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Comparison of Assurance Gold Salmonella EIA, BAX for Screening/Salmonella, and GENE-TRAK Salmonella DLP Rapid Assays for Detection of *Salmonella* in Alfalfa Sprouts and Sprout Irrigation Water

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The Assurance Gold Salmonella EIA, BAX for Screening/Salmonella, and GENE-TRAK Salmonella DLP rapid assays were compared with official cultural methods described in the *Bacteriological Analytical Manual* (BAM) for analysis of alfalfa sprouts and sprout irrigation water for the presence of *Salmonella*. The lower limits of detection of 4 serovars of *Salmonella* cells (*S. tennessee*, *S. muenchen*, *S. mbandaka*, and *S. cubana*) in pure culture were determined as approximately log₁₀ 2, 5, and 6 for the BAX, GENE-TRAK, and Gold EIA, respectively. Despite its low detection limit, the BAX did not perform as well as the other assays in analyzing contaminated sprouts and sprout irrigation water. For 4 different lots of sprouts and sprout irrigation water samples inoculated with the 4 serovars at low [1–2 colony forming units (CFU/g)] and high (68–180 CFU/g) levels, the BAX detected *Salmonella* in 58/64 (90.6%) of the samples, compared with 64/64 (100%) by the GENE-TRAK, Gold EIA, and BAM methods. Assay performance was also compared for analysis of naturally contaminated sprouts and sprout irrigation water with 3 lots of alfalfa sprouted seeds associated with different salmonellosis outbreaks. Positive assay results for the naturally contaminated samples were Gold EIA 41, GENE-TRAK 36, BAM 33, and BAX 13.

ence of *Salmonella* and *Escherichia coli* O157:H7, the 2 primary infective agents associated with sprout consumption.

Production of sprouts involves seed germination and sprouting in warm, moist conditions that encourage rapid growth of indigenous bacteria to levels as high as 10⁹ colony forming units (CFU)/g (4, 5). If pathogenic bacteria are present, they also are likely to undergo rapid growth (6–12). The sprouts are periodically irrigated during production, and the irrigation water collects bacteria, including pathogens, as it passes through the sprouts. In fact, this “spent” irrigation water was shown to contain bacterial populations at levels within approximately 1 log of the populations in the sprouts themselves (13). Pathogens are similarly distributed to the spent irrigation water, as shown in sprouting studies of both artificially inoculated seeds (12, 13) and naturally contaminated seeds associated with salmonellosis outbreaks (11).

In its Guidance to Industry (3), FDA has recommended that spent irrigation water be used as an analytical sample in preference to the sprouts themselves, because it is easy to sample and contains bacterial populations similar to those present in the sprouts. Sampling of spent irrigation water is recommended as early as 48 h from the start of production, when bacterial populations, including pathogens, tend to reach maximum levels (11–13). Rapid assays as specified (3) may be used for testing, allowing quick turnover of results within the normal production schedule and before the release of product. In this way, distribution of contaminated product may be prevented.

The rapid immunochemical-based assays suggested for testing spent irrigation water samples are the Assurance Gold Salmonella EIA, the Visual Immunoprecipitate (VIP) Assay for Salmonella, the VIP for EHEC, and the Reveal for *E. coli* O157:H7 (3). We compared nucleic acid-based assays for testing both sprouts and spent irrigation water, specifically, the BAX for Screening/Salmonella and the GENE-TRAK Salmonella DLP Assay. These 2 assays were compared with the Assurance Gold Salmonella EIA and official methods based on conventional culture for detection of *Salmonella* in both artificially inoculated and naturally contaminated samples.

