Twenty-Four-Hour Immunofluorescence Technique for the Detection of Salmonellae in Nonfat Dry Milk

R. H. REAMER AND R. E. HARGROVE

Dairy Products Laboratory, Agricultural Research Service, Beltsville, Maryland 20705

A detection procedure was developed in which a newly devised lysine-iron medium was used as a one-step selective and enrichment medium for detection of salmonellae by the fluorescent-antibody technique. Incubation was conducted in two steps: initially at 30 C for 5 hr to resuscitate sublethally stressed cells, followed by incubation at 39 C for 17 hr. Twenty-seven strains of salmonellae from groups A-I were utilized in the development of this procedure which was sensitive enough to detect one *Salmonella* bacterium in 100 g of nonfat dry milk.

Rapid and reliable means are urgently needed to detect contamination of food by salmonellae. This is especially true of foods like dairy products where wide use and a significant number of very susceptible consumers (infants and aged) require the most careful quality control. Because of the very low incidence of contamination in dairy products, particularly nonfat dry milk (NDM), a rapid screening procedure would minimize the holding period required to assure a safe product for shipment.

Numerous methods utilizing the principal biochemical characteristics of salmonellae such as inability to split lactose, production of hydrogen sulfide, and possession of a lysine decarboxylase (1, 4, 5), as well as modification of media plus serology (12), have been proposed for speeding up salmonellae detection. Specialized glassware utilizing carbohydrate fermentation and the motility of the organism also have been recommended (2). The fluorescent-antibody technique also has been proposed in many procedures for detecting organisms in meat (6), eggs (11), miscellaneous foods (8), and NDM (10).

The purpose of this work was to determine whether a newly proposed presumptive type selective broth medium (7) for salmonellae could be used as a combination pre-enrichment and selective medium for a fluorescent-antibody procedure to determine the presence of salmonellae in NDM within 24 hr.

MATERIALS AND METHODS

Salmonella serotypes. Serotypes from groups A through I were utilized including those serotypes most frequently isolated from dairy products, viz. *S. anatum*, *S. blockley*, *S. bredeney*, *S. cubana*, *S. derby*, *S. enteritidis*, *S. meleagridis*, *S. montevideo*, *S. new brunswick*, *S. newington*, *S. oranienburg*, *S. senftenberg*, *S. tennessee*, *S. thompson*, *S. typhimurium*, and *S. worthington*.

Other Enterobacteriaceae. Representative strains of *Enterobacter*, *Escherichia*, *Shigella*, *Citrobacter*, and *Proteus* were included to determine specificity of the method for salmonellae.

Medium. The lysine-iron-neutral red-broth presumptive medium as proposed by Hargrove et al. (7) was used to differentiate pure cultures and to detect salmonellae in NDM. The bases of this presumptive medium were *p*H response to an indicator dye and formation of H_2S with novobiocin added as a selective agent.

Salmonella antiserum. The *Salmonella* antiserum used was a commercially available globulin fraction of type O polyvalent antiserum (Sylvania Co., Milburn, N.J.). It had been absorbed with strains of *Escherichia coli* and *Citrobacter freundii* and conjugated with fluorescein isothiocyanate and contained antibodies against groups A through I. This antiserum stained both somatic and flagellar sites.

Fluorescent-antibody microbiology. An advanced laboratory microscope was used throughout this work (American Optical Co., Buffalo, N.Y.; model LIOTU-FDW). Brighter fluorescence was observed with two oil-immersion objectives (×50 and 100) which contained iris diaphragms. The ×100 objective was used for the final evaluation of the

slide.

Differentiation of pure cultures. Pure cultures were inoculated into tubes of medium and were incubated at 37 C for 18 hr, at which time color was recorded and samples were taken for the fluorescent-antibody procedure.

Test samples. Since no significant number of commercially contaminated samples was available, samples of NDM powders were prepared by adding known numbers of salmonellae to them. Twenty-four-hour broth cultures were diluted as necessary and blended with NDM in a mortar and pestal; additional NDM was added to obtain the approximate final concentration of salmonellae desired. Thorough mixing was carried out by mixing with large, sterile spoons and subsequent extensive shaking of powder in polyethylene bags. Powders were stored at 4 C. These were then assayed for approximate number of viable salmonellae by addition of trypsin to NDM reconstituted in lactose broth and assayed by conventional procedures.

