New Method of Isolating Salmonellae from Milk

JOY G. WELLS, GEORGE K. MORRIS, AND PHILIP S. BRACHMAN

Epidemiology Program, Center for Disease Control, Atlanta, Georgia 30333

Received for publication 21 September 1970

The use of a cotton gauze swab and subsequent culture of the swab was found to be a more sensitive method for isolating Salmonella from liquid milk than the revised procedure of North. The swab method was found to be as sensitive as the North procedure for recovering Salmonella when incubated at 37 C but more sensitive when incubated at 43 C. Incubation of the swab cultures at the elevated temperature of 43 C gave good results when Salmonella was present at levels as low as one per liter. Swabs exposed to milk contaminated with 100 Salmonella per liter remained positive even when subsequently washed for 2 hr in noncontaminated milk. Bismuth sulfite agar and Brilliant Green sulfadiazine agar were equally effective for isolating Salmonella from broth cultures; use of both media resulted in maximal isolations.

In 1965 and early 1966, an interstate outbreak of gastroenteritis occurred involving 29 laboratory-confirmed cases of Salmonella newbrunswick (2). Epidemiological and laboratory evidence implicated instant nonfat dried milk as the source of infection. S. newbrunswick was isolated from shelf samples of the product and from the milk drying plant in which it was produced. A study of the plant indicated an environmental situation that allowed continual perpetuation of salmonellae within the plant; it was assumed that the initial source of salmonellae was the raw milk supply.

As a result of the S. newbrunswick epidemic, a protocol for the examination of milk for salmonellae was recommended by members of a joint committee from the Center for Disease Control, the Food and Drug Administration, and the Division of Environmental Engineering and Food Protection (13). It is a revision of the procedure of North (11).

Julseth and Deibel (6) have shown that in the case of nonfat dry milk, nine salmonellae per liter of reconstituted milk can multiply to a level sufficient to cause disease. Thus it appears that a method of analyzing milk should be sufficiently sensitive to detect fewer than 10 salmonellae per liter of milk.

The purpose of this study was to evaluate the recommended method of analyzing raw whole milk for salmonellae and to develop a procedure more applicable to field conditions.

MATERIALS AND METHODS

Preparation of Salmonella inocula. S. typhimurium isolated in this laboratory from a frozen dessert product was used throughout this study. The inoculum was prepared from a culture grown in Trypticase Soy Tryptose (TST) broth [15 g of Trypticase Soy (BBL) and 13 g of Tryptose Broth (Difco) per liter] for 18 to 24 hr at 37 C. After incubation, 1 ml of the culture was transferred to 8 ml of fresh TST broth, and the inoculated TST was incubated at 37 C for 4 to 6 hr. The growth was quantitated by reading turbidity at 500 nm on a Spectronic-20 colorimeter (Bausch & Lomb). The culture was diluted to a 40% transmission with TST broth, and plate counts on MacConkey agar (Difco) showed this to contain 5 \times 10^9 salmonellae per ml. Final dilutions to obtain the desired inoculum for tests were made in normal saline.

Milk. Raw milk used in this study was obtained from a local dairy which produces grade A milk. Total bacterial and coliform counts were determined on the raw milk prior to each experiment by making appropriate dilutions of milk in normal saline, inoculating 0.1 ml of each dilution to duplicate plates, and spreading the inoculum on the plates with a sterile glass rod. Tryptone glucose extract (TGE) agar (Difco) was used to determine total counts. The plates were read after 24 hr of aerobic incubation at 37 C. The coliform count was defined in this study as the number of lactose-fermenting colonies present on MacConkey agar after 24 hr of aerobic incubation at 37 C.

Conventional method. The currently accepted method of isolating salmonellae from liquid milk is a modification of the procedure of North (11). The method consisted of adding 20 mg of Brilliant Green per liter of liquid milk. After 24 hr of incubation at 37 C, a loopful of this pre-enrichment was streaked to Brilliant Green agar (Difco) containing 80 mg of sodium sulfadiazine per liter of agar (BGS) and bismuth sulfite agar (BS, Difco). At the time of plating, 10 ml of the milk was subcultured to 100 ml of tetraphionate broth (Difco) containing 10 mg
Brilliant Green per liter (TET), and this enrichment was streaked after 24 hr of incubation at 37 C to BGS and BS.

Swab culture method. Moore (8) found that suspending a cotton gauze swab in the flowing sewage in a sewer for 1 to 3 days with subsequent culture of the swab was a useful technique for isolating salmonellae.