The large number of foodborne illnesses linked to raw vegetable sprouts (1) led in 1999 to industry recommendations from the National Advisory Committee for the Microbiological Criteria for Foods and from the U.S. Food and Drug Administration (FDA) which were aimed at increasing the safety of sprouts (2, 3). Among the recommendations was the testing of every sprout production batch for the pres-

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Table 1. Lower limits of detection^a of 4 *Salmonella* serovars in pure culture by the Assurance Gold *Salmonella* EIA, BAX for Screening/*Salmonella*, and GENE-TRAK *Salmonella* DLP assays

<i>Salmonella</i> serovar	Detection by assay ^b					
	Assurance Gold EIA		BAX		GENE-TRAK	
	log ₁₀ CFU	Result	log ₁₀ CFU	Result	log ₁₀ CFU	Result
<i>S. tennessee</i>	6.43	+	TNP	TNP	6.38	+
	5.43	—	5.38	+	5.38	+
	4.43	—	4.38	+	4.38	—
	3.43	—	3.38	+	3.38	—
	2.43	—	2.38	+	2.38	—
	1.43	—	1.38	—	1.38	—
	TNP	TNP	0.38	—	TNP	TNP
<i>S. muenchen</i>	6.48	+	TNP	TNP	6.34	+
	5.48	—	5.15	+	5.34	+
	4.48	—	4.15	+	4.34	—
	3.48	—	3.15	+	3.34	—
	2.48	—	2.15	+	2.34	—
	1.48	—	1.15	—	1.34	—
	TNP	TNP	0.15	—	TNP	TNP
<i>S. mbandaka</i>	6.40	+	TNP	TNP	6.34	+
	5.40	—	5.36	+	5.34	+
	4.40	—	4.36	+	4.34	—
	3.40	—	3.36	+	3.34	—
	2.40	—	2.36	+	2.34	—
	1.40	—	1.36	—	1.34	—
	TNP	TNP	0.36	—	TNP	TNP
<i>S. cubana</i>	6.40	+	TNP	TNP	6.18	+
	5.40	—	5.36	+	5.18	+
	4.40	—	4.36	+	4.18	—
	3.40	—	3.36	+	3.18	—
	2.40	—	2.36	+	2.18	—
	1.40	—	1.36	—	1.18	—
	TNP	TNP	0.36	—	TNP	TNP

^a Determinations of lower detection limits of the assays were made once for each serovar.^b + = Positive assay result; — = negative assay result; TNP = testing not performed at the corresponding log₁₀ CFU level.

Experimental

Bacteria and Media

Sprout-associated outbreak *Salmonella* serovars of *S. tennessee*, *S. muenchen*, *S. mbandaka*, and *S. cubana* were cultured at 35°C for 18–24 h in brain heart infusion (BHI) broth, unless otherwise indicated. Cell numbers were determined after plating onto plate count agar (PCA) and incubating the plates at 35°C for 18–24 h. Culture media were obtained from Difco Laboratories (Detroit, MI) and Becton Dickinson Microbiology Systems (Sparks, MD), unless otherwise indicated.

Alfalfa Seed Sprouting

Sprouts were grown from alfalfa seeds in sterile glass jars. Alfalfa seeds were added to each jar, soaked in 500 mL sterile tap water for 3 h, and then drained, capped, and incubated in the dark at 25°C. The sprouting seeds were rinsed once each day by swirling in 500 mL sterile tap water. Sprouting was allowed to occur for 2–3 days; sprouts were then stored at 4°C for up to 3 days until needed. On day of use, the sprouts were rinsed once more with sterile tap water. Both sprouts and final irrigation water were sampled for use in the rapid detection assay comparisons.

Table 2. Detection of inoculated *Salmonella* in sprouts and sprout irrigation water by BAM, Gold EIA, BAX, and GENE-TRAK assays

Seed lot	Serovar ^a	Sample ^b	Inoculation level ^c	Detection by assay ^d			
				BAM	Gold EIA	BAX	GENE-TRAK
A	T	Sprouts	High	+	+	+	+
			Low	+	+	-	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	-	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	-	+
		Water	High	+	+	+	+
			Low	+	+	-	+
	C	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
B	T	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	-	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	C	Sprouts	High	+	+	+	+
			Low	+	+	-	+
		Water	High	+	+	+	+
			Low	+	+	+	+
C	T	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+

Table 2. (continued)

Seed lot	Serovar ^a	Sample ^b	Inoculation level ^c	Detection by assay ^d			
				BAM	Gold EIA	BAX	GENE-TRAK
D	C	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	T	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	C	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
Totals, sprouts: No. positives / 32 tested				32	32	27	32
Totals, water: No. positives / 32 tested				32	32	31	32
Totals, all samples: No. positives / 64 tested (%)				64/64 (100)	64/64 (100)	58/64 (90.6)	64/64 (100)

^a T = *S. tennessee*; Mu = *S. muenchen*; Mb = *S. mbandanka*; C = *S. cubana*.

