Journal of Food Protection, Vol. 70, No. 4, 2007, Pages 835–840

Evaluation of the BAX Gel and Fluorometric Systems for the Detection of Foodborne *Salmonella*

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MS 06-543: Received 18 October 2006/Accepted 21 November 2006

ABSTRACT

The present study compared the sensitivity of the BAX automated fluorometric and the recently discontinued BAX gel electrophoresis systems with a standard culture method to detect *Salmonella* in 333 high-moisture and 171 low-moisture foods. A total of 95 naturally contaminated foods, including 63 high-moisture and 32 low-moisture foods, were detected by the standard culture method. No contaminated samples were identified exclusively by the BAX systems. By means of the analytical protocol stipulated by the manufacturer, the BAX fluorometric system detected 36 (57.1%) and 29 (90.6%) of the contaminated high- and low-moisture foods, respectively. Similar results were obtained with the BAX gel electrophoresis system, which identified 40 (63.5%) and 26 (81.3%) of the contaminated high- and low-moisture foods. The rate of false-positive reactions with the BAX systems was low. Our results indicate that the low sensitivity of the BAX systems with high-moisture foods, notably raw meats and poultry products, was serovar-independent. The high levels of background microflora that commonly occur in raw meat and on fresh fruit and vegetable products, and the high successive dilutions of test materials for PCR analysis, suggestively undermined the sensitivity of the gel and the fluorometric BAX assays. The potential benefits of immunomagnetic separation of *Salmonella* in preenrichment cultures, of selective broth enrichment following preenrichment to markedly reduce levels of background microflora in PCR test materials, and the use of larger portions of test materials in PCR analyses should be investigated.

The continued prominence of *Salmonella* as one of the leading causes of human foodborne disease in many countries stems from the ubiquity of this invasive pathogen in the natural environment, from its prominence in various agricultural sectors, and from the expansive global trade of foods and food ingredients *(5–7).* This reality predicates the need for rapid, reliable, and cost-effective methods for the detection of this important human bacterial foodborne pathogen. Recent years have witnessed great advances in the development of nucleic acid hybridization techniques for the presumptive identification of salmonellae in foods and agricultural products. These methods flag potentially contaminated samples 1 to 2 days sooner than conventional culture methods, which generally require 4 working days to obtain negative or presumptively positive results. The sensitivity of many commercial methods based on fluorometric microplate DNA-rRNA hybridization and PCR reactions has compared favorably with that of standard culture methods for the rapid detection of foodborne *Salmonella (5).*

The present study evaluates the performance of the Qualicon BAX automated fluorometric and the BAX gel electrophoretic systems against the Health Canada MFHPB-20 culture method for the detection of salmonellae in naturally contaminated foods and animal feeds.

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MATERIALS AND METHODS

Food samples. Naturally contaminated (noninoculated) test products were obtained from local retail outlets or as a result of monitoring and compliance activities of Canadian government agencies during the 2002 to 2004 study period. Fresh products were generally analyzed within 1 day of receipt, whereas frozen foods were thawed for 24 to 48 h in the refrigerator (4°C) prior to analysis. A total of 333 high-moisture foods, consisting mainly of raw poultry and other raw meat samples (Table 1), and 171 low-moisture foods (Table 2) were analyzed as described in Figure 1.

Sample analyses. The performance of the BAX-PCR gel electrophoretic and the automated microplate fluorometric methods (DuPont Qualicon, Wilmington, Del.) for the detection of *Salmonella* was evaluated against a standard culture method *(8)* from which preenrichment cultures provided a common test material for the three assay methods. Each of three analysts involved in this validation study worked independently in separate laboratories and consistently analyzed test samples by the same method. A total of 15 or fewer samples were tested in each weekly experimental trial. Coded aliquots of preenrichment cultures arising from the standard culture analysis of test products were provided to the appropriate analysts for immediate assay by the BAX gel electrophoretic and the automated fluorometric methods. Analytical results obtained by the BAX systems and the standard culture method were submitted regularly to the project coordinator for collation and analysis.

