Rapid Quantitative Method for Salmonella Detection in Polluted Waters

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A procedure has been developed for the enumeration of salmonellae in polluted waters using several modifications of existing techniques. Confirmation of salmonellae is achieved within 48 hr. This procedure includes selective enrichment in mit-Tetrathionate Broth (22 ± 1 hr), plating on Brilliant Green Sulfag Agar (20 ± 1 hr), and confirmation by flagellar (H) agglutination of the growth in a mannos-containing medium (6 ± 1 hr). An incubation temperature of 41.5°C was used throughout this procedure. Dilution to extinction techniques (most probable number) were employed to enumerate salmonellae. Large sample volumes were concentrated through the use of membrane filters. This technique proved to be rapid and reliable for the enumeration of salmonellae in water, waste water, and waste-water sludges.

In the past many different techniques and selective media have been developed to recover salmonellae from foods, animal feeds, fecal specimens, and natural waters. However, complete identification of salmonellae is often a time-consuming procedure in the diagnostic laboratory. Since most salmonellae are motile, Hajna and Damon (6) suggested that polyvalent Salmonella flagellar agglutination could be used as a rapid screening test for the presence of Salmonella. Spicer (12) developed a simplified method for identifying the flagellar antigens of the most commonly encountered salmonellae. He used four main polyvalent Salmonella flagellar antisera and two additive antisera for identifying more than 17 Salmonella flagellar antigens. Edwards and Ewing (2) modified the Spicer antisera to achieve greater specificity and to minimize the incidence of cross-reactions. Silliker et al. (10) used Spicer-Edwards H antisera as a primary screening procedure for detecting salmonellae in food products. These investigators showed that organisms agglutinating in polyvalent Salmonella flagellar antisera could be tentatively classified as salmonellae with a good degree of certainty and that further biochemical tests would confirm the preliminary results. Sperber and Deibel (11) employed Spicer-Edwards antisera (with some modifications) to detect salmonellae in foods and animal feeds within 50 hr of initiating the analysis. They reported their procedure to be as sensitive as the more time-consuming, conventional method which required at least 4 to 5 days. These authors also developed a mannoscontaining medium (M-broth) to minimize the incidence of nonspecific agglutination.

The purpose of this study was to develop a rapid and relatively simple technique to enumerate salmonellae in waste water, polluted water, and waste-water sludges. The procedures employed flagellar agglutination as a rapid screening test to detect salmonellae in the unique environment of polluted water.

MATERIALS AND METHODS

Samples. Samples of polluted water, waste-water, and sludges were collected at selected points in the Madison, Wis., area. Sterile, 1-liter polyethylene bottles were used to collect the samples, and analyses were performed immediately upon return of the samples to the laboratory. The sludge samples were diluted and homogenized for 1 min in a Waring Blendor before seeding into enrichment broth.

Sample concentration. Multiple tube, serial dilution techniques were employed to quantitate salmonellae in the samples. Large volumes of water were more conveniently tested by concentrating the sample prior to enrichment. This was accomplished for the 100-ml samples by filtering them through a membrane filter (HA 0.45-μm pore size; Millipore Corp., Bedford, Mass.). The filter was then placed into 100 ml of single-strength enrichment medium. For waters high in suspended matter, a microfiber prefiter (type AP 25; Millipore Corp.) was placed over the membrane filter, and approximately 2 to 3 g of sterilized diatomaceous earth (Celite no. 503) was added asep-
tically to the sample prior to filtration. After filtration, the membrane filter, prefilter, and diatomaceous earth mat were placed into a culture flask containing 100 ml of single-strength enrichment medium. The flasks were gently shaken and then incubated at 41.5 ± 0.5°C for 22 ± 1 hr.

**Selective enrichment procedures.** Serial dilutions of water samples were inoculated into tubes containing m-Tetrathionate Broth (Difco). One-milliliter samples and subsequent lower decimal dilutions were inoculated into 10 ml of single-strength broth, and 10-ml samples were inoculated into 10 ml of double-strength broth. One hundred-milliliter samples were handled as described above. All samples were incubated at 41.5 ± 0.5°C for 22 ± 1 hr.