^b A single test was performed for each sample at each inoculation level.

^c High, 68–180 CFU/g; low, 1–2 CFU/g.

^d + = Positive assay result; – = negative assay result.

Sample Preparation

Sprouts (25 g) and irrigation water (25 mL) samples were added to 225 mL appropriate enrichment broth, as specified by the assay protocol. The sprouts were homogenized for 2 min in sterile bags using a Stomacher Model 400 blender (Tekmar Co., Cincinnati, OH) set at medium speed. Irrigation water samples were swirled to mix. The mixtures were allowed to stand at ambient temperature for 60 min, and were then adjusted to pH 6.8 ± 0.2.

Assay Protocols

(a) *Assurance Gold Salmonella EIA*.—The package instructions indicating “Raw Foods” was followed, as specified by the manufacturer (BioControl Systems, Inc., Bellevue, WA). The enrichment protocol was, as follows: sample pre-enrichment in 225 mL buffered peptone water supplemented with novobiocin (BPW + n) at 18 µg/mL for 22–26 h at 35°C; selective enrichment by transfer of 0.1 mL BPW + n pre-enrichment to 10 mL Rappaport-Vassiliadis (RV) broth

and 1.0 mL pre-enrichment to 10 mL tetrathionate (TT) broth, with incubation at 42°C for 5–8 h; and post-enrichment by transfer and combination of 0.5 mL RV and 1 mL TT into a single tube containing 10 mL prewarmed trypticase soy broth supplemented with novobiocin (TSB + n) at 10 µg/mL with incubation for 16–20 h at 42°C. The post-enrichments were extracted and the assay was performed according to package instructions, using reagents as specified and supplied by the manufacturer. An OTC 40 Manual EIA Plate Reader (Organon Teknika Corp., Durham, NC) was used for color development determination in the microtiter plate wells and for calculation of the cut-off value.

(b) *BAX for Screening/Salmonella*.—The enrichment protocol, as specified by the manufacturer (Qualicon, Inc., Wilmington, DE), was, as follows: sample pre-enrichment in 225 mL lactose broth for 22–26 h at 35°C; post-enrichment by transfer of 10 µL pre-enrichment to 500 µL BHI broth and incubate for 3 h at 35°C. The cells were then lysed, and the DNA was amplified by polymerase chain reaction (PCR), using re-

Table 3. Detection of *Salmonella* in sprouts and sprout irrigation water from outbreak-associated seeds by BAM, Gold EIA, BAX, and GENE-TRAK assays

Seed lot	Replicate	Sample	Detection by assay ^a			
			BAM	Gold EIA	BAX	GENE-TRAK
X	1	Sprouts	+	+	+	+
		Water	+	+	-	+
	2	Sprouts	+	+	-	+
		Water	+	+	-	+
	3	Sprouts	-	+	-	+
		Water	+	+	-	+
	4	Sprouts	+	+	-	+
		Water	+	+	+	+
	5	Sprouts	+	+	-	+
		Water	+	+	-	+
	6	Sprouts	+	+	+	+
		Water	+	+	-	+
	7	Sprouts	+	+	-	+
		Water	+	+	-	+
	8	Sprouts	+	+	-	+
		Water	+	+	-	+
	9	Sprouts	-	-	-	-
		Water	-	-	-	-
	10	Sprouts	+	+	-	+
		Water	+	+	-	+
Y	1	Sprouts	-	-	-	-
		Water	-	-	-	-
	2	Sprouts	+	+	-	+
		Water	+	+	+	+
	3	Sprouts	-	-	-	-
		Water	-	-	-	-
	4	Sprouts	-	+	-	-
		Water	+	+	-	+
	5	Sprouts	+	+	-	+
		Water	+	+	-	+
	6	Sprouts	+	+	-	+
		Water	+	+	-	+
	7	Sprouts	+	+	+	+
		Water	+	+	+	+
	8	Sprouts	-	+	-	-
		Water	-	+	-	-
	9	Sprouts	-	+	-	-
		Water	+	+	+	+
	10	Sprouts	+	+	+	+
		Water	+	+	+	+
Z	1	Sprouts	-	+	+	+
		Water	-	+	+	+
	2	Sprouts	+	+	+	+
		Water	+	+	+	+

