, the least volume of sample which shows the presence of salmonellae was determined. In the Big Sioux River this was one ml according to the previous study (32). Therefore, the sample sizes were limited to 1, 0.1, and 0.01 ml, and these samples were filtered through a membrane filter. The procedure for this experiment was exactly the same as for the isolation method previously discussed in an earlier part of this study.

Sensitivity Test for NRLIC Broth The composition of NRLIC broth is L-lysine 10 g, tryptone 5 g, yeast-extract 3 g, lactose 5 g, glucose 2 g, ferric ammonium citrate 0.5 g, sodium thiosulfate 0.1 g, L-cystine 0.3 g, neutral red 0.025 g, and one liter of distilled water (11).

The specific selectivity of NRLIC broth was tested under known mixed culture conditions. In this experiment Salmonella enteritidis serotype Typhimurium was used as a positive control (A), and E. coli, Shigella sonneii, Proteus mirabilis, and Citrobacter freundii were used as negative controls (B). For a mixed culture (C) S. enteritidis serotype Typhimurium, E. coli, S. sonneii, P. mirabilis, and C. freundii were mixed together. Each pure culture was incubated in BHI broth (Figure 3, I) at 41.5 C overnight. Each culture was subcultured for four hours at 41.5 C (Figure 3, II). Then these cultures were diluted as shown on the diagram in Figure 3.

Enumeration of Salmonella by NRLIC Broth Method The quantitation procedures for Salmonella from the Big Sioux River are shown in a flow diagram (Figure 4). In this experiment the MPN counts of salmonellae in surface water samples and in sedimentation layer samples were compared. These counts of salmonellae were compared to the counts of total coliform, fecal coliform, and fecal streptococci from the same water samples. Water samples of 10, 1, 0.1, and 0.01 ml, were placed directly into the NRLIC broth without pre-enrichment. These samples were incubated for 18 to 24 hours at 41.5 C. This was a presumptive test (Figure 4, I) of salmonellae quantitation by the MPN method, and the results of this were calculated from a standard five tube MPN table (42). The typical Salmonella reaction in NRLIC broth was shown by a pale yellow color and a massive black precipitate (Figure 4, Ia). If the medium, after the incubation period, shows a yellow color (Figure 4, Ib) without a black precipitate, it should then be reincubated for another 24 hours. By

(A) Positive control -- Typhimurium (0.2 ml) diluted to $10^{-14}$

(B) Negative control -- E. coli, S. sonneii, P. mirabilis, and C. freundii (0.25 ml of each) diluted to $10^{-14}$

(C) Mixed cultures -- All five cultures (0.2 ml of each) diluted to $10^{-14}$. After the appropriate dilutions, positive and negative controls and mixed cultures were then transferred to NRLIC broth (Figure 3, III) for 18 to 24 hours at 41.5 C.

