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Rapid Cultural Methods for Detection of *Salmonella* in Feeds and Feed Ingredients

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ABSTRACT

Efficacy of standard, 6-h standard and direct enrichment methods for detection of *Salmonella* in naturally contaminated feeds and feed ingredients was compared. Analysis by the standard method involved preenrichment of feed slurries in nutrient broth, selective enrichment in tetrathionate brilliant green (43°C) and selenite cystine (35°C), and isolation of presumptive isolates on bismuth sulfite and brilliant green sulfa agar media. Sample analysis by the 6-h standard method was identical to the above except that incubation of enrichment broths was reduced to 6 h; for direct enrichment, preenrichment in nutrient broth was omitted. Of 287 samples tested, 75 were found to contain salmonellae by the three methods combined. Ability of the standard and 6-h standard methods to identify the same 58 contaminated samples underlines the reliability of the 6-h standard method for the more rapid detection of *Salmonella* in animal feeds. Identification of 68 positive samples by direct enrichment presumably resulted from equilibration (3 to 4 h) of feed slurries at reduced water activity before analysis. Addition of novobiocin (40 μg/ml) to selective enrichment broths did not facilitate isolation of *Salmonella* through repression of competitive flora. Productivity of the six enrichment-plating combinations used in this study was comparable, and no single medium played a determinant role in recovery.

Recent increases in human cases of salmonellosis (*4,13,17,24,30*) underline the ubiquitous distribution of salmonellae and need for effective control measures to protect the health of consumers and prevent contamination of their food supply. Animal feeds constitute an important reservoir of *Salmonella* infection and contribute largely to the spread of disease in human and animal populations (*1,3,25,39,40*). Control of the bacteriological quality of feeds and feed ingredients poses a major challenge because of the size of the industry and rapid turnover of production lots to satisfy domestic and foreign markets. Rapid and sensitive analytical methods for detection of *Salmonella*, therefore, become economically attractive to the feed industry. Standard cultural methods of analysis generally recommend non-selective enrichment (preenrichment) of feed samples, enrichment in selective broths and plating on differential agar media (*16,20,21,36*); direct enrichment (no preenrichment) of highly contaminated feeds is also advocated (*37*).

The present study evaluates the reliability of direct enrichment (*6,9,10*) and a 6-h standard method for the rapid identification of *Salmonella* in feeds and feed ingredients.

MATERIALS AND METHODS

A total of 269 samples of animal feeds and feed ingredients was randomly collected during routine monitoring of Canadian rendering plants and feed mills by officials of the federal Department of Agriculture; 18 additional samples from lots previously found to contain *Salmonella* were also included in this study. Feed samples (500 g) were packed in plastic Whirl-Pak bags and stored at room temperature pending shipment to the laboratory for analysis.

A 225-g slurry of feed was prepared with approx. 1.5 volumes of sterile distilled water and homogenized 60 s in a blender. Replicate 25-g samples of the slurry were withdrawn with a spoon and tested in parallel by each of the following methods: (a) standard, (b) 6-h standard and (c) direct enrichment. For the standard and 6-h standard method analyses, a single 25-g portion of slurry was preenriched overnight at 35°C in 225 ml of nutrient broth (NB). Replicate 1-ml portions of the preenrichment culture were selectively enriched in tetrathionate brilliant green (TBG) and selenite cystine (SC) broths with and without added novobiocin (N) to a final concentration of 40 μg/ml. Enrichment broths were incubated at 35°C (SC_{35}, SCN_{35}, TBG_{35}, $TBGN_{35}$) and 43°C (TBG_{43}, $TBGN_{43}$). After 6 h of incubation, one loopful of each SC and TBG enrichment culture was plated on bismuth sulfite (BiS) and brilliant green sulfa (BGS) agar media and incubated for 16 to 18 h at 35°C (6-h standard); the remaining portions of enrichment cultures were incubated overnight and streaked on both agar media (standard method). Presumptive isolates on plating media were screened biochemically on triple sugar iron and lysine iron agars, and serologically with polyvalent and single grouping somatic antisera. Serotyping was completed at the National Enteric Reference Center (Health and Welfare Canada). For direct enrichment, six replicate 25-g portions of the feed slurry used for the standard and 6-h standard methods were selectively enriched overnight at 35 or 43°C in 225 ml of each of the six enrichment broths described above. The need to prepare fresh TBG and SC daily resulted in a 3 to 4-h delay in initiating sample analysis by the six direct enrichment conditions. During this time, feed slurries were left standing at room temperature in blender jars.

The number of *Salmonella* in the original slurry material was determined by the 3-tube most probable number (MPN) technique. Triplicate

10-, 1.0- and 0.1-g portions of the slurry were preenriched overnight (35°C) in 9 volumes of NB, except the 0.1-g samples which were preenriched in 9 ml of NB. One-ml portions from each of the nine preenrichment cultures were selectively enriched overnight in 9 ml of TBG (43°C) and streaked on BiS alone. Presumptive isolates were screened as previously described.