**Plating procedures.** After selective enrichment, one loopful of enrichment broth was streaked onto Brilliant Green Sulfag (BG Sulfag) Agar (Difco) and incubated at 41.5 ± 0.5°C for an additional 20 ± 1 hr.

**M-broth enrichment.** Two or more suspected *Salmonella* colonies were picked from the BG Sulfag Agar plates and each was placed into individual 1-ml samples of M-broth (11). The M-broth tubes were incubated at 41.5 ± 0.5°C for 6 ± 1 hr.

All media were prewarmed to 41.5°C before inoculation. This was particularly essential to the M-broth enrichment step which was usually incubated for only 6 hr.

**Confirmation by modified H agglutination test.** One drop of Formalin-salt solution (3.3 ml of Formalin and 4.62 g of NaCl to 100 ml of distilled water) was added to each M-broth culture, and 0.1 ml of pooled H antisera (11) was subsequently added. The tubes were gently shaken and then incubated for up to 60 min in a water bath at 50°C. Any visible amount of flocculent precipitate (H agglutination) detected in the tube was regarded as a positive test for salmonellae. If pure isolate was required for further biochemical confirmation or complete serotyping, one loop of M-broth culture was streaked onto appropriate selective agar prior to the addition of Formalin-salt solution. Further confirmation of positive H-agglutinated cultures was determined by biochemical tests on Triple Sugar Iron Agar (Difco), Lysine Iron Agar

**TABLE 1. Sensitivity study of the quantitative procedure**

| Species tested          | Spread plates          | Proposed quantitative procedure          |
|-------------------------|------------------------|------------------------------------------|
|                         | Mean*                  | 95% Confidence limit                      | MPN* index per 100 ml | 95% Confidence limit                      |
|                         | Lower                  | Upper                                    | Lower                  | Upper                                    |
| *Salmonella typhimurium*| 6.0                    | 4.1                                      | 7.9                    | 9.3                                      |
| *S. typhimurium*        | 45.8                   | 38.6                                     | 53.2                   | 64.0                                     |
| *S. heidelberg*         | 4.8                    | 3.2                                      | 6.3                    | 4.3                                      |
| *S. heidelberg*         | 24.0                   | 21.0                                     | 27.0                   | 15.0                                     |
| *S. Newport*            | 3.4                    | 2.1                                      | 4.7                    | 4.3                                      |
| *S. Newport*            | 9.0                    | 6.7                                      | 11.8                   | 9.3                                      |
| *S. Enteritidis*        | 50.3                   | 47.7                                     | 52.8                   | 24.0                                     |

* Mean of ten spread plates on Brilliant Green Sulfag Agar.

**TABLE 2. Salmonella recovery in water from selected processes at Madison Metropolitan Sewage Treatment Plant**

| Date       | Salmonellae per 100 ml sample* |
|------------|--------------------------------|
| Raw        | Primary clarifier effluent      |
|            | Trickling filter effluent       |
|            | Activated sludge effluent       |
|            | Final effluent after chlorination |
| 11-8-69    | 110                             | 15                          | 46                       | <0.4                        |
| 3-24-70    | 11,000                          | 150                         | 91                       | 93                          | <0.4                        |
| 4-15-70    | 240                             | 240                         | 21                       | 14                          | <0.4                        |
| 4-30-70    | 2,400                           | 390                         | 21                       | 91                          | <0.4                        |
| 6-4-70     | 230                             | 230                         | 91                       | 750                         | <0.4                        |
| 7-9-70     | 930                             | 240                         | 460                      | 240                         | <0.4                        |
| 8-18-70    | 11,000                          | 930                         | 210                      | 930                         | <0.4                        |
| 9-16-70    | 11,000                          | 2,400                       | 4,600                    | 2,400                       | <0.4                        |

* Most probable number; four dilutions in triplicate.

(Difco), and Urea Agar (Difco). Additional biochemical testing and complete serotyping of isolates were performed, as required, by courtesy of the Wisconsin State Laboratory of Hygiene.

**Quantitation of salmonellae.** Densities of salmonellae in samples were obtained by the use of multiple tube serial dilution techniques using most probable number (MPN) tables (1). Decimal dilutions of samples in triplicate were normally used throughout these studies.

