3160

Increased Sensitivity of Immunofluorescent Assay for *Salmonella* in Nonfat Dry Milk

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The necessity of developing a quick, sensitive, and reliable test for *Salmonella* in nonfat dry milk (NDM) is evident from the recent tracing of *Salmonella* outbreaks to this product. Normally, coagulation of casein occurs when assaying NDM under regular cultural conditions, raising the possibility of trapped bacteria. After 20 hr of incubation of NDM in preenrichment lactose broth, enrichment was achieved by using Selenite-Cystine Broth. Smears from the enrichment broth were examined by the fluorescent-antibody technique (FAT) with a commercially available polyvalent O globulin conjugated with fluorescein. Standard cultural methods (SCM) were performed for comparison with FAT. Sensitivity of FAT was definitely improved by the use of trypsin. Casein coagulation of NDM can be avoided by addition of trypsin to samples during initial preenrichment in lactose broth. Samples containing approximately one *Salmonella* per 10 g were easily detected by FAT with trypsin-treated samples. The method required only 42 hr to complete. Additionally, the use of trypsin enhanced recovery of *Salmonella* by use of SCM, as evidenced by alteration in the observed coliform to *Salmonella* ratios.

Recently, the transmission of salmonellosis to humans by nonfat dry milk (NDM) was demonstrated by its involvement in outbreaks of human disease and by the isolation of many *Salmonella* serotypes from products and from the environment of several drying plants in the United States (7, 8).

Generally, standard cultural methods (SCM) are used for the isolation of *Salmonella* from NDM powder and other dairy products (1, 5). Positive identification usually requires 4 days, and there is general agreement that more rapid and reliable means of isolation and identification are needed. Recent work with the fluorescent-antibody technique (FAT) suggests that this method can be of great value when used for the rapid detection of *Salmonella* in food and food products. Georgala and Boothroyd (2) showed the indirect FAT to be adaptable for detecting naturally occurring *Salmonella* in carcass and boneless meat in 18 hr. The main disadvantage of the method was the number of false positives encountered. Silliker et al. (6) assayed dried food samples of whole egg, yolk, and albumin by the indirect FAT by use of commercially available *Salmonella* typing antisera (Difco, the Spicer-Edwards set) for flagellar antigen. Absolute correlation was obtained between FAT and SCM when dried egg samples were used but not when examining liquid egg products.

Insalata et al. (4) used commercially prepared isothiocyanate-conjugated antisera (Sylvana Co., Milburn, N.J.) with excellent results for detection of *Salmonella* in foods intentionally inoculated with several *Salmonella* species. The direct FAT was employed by using both O and H antisera.

The purpose of this study was twofold: (i) to ascertain the specificity of a commercially available labeled antiserum and (ii) to ascertain whether the technique can be modified for the assay of *Salmonella* in NDM.

MATERIALS AND METHODS

Salmonella serotypes. The following serotypes, from a broad variety of sources, were used for preliminary screening of the commercial antisera: five strains of *S. typhimurium*, two strains each of *S. senftenberg*, *S. derby*, *S. choleraesuis*, *S. thompson*, and one strain each of *S. chester*, *S. oranienburg*, *S. enteritidis*, *S. anatum*, *S. meleagridis*, *S. bredeney*, *S. tennessee*, and *S. blockley*. Serotypes for final characterizations of the commercial antisera were chosen on the basis of those which had been most frequently isolated from NDM. These were the following: *S. anatum* (9270), *S. cubana* (12007), and *S. tennessee* (10722) from the American Type Culture Collection, *S. montevideo* and *S. new brunswick*

from the Food and Drug Administration, and *S. oranienburg* and *S. worthington* from the U.S. Department of Agriculture, Agricultural Research Service, Western Utilization Research and Development Division.

Coliform bacteria. The coliform bacteria used in screening the commercial antisera were isolated from raw milk on violet red bile agar incubated at 37 C. After two standard purification procedures, these coliforms were characterized by their indole, methyl red, Voges-Proskauer, citrate reactions and by their cultural characteristics on eosin methylene blue and Brilliant Green (BG) agars. Test strains included 4 *Escherichia coli*, 10 *Enterobacter aerogenes*, and 1 *Proteus* species.

Dry milk samples. The *Salmonella*-contaminated NDM samples were prepared by two procedures. (i) Skim milk concentrates were inoculated with broth cultures of *Salmonella* or coliforms, or both, and were spray-dried. The relative number of detectable salmonellae was approximately 1 per g. This value remained stable throughout the 14-month work period. (ii) Low-heat grade A commercial NDM was inoculated with pure cultures of *Salmonella* ground in a mortar with a pestle, placed in plastic bags, and shaken well. The level of salmonellae added varied from 100 per g to 1 per 25 g. The detectable level was essentially the same and remained so throughout a work period of 5 months. The powders were then stored at 4 C.