Differences in the productivity of the three test methods were analyzed statistically by Cochran's Q test (*12*).

RESULTS AND DISCUSSION

Of 287 samples tested, 75 (26%) were found to contain *Salmonella* by the three test methods combined (Table 1). Levels of contamination ranged from <3 to 1100 salmonellae/100 g, with a median value of 9.1. A report on the incidence of *Salmonella* in Canadian feed products in 1980-81 showed contamination levels of 21.9 and 4.8% in rendered feed ingredients and finished feeds, respectively (unpublished data). These values are not markedly different from those published in other national studies (*28,32,40*). Contamination of approximately 50% of meat meal and meat and bone meal samples (Table 1) underlines the importance of these products as contaminating ingredients in finished feeds and likely reflects contamination of raw carcass meats and cross-contamination of the rendering plant environment (*2,25,40*). Of 26 serovars identified (Table 2), *Salmonella montevideo, Salmonella senftenberg, Salmonella tennessee* and *Salmonella schwarzengrund* occurred with the highest frequency of isolation.

Research efforts to channel selective enrichment cultures away from the sequential plating and biochemical screening steps to more rapid diagnostic tests, including enrichment serology (*34*), immunofluorescence (*35*), enzyme-linked immunosorbent assay (*26*) and lysine-iron-cystine-neutral red broth (*5,15*), have met with limited success. Recent work clearly showed that method brevity through incubation of preenrichment cultures for short (4 to 6 h) periods of time is not feasible (*6*). Ability of the standard and 6-h standard methods to detect the same 58 contaminated feed samples (Table 3) underlines the reliability of the 6-h selective enrichment step as a novel, more rapid analytical approach for the presumptive identification of

TABLE 1. Salmonella *in animal feeds and feed ingredients.*

Feed or feed ingredient	No. tested	No. positive (%)	Level of contamination[a]
Meat meal	112	50 (45)	<3-1100 (9.1)
Poultry supplement	38	2 (5)	9.1[b] (9.1)
Hog supplement	32	5 (16)	<3-23 (<3)
Meat and bone meal	30	15 (50)	<3-460 (3.6)
Fish meal	28	3 (11)	<3-21 (9.1)
Feather meal	20	0 (0)	NA[c]
Dairy ration	19	0 (0)	NA
Blood meal	4	0 (0)	NA
Milk substitute	3	0 (0)	NA
Sunflower meal	1	0 (0)	NA
TOTAL	287	75 (26)	

[a]Range of salmonellae per 100 g of product determined by the most probable number technique; values in brackets are the median.
[b]Same level of contamination encountered in both samples.
[c]NA, not applicable.

TABLE 2. Salmonella *serovars in feeds and feed ingredients.*

Feed or feed ingredient	Total samples positive	Serovar (No. of isolations)
Meat meal	50	*S. montevideo* (10); *S. mbandaka* (5); *S. johannesburg* (4); *S. senftenberg* (4); *S. cerro* (3); *S. orion* (3); *S. tennessee* (3); *S. california* (2); *S. drypool* (2); *S. kentucky* (2); *S. schwarzengrund* (2); *S. agona* (1); *S. bareilly* (1); *S. binza* (1); *S. eimsbuettel* (1); *S. halmstad* (1); *S. livingstone* (1); *S. nienstedten* (1); *S. saint-paul* (1); *S. thompson* (1); *S. worthington* (1).
Meat and bone meal	15	*S. schwarzengrund* (3); *S. alachua* (2); *S. senftenberg* (2); *S. bredeney* (1); *S. cerro* (1); *S. eimsbuettel* (1); *S. nienstedten* (1); *S. tennessee* (1); *S. thomasville* (1); *S. typhimurium* (1); *S. worthington* (1).
Hog supplement	5	*S. agona* (1); *S. johannesburg* (1); *S. montevideo* (1); *S. schwarzengrund* (1); *S. typhimurium* (1).
Fish meal	3	*S. tennessee* (2); *S. senftenberg* (1).
Poultry supplement	2	*S. newport* (1); *S. nienstedten* (1).

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Salmonella. Our findings are consistent with results of a recent study utilizing a 6-h selective enrichment period for detection of salmonellae by a hydrophobic grid membrane filtration (HGMF) technique (*11*). Experimental work currently in progress may support extension of abbreviated enrichment to foods other than animal feeds.