**Sensitivity test of the quantitative procedure.** Four frequently encountered species of salmonellae found in surface waters, *Salmonella typhimurium, S. heidelberg, S. Newport,* and *S. Enteritidis,* were employed in the evaluation of the sensitivity of the quantitative procedure outlined above. Stock cultures of these four species were obtained from the Wisconsin State Laboratory of Hygiene. The cultures were inoculated into nutrient broth for 18 hr at 37°C and then diluted with phosphate buffer (42 mg of KH₂PO₄ to 1 liter, adjusted to pH 7.2 with NaOH) to produce suspen-
TABLE 3. Salmonellae recovery in anaerobically digested sludge at Madison Metropolitan Sewage Treatment Plant

| Date   | Raw sludge | Digested sludge |
|--------|------------|-----------------|
|        | Total solids (g/liter) | Volatile solids (%) | Salmonellae* per gram of total solids | Total solids (g/liter) | Volatile solids (%) | Salmonellae* per gram of total solids |
| 6-3-70 | 41.05      | 76               | 22,400                      | 22.04          | 67               | 680                      |
| 7-30-70| 34.87      | 76               | 5,340                       | 20.68          | 66               | 220                      |
| 9-1-70 | 32.17      | 74               | 2,780                       | 16.08          | 68               | 90                       |
| 9-16-70| 25.61      | 75               | 3,350                       | 16.68          | 67               | 960                      |

* Most probable number; four dilutions in triplicate.

TABLE 4. Salmonella serotypes isolated from wastewater at Madison Metropolitan Sewage Treatment Plant

| Classification          | No. of samplesa |
|-------------------------|-----------------|
| Salmonella typhimurium   | 6               |
| S. derby                 | 5               |
| S. infantis             | 4               |
| S. enteritidis          | 3               |
| S. senftenberg          | 2               |
| S. anatum               | 1               |
| S. thompson             | 1               |
| S. newport              | 1               |
| S. heidelberg           | 1               |

a Samples were collected from August 29, 1969, to June 8, 1970.

sions containing 10 to 1,000 organisms per ml. Ten spread plates were prepared for each culture using BG Sulfag Agar by spreading 0.1 ml of suspension on the agar surface. These plates were incubated at 41.5 °C for 20 ± 1 hr and counted using a Quebec Counter. Subsequently, 1 ml of the diluted bacterial suspensions was inoculated into 1 liter of lake water. The lake water suspensions were then analyzed in accordance with the procedures delineated above to obtain an MPN estimate for each culture.

RESULTS

Sensitivity of quantitative procedure. The results of the spread plate and MPN procedure for the four stock cultures of salmonellae are presented in Table 1. Statistical analysis by use of the t test (3) was made to evaluate the 95% confidence intervals for each set of spread plate counts. The 95% confidence intervals for MPN estimates were provided in MPN tables (1). Examination of Table 1 suggests that the proposed quantitative procedure provides estimates of salmonellae within acceptable limits based on spread plate counts, at least for the four serotypes of salmonellae tested herein. That the MPN confidence limits were wide is the inherent characteristic of the three-tube-test. Narrower limits could be achieved through more tube plantings, but the benefits for greater accuracy in results would have to be weighed against the costs for obtaining them.

Recovery of salmonellae in municipal wastewater and polluted streams. The quantitative method outlined above is being used in extensive studies to determine the sources and occurrence of salmonellae in polluted waters. A brief resume of some of the findings to date is presented herein to demonstrate the usefulness of the procedure. A more complete analysis of these findings will be presented in detail at a later date.