For the culture method (MFHPB-20), a test sample (100 g) was preenriched for 24 h at 35°C in 9 volumes of buffered peptone water or other preenrichment broth medium, such as nonfat dry

TABLE 1. *Cultural detection of* Salmonella *in high-moisture foods with MFHPB-20*

Food	No. of samples		*Salmonella* serovars (*n*)
	Tested	Positive	
Raw meats			
Chicken	116	33	Hadar (2); Heidelberg (16); Kentucky (7); Saint-Paul (1); Thompson (6); 1,4,5,12:i:− (1)
Turkey	38	9	Heidelberg (7); Senftenberg (1); Wein (1)
Pork	73	10	Brandenburg (1); Bareilly (1); Derby (3); Heidelberg (1); Infantis (2); Ohio (1); Typhimurium (1)
Veal	10	1	Typhimurium (1)
Lamb	11	0	
Beef	15	3	Johannesburg (1); Kentucky (1); Thompson (1)
RTE[a] meats	4	0	
Eggs and egg products	13	4	Heidelberg (2); Mbandaka (1); 1,4,5,12:i:− (1)
Fresh vegetables	16	1	Thompson (1)
Fish	11	0	
Fruit	11	1	Typhimurium (1)
Shellfish	8	1	Thompson (1)
Other[b]	7	0	
Total	333	63	

[a] RTE, ready-to-eat.
[b] Cheese (6) and black peppered pâté (1).

TABLE 2. *Cultural detection of* Salmonella *in low-moisture foods with MFHPB-20*

Food	No. of samples		*Salmonella* serovars (*n*)
	Tested	Positive	
Animal feeds	31	3	Agona (1); Cerro (1); Johannesburg (1)
Chocolate	13	2	Oranienburg (2)
Dried fruit	12	0	
Fish/shellfish[a]	6	0	
Pasta	4	0	
Sesame products	48	22	Brandenburg (3); Mbandaka (2); Idikan (2); Liverpool (2); Montevideo (8); Oranienburg (2); Senftenberg (3)
Spices	51	5	Enteritidis (1); Hvittingfoss (1); Matadi (2); Newport (1)
Other[b]	6	0	
Total	171	32	

[a] Dried and smoked products.
[b] Rice (2), nuts (2), fried dough (1), and powdered milk (1).

milk and brilliant green water, as stipulated in the culture method (Fig. 1). Replicate portions (1 ml) from each preenrichment culture were selectively enriched in 9 volumes of tetrathionate brilliant green and selenite cystine broth media for 24 h at 43 and 35°C, respectively. Replicate portions (1 ml) from each preenrichment culture were also withdrawn for the gel and the fluorometric BAX assays. Each selective enrichment culture was then plated onto bismuth sulfite and brilliant green sulfa agars, and suspect colonies were screened biochemically on triple sugar iron and lysine iron agar slants. Presumptive *Salmonella* isolates were confirmed serologically with polyvalent and single grouping somatic (O) and flagellar (H) antisera (Difco, Becton Dickinson, Sparks, Md., and PRO-LAB Diagnostics, Austin, Tex.). Bacteriological media were obtained from Difco, Becton Dickinson. Appropriate positive and negative controls were included in each experimental trial.

The BAX gel electrophoretic assay was performed as described in the manufacturer's package insert, with PCR reagents and tubes provided in the DuPont Qualicon BAX for Screening/Salmonella kit. A portion (10 μl) of a 24-h preenrichment culture of all test products was inoculated into brain heart infusion (BHI) broth (500 μl) and incubated for 3 h at 35°C. These BHI (3-h) cultures were also assayed by the automated BAX fluorometric method as described in the following paragraph. A portion (5 μl) of the BHI culture was then dispensed into a lysis tube containing a protease solution (200 μl) and heated for 20 min at 37°C and then for 10 min at 95°C. Reaction tubes were then cooled in a lysate cooling block. A portion (50 μl) of a lysate was dispensed into a Dupont Qualicon PCR tube containing a proprietary reagent tablet that provides the necessary primers, nucleotides, polymerase, and internal positive control for DNA amplification. The reaction mixture was amplified in a Gene-Amp System 9600 (Perkin-Elmer Corp., Norwalk, Conn.) by the BAX-70 program.