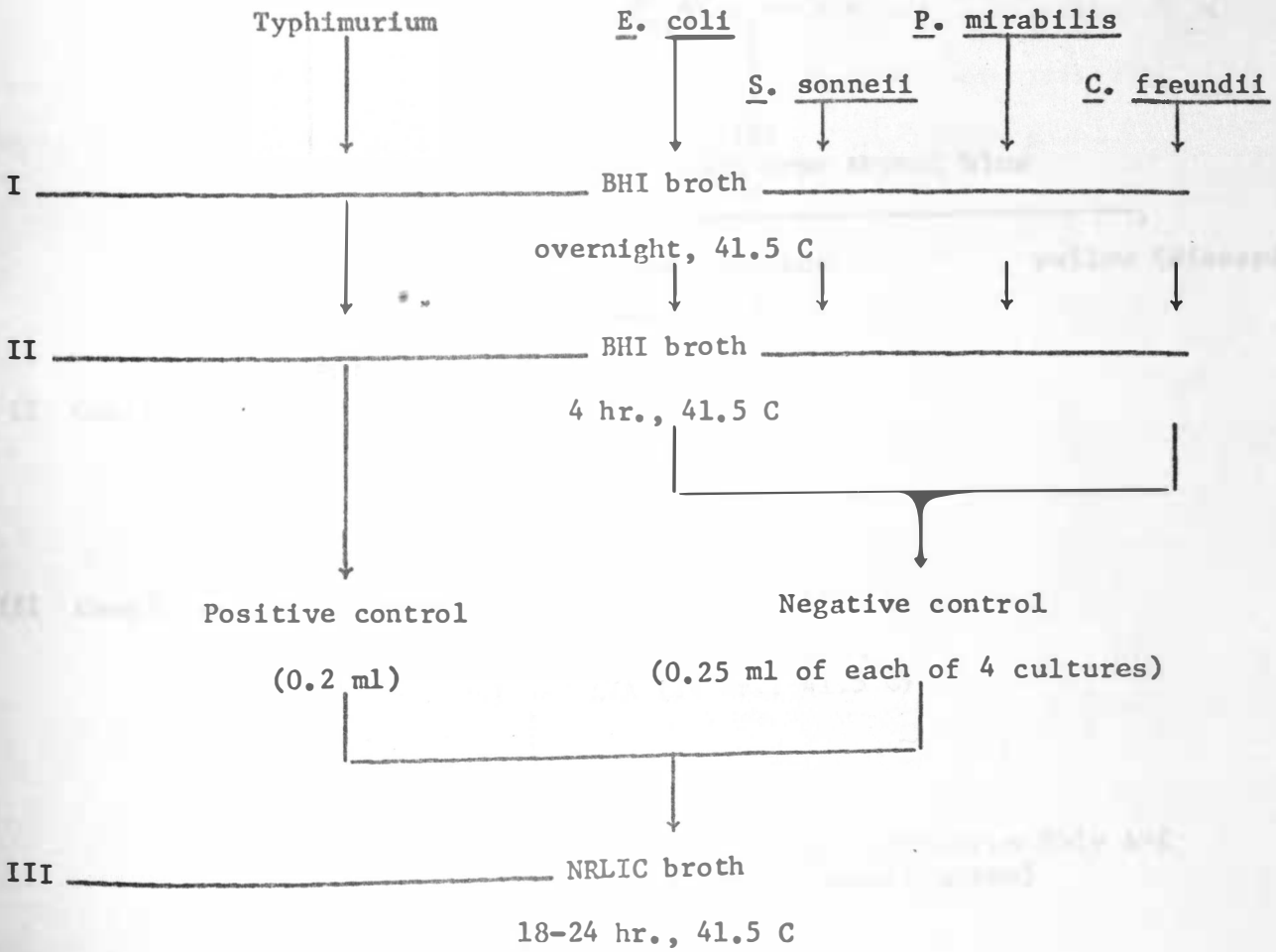


Figure 3. Sensitivity test for Salmonella enteritidis serotype Typhimurium in NRLIC broth.