This method was modified in this study for isolating salmonellae from liquid milk. For laboratory evaluation, the swab, which was a piece of cotton gauze 4 ft by 6 inch, was folded (eight times), tied in the middle with a wire, and suspended in a liter of milk. The milk was then stirred on a magnetic stirrer at about 100 rev/min for 10 min. The swab was removed from the milk, placed in 150 ml of TET broth, and after 24 and 48 hr of incubation a loopful was streaked to BGS and BS.

Serial transfer experiment. Thirteen beakers, each containing a liter of raw milk, were used. The second beaker was inoculated with the test organism. The gauze swab was suspended in the first beaker (uninoculated), stirred on a magnetic stirrer for 10 min, and then passed sequentially through the remaining 12 beakers in the same manner. This was to simulate a condition in which a swab in a flowing stream of milk would be exposed temporarily to contaminated milk (beaker 2) and washed in noncontaminated milk for a prolonged period (the remaining 11 beakers). After removal from the last beaker, the gauze swab was cultured by the procedure described above.

Secondary enrichment. Subcultures, after 1 week of incubation at room temperature, were made in several of the experiments. The initial tetrathionate enrichment broth, after the first culturing, were left at room temperature for 1 week, and then 1 ml was transferred to 9 ml of fresh tetrathionate enrichment broth (secondary enrichment). The latter was incubated for 24 hr, and then a loopful was streaked to BGS and BS.

Isolation and identification. The BGS and BS plates were incubated at 37 C: the BGS for 24 hr and the BS for 48 hr. Three salmonella suspect colonies were picked from each positive BGS or BS plate and transferred to triple sugar iron (TSI, Difco) agar slants. All TSI cultures having typical salmonella reactions after 24 hr of incubation were subjected to serological and, when indicated, biochemical tests. Details of the procedures followed the techniques described by Galton, Morris, and Martin (3).

Statistical analyses. Statistical analyses were conducted with probabilities based on exact binomial confidence limits. Probabilities of 0.05 or less were considered significant.

RESULTS

The efficiency of the conventional method for recovering salmonellae from raw liquid whole milk was evaluated by inoculating known numbers of S. typhimurium into the milk, and the results indicated that an inoculum of over 1,000 organisms was required to recover salmonellae by this method. These data indicated that the sensitivity of the conventional method was inadequate for routine surveillance of milk for salmonella contamination.

Subculturing a primary broth to a secondary broth has been shown to be advantageous for isolating salmonellae in a procedure where a lactose broth is subcultured to a selective broth (12) and in a procedure where tetrathionate broth is subcultured to another tetrathionate broth (5; G. K. Morris, J. G. Wells, and C. G. Dunn, unpublished data). Therefore, it was decided to evaluate the usefulness of a secondary tetrathionate enrichment inoculated (1 ml to 9 ml) from the primary tetrathionate broth utilized in the conventional method. Also, since the cotton gauze swab described by Moore (8) for sampling sewer effluent would be very applicable to field conditions for sampling raw milk, it was decided to evaluate in the laboratory the efficiency of this swab for isolating salmonellae from milk as compared with the conventional method. The results of the comparisons indicated that the swab culture technique was approximately equivalent to the conventional method for isolating salmonellae (Table 1). The secondary tetrathionate enrichment of the conventional method yielded the best results, followed by the primary tetrathionate broth, whereas no isolates were obtained by plating the pre-enrichment. A secondary enrichment also appeared to be advantageous when using the swab culture technique.

Total bacterial and coliform counts determined on the raw milk prior to each experiment indicated that the sensitivity of the method was influenced by these counts. The total counts ranged from 9,000 to 172,000 per ml, and the coliform counts ranged from less than 10 to 4,000 per ml.

| No. of salmonellae added | Recovery by conventional method | Recovery by swab culture technique |
|--------------------------|---------------------------------|-----------------------------------|
|                          | Pre-enrichment | Primary enrichment | Secondary enrichment | Pre-enrichment | Primary enrichment | Secondary enrichment |
| 1,000                    | 0/4a           | 2/4                | 2/4                | 2 3 3/3        | 2 3 3/3          |
| 100                      | 0/4            | 1/4                | 2/4                | 1 3 2/3        | 1 3 2/3          |
| 10                       | 0/4            | 0/4                | 0/4                | 0 3 0/3        | 0 3 0/3          |
| 0a                       | 0/4            | 0/4                | 0/4                | 0/3 0/3        |
| Total                    | 0/16           | 4/16               | 6/16               | 3/12 6/12      |
| Per cent                 | 0              | 25                 | 38                 | 25             | 30               |

* Number positive/number examined.
* These were uninoculated control samples; hence, they were not included in totals.