FAT. NDM samples were diluted 1:10 in lactose broth. Trypsin (Trypsin 1-300, Nutritional Biochemicals Corp. Cleveland, Ohio) was added at a final concentration of 1:10,000 (w/v). (In developing the procedure, samples of contaminated powder varied in weight from 0.1 to 40 g.) The samples were incubated immediately in a water bath at 37 C for 2 hr, during which time the casein was generally broken down and sample turbidity was substantially reduced. This was followed by 20 hr of incubation at 37 C. A 10-ml amount of Selenite-Cystine Enrichment Medium (Difco) was inoculated with 2 ml of the pre-enrichment broth and incubated for 20 hr at 37 C, after which sample tubes were gently mixed and 0.05 ml of each sample was placed onto etched circles of a nonfluorescent glass slide (Aloe Scientific, St. Louis, Mo.). After drying, the slides were fixed in Haglund's (3) solution (absolute alcohol, chloroform, and Formalin, 60:30:10) for 30 sec, touched off on absorption paper, transferred to absolute alcohol for 30 sec, and air-dried.

Each smear was stained with the polyvalent 0 antiserum at a 1:2 dilution by placing 0.05 ml over the smear area. The slides were incubated for 30 sec under inverted 150-mm glass petri dishes containing moist filter paper.

The incubation period was followed by a rinse in normal saline, 10 min in phosphate-buffered saline, and a rinse in distilled water. After air drying, the slides were mounted in a buffered glycerol FA mounting fluid (Difco) and covered with a no. 0 Corning cover glass.

SCM. BG agar was streaked with a loop of the same 20-hr enrichment media used for the FAT and incubated at 37 C for 20 hr. Typical colonies on BG were then picked to triple sugar iron agar which was incubated for 24 hr. Tubes exhibiting a standard *Salmonella* reaction were further inoculated to lysine iron (LI) agar and urea agar to rule out *Proteus* and *Citrobacter* and were incubated for 24 hr at 37 C. Nutrient agar slants were inoculated from typical LI slants and were tested on the following day for agglutination with *Salmonella* poly O typing sera (Difco).

Salmonella antiserum. The *Salmonella* antiserum used was a commerically available globulin fraction of type O polyvalent antisera. It was absorbed with strains of *E. coli* and *C. freundii* and conjugated with fluorescein isothiocyanate (Sylvana Co.). It contains antibodies against groups A through H. In preliminary work, it was noted that this antisera stained both somatic and flagellar sites.

Fluorescent-antibody microscopy. Fluorestar microscope (American Optical Co., Buffalo, N.Y., LIOTU-FDW) with built-in base illuminator and transformer was used throughout this work. Brighter fluorescence was observed by using two oil immersion objectives

TABLE 1. *Specificity of a commercially available fluorescent conjugated Salmonella poly O antiserum*

Salmonella organism[a]	Degree of fluorescence[b]	
	Somatic	Flagellar
Group B		
S. bredeney	1+	2+
S. chester	1+	1+
S. derby	2+	1+
	2+	1+
S. typhimurium	1+	2+
	2+	1+
	1+	1+
	1+	0
	1+	1+
Group C_1		
S. choleraesuis var. *kunzendorf*	2+	2+
S. choleraesuis	1+	0
S. thompson	2+	0
S. thompson var. *berlin*	2+	2+
S. oranienburg	2+	2+
S. tennessee	2+	2+
Group C_2		
S. blockley	1+	1+
Group D		
S. enteriditis	1+	0
Group E_1		
S. anatum	2+	2+
S. meleagidis	2+	2+
Group E_4		
S. senftenberg	2+	1+
	1+	1+

[a] Pure cultures in Trypticase Soy Broth for 24 hr at 37 C.

[b] Degree of fluorescence: 1, bright fluorescence; 2, very bright fluorescence.

(50 and 100 ×) with iris diaphragm compared to standard objectives. The filter system consisted of a Shott OG-1 barrier filter, a BG 12 exciter filter, and a large, glass heat-absorbing filter.