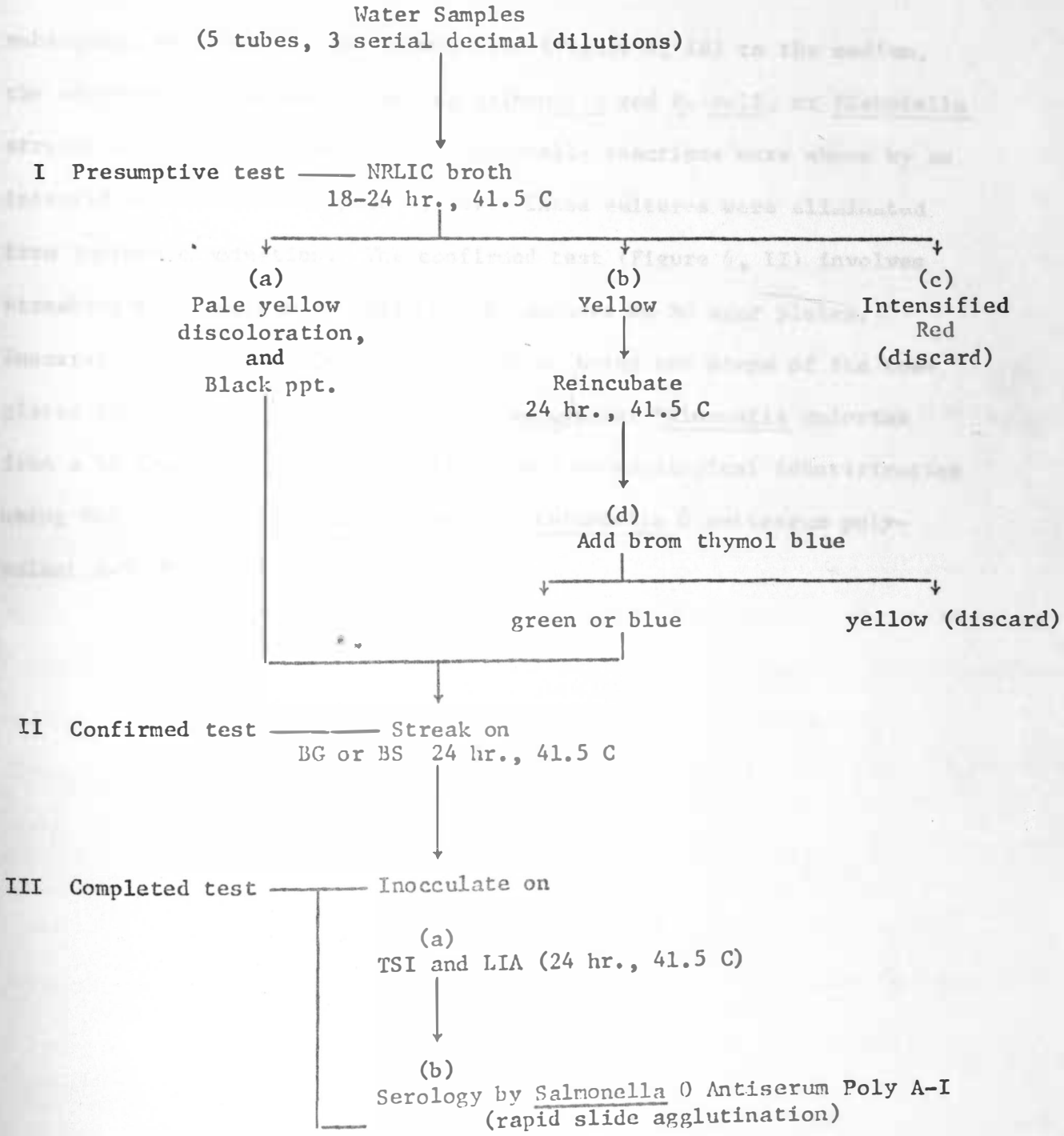


Figure 4. Enumeration of Salmonella by NRLIC broth MPN method.

subsequent addition of brom thymol blue (Figure 4, Id) to the medium, the non-hydrogen sulfide producing Salmonella and E. coli, or Klebsiella strains were differentiated. Non-salmonella reactions were shown by an intensified red color (Figure 4, Ic). These cultures were eliminated from further examination. The confirmed test (Figure 4, II) involves streaking two loopfuls of NRLIC broth cultures on BG agar plates. Enumeration of salmonellae was finished by using two steps of the completed test (Figure 4, III): by picking typical Salmonella colonies from a BG agar plate (Figure 4, IIIa), and by serological identification using the slide agglutination test with Salmonella O antiserum polyvalent A-I (Figure 4, IIIb).

# RESULTS AND DISCUSSION

This section is divided into two parts: the determination of the distribution of Salmonella spp. and the development of a quantitative method for the Salmonella in the Big Sioux River.

## Isolation of Salmonella

During the period of April 1970 to February 1971, 20 different water samples of the Big Sioux River were collected. From each of these samples, approximately 20 isolates were picked. The isolates were tested by the method described in Figure 2 of Materials and Methods.

This improved method of isolating Salmonella which was developed by Pierce (32) was found to be very effective for the qualitative detection of salmonellae. It was also true that the combination of tetrathionate (TT) broth with brilliant green (BG) agar gave higher recovery of Salmonella spp. than selenite-cystine (SC) broth and bismuth sulfide (BS) agar combinations.

Of these isolates picked, 381 were found to be Salmonella spp. because they were agglutinated by Salmonella polyvalent A-I antiserum. However, because some of the 381 isolates were found to be contaminated, all of these isolates were repeated. They were also retested with Salmonella O antiserum polyvalent A-I.

Serological Identification of Salmonella The serological identification of salmonellae was carried out in three separate steps.

The first was to detect the presence of salmonellae somatic antigen. This was done by using Salmonella O antiserum polyvalent A-I (Difco).

The second step was to characterize each of the 381 isolates into their individual Salmonella somatic antigen groups, B, $C_1$, D, $E_1$, G, and H.

The commercial antisera were first tested with known cultures to determine the titer. The titers of the commercial antisera are indicated in Table 1.

All of the 381 isolates that were confirmed by Salmonella O antiserum polyvalent A-I were tested for their somatic antigen group. The results from these tests are shown in Table 2.