Table 3. (continued)

Seed lot	Replicate	Sample	Detection by assay ^a			
			BAM	Gold EIA	BAX	GENE-TRAK
	3	Sprouts	—	—	—	—
		Water	—	—	—	—
	4	Sprouts	—	—	—	—
		Water	—	—	—	—
	5	Sprouts	—	—	—	—
		Water	—	—	—	—
	6	Sprouts	—	—	—	—
		Water	—	—	—	—
	7	Sprouts	—	—	—	—
		Water	—	—	—	—
	8	Sprouts	—	—	—	—
		Water	—	—	—	—
	9	Sprouts	—	—	—	—
		Water	—	+	—	—
	10	Sprouts	+	+	—	+
		Water	+	+	—	+
Totals, sprouts: No. positives / 30 tested			15	20	6	17
Totals, water: No. positives / 30 tested			18	21	7	19
Totals, all samples: No. positives / 60 tested (%)			33 (55)	41 (68)	13 (22)	36 (60)

^a + = Positive assay result; — = negative assay result.

agents supplied by the manufacturer, as specified by the package instructions and User's Guide (14). Amplification was performed in a GeneAmp 2400 PCR thermal cycler (Applied Biosystems, Foster City, CA) using the BAX-70 program (14), as follows: 2 min at 94°C, followed by 38 cycles of 15 s at 94°C, 3 min at 70°C, and finally cooling to 25°C. The amplified products were loaded onto precast 2% SeaKem Gold agarose gels containing ethidium bromide (BioWhittaker Molecular Applications, Rockland, ME), electrophoresed in 0.5X Tris-borate-EDTA (TBE) buffer at 180 V for 25 min (14), and visualized by UV transillumination. Results were interpreted based on the presence of a 200 base pair (bp) internal control band and an 800 bp target band for each sample. The presence of the target band, regardless of the control band, indicated a positive result for *Salmonella*. Lack of the target band with a positive control band indicated a negative result, and absence of both bands indicated an indeterminate result.

(c) *GENE-TRAK Salmonella DLP assay*.—The package instructions indicating "All Other Food Products" was used, as specified by the manufacturer (GENE-TRAK Systems Corp., Hopkinton, MA). The enrichment protocol was, as follows: sample pre-enrichment in 225 mL lactose broth for 22–26 h at 35°C; selective enrichment by transfer of 0.1 mL pre-enrichment broth to 10 mL RV broth and 1.0 mL pre-enrichment broth to 10 mL TT broth, with incubation at 42°C for 22–26 h; and post-enrichment by transfer of 1.0 mL RV and 1.0 mL TT each into separate tubes containing 10 mL

GN enrichment broth (EM Science, Gibbstown, NJ), with incubation for 6 h at 35°C. The cells were then lysed, and the nucleic acid hybridization reaction was performed, using reagents and dipsticks supplied by the manufacturer, as specified by the package instructions. A GENE-TRAK photometer was used for color development determination and for calculation of the cut-off value.