For the electrophoretic detection of *Salmonella*-specific amplicons, a portion (15 μl) of the amplified sample and mass ladder was loaded into gel wells and separated by electrophoresis at 180 V for 25 min. The presence of an ethidium bromide–stained fluorescent band at a mass value of 800 bp presumptively identified the presence of *Salmonella* in the test sample. A presumptive-positive BAX result was confirmed by the isolation of viable salmonellae from the homologous preenrichment culture by the standard culture method *(8).*

The DuPont Qualicon procedure for the automated BAX fluorometric detection of foodborne salmonellae was as described in the *User Guide—BAX System/PCR Assay with Automated Detection* (2000 to 2002). Notably, this procedure stipulates that all test products, except for raw meats and poultry products, be regrown in BHI broth for 3 h at 37°C to ensure that the BAX threshold level of 10^4 salmonellae per ml of broth culture has been attained. To obtain a detailed evaluation of the BAX fluorometric assay, the preenrichment culture (A/BAX-direct) and the BHI culture (A/BAX-BHI) of all test products, including raw meats and poultry products, were assayed by the automated BAX fluorometric method (Fig. 1). Briefly, separate portions (5 μl) from the preenrichment and BHI (3-h) cultures of each test product were lysed in 200 μl of protease solution. A portion (50 μl) of each lysate was then dispensed into a separate PCR tube containing a proprietary PCR reagent tablet. The lysate tube was then placed in a DuPont Qualicon cycler-detector for automated fluorometric PCR analysis. The instrument prints negative (green) and presumptively positive (red) results within 3.5 h following initiation of the automated sample analysis.

The endpoint dilution technique was used to enumerate salmonellae in paired preenrichment (24-h) and BHI (3-h) cultures of five sesame seed products that had previously yielded false-negative results by the direct BAX assay but positive results following regrowth in BHI broth. These samples of tahineh (3) and