According to these results, almost half of the isolates from the Big Sioux River were group B. This was understandable because group B is the most common Salmonella somatic group (25) and includes S. enteritidis serotype Typhimurium. Group $C_1$ was the second largest in number with group $E_1$ following. All of group G were isolated from the samples taken from waste water discharge from the Armour Meat Packing Plant in Huron, South Dakota. This was of special interest to this study because group G were not present in the isolates from the Big Sioux River. Only one isolate of group H was obtained from the Big Sioux River.

Identification of Serotypes The third step was to identify the serotype of each Salmonella isolate. Typhimurium serotyping was done at the Division

Table 1. Determination of Titer for Group Antiserum

| | *Salmonella* O Antiserum Group | | | | |
|---|---|---|---|---|---|
| | B | $C_1$ | D | $E_1$ | G |
| Typhimurium | + (1:64) | - | - | - | - |
| Oranienburg | - | + (1:128) | - | - | - |
| Javiana | - | - | + (1:64) | - | - |
| Anatum | - | - | - | + (1:64) | - |
| Cubana | - | - | - | - | + (1:64) |

Table 2. Slide Agglutination Test Results

| | | Total number positive | Percent positive |
|---|---|---|---|
| *Salmonella* group | B | 175 | 46.0 |
| | $C_1$ | 116 | 30.5 |
| | $E_1$ | 80 | 21.0 |
| | G | 9 | 2.3 |
| | H | 1 | 0.2 |
| Total isolates | | 381 | 100.0 |

of Laboratories, South Dakota State Department of Health, Pierre, South Dakota. However, most of the identification of isolates was done at CDC (Center for Disease Control) because of the availability of the antisera. Groups $C_1$, $E_1$, G and part of B were identified as shown in Table 3.

Future application of serotyping the Salmonella is to trace the source of the Salmonella contamination in lakes and rivers. A recent National Academy of Science publication (29) reported that certain serotypes are primarily adapted to man including S. typhi, and S. enteriditis serotype paratyphi A and B. Other serotypes are primarily adapted to animal including Pullarum, Gallinarum, and Dublin. The most common isolates from river water, S. enteriditis serotype Typhimurium is the most frequent pathogen for both man and farm animals.

## Enumeration of Salmonella

The second objective of this study was to develop a quantitative method for Salmonella spp. in water samples.

To measure the degree of Salmonella contamination in a water sample, quantitation of salmonellae is necessary. For this purpose, previous workers used a modified method to estimate the level of Salmonella contamination in food samples, (1, 4, 19, 23, 31, and 44). This modified method usually involves enriching bacteria from a known quantity of sample and their subsequent isolation on a selective agar medium. However, this method takes at least three days to complete because the enrichment medium does not prove the presence of salmonellae even after the incubation period and biochemical tests must be run.

Table 3. Serotypes that were Identified by the Center for Disease Control

| Salmonella Somatic Antigen | Serotype | No. of Culture |
|---|---|---|
| Group B | Typhimurium* | 47 |
| | Heidelberg | 2 |
| | Derby | 1 |
| Group $C_1$ | Infantis | 10 |
| | Oranienburg | 17 |
| | Thompson | 2 |
| Group $E_1$ | Anatum | 3 |
| | Meleagridis | 1 |
| Group G | Cubana | 3 |

*Identification of serotype Typhimurium was done at Division of Laboratories, South Dakota State Department of Health, Pierre, South Dakota.

A rapid, one step quantitative method was needed to demonstrate the presence of salmonellae in water samples. In other words, this method for Salmonella spp. would be equivalent to the Standard MPN index determination which uses lactose broth for a presumptive test of the coliforms. A Salmonella method was not possible until Hargrove, McDonough, and Reamer (11) developed a very selective presumptive test medium, neutral red lysine iron cystine broth (NRLIC). Hargrove et al. used this medium in a presumptive test procedure for qualitative detection of Salmonella in dairy products.