(d) *Salmonella official methodology*.—Official methods by conventional culture, as specified in the *Bacteriological Analytical Manual* (BAM; 15), were used as standard methods with which rapid detection assays were compared. Samples were pre-enriched in lactose broth for 22–26 h at 35°C. The selective enrichment procedure for "Foods with a High Microbial Load" was used, as follows: 0.1 mL lactose broth pre-enrichment was transferred to 10 mL RV broth and another 1.0 mL was transferred to TT broth, with incubation at 42°C for 22–26 h. The RV and TT selective enrichments were then streaked to xylose lysine desoxycholate (XLD), bismuth sulfite (BS), and Hektoen enteric (HE) agar plates. After incubation of the plates at 35°C for 24–48 h, the presence of typical colonies was recorded. If necessary, colony presence was confirmed in tubes of triple sugar iron agar and lysine iron agar. This procedure was considered sufficient for *Salmonella* confirmation because, taken together, the steps would exclude isolation of all genera of the *Enterobacteriaceae* except *Salmonella* and *Edwardsiella*. Any *Edwardsiella* colonies that may have been present would not have been mistaken morpho-

logically for those of *Salmonella* serovars used as artificial inoculants or present as natural contaminants of the seeds.

Determination of Assay Lower Detection Limits

The 4 *Salmonella* serovars were cultured to mid-exponential phase in TSB + n, BHI, and GN broths. Serial 10-fold dilutions of each were prepared for determining the lower detection limits of *Salmonella* in pure culture in each of the rapid assays. Various dilutions of the cultures were placed on ice until used directly in the assay and tested according to package instructions. The appropriate final enrichment broths for the assays were used as diluents, i.e., TSB + n for the Assurance Gold EIA; BHI broth for the BAX for Screening/*Salmonella*; GN broth for the GENE-TRAK *Salmonella* DLP. Cell numbers were determined from these dilutions by plating onto PCA as described above.

Testing of Artificially Inoculated Samples

Sprouts were produced from 4 different alfalfa seed lots, A, B, C, and D, obtained from commercial suppliers. For each lot, seeds (100 g) were added to glass jars (2 L capacity), sprouted, and stored as described above. On the day of testing, sprouts (25 g) and irrigation water (25 mL) samples were prepared as described above in 225 mL of the various pre-enrichment broths.

The 4 *Salmonella* serovars were each cultured, serial 10-fold dilutions were made in Butterfield's phosphate buffer (BPB; 15), and cell concentrations were determined after plating on PCA. Appropriate dilutions (0.1 mL) of each serovar were added to the pre-enrichment broths to achieve high- and low-level inoculations of ca $\log_{10}$ 2 and 1, respectively. Uninoculated control samples of the sprouts and irrigation waters were also prepared. The enrichment protocols and detection assays were performed as described above for the control and artificially inoculated samples. All assays were initiated on the same day using portions of the same inoculated samples.

Testing of Naturally Contaminated Samples

Sprouts were produced from 3 different alfalfa seed lots, X, Y, and Z, implicated in 3 different salmonellosis outbreaks. For each lot, seeds (200 g) were added to each of 10 glass jars (2 L capacity), sprouted, and stored as described above. On the day of testing, sprouts (25 g) and irrigation water (25 mL) samples from each of the 10 replicate jars were prepared as described above in 225 mL of the various pre-enrichment broths. The enrichment protocols and detection assays were performed as described above. All assays were initiated on the same day using portions of the same samples from the outbreak-associated lots. A pair-wise comparison of results was performed using the McNemar test (16) to compare *Salmonella* detection percentages of the 4 methods for analysis of sprouts and rinse water from the naturally contaminated seed lots.

Results and Discussion

For purposes of discussion, the names of the various assays are abbreviated, as follows: Assurance Gold *Salmonella* EIA,

Gold EIA; BAX for Screening/*Salmonella*, BAX; GENE-TRAK *Salmonella* DLP assay, GENE-TRAK; and *Salmonella* official methodology, BAM.

Lower Detection Limits of Assays

The rapid assays were compared for lower limits of cell detection of 4 outbreak *Salmonella* serovars grown in pure culture (Table 1). The number of cells tested in each assay was computed based on assay sample volumes and plate counting results from individual cultures. Although assays were performed on separate days using separate cell cultures, results could be compared because the 4 serovars grew to similar population levels each time. Each assay demonstrated a consistent lower detection limit at approximately the same $\log_{10}$ CFU level for all 4 *Salmonella* serovars.