TABLE 1. Recovery of S. typhimurium from raw milk by direct analysis and by indirect analysis with swab culture technique.
Table 2. Influence of incubation temperatures on the recovery of S. typhimurium by using the swab culture technique

| No. of salmonellae added | Recovery after primary enrichment | Recovery after secondary enrichment |
|--------------------------|----------------------------------|-----------------------------------|
|                          | 37 C | 43 C | 37 C | 43 C | 37 C | 43 C |
| 1,000                    | 3/6a | 6/6  | 5/6  | 6/6  | 5/6  | 6/6  |
| 100                      | 2/6  | 6/6  | 4/6  | 6/6  | 4/6  | 6/6  |
| 10                       | 0/6  | 4/6  | 1/6  | 4/6  | 1/6  | 4/6  |
| 1                        | 0/6  | 2/6  | 0/6  | 3/6  | 0/6  | 3/6  |
| 0                        | 0/6  | 0/6  | 0/6  | 0/6  | 0/6  | 0/6  |
| Total                    | 5/24 | 18/24| 10/24| 19/24| 9/24 | 19/24|
| Per cent                 | 20   | 75   | 42   | 79   | 42   | 79   |

- Number positive/number examined.
- Uninoculated controls.

Table 3. Recovery of S. typhimurium from raw milk by direct analysis and by indirect analysis by using the swab culture technique incubated at 43 C

| No. of salmonellae added | Recovery by conventional method | Recovery by swab culture technique |
|--------------------------|---------------------------------|-----------------------------------|
|                          | Pre-enrichment | Primary enrichment | Secondary enrichment | Primary enrichment | Secondary enrichment |
| 1,000                    | 0/4a | 3/4 | 4/4 | 1/4  | 4/4 | 4/4 |
| 100                      | 0/4  | 1/4 | 1/4 | 0/4  | 0/4 | 0/4 |
| 10                       | 0/4  | 0/4 | 0/4 | 0/4  | 0/4 | 0/4 |
| 1                        | 0/4  | 0/4 | 0/4 | 0/4  | 0/4 | 0/4 |
| 0                        | 0/4  | 0/4 | 0/4 | 0/4  | 0/4 | 0/4 |
| Total                    | 0/16 | 4/16| 5/16| 11/16| 12/16| 12/16|
| Per cent                 | 0    | 25  | 31  | 69   | 75  | 75  |

- Number positive/number examined.
- Uninoculated controls.

A more sensitive test for Salmonella was observed in the milk with lower bacterial counts.

Serial transfer experiments were conducted to determine whether salmonellae were retained by the gauze swab after washing in noncontaminated milk. After 2 hr of serial washing (12 beakers of milk at 10 min each), salmonellae were recovered from the swab even when the swab was inoculated with as few as 100 salmonellae prior to washing. There have been reports of an increased sensitivity in the isolation of salmonellae from various type samples by utilization of the elevated incubation temperature of 43 C (1, 4, 10). All of the milk in studies previously discussed were incubated at 37 C. Incubation temperatures of 37 and 43 C were compared by utilizing the swab culture technique and a secondary enrichment (Table 2).

There were more recoveries made at the 43 C incubation temperature at the inoculum levels of 1,000, 100, and 10 salmonellae per liter. In addition, at 43 C frequent recoveries were made from samples at the level of one salmonella per liter, whereas there were no recoveries made at this level at the 37 C incubation temperature. There was not a statistically significant increase in the recovery rate at either temperature between the primary and secondary enrichment, but recoveries obtained by 43 C incubation were greater than those at 37 C by both primary and secondary enrichment ($P < 0.01$).

Since incubating at 43 C increased the sensitivity of the swab culture technique, an experiment was conducted to determine whether the swab culture technique was more sensitive than the conventional method at this temperature.

The results indicated that the swab culture technique was superior ($P < 0.001$) to the conventional method for isolating salmonellae (Table 3).

A comparison was made of the method currently recommended, the conventional method incubated at 37 C, and the method of choice as indicated by these studies, the swab culture technique incubated at 43 C (Table 4). The swab culture technique incubated at 43 C was superior to the conventional method incubated at 37 C ($P < 0.01$). The conventional method yielded salmonellae from 10 of 24 samples, whereas the swab culture recovered salmonellae from 18 of 24 samples. In addition, two recoveries by the latter procedure were made at the one salmonella per liter level, whereas no recoveries were made at this level by the conventional method.