Municipal wastewater waters and sludges. Samples of raw and treated waste waters and digested and raw waste sludge have been collected routinely at the Metropolitan Sewage Commission Wastewater Treatment Plant, Madison, Wis. The plant which treats approximately 40 million gal of domestic and industrial waste water per day employs both activated sludge and trickling filtration treatment processes in parallel after primary sedimentation. Results from 24-hr composite samples are presented in Table 2. Grab samples of raw and digested sludge were also examined at this plant, and the results of four analyses appear in Table 3. In each survey

TABLE 5. Occurrence of salmonellae and fecal coliforms in Six Mile Creek, Wisconsin

| Distance from STP outfall (ft) | Salmonellae in 100 mlb | Fecal coliforms in 100 mlc |
|--------------------------------|------------------------|--------------------------|
| 0                              | 2.3                    | 1,470,000                |
| 800                            | 2.0                    | 380,000                  |
| 2,500                          | 4.3                    | 2,600                    |
| 4,500                          | 1.5                    | 810                      |
| 10,500                         | <0.4                   | 540                      |
| 15,500                         | <0.4                   | 460                      |

b Date: July 21, 1970.

c Waunakee Sewage Treatment Plant outfall.

d Most probable number; four dilutions in triplicate.

e Fecal coliforms (44.5 C), Geldreich et al. (5).
TABLE 6. Occurrence of salmonellae and fecal coliforms in Yahara River, Wisconsin

| Distance from STP outfall (ft) | Salmonellae per 100 ml | Fecal coliforms per 100 ml |
|------------------------------|-----------------------|--------------------------|
| 0                            | 240                   | 540,000                  |
| 100                          | 3.9                   | 6,300                    |
| 500                          | 7.5                   | 1,550                    |
| 1,000                        | 2.3                   | 1,050                    |
| 2,000                        | <0.4                  | 1,550                    |
| 3,000                        | 1.5                   | 14,500                   |
| 4,000                        | <0.4                  | 643                      |

* Most probable number; four dilutions in triplicate.

The method used was modified (0.3 salmonellae per 100 ml).

DISCUSSION

A method has been sought to give a rapid, low-cost estimate of salmonellae densities in polluted waters. Traditional methods and modifications of these methods recently reported in the literature have been examined to establish a reliable method to meet these needs. In general, a modification of the accelerated procedure using broth cultures and serological reactions developed by Sperber and Deibel (11) was adopted.

Pre-enrichment procedures were not employed in the modified technique for several reasons. Use of nonspecific broths such as lactose resulted in excessive overgrowth of coliforms and other microorganisms associated with polluted waters. Only a few positive samples were subjected to complete serotyping. Results of the serotyping are given in Table 4.

Polluted stream waters. Salmonellae have been frequently isolated in polluted streams and tidal waters. This quantitative technique is presently being used to study salmonellae die-off in streams receiving unchlorinated waste-water effluents. Examples of two surveys are presented in Table 5 and 6. For these two streams, there was not significant tributary flow into the streams below the treatment plant outfalls, thus dilution was not a major contributor to apparent die-off. Studies on other streams carrying chlorinated effluents invariably resulted in quantitative results below the sensitivity of this procedure (0.3 salmonellae per 100 ml).

TABLE 7. Effect of selective enrichment media on recovery of salmonellae per 100 ml

| Sample                  | SBG Sulfa (Difco) | Tetra-thionate (Difco) | m-Tetra-thionate (Difco) |
|-------------------------|------------------|-----------------------|-------------------------|
| Raw sewage              | 150              | 93                    | 460                     |
| Activated sludge effluent | 2.1             | 43                    | 240                     |
| Trickling filter effluent | 0.9             | 150                   | 2,400                   |

* Using procedures outlined in Materials and Methods section.

Selenite Brilliant Green Sulfa.

TABLE 8. Recovery of salmonellae in waste waters by enrichment-serology (ES) procedure and proposed procedure

| Sample                  | Method A | Method B |
|-------------------------|----------|----------|
|                         | No. of tubes giving positive reaction out of | MPN index per 100 ml | No. of tubes giving positive reaction out of | MPN index per 100 ml |
|                         | 3 of 10 ml each | 3 of 1 ml each | 3 of 0.1 ml each | 3 of 10 ml each | 3 of 1 ml each | 3 of 0.1 ml each |
| Raw sewage              | 0        | 0        | 2              | 6              | 2              | 1              | 0              | 15          |
| Trickling filter effluent | 2        | 2        | 0              | 21             | 3              | 2              | 0              | 93          |
| Activated sludge effluent | 1        | 1        | 0              | 7              | 2              | 1              | 0              | 15          |
| Raw sewage              | 0        | 3        | 1              | 13             | 3              | 3              | 2              | 1100        |
| Trickling filter effluent | 1        | 0        | 0              | 4              | 2              | 0              | 0              | 9           |
| Activated sludge effluent | 2        | 2        | 0              | 21             | 3              | 2              | 0              | 93          |

* ES procedure described by Sperber and Deibel (11): selective enrichment, M-broth enrichment, and H agglutination test.