A significantly ($P = 0.02$) greater number of contaminated samples were detected by direct enrichment than by the standard or 6-h standard methods (Table 3). Although detection of 7 contaminated samples by the latter two methods only is consistent with preenrichment-facilitated recovery of stressed *Salmonella*, identification of 17 samples by direct enrichment alone was unexpected and contrasts with earlier reports on the reduced sensitivity of this analytical approach (*6,9*). It was noted that most samples found to contain salmonellae by direct enrichment alone exhibited low levels of contamination, ranging from <3 to 9.1 salmonellae/100 g (Table 4). Presence of few salmonellae in these samples should have reflected poorly on the sensitivity of direct enrichment through reduced growth or death of injured cells. It is suggested that the 3 to 4-h delay in initiating analysis of feed slurries by direct enrichment, necessitated by a labor-intensive experimental protocol and need to prepare fresh TBG and SC daily, resulted in an unintentional but consistent equilibration of the slurries at low water activity. The situation recalls earlier reports on increased recovery of *Salmonella* upon reconstitution of milk powder at a sample-to-preenrichment broth ratio of 1:2 (wt/vol) and importance of osmotic pressure and temperature during reconstitution of dried milk products (*29,38*). Greater productivity of direct enrichment in the present study could not be attributed to feed type (Table 4) or serovars (data not shown).

Novobiocin concentrations of 5 to 80 μg/ml have been used with limited success to increase the selectivity of liquid (*18,22,34*) and solid (*19,23,27*) media through inhibition of competitive flora, notably *Proteus* and *Citrobacter* spp. In the present study, addition of novobiocin (40 μg/ml) to SC and TBG failed to markedly increase sensitivity of the standard and 6-h standard methods, but apparently compensated for the low selectivity of SC_{35} and TBG_{35} in direct enrichment (Fig. 1). The apparent contradiction between the low sensitivity (Fig. 1) and greater productivity (Table 3) of direct enrichment arises from detection of

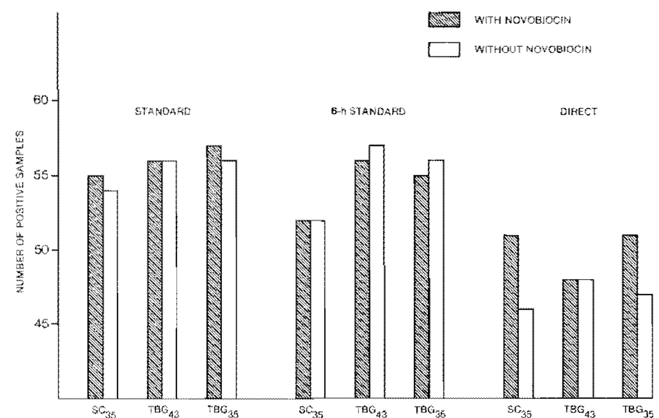

Figure 1. *Productivity of selective enrichment media with and without added novobiocin.*

TABLE 3. Salmonella *recovery patterns by different analytical methods.*

Recovery pattern			Frequency of result	
Standard (58)[a]	6-h Standard (58)	Direct enrichment (68)	With novo[b]	Without novo
+	+	+	51	47
+	+	−	6	10
+	−	+	0	0
−	+	+	0	1
−	−	+	13	13
−	+	−	0	0
+	−	−	1	0
−	−	−	216	216

[a]Samples positive by each method; a total of 75 samples were identified by the three methods combined.
[b]Novo, novobiocin.

TABLE 4. *Characteristics of samples positive by direct enrichment alone.*

Sample	Total	No. of positive samples		Level of contamination (MPN/100 g)		
	No.	With novo[a]	Without novo	<3	≤3.6	≤9.1
Meat meal	10	7	8	7	1	2
Meat and bone meal	4	4	3	2	2	0
Hog supplement	2	1	1	2	0	0
Poultry supplement	1	1	1	0	0	1
TOTAL	17	13	13	11	3	3

[a]Novo, novobiocin.

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positive samples by only one or two of the six direct enrichment conditions (data not shown).

Selectivity of enrichment conditions plays a determinant role in the successful recovery of salmonellae in high but not in low moisture foods (*7,14,31*). Performance of selective enrichment media was comparable within each of the three test methods, with greater variations within direct enrichment (Fig. 1). The BiS and BGS agar media were equally productive, except for SC_{35} plated on BGS which gave a substantially lower recovery rate (Table 5). These results are consistent with earlier findings on the indeterminate role of enrichment media and incubation temperatures for detection of *Salmonella* in low moisture foods (*8,14*).

The present study clearly establishes the equivalence of short (6 h) selective enrichment with standard cultural procedures for the rapid isolation of *Salmonella* in rendered animal products and finished feeds. The apparent interplay between rehydration of feeds at low water activity and increased detection of salmonellae is currently under investigation.

TABLE 5. *Productivity of selective enrichment/plating conditions.*

Analytical method	Total No. positive	*Salmonella* positive samples											
		BiS						BGS					
		With novo[a]			Without novo			With novo			Without novo		
		SC_{35}	TBG_{43}	TBG_{35}	SC_{35}	TBG_{43}	TBG_{35}	SC_{35}	TBG_{43}	TBG_{35}	SC_{35}	TBG_{43}	TBG_{35}
Standard	58	54	55	56	51	53	55	51	55	53	41	55	54
6-h Standard	58	51	55	54	49	56	55	45	53	52	34	53	50
Direct enrichment	68	49	44	49	43	47	45	50	43	48	40	46	44
TOTAL	184	154	154	159	143	156	155	146	151	153	115	154	148

[a]Novo, novobiocin.

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