A SELECTIVE PLATING AGAR FOR DIRECT ENUMERATION OF *SALMONELLA* IN ARTIFICIALLY CONTAMINATED DAIRY PRODUCTS[1]

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ABSTRACT

A selective plating medium was developed that allows direct enumeration of salmonellae in dairy products such as nonfat dry milk, and Cheddar and cottage cheese. The agar medium developed was a modification of the Lysine-Iron-Cystine broth of Hargrove et al., 1971. Strains of species of the genera *Escherichia, Enterobacter, Citrobacter, Proteus, Shigella, Pseudomonas,* and *Bacillus* were easily differentiated from salmonellae by colony color and color of surrounding area or absence of growth. The antibiotic, novobiocin, used as a selective agent, inhibited growth of some *Proteus, Shigella,* and *Escherichia;* however the antibiotic was most effective against *Bacillus,* without having any observable effect on salmonellae. The medium was sufficiently sensitive and selective to permit detection of as few as 1-2 salmonellae per gram of product in the presence of naturally occurring bacteria and should be of considerable value in following the *Salmonella* content of artificially contaminated foods during processing and also during storage.

Several different types of bacteriological media have been proposed for isolation and identification of *Salmonella* in foods. Most of these were designed for selective preenrichment or for differential growth on surface agars. Methods for direct quantitative enumeration consist almost entirely of a most probable numbers procedure (MPN) *(6)*. Some workers contend that if dried foods are to be examined, preenrichment and quantitative estimation by MPN is essential *(2)* and perhaps the only way of estimating the degree of contamination. Sperber and Deibel recently reported an accelerated procedure for *Salmonella* detection in dried foods *(5)*. There appears to be a definite need for a selective direct plating procedure which would permit a more rapid and reliable estimation of *Salmonella* particularly with artificially contaminated foods. The advantages of direct plating of a food, is that as a research tool, one can follow the effect of processing and storage on the *Salmonella* bacteria even in the presence of other naturally occurring bacteria.

In a recent study reported by Hargrove et al. *(1)* a lysine-iron-cystine broth was used effectively for presumptive detection of *Salmonella* in dairy products and as a one-step preenrichment and selective medium in a 24-h immunofluorescent procedure for salmonellae in non-fat dry milk (NDM) which is capable of detecting one *Salmonella* per 100 g (4). The present study reports the adaptation of this medium as a selective plating agar medium for the direct enumeration of *Salmonella.*

MATERIALS AND METHODS

Pure cultures of salmonellae and related types were used in developing the plating medium; these included 36 *Salmonella* serotypes and representative strains of the species of the genera *Escherichia, Enterobacter, Citrobacter, Proteus, Shigella, Pseudomonas,* and *Bacillus.*

In preliminary screening tests several standard and selective agar media for *Salmonella* were compared in the direct enumeration of stock cultures of salmonellae in milk. Media compared were Bismuth Sulfite agar (Fisher), Brilliant Green agar (Difco), Salmonella-shigella agar (Baltimore Biological Laboratories), MacConkey agar (Difco), and a lysine iron agar prepared from Hargrove's lysine-iron-cystine broth *(1)*. Total plate counts were compared with those obtained with Standard Plate Count agar (Difco).

The type and degree of injury that normally occurs to *Salmonella* in processed foods should be closely simulated by the method of preparation of contaminated product. Organisms used were fresh 18 h cultures grown on trypticase soy broth. Dilutions of these were inoculated into pasteurized whole milk (Cheddar cheese) and/or skim milk (non-fat dry milk) before manufacturing into specific dairy products in our pilot plant. Fresh skim milk for Cottage cheese was fortified to 11% solids with NDM and pasteurized. Products tested include nonfat dry milk (NDM) and Cheddar and cottage cheese.

Medium

The lysine-iron-cystine broth medium as proposed by Hargrove et al. *(1)* was slightly modified for use as a plating medium. The significant changes were the substitution of brom cresol purple as a pH indicator and an increase of the cystine content from 0.1 to 0.2 g per liter. The medium which was formulated and which showed the greatest selectivity for all *Salmonella* strains is listed below:

L-Lysine, 10 g; Bacto tryptone, 5 g; yeast extract, 3 g; lactose, 5 g; salacin, 1 g; glucose, 1 g; L-cystine, 0.2 g; ferric ammonium citrate, 0.5 g; sodium thiosulfate, 0.5 g; brom cresol purple, 0.02 g; agar, 15 g; distilled water, 1000 ml. The medium was adjusted to pH 6.4-6.5, dispensed in flasks, and sterilized at 121 C for 15 min.