RESULTS AND DISCUSSION

Screening results of several strains of different serotypes of *Salmonella* are presented in Table 1. These data indicate that the commercially available conjugated antiserum used was quite specific. All of the *Salmonella* fluoresced somatically; however, none of the species of coliform bacteria tested (*E. coli*, *E. aerogenes*, and *Proteus* species) fluoresced somatically. Note that, although the antiserum is designated as a poly O antiserum, it also stained flagellar (H) antigens. The observation of completely stained bacteria, i.e., flagellar and somatic, comprises an optimal assay. As used here, specificity means ability to be nonreactive in the absence of *Salmonella*. *Salmonella* serotypes included in this screening were from groups B, C_1, C_2, D, E_1, and E_4.

TABLE 2. *Survey of pure cultures and contaminated NDM by using species of the most commonly isolated Salmonella occurring in NDM*

Organism	Antigenic group	Fluorescence				SCM
		Pure culture antisera dilution		NDM antisera dilution		
		1:2	1:8	1:2	1:8	
S. anatum	C_1	+	+	+	+	+
S. montevideo	C_1	+	+	+	+	+
S. oranienburg	C_1	+	+	+	+	+
S. tennessee	C_1	+	+	+	+	+
S. newington	E_2	+	+	+	+	+
S. cubana	G[a]	+	−	+	−	+
S. worthington	G	+	−	+	−	+

[a] Group G accounts for approximately 10% of *Salmonella* isolants from NDM.

Serotypes of *Salmonella* which have been most frequently isolated from NDM were tested as pure cultures and in artificially contaminated NDM (Table 2). The serotypes included here were C_1, E_2, and G. All serotypes tested (including those in Table 1), except group G, were easily detected at a 1:8 dilution of antisera. Group G, however, could not be detected at antisera dilutions below 1:2. For this reason, the 1:2 dilution was used in all assays to be certain of including group G species. These data demonstrate (Table 2) that those *Salmonella* species fluorescing in pure culture also fluoresce when contained in NDM. It should also be noted that the FAT result which required 42 hr was confirmed at 96 hr by the SCM.

The FAT and SCM were compared in the assay of *S. new brunswick* in NDM. Although the FAT result was a 42-hr assay, it gave the same end point as did the SCM result of 96 hr.

The effect of trypsin digestion of casein on coliforms and *Salmonella* is shown in Table 3. With NDM powder 1, which contains coliforms and *S. new brunswick*, trypsin treatment increased the sensitivity of both the FAT and the SCM and also somehow eliminated coliforms. Other pre-

TABLE 3. *Effect of trypsin digestion of casein on coliforms and Salmonella*

Determination	Amt of NDM	FAT		SCM			
		Trypsin	No trypsin	Trypsin		No trypsin	
				Salmonella	Coliform	*Salmonella*	Coliform
	g						
Powder 1[a]	1.00	TNTC[b]	20	+	+	+	+
	0.50	70	3.0	+	+	−	+
	0.25	20	0.5	+	−	−	+
	0.10			−	−	−	+
Powder 2[c]	40.0	TNTC	TNTC	+	−	+	−
	25.0	TNTC	TNTC	+	−	+	−
	10.0	TNTC		+	−	−	−

[a] Powder containing coliforms, some sporeformers, and *S. new brunswick*. Duplicate sets of base dilutions were prepared, each divided into two equal volumes and made up to 100 ml for assay with and without trypsin.

[b] Indicates the number of organisms per microscopic field (oil immersion). TNTC, too numerous to count.

[c] Powder containing approximately one *S. tennessee* per 25 g. Duplicate sets of base dilutions were prepared, each divided into two equal volumes and made up to 250 ml for assay with and without trypsin.

liminary experiments indicate that trypsin affects the coliforms and has no adverse effect on *Salmonella*. An increase in sensitivity was seen with the FAT; an average of 20 bacteria per field were found with the trypsin NDM and less than 1 per field was found wlth the non-trypsin NDM. This was probably caused by the freeing of bacteria from casein clumps, with subsequent multiplication enabling easy detection with the FAT.

Trypsin-treated NDM assayed under the 96-hr SCM method picked up two more *Salmonella*-positive samples than did the non-trypsin-treated NDM. This could result primarily from coliform overgrowth in the non-trypsin-treated samples. It is reasonably certain that NDM powders containing *Salmonella* species also contain coliforms, as they are both found in products produced under improper sanitation conditions. When assaying NDM in quantities necessary for quality control work, the contribution of trypsin treatment is seen both in the FAT and SCM (powder 2, Table 3). Note that without trypsin treatment the 10-g sample was negative. Samples of this size form a large casein clump without trypsin. This suggests that, unless NDM is properly prepared for assay, samples tested having a low *Salmonella* count may be called negative.

Work is in progress at this laboratory to define the mechanism of the observed trypsin effect on coliforms.

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