After inoculation with pure cultures of salmonellae and incubation at 41.5 C for 18 hours, this medium changed in color from red to pale yellow. Most Salmonella spp. decolorized the neutral red and formed a massive black precipitate which obscured the yellow color in 18 hours. There were only three serotypes which did not show the characteristic black precipitate: S. enteritidis serotypes Sendai, Abortivoequina, and S. choleraesuis (diaphasic) (11). E. coli and Klebsiella strains, like Salmonella spp., decolorized the medium from red to yellow without a change in pH; and neither produced hydrogen sulfide. Non-hydrogen sulfide producing Salmonella spp., E. coli, and Klebsiella spp. produced the same yellow color after incubation. They could be differentiated after an additional 24 hour incubation period and subsequent addition of brom thymol blue to the medium. Only Salmonella spp. gave an alkaline reaction changing brom thymol blue from yellow to green or blue. E. coli and Klebsiella strains lowered the pH of the medium which remained yellow. Cultures of Shigella, Enterobacter, Citrobacter, Proteus, and Pseudomonas

usually remained bright red in color after incubation for 18 hours. They did not blacken the medium. Because the cultures lowered the pH of the medium, they intensified the red color.

This differential test is based on the final pH as indicated by neutral red and by brom thymol blue, and the production of iron sulfide (11) which blackens the medium (11).

A method was developed in this thesis study using the NRLIC broth not just as a qualitative presumptive test but as a medium for enumeration of salmonellae. By using five tubes in decimal dilution, the results from the NRLIC were read directly from the five tube MPN table (42). BG agar medium was used as a confirmed test method, and biochemical test and serological characterization were used as the completed test.

End Point Determination of Salmonella in River Water The end point determination experiment was run to determine the least volume of sample which shows the presence of salmonellae. In this experiment 1, 0.1, and 0.01 ml samples were filtered and pre-enriched in brain heart infusion (BHI) broth. Conventional methods of isolating Salmonella were used and confirmed by Salmonella O antiserum polyvalent A-I. Salmonella were present in 0.1 ml samples of the Big Sioux River. One one-hundredth ml samples did not have the presence of salmonellae. This experiment was performed four more times, and all experiments showed the same results. The results were as tabulated in Table 4.

Sensitivity Tests for NRLIC Broth The specificity of this medium, which has a very distinct reaction for salmonellae, was tested with controlled

Table 4. End Point Determination of Salmonellae in River Water

| Sample Volume | 1 ml | 0.1 ml | 0.01 ml |
|---|---|---|---|
| Experiment No. | Number of Positive Tubes per 10 Tubes | | |
| 1 | 10/10 | 3/10 | 0/10 |
| 2 | 10/10 | 2/10 | 0/10 |
| 3 | 10/10 | 3/10 | 0/10 |
| 4 | 10/10 | 3/10 | 0/10 |
| 5 | 10/10 | 3/10 | 0/10 |

pure and mixed cultures to determine the specific selectiveness of NRLIC broth. Five different enteric bacteria were used for this experiment; these were Salmonella enteritidis serotype Typhimurium, E. coli, Shigella sonneii, Proteus mirabilis, and Citrobacter freundii. All the cultures were grown in BHI broth separately and incubated at 41.5 C overnight. They were then transferred to fresh BHI broth at 41.5 C for four hours in order to obtain the cultures at their exponential growth phase. These cultures were then diluted appropriately and transferred to NRLIC broth. The results of this experiment are in Table 5.

The positive control with pure Salmonella cultures showed a positive reaction through the $10^{-12}$ dilution. The densities of blackening throughout the media were the same except at the $10^{-12}$ dilution; here, the blackening of the medium was less than the other dilutions. The negative control did not have any trace of hydrogen sulfide production or blackening of the media. However, the $10^{-2}$ and $10^{-4}$ dilution showed a

Table 5. Sensitivity Tests for NRLIC Broth

| Dilutions | *Positive control | **Mixed cultures | ***Negative control |
|---|---|---|---|
| $10^{-2}$ | black + | black + | yellow - |
| $10^{-4}$ | black + | black + | yellow - |
| $10^{-6}$ | black + | black + | red - |
| $10^{-8}$ | black + | black + | red - |
| $10^{-9}$ | black + | black + | red - |
| $10^{-10}$ | black + | black + | red - |
| $10^{-11}$ | black + | red - | red - |
| $10^{-12}$ | black + | red - | red - |
| $10^{-13}$ | red - | red - | red - |
| $10^{-14}$ | red - | red - | red - |