Novobiocin (Upjohn) was added aseptically to the cooled

[1]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

TABLE 1. GROWTH OF REPRESENTATIVE PURE CULTURES IN SELECTIVE MEDIUM[1]

Organism	LICA growth	Colony appearance	Medium reaction
Salmonella cubana	+++	Black-lens	Alkaline-purple
Salmonella montevideo	+++	Black-lens	Alkaline-purple
Salmonella oranienburg	+++	Black-lens	Alkaline-purple
Salmonella tennessee	+++	Black-lens	Alkaline-purple
Salmonella worthington	+++	Black-lens	Alkaline-purple
Salmonella senftenberg	+++	Brown-lens	Alkaline-purple
Salmonella choleraesuis	+++	Brown-lens	Alkaline-purple
Salmonella paratyphi A	+	Black-lens	Purple
*Salmonella tennessee***	+++	Black-lens	Acid-yellow
Escherichia coli	±*	White	Acid-yellow
Proteus vulgaris	±	White	Slightly acid
Proteus morganii	–	–	–
Proteus mirabilis	+++	White	Acid-yellow
*Bacillus megaterium****	–	–	–
Bacillus cereus	–	–	–
Bacillus subtilis	–	–	–
Bacillus sterothermophilus	–	–	–
Aerobacter sp.	+++	White	Acid-yellow
Citrobacter sp.	+++	White	Acid-yellow
Pseudomonas sp.	±	White	Acid and alkaline
Shigella sp.	–	–	–

[1]The newly developed lysine-iron-cystine agar (LICA).
*± Some strains inhibited.
** Lactose positive.
*** Bacillus strains tested at 10^6 organism/plate.
+++ = 300-100 colonies corresponding with counts on Plate Count Agar of between 300 and 100 colonies.
+ = Few colonies 3-30, control plates were between 300-100 colonies.

(45 C) melted agar just before plating to give a final concentration of 5 μg/ml. As previously shown *(1) novobiocin* selectively inhibited growth of undesirable gram-positive microorganisms, particularly sporeformers. Selected strains of *Escherichia coli, Proteus, Pseudomonas,* and *Shigella* were also inhibited.

Trypsin (Nutritional Biochemical 1-3000) was added to the medium or plates containing .25 to 1 g of dairy product to digest milk casein *(3)*. Stock sterile trypsin solution was previously described *(1)* and usually 1-2 ml of the trypsin solution was added to the cooled melted agar medium.

Plating procedure

Because of the spreading growth of *Salmonella* in the medium, agar plates were poured in three stages.

An underlayer (10-15 ml of single strength agar) was added to sterile petri dishes and allowed to solidify. The sample dilution was added and poured with a second layer of agar (10-15 ml) and allowed to solidify. (Double strength agar was used for the second agar layer when the sample aliquot exceeded 1 ml). Plates were then covered with a third "sealing" layer of agar. Plates were incubated at 37 C for 24 h; only the black colonies were recorded, and then all plates were reincubated for an additional 24 h to detect slight H_2S producers.

NDM and cheese samples were prepared for plating as follows:

A 25% (W/V) milk concentrate was prepared from NDM powder by adding sterile distilled water (85 ml) to 25 g of powder. The mixture was blended in a sterile blender and the volume was adjusted to 100 ml before plating. A maximum of 4 ml of the concentrate could be added to a 100-mm diameter glass petri plate. The concentrate was used to make

TABLE 2. COMPARISON OF DIRECT PLATE COUNT[1] AND MPN FOR ESTIMATING SALMONELLAE IN NDM

NDM Lot. no.	Age (Weeks)	Contaminating *Salmonella*	*Salmonella*/g Direct count[2]	MPN
1 A	1	S. *newbrunswick*	320	460
	2	"	296	460
	3	"	220	240
	4	"	80	43
	6	"	39	43
1 B	1	S. *cubana*	480	460
	2	"	445	460
	3	"	310	240
	4	"	150	93
	6	"	80	93
1 C	1	S. *newbrunswick*	80	75
	2	"	34	43
	3	"	34	43
	4	"	30	43
	6	"	2	0
1 D	1	S. *cubana*	44	43
	2	"	39	43
	3	"	31	43
	4	"	9	9.1
	6	"	1	3.6

[1]Using the newly developed lysine iron cystine agar.
[2]Average value of triplicate platings of samples.

TABLE 3. ASSAY OF *Salmonella*-CONTAMINATED COTTAGE CHEESE DURING PROCESSING

Point in manufacture	Lysine-iron cystine count/ml-g	MPN[2]/ml-g
Cheese milk	21×10^4	24×10^4
Milk coagulated (before cutting)	25×10^4	24×10^4
Whey before cooking[1]	27×10^3	32×10^3
Whey before drain	0	0
Curd-uncreamed	0	0

[1]Cooked at 120 F for 20 min.
[2]5 tube MPN.

TABLE 4. ASSAY OF SALMONELLAE IN ARTIFICIALLY CONTAMINATED CHEDDAR CHEESE DURING MANUFACTURE AND STORAGE USING THE LYSINE-IRON-CYSTINE SELECTIVE PLATING AGAR

Time	Make status	Number of cells 11 ml-g
2 h 15 min.	Inoculated pasteurized milk	33×10^8
3 h 15 min.	Whey-end of cooking (100 F)	23×10^1
3 h 15 min.	Whey-drained	47×10^1
24 h	Curd-drained	60×10^4
2 wk	Begin (curing) storage at 50 F	54×10^4
6 wk	Curing 50 F	23×10^4
14 wk	"	19×10^4
18 wk	"	24×10^3
26 wk	"	11×10^3
30 wk	"	15×10^3
34 wk	"	2×10^1
0	"	0

the appropriate tenfold dilutions of the powder.

