

A SELECTIVE MEDIUM AND PRESUMPTIVE PROCEDURE FOR DETECTION OF SALMONELLA IN DAIRY PRODUCTS

R. E. HARGROVE, F. E. McDONOUGH, AND
R. H. REAMER

*Dairy Products Laboratory
Eastern Utilization Research and Development Division
Agricultural Research Service U. S. Department of Agriculture
Washington, D. C. 20250*

(Received for publication July 6, 1970)

ABSTRACT

A culture medium and testing procedure were developed to detect and differentiate *Salmonella* in pure culture study and for presumptive detection of *Salmonella* in dairy products. Most pure cultures of *Salmonella* were easily differentiated from other members of the *Enterobacteriaceae* group after 18 hr incubation at 37 C in a neutral red-lysine-iron-cystine broth. *Salmonella* changed neutral red in the medium from red to yellow and most strains turned the medium black through formation of a massive black precipitate. Species of *Enterobacter*, *Citrobacter*, *Proteus*, *Shigella*, and *Pseudomonas* intensified the red color of the medium and failed to blacken it. Species of *Klebsiella* and *Escherichia* usually changed the medium from red to yellow in 18 hr but none developed a black precipitate. The few nonhydrogen sulfide producing strains (no medium blackening) of salmonellae were differentiated from *Escherichia* and *Klebsiella* sp. by continued incubation to a total of 42 hr followed by the use of a second indicator, brom thymol blue. Only salmonellae gave an alkaline reaction and converted the medium from yellow to green or blue. The medium provided for rapid detection of most salmonellae after 18 hr incubation, as characterized by medium blackening or color change from red to yellow. Related enteric bacteria, other than *Arizona* strains were readily differentiated.

For dairy products a slight modification in medium formula and use of novobiocin and trypsin were required. Novobiocin selectively inhibits growth of most interfering spore formers and gram-positive bacteria and also certain strains of *Escherichia coli* and *Proteus*. Trypsin was used to digest casein added with the dairy product sample. A positive presumptive test for *Salmonella* in dairy products was indicated in the medium by a color change from red to yellow and/or production of a massive black precipitate of iron sulfide after 24 hr incubation. Absence of salmonellae was indicated by no color change or no medium blackening. Results from testing several dairy products indicated that the procedure may be of value in the rapid screening of these foods for salmonellae. Although confirmation and serological identification are still essential, the test eliminates preenrichment and gives presumptive evidence of salmonellae contamination after 24 hr.

Recent outbreaks of food poisoning have resulted in the intensive scrutiny of all food products for salmonellae by public health officials and have prompted food processors to reevaluate processing procedures and factors that affect product quality.

Rapid and reliable means are urgently needed whereby contamination can be detected during processing and/or in the finished product. A food processor, especially where a perishable product is con-

cerned, cannot afford to wait from 4 to 7 days for analyses.

It is generally agreed that methods to detect and enumerate *Salmonella* are complex and time consuming. Recently, efforts to simplify these procedures have been reported (1, 8, 12). Many of the liquid media in current use were designed for isolating these organisms from feces and are by nature somewhat toxic to the bacteria. The liquid preenrichment medium prescribed by North (10) is widely used but its use may be questioned as it tends to favor lactose fermenting types.

Since most salmonellae decarboxylate lysine and produce hydrogen sulfide, it appeared that these biochemical characteristics could be utilized to develop a rapid presumptive test. Several decarboxylase media have been proposed for differentiating members of the *Enterobacteriaceae* group (2, 5). Edwards and Fife (4) proposed a lysine-iron agar for *Arizona* strains; however, it failed to distinguish between shigellae and many strains of *Escherichia coli*.

The objectives of the present study were threefold: (a) to develop a culture medium that could be used to differentiate *Salmonella* from other *Enterobacteriaceae* and also from organisms that are commonly found in dairy products, (b) to adapt the medium for use as a presumptive test for *Salmonella* in dairy products, and (c) to combine preenrichment with the presumptive test, thus shortening the period required for identification. Future work would attempt to use the fluorescent antibody technique (FAT) for confirming results from the presumptive test.