The BS agar and BGS agar were equally effective for isolating salmonellae in this study. However, recoveries on the two media were not

Table 4. Recovery of S. typhimurium from raw milk by the conventional method (incubated at 37 C) and by the swab culture technique (incubated at 43 C)

| No. of salmonellae added | Recovery by conventional method (37 C) | Recovery by swab culture technique (43 C) |
|--------------------------|---------------------------------------|----------------------------------------|
| 1,000                    | 4/6a                                  | 6/6                                    |
| 100                      | 3/6                                   | 6/6                                    |
| 10                       | 3/6                                   | 4/6                                    |
| 1                        | 0/6                                   | 2/6                                    |
| 0                        | 0/6                                   | 0/6                                    |
| Total                    | 10/24                                 | 18/24                                  |
| Per cent                 | 42                                    | 75                                     |

- Data do not include secondary enrichment results.
- Number positive/number examined.
- Uninoculated controls.
always made from the same sample. Recoveries on the BS agar alone were frequently made from samples with low salmonella inocula. The raw milk analyzed in these experiments appeared to contain natural flora that was highly adapted for fermenting lactose, and with plating medium such as BGS, which contains lactose as a differential agent, the indicator system in the plate was frequently overwhelmed by these lactose fermentors, causing an acid condition over the entire plate. Under these conditions, small numbers of salmonella colonies did not give the typical alkaline reaction. BS agar does not contain lactose as a differential agent; thus small numbers of salmonella colonies were not overlooked because of these lactose fermentors. BGS agar, however, was advantageous in that it suppressed these coliforms better than BS agar.

**DISCUSSION**

Efficient methods of isolating salmonellae from raw milk are necessary to facilitate surveillance of the milk industry for salmonella and to aid in investigating salmonella outbreaks in which milk products are implicated as the source. Milk products are still a problem as a source of salmonella as indicated by U. S. Department of Agriculture data reported in the Center for Disease Control Salmonella Surveillance Report (14). During 1969, salmonellae were isolated from 44 of 1,697 product samples of dry milk and from 89 of 196 environmental samples. The raw milk from which this milk was manufactured must be considered as a possible source of contamination. Although workers have recovered salmonellae from nonfat dried milk and other milk products with relative frequency, there has been little success in isolating salmonellae from the raw milk when attempts were made to trace the contamination back to its source. The conclusions from these investigations usually are that the problem is one of perpetuation of salmonella in the plants rather than contaminated raw milk supplies. Although perpetuation in the plant may be a major part of the problem, the raw milk cannot be ruled out as a source of contamination without a sufficiently sensitive method of detecting salmonella, especially at very low levels. Contaminated milk from one cow when combined with milk from hundreds of other cows in a bulk tank truck would be diluted to the point that the number of salmonellae present would be extremely low, possibly in the range of 1 to 10 salmonellae per liter. We have shown in this study that the conventional method is inadequate to detect this level of salmonellae. To make a recovery by the conventional method, the presence of $10^5$ salmonellae per liter of raw milk was frequently required.

In contrast, the swab culture technique at 43°C recovered salmonellae from liter quantities of raw milk containing 1 to 10 salmonellae. These results indicate that the swab culture technique has an advantage over the present method for isolating *S. typhimurium* from raw milk. In addition, larger quantities of milk can be examined by the swab technique than by the conventional method. Salmonellae were recovered from the swab inoculated with 100 organisms after 12 successive washings of 10 min each while stirring on a magentic stirrer (2 hr total washing time). This indicates that a swab can be suspended in a flowing stream of milk at a milk drying plant or suspended in milk delivery trucks; thus large quantities of milk can be examined with one swab. In contrast, only 1-liter samples can be conveniently examined by the conventional method. Additionally, many problems are encountered when shipping refrigerated quantities of liquid milk to the laboratory for examination, whereas swabs are easily shipped and require no refrigeration if shipped in tetrahionate broth.

The secondary enrichments appeared to yield a better recovery of salmonellae than the initial enrichments with both the conventional method and the swab culture technique. This difference appeared to be greater for the swab culture technique at 37°C than at the 43°C incubation temperature, indicating that the secondary enrichment is of little value when using the swab culture technique at 43°C. The secondary enrichment would appear to be most advantageous when samples are held at room temperature for a period of time before examination (such as one might expect under field conditions).