*Positive control -- S. enteritidis serotype Typhimurium

**Mixed cultures -- Typhimurium, E. coli, S. sonneii, C. freundii, and P. mirabilis

***Negative control -- E. coli, S. sonneii, C. freundii, and P. mirabilis

yellow color while the others showed a red color. This indicated that at a lower dilution, the E. coli reaction overcame the other enteric bacteria reaction; but at higher dilutions, the other three enterics demonstrated a stronger reaction by intensifying a red color. This result was due to the decreased pH in the medium. The known mixed cultures which showed positive reactions to $10^{-10}$ and any dilution greater than $10^{-10}$ showed a red color reaction. This indicated that Salmonella cells present in this mixed culture were able to grow and produced a typical reaction up to the $10^{-10}$ dilution. Dilutions higher than $10^{-10}$ showed the same reactions as the three other enterics: production of a red color reaction by lowering the pH of the medium. Those dilutions that showed positive reactions were streaked on BG agar plates, and biochemical and serological tests were carried out to make sure that these were Salmonella spp. Indeed, the Salmonella spp. were isolated from these positive dilutions.

Enumeration of Salmonella from River Water using NRLIC Broth Experiments were done to determine the least volume of river water sample that had Salmonella cells and the sensitivity of the NRLIC broth media. The previous experiments determined that Salmonella spp. were present in 0.1 ml sample of river water, and that the sensitivity of the medium was effective up through the $10^{-10}$ dilution using known mixed cultures. The practical application of this medium for enumeration of salmonellae by this method was the next step. The enumeration of Salmonella spp. in river water was carried out in two separate steps: one, quantitation of salmonellae in surface water, and two, quantitation of Salmonella in the sedimentation layer. The results of these experiments were compared to total coliform, fecal coliform, and fecal streptococcus densities.

Table 6. Enumeration of Salmonella from the River Water using NRLIC Broth

| | Surface Water | | | | Sedimentation Layer | | | |
|---|---|---|---|---|---|---|---|---|
| No. Expt. | 10 ml | 1 | 0.1 | MPN index /100 ml | 10 ml | 1 | 0.1 | MPN index /100 ml |
| 1 | 4 | 2 | 1 | 26 | 5 | 1 | 2 | 63 |
| 2 | 3 | 3 | 0 | 17 | 5 | 3 | 0 | 79 |
| 3 | 2 | 3 | 0 | 12 | 5 | 3 | 1 | 109 |
| 4 | 4 | 3 | 1 | 33 | 4 | 4 | 0 | 34 |

Table 7. Comparison of Surface Water Counts; Salmonella, Total Coliform, Fecal Coliform, and Fecal Streptococcus

| | MPN Index per 100 ml | | | |
|---|---|---|---|---|
| No. Expt. | Total Coliform | Fecal Coliform | Fecal Streptococcus | Salmonella |
| 1 | 172,000 | 31,000 | 79 | 26 |
| 2 | 348,000 | 49,000 | 33,000 | 17 |
| 3 | 918,000 | 4,000 | 4,300 | 12 |
| 4 | 230,000 | 7,000 | 3,300 | 33 |

Table 8. Comparison of Sedimentation Layer Counts; Salmonella, Total Coliform, Fecal Coliform, Fecal Streptococcus

| | MPN Index per 100 ml | | | |
|---|---|---|---|---|
| No. Expt. | Total Coliform | Fecal Coliform | Fecal Streptococcus | Salmonella |
| 1 | 71,600,000 | 542,000 | 79,000 | 63 |
| 2 | 9,300,000 | 3,000 | 930,000 | 79 |
| 3 | 7,900,000 | 1,300,000 | 490,000 | 109 |
| 4 | 12,300,000 | 60,000 | 50,000 | 34 |

According to these results the sedimentation layer had a greater number of salmonellae than the surface water samples. This is also true with total coliform, fecal coliform, and fecal streptococcus counts.

The numbers of salmonellae, total coliform, fecal coliform, and fecal streptococcus may not necessarily have a numerical relationship. Even though there was not a fixed relation in numbers, it is probable that samples with higher total coliform, fecal coliform, and fecal streptococcus counts may have higher counts of salmonellae; samples with lower total coliform, fecal coliform, and fecal streptococcus counts may have lower counts of salmonellae. Furthermore, some interesting phenomena may often occur with larger or smaller volumes of river water samples which showed typical reactions of other enteric bacteria, such as, species of Proteus, Shigella, Citrobacter, or E. coli. This phenomenon can be explained as follows: since the samples from river water were not pre-enriched in any medium, the Salmonella present in those samples had a tendency to have low viability as was described by Pierce (32). Thus samples, such as 10 ml or 0.01 ml did not show a uniformly positive reaction on NRLIC broth; when other dilutions, such as 1 ml and 0.1 ml samples showed a distinct positive Salmonella reaction. This indicated that at these dilutions the Salmonella either were not present or the Salmonella were too weak to grow in the presence of a greater number of other enteric organisms. This same phenomenon was shown by the sensitivity test. The negative control of that experiment showed a yellow color reaction at higher dilutions, for example $10^{-2}$ and $10^{-4}$, and a red color reaction at lower dilutions such as $10^{-6}$ . . . $10^{-14}$. Thus the NRLIC

broth medium was very sensitive to the proper dilutions of samples; with higher or lower dilutions the competition was so great that Salmonella spp. did not characteristically change the medium.