MATERIALS AND METHODS

In preliminary studies various media were compared for their ability to selectively differentiate salmonellae from other enteric bacteria on the basis of lysine utilization and hydrogen sulfide production. The medium of Falkow (5) was compared with a lysine-iron broth medium similar in composition to the lysine-iron agar formula proposed by Edwards and Fife (4) for the detection of *Arizona* cultures. Attempts were made to improve the selectivity of the lysine-iron broth by adding or substituting various dyes, carbohydrates, iron and sulfur compounds, and anti-metabolites in the formula.

*Detection and Differentiation of Cultures**Medium*

The medium which showed the greatest potential for differentiating salmonellae from other genera contained: L-lysine — 10 g, tryptone — 5 g, yeast extract — 3 g, lactose — 5 g, glucose — 1 g, salicin — 1 g, ferric ammonium citrate — 0.5 g, sodium thiosulfate — 0.1 g, L-cystine — 0.1 g, neutral red — 0.025 g, and distilled water — 1 liter. The medium was adjusted to pH 6.2, dispensed in 8 to 10 ml portions in screw cap tubes, and autoclaved at 121 C for 15 min.

Cultures

Pure cultures of salmonellae and related types were tested in the selective medium. These included 64 *Salmonella* cultures representing serological groups A through I. Particular attention was given to reactions obtained from those serotypes most frequently isolated from contaminated nonfat dry milks (NDM), namely, *Salmonella cubana*, *S. anatum*, *S. montevido*, *S. oranienburg*, *S. tennessee*, *S. worthington*, *S. newington*, and *S. new brunswick*. Representative strains of *Shigella dysenteriae*, *Shigella flexneri*, *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, *P. mirabilis*, *P. morgani*, *Paracolobactrum arizonae*, *P. aerogenoides*, *Providencia stuartii*, and species of *Klebsiella* and *Pseudomonas* were tested. Spore formers, such as *Bacillus subtilis*, *B. cereus*, *B. megaterium*, and *B. stearothermophilus*, which are frequently found in milk powders also were tested.

Test procedure and evaluation

Tubes containing 8 to 10 ml of test medium were usually inoculated with a 3 mm standard loop from an 18 to 24 hr broth culture. In tests with unknown isolates, growth of individual colonies on selective agars frequently served as an inoculum.

Cultures were incubated at 37 C for 18 hr and observed for neutral red color change, and medium blackening. Salmonellae were readily detected after 12 to 18 hr growth by neutral red color change and production of a massive black precipitate. A few diphasic salmonellae will not produce the black precipitate but do change the medium from red to yellow. Species of *Shigella*, *Enterobacter*, *Proteus*, *Citrobacter* and most gram-positive types intensify the red color of the medium and do not yield a black precipitate. Even those *Proteus* species such as *P. vulgaris* and *P. mirabilis* that produce hydrogen sulfide in TSI agar do not cause the medium blackening or change the medium color. Species of *Escherichia* and *Klebsiella* change the medium color from red to amber or yellow without blackening. Differentiation of the few nonblackening salmonellae strains in the medium, namely *S. sendai*, *S. abortusovine*, and diphasic *S. choleraesuis*, from species of *Escherichia* and *Klebsiella* required an additional period of incubation. Therefore, all cultures showing a medium color change from red to yellow after 18 hr at 37 C are reincubated an additional 24 hr at 37 C (total 42 hr). After the second incubation, 0.1 ml of 0.3% brom thymol blue solution is added to each tube, and the color recorded. Salmonellae strains produce an alkaline reaction, changing the medium from yellow to green or blue. Blue color is more intense at the top of the tube on standing but color differences are immediately obvious. *Klebsiella* and *Escherichia* species allow medium to remain bright yellow. Brom thymol blue solution (50% alcoholic) was prepared by mixing 0.3 g of brom thymol blue indicator powder (Nutritional Biochemical Corp.)¹ with 2 ml of 0.1N NaOH and diluting to 100 ml with 50% ethyl alcohol in distilled water.