Eleven-gram samples of cheese were emulsified in 99 ml of sterile distilled water containing 0.2% sodium citrate. A maximum of 5 ml of the cheese slurry could be added per plate. Dilute solutions of milk or cheese required no trypsin.

The MPN *(6)* was determined in lactose broth. Selenite broth also was used occasionally to determine whether coliform overgrowth was an interfering factor. After incubation at 37 C for 18 h, 0.05 ml from each MPN tube was streaked onto Brilliant Green agar. Growth from typical colonies was transferred to Lysine agar (Difco) and Triple Sugar Iron agar (TSI; Difco). Growth from tubes exhibiting typical *Salmonella* reactions was checked for agglutination with type 0 polyvalent *Salmonella* antisera (Difco).

RESULTS AND DISCUSSION

Except for lysine-iron and MacConkey's agar, all of the test media used for comparative enumeration of *Salmonella* in milk showed some degree of inhibition. Nearly all *Salmonella* grew rapidly in the medium and formed large, black lenticular colonies in 24 to 48 h. Typical results from pure cultures are shown in Table 1. A deeper purple zone about the colonies indicated an alkaline reaction due to the action of lysine decarboxylase. Strains of *Salmonella choleraesuis, Salmonella sendai,* and *Salmonella senftenberg* produced brownish rather than typically black colonies in 48 h. These strains are known to produce little or no hydrogen sulfide on TSI agar. A lactose-positive *Salmonella tennessee* gave an acid medium reaction but the colony was the typical black. Coliforms and *Citrobacter* utilized lactose and salacin and gave an acid reaction (yellow) in the medium around their white lenticular colonies; colony growth remained white without blackening. All the *Bacillus* species used were very sensitive to 5 μg novobiocin/ml and as many as 10^7 cells/ml were inhibited. Additionally many gram-positive organisms were inhibited by 5 μg of novobiocin/ml, as was *Shigella,* some strains of *E. coli,* and species of *Proteus and Pseudomonas. Salmonella paratyphi A* showed slight inhibition with 5 μg of novobiocin/ml. Most salmonellae grew readily in the medium with levels of antibiotic as high at 10 μg/ml.

An agar underlayer was essential before addition of the sample to prevent colony spreading on the bottom of the plates. Similarly, after plates were poured an overlayer was essential to prevent surface spreading. Without an overlayer *Salmonella* colonies were indistinguishable from coliforms and other gram-negative organisms, because all surface colony growth turned black and gave an alkaline reaction in the medium.

Trypsin was essential to clarify the medium when milk concentrates of dairy products were plated *(4).* Casein digestion and medium clearing was usually complete after 4 to 6 h incubation. A comparison of the direct plate count and the three tube MPN (a prepared table estimated value) procedures *(6)* in repeated sampling of four lots of NDM during storage is shown in Table 2. These data show that the direct plate count is as sensitive as the MPN at these levels of contamination. The decline in viable cells over a 6-week period can be seen in both the high and low contamination level. The medium and plating procedure was sufficiently sensitive and selective to permit detection of as few as 1-2 salmonellae per gram of product. While reliability of results from such low counts may be questionable, they were generally reproducible. In following the manufacture of *Salmonella* contaminated cottage cheese one can see it can easily be monitored with lysine-iron-cystine agar (LICA), and the agar can be used effectively to establish that the cooking stage (or sanitizing step) is effective (Table 3).

Artificially contaminated Cheddar cheese can be analyzed for *Salmonella* with LICA during manufacture and curing (storage at 50 F). Note that multipli-

cation of *Salmonella* has occurred during manufacture (Table 4). It is believed that this medium will be of considerable value to researchers interested in following the progress of *Salmonella* in artificially contaminated food products.

REFERENCES

1. Hargrove, R. E., F. E. McDonough, and R. H. Reamer. 1971. A selective medium and presumptive procedure for the detection of *Salmonella* in dairy products. J. Milk Food Technol. 34:6-11.

2. North, W. R., Jr. 1961. Lactose preenrichment method for isolation of *Salmonella* from dried egg albumin. Appl. Microbiol. 9:188-195.

3. Reamer, R. H., R. E. Hargrove, and F. E. McDonough. 1969. Increased sensitivity of immunofluorescent assay for *Salmonella* in non-fat dry milk. Appl. Microbiol. 18:328-331.

4. Reamer, R. H., and R. E. Hargrove. 1972. Twenty-four hour immunofluorescence technique for the detection of *Salmonella* in non-fat dry milk. Appl. Microbiol. 23:78-81.

5. 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.

6. Standard methods for the examination of water and waste water. 12th ed. 1965. Amer. Pub. Health Ass. Washington, D.C.