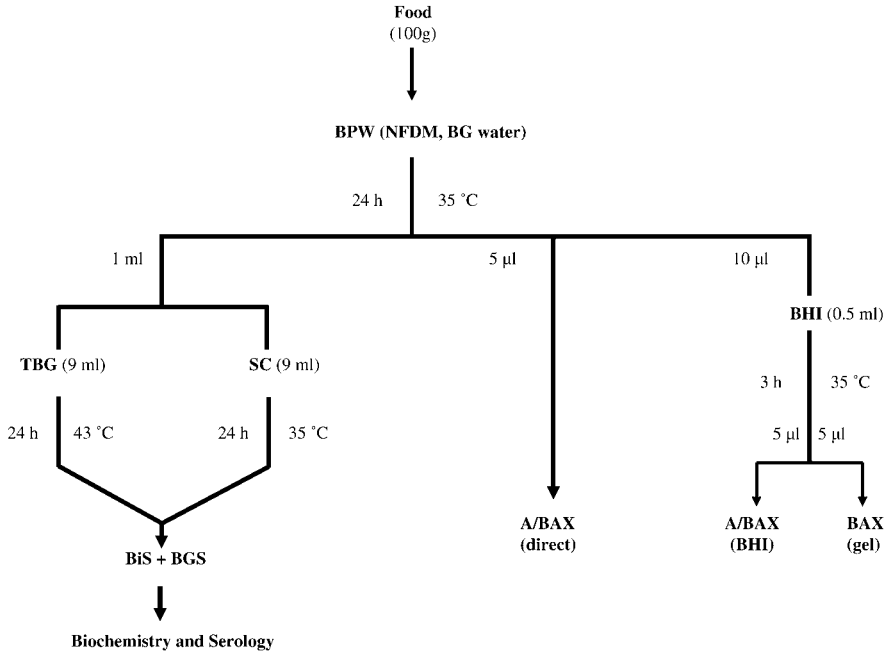

FIGURE 1. *Protocol for the evaluation of the BAX versus the MFHPB-20 method for the detection of foodborne* Salmonella. *BPW, buffered peptone water; NFDM, nonfat dry milk; BG, brilliant green water; TBG, tetrathionate brilliant green broth; SC, selenite cystine broth; BHI, brain heart infusion broth; BiS, bismuth sulfite agar; BGS, brilliant green sulfa agar; A/BAX, automated BAX assay.*

halva (2) were naturally contaminated with *Salmonella* Athinia, *Salmonella* Liverpool, *Salmonella* Mbandaka, *Salmonella* Montevideo, or *Salmonella* Senftenberg. Briefly, a portion (100 g) of each sesame seed product was preenriched in 900 ml of buffered peptone water for 24 h at 35°C. Portions (1.0 ml) from each member of a serial dilution series (10^{-2} to 10^{-8}) of each preenrichment culture were then analyzed individually by the MFHPB-20 method *(8).* Concurrently, portions (10 µl) from each of the same preenrichment cultures were inoculated into separate tubes containing BHI (0.5 ml) and incubated for 3 h at 35°C. Levels of salmonellae in the BHI cultures were determined by the endpoint dilution technique as described above.

RESULTS AND DISCUSSION

Of the 333 high-moisture (Table 1) and 171 low-moisture (Table 2) foods examined in this study by the gel and the automated fluorometric BAX methods and by the standard MFHPB-20 culture method, 63 (18.9%) and 32 (18.7%), respectively, were culturally positive for *Salmonella.* The frequency of foodborne isolations reported in the present study does not correspond to current levels of salmonellae in the Canadian food chain because many of the test samples, notably frozen meats and shelf-stable products, originated from our laboratory collection of naturally contaminated products. The prominence of *Salmonella* Heidelberg in raw poultry products (Table 1) was not totally unexpected, given the persistence of this serovar among the three most common serovars in domestic agricultural products in recent years *(10).* The notoriety of *Salmonella* Heidelberg in the Canadian registry of human cases of salmonellosis is also noteworthy *(11).* The infrequent isolation of *Salmonella* Newport in the present study and in a recent Canadian study *(13)* contrasts with the current importance of this serovar in the United States and its rapid spread from dairy herds to other sectors of the U.S. food industry and in the natural environment *(9, 17).* Ready-to-eat sesame seed products obtained from Canadian retail outlets in recent years originated mainly from countries of the Middle East (Table 2). These included tahineh (tahini), an oily paste product derived from the grinding of roasted sesame seeds, and halawa (halva), which is a sweetened tahineh-based condiment that may contain pistachios, other varieties of nuts, or chocolate flavoring. The solid chocolate manufactured in Germany was incriminated in a recent outbreak of human *Salmonella* Oranienburg infections in several European countries *(20).* Rendered products and complete feeds were obtained from the Canadian Food Inspection Agency as a result of domestic monitoring and compliance activities.

Naturally contaminated products were used throughout this study to challenge the sensitivity and specificity of the BAX-PCR methods under realistic conditions. Such products contain a variety of *Salmonella* serovars and heterogeneous populations of background microflora at different levels of occurrence and in different physiological states. The use of a common preenrichment culture in our experimental protocol enabled the direct performance assessment of the two BAX-PCR methods with the standard MFHPB-20 culture method. The stringency of the test conditions used in the present study contrasts sharply with that in other collaborative studies wherein the performance assessment of novel methods was based on the detection of salmonellae in a few dry foods, each inoculated at high and low levels of contamination with a single, frequently nonstressed *Salmonella* serovar. Moreover, the prominence of inoculated dry foods in many collaborative studies suggestively introduces a bias in the assessment of method performance because of the inherently low levels of background microflora in dry products that represent a lesser challenge to the sensitivity and specificity of novel test methods.