*Presumptive Procedure for Detecting Salmonella in Dairy Products**Dairy product samples*

Commercial samples of dried milks were kindly furnished by the Dairy Division, Consumer and Marketing Service, USDA and the Food and Drug Administration. Because some difficulty was encountered in collecting positive commercial products, many of the test products had to be prepared experimentally in the dairy pilot plant. The processing milk was artificially contaminated with salmonellae prior to product manufacture. Products prepared included NDM, milk concentrates, whey, raw and pasteurized milk, and Cottage and Cheddar cheese. Milk powders containing approximately 1 to 10 salmonellae per 100 g were prepared to determine the sensitivity of the proposed medium. Milk concentrates and raw milks containing salmonellae were prepared by seeding the milks with 18 hr broth cultures and diluting with milk to obtain a level of approximately 1 *Salmonella*/ml. Cheeses were prepared according to previously described methods and the salmonellae content was followed throughout storage and curing (6, 9). Cheeses containing less than 10 salmonellae/g were used. Whey from these cheeses containing low levels of salmonellae also were tested.

Salmonella strains added to or found in the dairy products included *S. senftenberg*, *S. typhimurium*, *S. cubana*, *S. anatum*, *S. new brunswick*, *S. oranienburg*, *S. montevido*, *S. newington*, and *S. choleraesuis*.

Medium for testing dairy products

When the test medium as developed for differentiation of pure cultures was applied to detect salmonellae in dairy products, certain adjustments in the medium and test procedure were essential. First, selectivity for salmonellae had to be increased in tests with mixed populations and growth of certain gram-positive types had to be suppressed. Second, casein carried over with dairy product samples interfered with color changes and this problem had to be overcome.

Novobiocin. A number of antibiotics were screened for ability to selectively inhibit growth of interfering microorganisms in the presence of *Salmonella*. These included novobiocin, erythromycin, kanamycin, neomycin, and sodium oxacillin. They were added aseptically to the medium at levels ranging from 3 to 20 µg/ml. Preliminary evidence indicated that novobiocin (novobiocin, sodium) albamycin^B, Upjohn Co.¹ was the most effective antibiotic in suppressing the growth of spore formers and species of *Proteus* and *Escherichia*. The optimum concentration of novobiocin in the test medium was determined with pure and mixed cultures of salmonellae and with salmonellae-positive dairy products. Concentrations tested were 2, 5, 10, and 15 µg/ml. A stock solution was prepared by adding 100 mg of crystalline novobiocin to 100 ml of sterile distilled water. The stock solution was stored at 4 C and discarded after two weeks.

Trypsin. The enzyme trypsin was tested in the medium as a clarifying agent for milk casein. Sterile stock solutions were prepared by mixing 1 g of trypsin (1-300) Nutritional Biochemical Corporation¹ in 100 ml of distilled water and filtering through a Seitz bacterial filter. This solution was usually dispensed in tubes, frozen at -17 C, and then thawed and used as needed. Levels of trypsin tested for casein digestion and possible toxic effect on the growth of salmonellae varied from 1 to 10 ml of stock solution per 100 ml of test medium.

¹Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

Medium formula. The medium developed for detecting salmonellae in dairy products was essentially the same as for pure cultures but was modified to contain: 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 distilled water — 1 liter. No adjustment of pH was required. The medium was usually dispensed in 500 or 1000 ml portions in large mouth flasks, autoclaved, and stored at 4 C until used. Freshly prepared autoclaved medium with the oxygen expelled was not used until the following day or until after oxygen equilibration because false-positives were encountered under these conditions. Stock solutions of novobiocin and trypsin were added aseptically to the medium just prior to testing or at the time of mixing with dairy samples. The concentration of novobiocin was 5 $\mu\text{g}/\text{ml}$ of medium or 0.5 ml of stock solution to each 100 ml. Stock trypsin was added at the rate of 2 ml for each 100 ml of medium.

Evaluation and sensitivity. The sensitivity of the procedure was established by testing milk powders known to contain low levels of sublethally impaired salmonellae (1-10 per 100 g). Additionally, milk powders known to contain salmonellae and related types such as *E. coli*, *Enterobacter*, *Citrobacter*, and *Proteus* were tested.