Assay of NDM. Generally, NDM is assayed on a 1:10 (weight of sample/volume of medium) basis; however, when 100-g samples were assayed to detect low levels of contamination, it was found that a 1:15 ratio gave better dispersion of the samples. Trypsin (30 ml of 1% solution) and novobiocin (5 μg/ml) were added as previously described (7). Based on studies of sublethally stressed cells (9) and optimum temperatures for salmonellae growth (3), the incubation was conducted in two steps: initially at 30 C for 5 hr, followed by 39 C for 17 hr.

The stepwise procedure for assaying NDM samples was as follows. (i) Lysine-iron broth medium was prepared by the formula of Hargrove et al. (7), dispensed in 1,500-ml portions in large-mouth Erlenmeyer flasks, and autoclaved 121 C for 15 min. (ii) One hundred grams of NDM powder was mixed aseptically in 1,500 ml of medium in a Waring Blendor. (iii) The broth and milk mixture was returned to the medium flask, and stock solutions of trypsin and novobiocin were added (30 and 7.5 ml, respectively) and mixed. (iv) Flasks were incubated at 30 C for 5 hr and then incubated at 39 C for 17 hr. (v) Flasks were examined for medium indicator-color changes and blackening after a total of 22 hr of incubation.

The incubated samples were gently mixed, and 0.05 ml of each sample was placed onto an etched circle of a nonfluorescent glass slide (Aloe Scientific, St. Louis, Mo.). The slides were air dried, fixed in Haglund's solution (a 60:30:10 absolute alcohol-chloroform-Formalin mixture) for 30 sec, touched off on absorbent paper, and transferred to absolute alcohol for 30 sec before air drying.

Each smear was stained with the polyvalent antiserum at a 1:2 dilution. The slides were incubated for 30 min under inverted 150-mm glass petri dishes containing moist filter paper. This incubation period was followed by a rinse in normal saline, 10 min in phosphate-buffered saline, and a rinse in distilled water. The air-dried slides were mounted in a buffered glycerol fluorescent-antibody mounting fluid (Difco) and covered with a no. 0 Corning cover glass.

Final evaluation of these slides was based on bacterial morphology, size, and degree of fluorescence by using ×100 objective with iris diaphragm. Although in these experiments each field usually contained numerous fluorescing cells, a single fluorescent cell in 25 fields examined would be considered as positive for salmonellae. Approximately 2 hr was required for completion of the fluorescent-antibody procedure.

RESULTS AND DISCUSSION

The 27 salmonellae serotypes from groups A through I that were used in our previous study (7) were tested to determine the specificity of the conjugated antiserum, and all gave a bright fluorescence of somatic and flagellar sites. Additionally, strains of *Bacillus*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Paracolobactrum*, *Proteus*, *Providencia*, *Pseudomonas*, and *Shigella* were tested. None of these organisms was positive with this antiserum. Our previous work established (10) that a salmonellae which fluoresced when grown in pure culture would fluoresce when grown in reconstituted NDM.

The data in Table 1 indicate that the prepared samples which had been shown to contain approximately one viable *Salmonella* cell per 50 to 100 g with trypsin-treated NDM assayed by conventional methods were also positive by this method. This is further amplified in Table 2 which also shows that with salmonellae known to be H_2S-negative the medium was still a good enrichment, and the fluorescent-antibody test would give positive identification.

An explanation of the lack of blackening by *S. paratyphi* A (WMN), a known H_2S producer, in this medium within that time is shown in Table 3. Novobiocin (5 μg/ml) restricted the growth of the two strains of *S. paratyphi* A as evidenced by the few cells observed on the fluorescent-antibody slides. Thus, the limited growth in 22 hr was probably insufficient to produce enough H_2S to show a blackening even though it was sufficient to give a positive fluorescent-antibody test. The variability on response among strains of *S. pullorum* may have been caused by temperature-sensitive enzymes since Tittsler (13) showed that 30 C is optimum for H_2S production of this strain. Neither *S. paratyphi* A nor *S. pullorum* has been reported in NDM.