In the present study, a total of 95 *Salmonella*-contaminated products were identified by the standard MFHPB-20 method (Table 3). No additional contaminated samples were detected by the PCR methods. Under the conditions of use stipulated by the manufacturer, the automated fluo-

TABLE 3. *BAX detection of foodborne* Salmonella

Food	No. of positive samples[a]				
	Culture[b]	BAX gel	BAX automated		BAX total (gel + automated)
			Direct	BHI (3 h)	
High moisture	63	40 (5)[c]	36[d] (1)	38 (4)	45
Low moisture	32	26	8[e]	29[d] (1)	30
Total	95	66[f] (5)	44 (1)	67[f] (5)	75

[a] Culturally confirmed.
[b] MFHPB-20 method.
[c] Number of false-positive reactions in parentheses.
[d] Positive samples per manufacturer's protocol.
[e] None of the 22 contaminated sesame products were detected.
[f] Methods detected salmonellae in the same 64 food samples.

rometric BAX system detected 36 (57.1%) and 29 (90.6%) of the naturally contaminated high- and low-moisture foods, respectively (Table 3). The recovery patterns for *Salmonella* by the PCR and culture methods (Table 4) and the identity of foods associated with false-negative results showed that erroneous results by the Dupont Qualicon protocol for the fluorometric detection of salmonellae were serovar independent (Table 5). Similar results were obtained with the now discontinued BAX gel electrophoresis system, which detected 40 (63.5%) and 26 (81.3%) of the *Salmonella*-contaminated high- and low-moisture foods, respectively. These results were not totally unexpected, given the use of common lysis and amplification protocols and patented PCR reagent tablets in the BAX gel and fluorometric assays. The two BAX detection systems generated very few false-positive reactions that were associated mainly with high-moisture foods (Table 3).

The identification of only 36 (57.1%) and 38 (60.4%) of the 63 culturally confirmed *Salmonella*-positive high-moisture foods by the direct and the BHI fluorometric assay, respectively, is of concern (Table 3). Although the underlying causes for these limiting results have yet to be identified, factors such as the high levels of background microflora in the preenrichment cultures of high-moisture foods and the attendant lytic release of nontargeted DNA could undermine the efficacy of PCR amplification by reducing the affinity of primers for their *Salmonella*-specific nucleotide sequences. Moreover, sequential dilutions and the use of microquantities of test materials during sample preparation and amplification steps could undermine the sensitivity of the BAX systems. Synergism between adverse contributing factors is of essence in the present context, as evidenced by the inability of a 50-fold dilution of total microflora associated with the regrowth of preenrichment cultures (10 μl) of high-moisture foods into 500 μl of BHI broth to significantly increase the sensitivity of the BAX fluorometric assay (Table 3). More specifically, a 50-fold dilution of preenrichment cultures marginally increased BAX sensitivity from 57.1% (direct assay) to 60.4% (BHI). These observations predicate the need to investigate the ability of traditional selective enrichment conditions to increase the sensitivity of the BAX fluorometric assay of

TABLE 4. *Recovery patterns of foodborne* Salmonella *by the BAX systems*

Food	Recovery patterns				No. of samples
	Culture	BAX			
		Gel[a]	A-Direct[b]	A-BHI[c]	
High moisture	+	+	+	+	31
	+	+	+	−	1
	+	+	−	−	3
	+	−	−	−	18
	+	−	+	−	3
	+	+	−	+	5
	+	−	−	+	1
	+	−	+	+	1
Subtotal					63
Low moisture	+	+	+	+	8
	+	+	−	−	1
	+	−	−	−	2
	+	−	−	+	4
	+	+	−	+	17
Subtotal					32

[a] Gel-based method after regrowth in BHI (3 h).
[b] Automated fluorometric assay of preenrichment culture.
[c] Automated fluorometric assay of BHI (3-h) culture following preenrichment.