Assay of dairy products

NDM. NDM powder was added to sterile medium in the proportion of 1 to 10 (w/v). Lesser amounts of powder to medium may be used without harming the sensitivity but this limit should not be exceeded. Both 50 and 100 g samples were tested in the development of the procedure. One hundred gram samples were slowly mixed with 1000 ml of medium in a sterile Waring blender and after initial slow mixing, the solutions of novobiocin (5 ml) and trypsin (20 ml) were added and mixed. Mixtures were then returned to the original flasks. To allow resuscitation of sublethally impaired cells in dried products such as milk powder, the mixed samples were immediately warmed to 30 C and held for 6 hr. After 6 hr preincubation, flasks were placed in a 39 C water bath and held for an additional 18 hr. After incubation (total 24 hr) the mixtures were observed for color changes and/or blackening.

Milk concentrates. Milk concentrates from condensing (25 to 50% solids) were added directly to flasks or bottles of medium containing the prescribed amounts of novobiocin and trypsin (0.5 ml and 2 ml/100 ml, respectively). Usually 10 g of 50% concentrate or 20 g of 25% concentrate was added per 100 ml of medium. (Lesser amounts of concentrate to medium may be used). Mixtures were incubated at 39 C for 24 hr and observed for color changes as with NDM. Preincubation at 30 C was not essential with liquid dairy products.

Cheddar and Cottage cheese. Cheese samples of 11 g were blended in a Waring blender with 100 ml of sterile distilled water containing 0.2 g sodium citrate. Usually 10 or 20 ml aliquots of the cheese slurry were added to 100 ml of test medium. Novobiocin and trypsin were added and incubated as previously described for milk concentrates.

Whey, raw and pasteurized milk. Whey and milk samples were added to the medium at the ratio of 1 to 10 or 10 ml per 100 ml of medium. Trypsin and novobiocin were added, mixed, and the mixtures incubated at 39 C.

After 24 hr incubation, flasks or bottles were removed from the water bath and recorded for medium color changes. A positive presumptive test for the presence of salmonellae was indicated by medium blackening and/or color change from red to yellow. A negative test was indicated by the absence of color change or blackening. All salmonellae will change the medium color from red to yellow in 24 hr and in

addition most will form a massive black precipitate. Growth from all positive samples should be positively identified as containing salmonellae.

Confirmation. Samples from the presumptive test at 24 hr were removed and confirmed for the presence or absence of salmonellae by standard cultural and serological procedures; e.g., growth on differential agars and serological tests with specific antisera. Limited tests were made in which confirmation was made by FAT (11). To determine the effectiveness of the presumptive medium, duplicate samples of the dairy products were assayed by conventional lactose pre-enrichment, cultural and serological procedures (Bacteriological Analytical Manual).

RESULTS AND DISCUSSION

Differentiation of cultures

The medium as developed in pure culture study proved extremely useful in the detection and differentiation of unknown strains of salmonellae from other genera of the *Enterobacteriaceae* group. Salmonellae were readily differentiated after 18 hr incubation from species of *Shigella*, *Citrobacter*, *Enterobacter*, *Proteus*, and *Pseudomonas*. Unknown colonies of salmonellae picked from selective agars were also readily identified. All test strains with the exception of a few *S. pullorum* grew rapidly in the neutral red broth in 12 to 18 hr at 37 C and changed it in color from red to yellow or black. Most salmonellae decolorized neutral red and formed a massive black precipitate which obscured yellow color in 18 hr. Medium blackening was usually evident after 7 to 8 hr incubation. Typical medium reactions obtained with representative *Salmonella* serotypes are shown in Table 1. Only three serotypes tested failed to produce the characteristic black precipitate, e. g., *S. sendai*, *S. abortusovae*, and diphasic *S. choleraesuis*. These strains could still be identified as salmonellae and differentiated from other genera by continued incubation for 24 hr followed by addition of brom thymol blue. Of two *S. paratyphi* A strains tested, one failed to produce H₂S and give the typical reaction of most salmonellae. Two of six strains of *S. pullorum* grew poorly at 37 C and could be differentiated more readily when grown at 30 C. Lactose-positive *Salmonella* produced typical reactions in the medium, e.g., decolorization and blackening. The reactions obtained with microorganisms other than salmonellae are shown in Table 2. Species of *Shigella*, *Enterobacter*, *Citrobacter*, *Proteus*, and *Pseudomonas* usually remained bright red in color after 18 hr incubation and did not blacken the medium. *Enterobacter*, *Proteus*, and *Citrobacter* species usually lower the medium pH, thus intensifying its red color. Even those *Proteus* strains which produce hydrogen sulfide on TSI agar, such as *P. mirabilis* failed to blacken the medium and remained red, thus easily distinguishing them from salmonellae.