The medium provides a very satisfactory one-step enrichment procedure for subsequent fluorescent-antibody examination by providing

TABLE 1. *Evaluation of proposed fluorescent-antibody test using nonfat dry milk containing low levels[a] of Salmonella[b]*

Organism	Amt of nonfat dry milk (g)	Medium color	Fluorescent antibody
Salmonella tennessee	25	Red	–
	50	Red	–
	100	Black	+[c]
S. senftenberg	25	Red	–
	50	Black	+
	100	Black	+
S. new brunswick	25	Red	–
	50	Black	+
	100	Black	+
S. cubana	25	Red	–
	50	Black	+
	100	Black	+
S. montevideo	25	Red	–
	50	Black	+
	100	Black	+

[a] Each sample was tested in four separate assays; the level of contamination in these samples ranged from 1 in 50 to 1 in 100 g.

[b] These samples were negative in 100-g amounts when examined by selenite enrichment without trypsinization. Our previous work (10) indicated that one *Salmonella* cell in as little as 10 g of nonfat dry milk may not be picked up because of entrapment in coagulated casein.

[c] In all of these positive slides, the number per field was too numerous to count.

"next day" results on NDM samples. Its routine application as a quality control procedure on the production from NDM plants should be valuable in avoiding long holding periods while awaiting test results.

TABLE 2. *Applicability of procedure to 100-g samples of nonfat dry milk contaminated with various bacteria[a]*

Sample contamination with[b]	Medium color	Fluorescent antibody
Uncontaminated nonfat dry milk	Red	–
Escherichia coli	Red	–
Salmonella tennessee	Black	+
Citrobacter freundii	Red	–
Proteus sp.	Red	–
S. new brunswick	Black	+
S. senftenberg	Black	+
S. montevideo	Black	+
S. cubana	Black	+
S. pullorum stock	Red	+
S. pullorum 2083-66	Black	+
S. pullorum 17-64	Red-orange	+
S. pullorum 64/64	Black	+
S. paratyphi A (WMN)	Red	+
S. paratyphi (ATCC 9283)	Red	+

[a] Six separate tests were performed with identical results.

[b] Level of salmonellae contamination in these samples ranged from 1 in 50 to 1 in 100 g.

TABLE 3. *Effect of novobiocin on Salmonella paratyphi A in nonfat dry milk[a]*

Salmonella	Amt of nonfat dry milk (g)	Novobiocin (5 μg/ml)	Medium color	Fluorescent antibody (no. of cells/field)
S. paratyphi A (WMN)	10	–	Black	TNTC[b]
	10	+	Red	3
	50	–	Black	TNTC
	50	+	Red	4
	100	–	Black	TNTC
	100	+	Red-orange	7
S. paratyphi A (ATCC 9283)	10	–	Yellow	TNTC
	10	+	Red	5
	50	–	Yellow	TNTC
	50	+	Red	6
	100	–	Yellow	TNTC
	100	+	Red	8

[a] Four separate experiments were performed with similar results.

[b] Too numerous to count.

LITERATURE CITED

1. Bachrach, U. 1959. An improved method for the determination of lysine decarboxylase activity of salmonellae. Amer. J. Clin. Pathol. **32**:580–581.
2. Banwart, G. J. 1968. Glassware apparatus for determining motile bacteria. Poultry Sci. **47**:1209–1212.
3. Chung, K. C., and J. M. Goepfert. 1970. Growth of *Salmonella* at low *p*H. J. Food Sci. **35**:326–328.
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. Clin. Pathol. **29**:598–600.
6. Georgala, D. L., and M. Boothroyd. 1964. A rapid immunofluorescence technique for detecting salmonellae in raw meat. J. Hyg. (Camb.) **62**:319–327.
7. Hargrove, R. E., F. E. McDonough, and R. H. Reamer. 1971. A selective medium and presumptive procedure for detection of *Salmonella* in dairy products. J. Milk Food Technol. **34**:6–11.
8. Insalata, N. F., S. J. Schulte, and J. H. Berman. 1967. Immunofluorescence technique for detection of salmonellae in various foods. Appl. Microbiol. **15**:1145–1149.
9. Ordal, Z. J. 1970. Current developments in detection of microorganisms in foods. Influence of environmental factors on detection methods. J. Milk Food Technol. **33**:1–5.
10. 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.
11. Silliker, J. H., A. Schmall, and J. Y. Chira. 1966. The fluorescent antibody technique as a means of detecting salmonellae in foods. J. Food Sci. **31**:240–244.
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.
13. Tittsler, R. P. 1931. Effect of temperature upon the production of hydrogen sulfide by *Salmonella pullorum*. J. Bacteriol. **21**:111–118.