Ideally, this phenomenon of "skip" should be prevented, and it is possible to do so by the use of a pre-enrichment medium such as BHI broth. But the pre-enrichment step may not allow the actual quantitation of salmonellae because during this step, the Salmonella count may increase unpredictably. Therefore, the pre-enrichment step was eliminated. Of course, it is true that when the qualitative detection of salmonellae from a sample is the sole purpose of an experiment, then the pre-enrichment procedure must be used to obtain better results; however, when enumeration rather than detection of salmonellae is the prime purpose, then the samples should be inoculated directly to NRLIC broth. Pursuing this reasoning, all the experiments for enumeration of salmonellae from the Big Sioux River were done without any pre-enrichment even though there were skips in some MPN counts. This method of enumeration of salmonellae from river water samples probably can be greatly accelerated by the use of fluorescent antibody techniques.

The ideal medium for the presumptive test in the MPN method of Salmonella spp. should satisfy the fact that the medium must be specific for the reactions of salmonellae so that the presence of salmonellae can be determined without extra days or incubation. Therefore, the real problem that had to be developed was an enrichment medium which could be used as a presumptive test medium.

The conventional method of enumerating Salmonella spp. was the dilution-enrichment-subculture method (i.e. TT broth or SC broth enrichment and subsequent streaking on BG or BS agar medium for typical Salmonella spp. colonies). This method has been used by Byrne et al. (4). Enumeration in this way would take at least three days of different tests just to confirm the presence of salmonellae. Recognizing the problem that the enrichment medium has to be improved, Taylor et al. (44) tried to modify the SC broth by substituting dulcitol or mannitol for lactose. However, he could not make any improvement because there were no differences with SC broth containing either dulcitol, or mannitol, or lactose. As one can see, the basic problem is the development of an enrichment medium. North (31) tried to increase the number of salmonellae counts in food samples by the use of the pre-enrichment medium, lactose broth. At present, this pre-enrichment method of quantitation is favored by researchers. But the use of the pre-enrichment method in the enumeration procedure has to be seriously thought out because the increase of salmonellae during the pre-enrichment step is unpredictable. Also, this method delays the result one more day.

The NRLIC broth is the answer to the best enrichment medium because it takes a very short incubation period of 18 to 24 hours to show the reactions of salmonellae. Also, the reactions of salmonellae are so definite that the results can be calculated directly from the Standard Method MPN table. Furthermore, one does not have to carry on the experiment when the results do not show any Salmonella sp. reaction; thus one can save time.

Therefore, NRLIC broth is applicable as a presumptive test medium in the standard multiple-tube test for enumeration of Salmonella spp. When the samples are inoculated into the NRLIC broth, this is considered the presumptive test. If the result from NRLIC broth shows a positive reaction, then the cultures from the NRLIC broth are streaked on BG agar; this step is the confirmed test of the standard test method. The completed test is used as the next step with TSI, LIA, and serology by Salmonella O antiserum polyvalent A-I.

Thus, the method of enumeration using NRLIC broth is more specific and more reliable than any other methods that are presently used. The counts of salmonellae from river water and sediment layers were compared to endorse the Hendricks (12) study which showed that Salmonella recovery for bottom sediments was observed to be higher than those from the surface water. He pointed out that it is difficult to explain the results, but that it may be possible that sedimentation and adsorption of the organisms to the sand and clay could concentrate bacteria on the stream bottom. Results from this research showed that the sediment layer had higher counts of salmonellae than the surface water samples. Thus, the results from this study confirm Hendricks' findings of higher recovery from the sediment layer. Also, Hendricks' explanation for this phenomenon seemed applicable to this study because the river flows slowly and the microorganisms settle to the bottom sediment.