TABLE 1. PURE CULTURE REACTIONS OF *Salmonella* IN TEST MEDIUM

Serotype	Serological group	Medium changes — 37 C		42 hr ^a Color
		18 hr Color	Black ppt.	
<i>Salmonella paratyphi</i> A	A	Y*	+ ± ^b	Y
<i>S. typhimurium</i>	B	BL**	+++	GB***
<i>S. schottmuelleri</i>	B	BL	+++	GB
<i>S. derby</i>	B	BL	+++	GB
<i>S. chester</i>	B	BL	+++	GB
<i>S. cholerae-suis</i>	C ₁	Y	—	GB
<i>S. cholerae-suis</i> var. <i>kunzensdorf</i>	C ₁	BL	+++	GB
<i>S. montevido</i>	C ₁	BL	+++	GB
<i>S. oranienburg</i>	C ₁	BL	+++	GB
<i>S. tennessee</i>	C ₁	BL	+++	GB
<i>S. blockley</i>	C ₂	BL	+++	GB
<i>S. newport</i>	C ₂	BL	+++	GB
<i>S. sendai</i>	D ₁	Y	—	GB
<i>S. typhi</i>	D ₁	BL	+++	GB
<i>S. enteritidis</i>	D ₁	BL	+++	GB
<i>S. pullorum</i>	D ₂	BL ^c	+++ ^d	GB
<i>S. anatum</i>	E ₁	BL	+++	GB
<i>S. meleagridis</i>	E ₁	BL	+++	GB
<i>S. newington</i>	E ₂	BL	+++	GB
<i>S. new brunswick</i>	E ₂	BL	+++	GB
<i>S. illinois</i>	E ₃	BL	+++	GB
<i>S. senftenberg</i>	E ₄	BL	++	GB
<i>S. worthington</i>	G	BL	+++	GB
<i>S. albuquerque</i>	H	BL	+++	GB
<i>S. gamnara</i>	I	BL	+++	GB

*Y = Yellow

**BL = Black from ppt — Neutral red decolorized to pale yellow

***GB = Green to blue

^aAfter adding brom thymol blue^b50% of the *S. paratyphi* A strains formed a black ppt.^cFour out of six strains gave blackening at 37 C — 2 grew poorly.

Many species of *Pseudomonas*, such as *P. fragi*, failed to grow in the medium at 37 C. Salmonellae and most *E. coli* and *Klebsiella* strains decolorized or irreversibly changed neutral red from red to yellow without a marked pH change, thus inactivating it as an indicator. None of the other test strains had this effect on neutral red. As indicated previously, the nonhydrogen sulfide strains of salmonellae required a longer incubation period and use of brom thymol blue to distinguish them from *E. coli* and *Klebsiella* species. Only salmonellae cultures gave an alkaline reaction changing brom thymol blue from yellow to green or blue. Lysine and lactose utilizing cultures such as *E. coli* and *E. aerogenes* sufficiently lowered medium pH and remained yellow. Thus culture differentiation in the medium is based upon pH changes as indicated by neutral red and brom thymol blue, decolorization of neutral red, and the production of iron sulfide or medium blackening. Cultures utilizing lysine are known to yield an alkaline reaction. Lac-