TABLE 5. *False-negative samples obtained with the BAX fluorometric system*[a]

Food	Total no. of samples	*Salmonella* serovars (*n*)
High moisture		
Raw chicken		
Cut up	1	Kentucky (1)
Minced	7	Heidelberg (4); Hadar (1); Kentucky (1); Thompson (1)
Giblets	9	Heidelberg (5); Thompson (3); Kentucky (1)
Raw turkey		
Cut up	1	Heidelberg (1)
Minced	4	Heidelberg (3); Wien (1)
Raw pork		
Sausage	3	Derby (1); Heidelberg (1); Infantis (1)
Raw beef		
Minced	1	Kentucky (1)
Coconut drink	1	Typhimurium (1)
Total	27	
Low moisture		
Sesame (halawa/tahineh)	2	Idikan (1); Mbandaka (1)
Meat meal	1	Cerro (1)
Total	3	

[a] By using the manufacturer's recommended protocol.

high-moisture foods. The immunomagnetic separation of salmonellae from preenrichment cultures with high-affinity antibodies adsorbed to beads prior to proteolytic lysis might also improve the sensitivity of the automated BAX assay.

In contrast, a 50-fold dilution of preenrichment cultures of low-moisture foods (Table 2) dramatically increased the identification of contaminated samples from 8 (25%) by the direct BAX assay to 29 (90.6%) by regrowth in BHI broth (Table 3). Notably, the latter favorable results were obtained by the analytical approach prescribed by the manufacturer for low-moisture foods. With a single exception (poultry feed), this unusual pattern of reactions was associated with the 22 contaminated sesame seed products in this study that were tested at different time periods (data not shown). These findings suggest that the greater BAX sensitivity with the BHI broth (3 h) compared to that obtained with preenrichment cultures resulted from one of the following three events: (i) a significant increase in the number of target salmonellae during BHI incubation; (ii) a dilution of a PCR inhibitor, such as the polyphenolics that occur in plant tissues *(14)*; or (iii) a dilution-dependent reduction of cell debris and nonsalmonellae DNA fragments that could decrease the affinity of primers for their targeted *Salmonella*-specific sequences. A quantitative investigation by the endpoint dilution technique showed that *Salmonella* populations did not increase in BHI (3 h) but, in fact, decreased by 1.0 to 2.0 log (data not shown), commensurate with the 50-fold dilution of preenrichment cultures associated with the BHI regrowth step. Moreover, the absence of growth in BHI was not unexpected, because 24-h nonselective preenrichment cultures are well within the stationary phase of growth, and subsequent inoculation into a fresh and different broth medium most likely engenders a lag phase of several hours in duration. The second plausible explanation for the increased BAX sensitivity with BHI cannot be entertained because proprietary positive signals from the internal BAX-positive control with all sesame seed products indicate the absence of an endogenous PCR inhibitor, unless the internal target-primer combination is less sensitive to external inhibitors than the PCR reactants that flag the presumptive presence of unique *Salmonella* DNA fragments. The possibility of such a diagnostic limitation with multiplex PCR-based assays has been recognized previously *(4, 12, 16, 21)*. The remaining hypothesis that the dilution of test samples markedly reduces the levels of cellular debris and foreign DNA and engenders more reliable PCR results is attractive. However, this hypothesis does not fit the results obtained with high-moisture foods, such as raw meats and poultry products, in which a 50-fold dilution of preenrichment cultures seemingly should have improved the performance of the BAX fluorometric assay but actually failed to markedly increase its sensitivity (Table 3). Clearly, these variable effects of sample dilution on BAX sensitivity require further investigation.