Incubation of the swab culture in tetrahionate at 43°C was found to be superior to incubation at 37°C. Other workers have observed success with the elevated temperature of 43°C for isolating salmonellae from sewage by using selenite broth (4) and feeds and poultry litter by using selenite-Brilliant Green-sulfapyridine broth (1). McCoy (7) found that tetrahionate broth incubated at 43°C was lethal to salmonellae. No evidence of such toxicity was noted in this study nor in other studies in this laboratory involving the isolation of salmonellae from fish meal and pork sausage by using tetrahionate broth (9, 10).

The use of duplicate plating media was advantageous in this study. Duplicate media, even if they were the same medium, would probably increase the chances of making a recovery, but it appears advantageous to use BS in conjunction with BGS in examining raw milk. Since raw milk
contains a large number of lactose fermentors which tend to upset the acid base balance in a medium that contains lactose, a medium that does not contain lactose as a differentiating agent such as BS is advantageous. BGS agar, however, was advantageous in that it suppressed coliforms better than BS agar.

The results of this study indicate that: (i) sampling raw milk with a cotton gauze swab and subsequent culture of the swab is a more sensitive method of isolating salmonellae than the conventional method, (ii) salmonellae are retained by the swab even after extensive washing, (iii) an incubation temperature of 43°C is better than 37°C for isolating salmonellae from the swabs, and (iv) both BGS agar and BS agar should be used as plating media.

ACKNOWLEDGMENT

We thank W. Jere Housworth, Center for Disease Control, for conducting the statistical analyses.

ADDENDUM IN PROOF

Subsequent to the completion of this study, 45 shipments of raw milk arriving at commercial milk plants were examined by the conventional method incubated at 37°C and the swab culture technique incubated at 43°C. S. typhimurium was recovered from one of these shipments. The isolation was made by the swab culture technique, but not by the conventional method.

LITERATURE CITED

1. Carlson, V. L., G. H. Snoeyenbos, B. A. McKie, and C. F. Smyser. 1967. A comparison of incubation time and temperature for the isolation of Salmonella. Avian Dis. 11: 217-225.
2. Collins, R. N., M. D. Treger, J. B. Goldsby, J. R. Boring, D. B. Coo hon, and R. N. Barr. 1968. Interstate outbreak of Salmonella new-brunswick infection traced to powdered milk. J. Amer. Med. Ass. 205:838-844.
3. Galton, M. M., G. K. Morris, and V. T. Martin. 1968. Salmonellae in foods and feeds—review of isolation methods and recommended procedures. Public Health Service Publ., National Communicable Disease Center, Atlanta, Ga.
4. Harvey, W. S., and T. H. Price. 1968. Elevated temperature incubation of enrichment media for the isolation of Salmonella from heavily contaminated materials. J. Hyg. 66:377-381.
5. Jameson, J. E. 1961. A study of tetrahionate enrichment techniques, with particular reference to two new tetrahionate modifications used in isolating Salmonella from sewer swabs. J. Hyg. 59:1-13.
6. Julseth, R. M., and R. H. Delbel. 1969. Effect of temperature on growth of Salmonella in rehydrated skim milk from a food-poisoning outbreak. Appl. Microbiol. 17:767-768.
7. McCoy, J. H. 1962. The isolation of Salmonella. J. Appl. Bacteriol. 25:213-224.
8. Moore, B. 1948. The detection of paratyphoid carriers in towns by means of sewage examination. Mon. Bull. Min. Health Public Health Lab. Serv. Directed Med. Res. Counc. (London) 7:241-248.
9. Morris, G. K., W. T. Martin, W. H. Shelton, J. G. Wells, and P. S. Brachman. 1970. Salmonellae in fish meal plants: relative amounts of contamination at various stages of processing and a method of control. Appl. Microbiol. 19:401-408.
10. Morris, G. K., and C. G. Dunn. 1970. Influence of incubation temperature and sodium heptadecyl sulfate (Tergitol No. 7) on the isolation of Salmonellae from pork sausage. Appl. Microbiol. 20:192-195.
11. North, W. R., Jr. 1960. Use of crystal violet or brilliant green dyes for the determination of Salmonellae in dried food products. J. Bacteriol. 88:861.
12. North, W. R. 1961. Lactose pre-enrichment method for isolation of Salmonella from dried egg albumen. Its use in a survey of commercially produced albumen. Appl. Microbiol. 9:188-195.
13. Salmonella Surveillance Rep. no. 49. 1966. National Communicable Disease Center, Atlanta, Ga.
14. Salmonella Surveillance Rep. no. 93. 1970. National Communicable Disease Center, Atlanta, Ga.