tose and salicin provided a carbohydrate source for microorganisms other than salmonellae which yield an acid reaction. Salicin also provided a carbohydrate source for slow or non-lactose utilizing strains such as *Proteus*. All *E. aerogenes*, *Klebsiella* and *Proteus* species, and most *E. cloacae* and *Escherichia* species fermented salicin. It has been reported by Edwards and Ewing (3) that all salmonellae produce hydrogen sulfide on TSI agar with the exception of *S. paratyphi* A, *S. sendai*, *S. berta*, *S. senftenberg*, *S. choleraesuis* (diphase), and *S. abortivoequina*. Addition of L-cystine to the neutral red medium intensified blackening with most species, and resulted in medium blackening with strains of *S. senftenberg* and *S. berta* and some *S. paratyphi* A. Apparently L-cystine is more easily utilized than sodium thiosulfate or adds to the sulfur source for these strains. Test strains of *P. arizonae* changed medium color and gave blackening, making it impossible to differentiate them from salmonellae. *Paracolobactrum aerogenoides* could be distinguished by indicator changes.

Several advantages were found in the use of the cultural test medium: (a) rapid results were obtained in 12 to 18 hr with most salmonellae, (b) one medium was sufficient for differentiation from related types, and (c) hydrogen sulfide producing strains of *Proteus* and *Citrobacter* were less likely to be confused with salmonellae than on TSI agar or lysine iron agar.

Assay of dairy products

The medium was effective in isolating and detecting salmonellae in most known positive dairy products. Dairy products containing salmonellae either turned the medium very black or changed its color from red to bright yellow depending upon the strain's ability to form hydrogen sulfide. A negative test was indicated by the absence of color change or medium blackening. Only a limited number of dairy products could be made with different *Salmonella* serotypes; however, all but a few made with *S. choleraesuis* caused medium blackening in 24 hr. A strain of *S. senftenberg* and lactose positive *S. tennessee* developed lesser amounts of iron sulfide. Milk powders prepared to contain approximately 1-10 salmonellae cells per 100 g were usually positive by the presumptive test. Medium sensitivity for dairy products was increased by changing the cystine content from 0.01 to 0.03% and raising the incubation temperature to 39 C. (Low levels of sublethally impaired salmonellae in NDM failed to give the characteristic reactions when samples were placed immediately at 39 C). It was found that a preincubation period of 6 hr at 30 C was sufficient to allow resuscitation of injured cells and then the samples could be incubated at 39 C. Test strains of *E. coli*, *E. aerogenes*, *P. vulgaris*, *P.*

TABLE 2. MEDIUM REACTION OF PURE CULTURES OTHER THAN SALMONELLAE

Strain	Medium changes — 37 C		
	Color	18 hr	42 hr ^a Color
<i>Shigella dysenteriae</i>	Red	—	red to brown ^b
<i>Shigella flexneri</i>	Red	—	red to brown
<i>Paracolobactrum arizonae</i>	Black*	+++	green or blue
<i>Paracolobactrum aerogenoides</i>	Yellow	+	yellow
<i>Escherichia coli</i>	Amber to Yellow	—	yellow
<i>Enterobacter cloacae</i>	Red	—	red to brown
<i>Enterobacter aerogenes</i>	Amber to Red ^{I**}	—	red to brown
<i>Citrobacter freundii</i>	Amber to Red ^I	—	red to brown
<i>Klebsiella pneumoniae</i>	Yellow	—	yellow
<i>Klebsiella ozaenae</i>	Yellow	—	yellow
<i>Proteus vulgaris</i>	Red ^I	—	red to brown
<i>Proteus mirabilis</i>	Red ^I	—	red to brown
<i>Proteus morgani</i>	Red	—	red to brown
<i>Providencia stuartii</i>	Red	—	red to brown
<i>Pseudomonas fragi</i>	Red ^{NG***}	—	red to brown
<i>Pseudomonas fluorescens</i>	Red ^{NG}	—	red to brown
<i>Pseudomonas aeruginosa</i>	Red	—	red to brown
<i>Bacillus cereus</i>	Red	—	red to brown
<i>Bacillus megaterium</i>	Red	—	red to brown
<i>Bacillus subtilis</i>	Red	—	red to brown

*Black from precipitate — Neutral Red decolorized

**I — Red color usually intensified

***NG — No growth

^aAfter addition of brom thymol blue

^bRed tubes at 18 hr need not be reincubated or treated with brom thymol blue

mirabilis, and *P. morgani* in the dairy products failed to give medium color changes or blackening after 24 hr incubation. Strains of *E. coli* did change the medium color to yellow with longer incubation periods (48 hr).