* Proposed procedure: selective enrichment, plating, M-broth enrichment, and H agglutination test.

* Most probable number.

* Date: March 17, 1970.

* Date: March 24, 1970.
waters. The pre-enrichment step requires an additional 24-hr incubation thereby lengthening the time required to complete an analysis. Finally, pre-enrichment is normally recommended as a means of recovering damaged cells from materials that have been treated to destroy pathogens. It is generally believed that salmonellae in polluted waters would not be weakened to the point where a pre-enrichment step would be necessary.

An ideal selective medium would permit the growth of all salmonellae while suppressing the proliferation of other closely related microorganisms. Unfortunately, there is no single medium which can entirely achieve this goal. Two of the media most commonly employed for selective enrichment of salmonellae are Selenite Cystine broth and Tetrathionate broth. McCoy (8) used both Tetrathionate and Selenite F broth in his study of salmonellae in raw sewage, sewage effluent, and sewage-polluted natural waters. Spino (13) used Tetrathionate broth as well as a Selenite Brilliant Green Sulfa enrichment medium to successfully isolate salmonellae from surface waters. He reported that incubation of both enrichment broths at 41.5°C often resulted in obtaining almost pure cultures of salmonellae. Kabler et al. (7) suggested the use of Tetrathionate broth without calcium carbonate for enrichment of salmonellae other than \( S.\ typhosa \). They reported about 80% recovery of salmonellae from mixed cultures containing coliforms. In the present study, it was confirmed that m-Tetrathionate Broth developed by Kabler and Clark (7) was superior to both Tetrathionate Broth (Difco) and SBG Sulfa Broth (Difco) for recovery of salmonellae in polluted waters (Table 7). In addition, incubation temperatures of 41.5°C as recommended by Spino (13) resulted in the highest densities of salmonellae.

In the modified method presented herein, plating of cultures directly from the selective enrichment step minimized undesirable overgrowth of microorganisms that normally occurred in enrichment-serology procedure developed by Sperber and Deibel (reference 11, Table 8). Numerous plating media have been developed for the isolation of salmonellae. Galton (4) reported that Brilliant Green agar, when properly prepared, was more inhibitory to other enteric organisms than other selective media and yet provided relatively easy detection of suspected salmonellae. Osborne and Stokes (9) showed that the addition of 0.1% sulfapyridine to Brilliant Green agar further enhanced its selectivity for salmonellae.

The M-broth enrichment step followed the plating procedures and was provided to stimulate the growth of flagella and to minimize the incidence of nonspecific agglutination. The use of polyvalent H antisera as a screening procedure has proven to be far more specific than biochemical tests. Experience with this method has indicated that preliminary classification of an organism as \( Salmonella \) on the basis of agglutination in pooled \( Salmonella \) H antisera is almost always confirmed by further biochemical tests and complete serotyping.

The technique proposed herein has proven to be highly sensitive to the recovery of salmonellae in polluted waters. As recognized by Sperber and Deibel (11) this technique will not detect all \( Salmonella \) species. The use of Selenite Cystine broth or other selective media in parallel with m-Tetrathionate Broth in enrichment procedures would improve recovery. In addition the three species \( S.\ agona \) (group B), \( S.\ quinhon \) (group X), and \( S.\ pullorum-gallinarum \) (group D) would not be detected by the pooled \( Salmonella \) H antisera (11). The two chief advantages of this procedure are the speed at which an analysis may be completed and the large volume of water sample that can be analyzed. This procedure also offers an economical advantage over other proposed methods of enumeration, costing approximately $1.20 per water sample (four dilutions in triplicate). Although the enumeration of salmonellae by MPN techniques is not accurate enough for laboratory research, it has proven to be adequate for field analysis. In concert with determination of fecal coliforms, it is anticipated that this method will prove useful in evaluating and monitoring polluted water sources.

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