The greater stringency of our experimental testing conditions, as described above, likely accounts for differences in our results from those reported in earlier evaluations of the BAX-PCR gel and fluorometric assays. Our study and earlier reports confirm that raw meats and other products containing high levels of background microflora present a major challenge to PCR technologies in their presumptive identification of foodborne *Salmonella*. The results of a major collaborative AOAC International study involving 16 laboratories showed a reduced sensitivity of the fluorometric BAX assay with high-moisture products *(15)*. For example, the BAX assay misidentified 7 (15.9%) of 44 naturally contaminated samples of raw ground chicken when compared to the U.S. Department of Agriculture culture method. Raw tilapia fish and fresh orange juice each inoculated with single *Salmonella* serovars accounted for the remaining five false-negative results in this AOAC International study. In a study involving raw chicken carcass rinses inoculated at high levels with a cocktail of four unstressed *Salmonella* serovars, the BAX fluorometric assay and the U.S. Department of Agriculture culture method identified, respectively, 111 (91.0%) and 113 (92.6%) of the 122 artificially contaminated samples *(2)*. Under similar analytical conditions and with similarly inoculated samples, the BAX and the U.S. Department of Agriculture methods each identified 60 (100%) ready-to-eat chicken hot dogs (*2*). The foregoing studies reiterate the limited ability of the BAX system to detect *Salmonella* in preenrichment cultures of foods containing high levels of background microflora that likely impede the growth of salmonellae to threshold levels (10^4/ml) required for successful PCR detection. Poor *Salmonella* growth during nonselective preenrichment could result from the production of metabolic inhibitors by background microflora or from bacterial competition for the limited nutrients in broth media when injured or stressed salmonellae are greatly outnumbered by competitive microorganisms.

Results of earlier studies with the BAX gel electrophoresis system are of scientific interest because the gel electrophoresis and the automated fluorometric BAX methods are based on common cultural and PCR amplification conditions for the detection of *Salmonella*. In a comparative study of the BAX gel system with a conventional culture method involving preenrichment in buffered peptone water and selective enrichment in selenite cystine (35°C) and Rappaport-Vassiliadis (42°C) for 24 h, each method identified 123 (85.4%) of the 144 raw meats and dairy products inoculated with high levels of unstressed *Salmonella (3)*. In another study, the BAX gel system identified 129 (99.2%) of 130 culture-positive samples of fresh fruits and vegetables inoculated with generally high levels of a single unstressed strain of *Salmonella* Enteritidis *(14)*. A subsequent evaluation of the BAX gel assay with naturally contaminated alfalfa sprouts and irrigation waters from outbreak-associated seeds yielded a false-negative rate of 78.3% when compared with the U.S. Food and Drug Administration *Bacteriological Analytical Manual* method and a false-negative rate of only 9.4% when inoculated alfalfa sprouts and irrigation waters were tested under homologous conditions *(18)*. Conditions used in the preparation of *Salmonella* inocula for fresh mushrooms and alfalfa sprouts, and the preenrichment of inoculated samples in a broth medium containing selective bile salts and novobiocin, markedly affected the sensitivity of the BAX system *(19)*.

The automated BAX fluorometric system for the detection of foodborne *Salmonella* with a threshold sensitivity of approximately 10^4 salmonellae per ml of preenrichment culture was granted AOAC First Action in 2003 and Final Action status in June 2006. The BAX method is currently used by the Food Safety and Inspection Service of the U.S. Department of Agriculture for the detection of salmonellae in selected agricultural products *(1).* Although several studies underline the high sensitivity of the BAX gel and fluorometric methods for the detection of *Salmonella* in dry foods, discrepant results with raw meats, fresh fruits, and vegetables, as well as with other foods containing high levels of background microflora, are of major concern *(15, 18, 19)* (Table 3) and predicate the need to investigate alternate enrichment culture conditions and PCR analytical protocols for the improved sensitivity of the BAX system with high-moisture foods. Clearly, the preparation of samples, the stringency of test conditions, and the sensitivity of the reference method can markedly alter the reliability of the performance assessments of novel methods. The claimed equivalency of a novel method with a reference method of limited sensitivity may in fact be stating that both methods suffer from an equal lack of sensitivity.

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