The antibiotics, novobiocin and erythromycin at levels of 5 and 10 µg/ml, respectively were equally effective in isolating *Salmonella* in the presence of gram-positive microorganisms; however, novobiocin was slightly superior in suppressing growth of many *E. coli* and *Proteus* strains. Advantages of novobiocin in isolation of salmonellae from fecal samples have been shown by Jeffries (7). Test strains of *B. subtilis*, *B. megaterium*, *B. cereus*, and *B. stearothermophilus* were inhibited with 5 µg/ml of novobiocin. All species of *Shigella* and many strains of *E. coli*, *Proteus*, and *Pseudomonas* were suppressed by 5 µg of novobiocin in the medium. Novobiocin was required in the medium before a positive test could be obtained when NDM samples contained *Salmonella* and *E. coli* in a ratio of 1 to 10 per gram of powder. Obviously large numbers of lactose fermenters such as *E. coli* may overgrow salmonellae unless suppressed. Trypsin was effective as an agent to digest casein and to clarify the medium and thus enhanced detection of color changes and medium blackening with all dairy products tested. Digestion of casein was usually complete after 12 hr. Addition of trypsin to

the medium did not appear to inhibit growth or recovery of stressed cells of salmonellae (heating and drying) in NDM or other dairy products. Five to 10 times the prescribed level of trypsin in the medium had no effect on the growth of salmonellae or medium reactions.

Detection of salmonellae in dairy products was based upon irreversible color changes of the neutral red and blackening of the medium by salmonellae. Excessive aeration of cultures and the use of anaerobic conditions are to be avoided when conducting the presumptive test. For example, false-positive reactions were frequently encountered, (medium change from red to yellow), when freshly autoclaved medium with oxygen expelled was used. As a result autoclaved medium was not used until the following day. Shaking and excessive aeration during incubation prevented blackening and appeared to inhibit growth of stressed cells of salmonellae in NDM. The number of salmonellae in some commercial NDM samples was obviously low because difficulty was encountered in detection even with repeated sampling by standard cultural procedures. NDM made in the pilot plant to contain approximately 1 *Salmonella* and 10 *E. coli* per gram was useful as a positive control and in demonstrating the effect of medium variables. Limited trials have shown that the test is sufficiently sensitive to detect between 1 and 10

TABLE 3. DAIRY PRODUCTS ANALYZED FOR *Salmonella*

Product	No. of samples	Standard cultural procedures		Presumptive test	
		+	-	+	-
NDM Commercial	21	9	12	8	13
NDM ^a	10	10	0	10	0
NDM ^b	2	2	0	2	0
NDM ^c	2	0	2	0	2
NDM ^d	4	0	4	0	4
NDM ^e	1	1	0	1	0
Milk concentrate (25 to 50%)	3	3	0	3	0
Cheddar cheese	16	14	2	15	1
Cottage cheese	5	5	0	5	0
Raw milk	15	14	1	12	3
Whey	21	20	1	20	1