The results from the enumeration of Salmonella in surface water and the sediment layer were compared to the numbers of indicator organisms in surface water and sediment layer to determine whether the total

coliform counts will represent the numbers of Salmonella present in water samples. Results from this thesis study concluded that the counts of Salmonella did not have any numerical relation with the counts of total coliform, fecal coliform, and fecal streptococcus. A similar study was done by Slanetz et al. (36 and 37) in estuary water, and concluded that there were certain numerical relationships among total coliform, fecal coliform, and fecal streptococcus. However, they found that there were no numerical relationships between counts of Salmonella and other indicator organisms. Furthermore, they were able to isolate Salmonella spp. from a sample that contained no detectable fecal coliform or fecal streptococcus.

The quality of water samples cannot be determined by coliform counts only because samples with low or no coliform counts are not necessarily free of pathogenic organisms such as Salmonella. Therefore, to estimate the degree of bacterial contamination in water, the quantitation of Salmonella must be considered as a separate requirement for measuring the bacterial contamination.

The first objective of this study was to determine the distribution of Salmonella spp. in the Big Sioux River. This was accomplished by using a sensitive BHI broth medium as a pre-enrichment method. The BHI broth medium had increased the low viability of the Salmonella spp. in the river water; thus, it promoted a better recovery of Salmonella spp. from water samples. By the use of BHI broth medium, Salmonella spp. were routinely isolated from 0.1 ml of the river water samples. The purpose of this

distribution study was to investigate and to collect different Salmonella serotypes so that the origin of their source could be determined, because certain serotypes are primarily adapted to men, and some serotypes are primarily adapted to animals.

The second objective of this study was to develop a quantitation method for Salmonella spp. from the water samples. Since there was no specific method for the enumeration of Salmonella from water samples, it was necessary to develop a new method. The development of an effective quantitation method for Salmonella spp. was accomplished by the use of NRLIC broth. The NRLIC broth was originally developed by Hargrove et al. (11) as a qualitative medium for the detection of Salmonella in dairy products. However, this medium proved to be very sensitive for Salmonella spp. in water samples. Therefore, this medium was used as a presumptive test medium in the MPN method for the enumeration of Salmonella from water samples. The sensitivity of this medium was based on the color changes of the medium due to the changes of pH. Another factor for effectiveness may be due to the use of higher temperature of 41.5 C (32). The great advantage of this enumeration method is that the procedure of this is the same as the total coliform count method which is well known to the sanitary bacteriologist.

# CONCLUSIONS

1. This study confirmed that Pierce's (32) improved method of isolating Salmonella spp. by brain heart infusion (BHI) broth pre-enrichment is very effective in qualitative detection of Salmonella spp. from water.

2. The combination of tetrathionate broth and brilliant green agar resulted in higher recovery of Salmonellae isolates from water than the combination of selenite cystine broth and bismuth sulfide agar.

3. Of 381 cultures of Salmonella isolated from the Big Sioux River water samples which were all O antiserum polyvalent A-I positives when tested for individual somatic antigen group, there were 175 isolates in group B (46 percent), 116 in group $C_1$ (30.5 percent), 80 in group $E_1$ (21 percent), 9 in group G (2.3 percent), and 1 in group H (0.2 percent).

4. The commonly used enrichment-subculture method of isolating and quantitating Salmonella spp. was not reproducible because the number of salmonellae in the initial sample could not be enumerated after the culture had been enriched and subcultured.

5. The neutral red lysine iron cystine (NRLIC) broth developed by Hargrove et al. (11) for qualitative detection of salmonellae in dairy products was demonstrated to be effective in detection of salmonellae from water samples.

6. An improved method of quantitation of salmonellae was developed using NRLIC broth and the MPN index of the Standard Method (42) which gave a distinct color reaction for Salmonella spp.

7. The NRLIC broth when used with known mixed cultures was sensitive to a dilution of $10^{-10}$.

8. A comparison of the numbers of cultures found in the bottom sediment layer and the river water demonstrated that Salmonella spp. counts were about one logarithm higher in the sediment layer.

9. The Salmonella counts did not have any numerical relationship with total coliform, fecal coliform, and fecal streptococcus counts.

10. Quantitation of salmonellae in river water by the MPN NRLIC broth method was found to be reliable.

11. Salmonella spp. isolated from the Big Sioux River included S. enteritidis serotypes Anatum, Cubana, Derby, Heidelberg, Infantis, Meleagridis, Oranienburg, Thompson, and Typhimurium.

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