^aPrepared with *Salmonella* serotypes producing hydrogen sulfide

^bPrepared with *S. choleraesuis* and *S. senftenberg*

^cPrepared with *E. coli*

^dPrepared with species of *Proteus*

^ePrepared with *S. new brunswick* and *E. coli*

salmonellae per 100 g sample. The procedure was slightly more sensitive when lesser amounts of powder to medium could be used, for example, 50 g of NDM to 1 liter of medium. Stressed cells of salmonellae in NDM were found to recover as fast or faster in the neutral red broth than in reconstituted milk. Recovery was determined by a direct plating procedure. As might be expected, the procedure was more sensitive for detection of salmonellae in liquid products and cheese than in NDM. In tests with raw milk, 1 *Salmonella*/ml could easily be detected when the total bacterial count did not greatly exceed 200,000/ml. In a few instances salmonellae were detected when the total count exceeded 1 million/ml. Raw milks of high counts obviously contained large numbers of lactose fermenters which overgrew salmonellae even when the medium contained novobiocin. *Salmonella* was readily detected in pasteurized milk and wheys. Lactic streptococci in the wheys were inhibited by novobiocin.

Results of tests with dairy products are shown in Table 3. Although 100% correlation was not obtained between the 24-hr presumptive test and the 4-day completed cultural and serological procedure, the number of positive samples at 24 hr was extremely high. Results to date indicate that the presumptive test cannot be solely relied upon for *Salmonella* detection. Even though confirmation of *Salmonella* in the growth medium is still required, the total time required for the completed test is reduced. The medium eliminates the usual preenrichment and is

more selective for the growth of *Salmonella* than reconstituted milk.

Full evaluation of the neutral red lysine-cystine medium and test procedure cannot be determined until many products have been assayed and the results compared with those of established test procedures. However, results from the various dairy products tested to date would indicate that the isolation procedure has definite potential as a rapid screening procedure that may be used by processors and technicians.

Preliminary tests with the FAT indicate that the growth from the presumptive test may be applied directly for FAT confirmation. Iron sulfide deposits were generally removed during the normal rinse procedures and did not interfere with fluorescence. Presumptive evidence of *Salmonella* and confirmation by FAT was obtained in a total of 26 hr. Results of FAT tests will be reported at a later date.

REFERENCES

1. Abrahamsson, K., G. Patterson, and H. Reimann. 1968. Detection of *Salmonella* by single-culture technique. *Appl. Microbiol.* 16:1695-1698.
2. Bachrach, U. 1959. An improved method for the determination of lysine decarboxylase activity of salmonellae. *Amer. J. Clinical Pathol.* 32:580-581.
3. Edwards, P. R., and W. H. Ewing. 1967. Identification of *Enterobacteriaceae*. Burgess Publishing Co., Minneapolis, Minnesota.
4. Edwards, P. R., and M. A. Fife. 1961. Lysine-iron agar for the detection of *Arizona* cultures. *Appl. Microbiol.* 9:478-480.
5. Falkow, S. 1958. Activity of lysine decarboxylase as an aid in the identification of salmonellae and shigellae. *Amer. J. Clinical Pathol.* 29:598-600.
6. Hargrove, R. E., F. E. McDonough, and W. A. Mattingly. 1969. Factors affecting survival of *Salmonella* in Cheddar and Colby cheese. *J. Milk Food Technol.* 32:480-484.
7. Jeffries, L. 1959. Novobiocin-tetrathionate broth: A medium of improved selectivity for the isolation of salmonellae from faeces. *J. Clinical Pathol.* 12:568-571.
8. King, Sylvia, and W. I. Metzger. 1968. A new plating medium for the isolation of enteric pathogens. I. Hektoen enteric agar. *Appl. Microbiol.* 16:577-578.
9. McDonough, F. E., R. E. Hargrove, and R. P. Tittsler. 1967. The fate of salmonellae in the manufacture of cottage cheese. *J. Milk Food Technol.* 30:354-356.
10. North, W. R., Jr. 1961. Lactose preenrichment method for isolation of *Salmonella* from dried egg albumin. *Appl. Microbiol.* 9:188-195.
11. Reamer, R. H., R. E. Hargrove, and F. E. McDonough. 1969. Increased sensitivity of immunofluorescent assay for *Salmonella* in nonfat dry milk. *Appl. Microbiol.* 18:328-331.
12. Sperber, W. H., and R. H. Deibel. 1969. Accelerated procedure for *Salmonella* detection in dried foods and feeds involving only broth cultures and serological reactions. *Appl. Microbiol.* 17:533-539.