Of the 3 rapid assays compared, the BAX demonstrated the lowest limit of detection, approximately $\log_{10}$ 2 CFU. The lower detection limits of the other 2 assays exceeded the BAX by 3–4 log levels. In practical terms, these data indicate that, under the best conditions (i.e., pure cultures in enrichment media prescribed by the assay manufacturers), at least 10^2 CFU are needed for BAX to register a positive result, and at least 10^5 and 10^6 CFU are needed by the GENE-TRAK and Gold EIA, respectively. These comparative results were expected. Detection by the BAX assay is based on exponential amplification of specific DNA regions, of which only a few molecular copies are necessary, theoretically, to obtain a positive signal. The GENE-TRAK had a lower detection limit than the Gold EIA by about 1 log. It is difficult to speculate on the reasons for the differences in detection limit of the GENE-TRAK and Gold EIA assays. The GENE-TRAK assay detects the presence of ribosomal RNA molecules through hybridization to 2 DNA probes: a capture probe bound to a dipstick, and an enzyme-labeled DNA probe, which catalyzes color formation upon addition of a chromogenic substrate. The Gold EIA uses a sandwich format to detect cellular antigens through antibody binding and a chromogenic enzyme-substrate reporter system. The number of target molecules present, probe affinity, and efficiency of the assay format all play a role in influencing lower detection limits.

Assay Performance: Inoculated Sprouts and Irrigation Water Samples

The Gold EIA, BAX, and GENE-TRAK assays were compared with the BAM method for their ability to show as positive the sprouts and irrigation water samples that had been artificially inoculated with *Salmonella* (Table 2). Four different lots of alfalfa seeds were sprouted, and the sprouts and water samples were inoculated at high (68–180) and low (1–2) levels with each of the 4 *Salmonella* serovars. In all, 64 inoculated samples were tested by each assay. Uninoculated control samples from each of the 4 lots were negative by all assays.

The BAM official methodology identified all 64 samples as positive, as did the Gold EIA and the GENE-TRAK assays. The BAX identified 58 out of 64 (90.6%) samples as positive, and showed a higher percentage of detection of positives among water samples (31 of 32, or 96.9%) than the sprout

samples (27 of 32, or 84.4%). This result was surprising, in light of the superior detection limit exhibited by the BAX compared with the other assays (Table 1). At least 2 contributing factors may explain the inferior performance of the BAX: (1) The BAX protocol calls for a short enrichment in nonselective media, whereas the other assays have more extensive enrichment procedures that include selective media. Nonselective enrichment is likely to be less successful in increasing the numbers of *Salmonella* against the high microbial load that is naturally present in sprouts. Therefore, the BAX enrichment protocol could result in fewer *Salmonella* cells in the assay sample volume. Given the detection limit of the BAX (Table 1), the low cell number might not be a problem, except for contributing factor (2): PCR inhibitors that may be present in a sample can limit the efficiency of DNA amplification and compromise the sensitivity of the reaction. Because of the simple BAX enrichment scheme, these PCR inhibitors may not be diluted sufficiently in the reaction. The lower detection rate in the sprouts (84.4%) relative to the irrigation water (96.9%) may also be explained by these 2 contributing factors.

Assay Performance with Naturally Contaminated Sprouts and Irrigation Water Samples

The Gold EIA, BAX, and GENE-TRAK assays also were compared with the BAM method for their ability to detect *Salmonella* in sprouts grown from seeds associated with salmonellosis outbreaks (Table 3). Ten replicate jars of sprouts were produced from each of 3 seed lots incriminated in different outbreaks, and the sprouts and irrigation water from each were assayed by the various methods. Because of the uncertainty of the distribution of *Salmonella* cells in these naturally contaminated samples, the identities of the true positives and true negatives among the samples were uncertain. Based on the number of positive replicates (sprouts or water) as confirmed by the official BAM method, the highest contamination incidence appeared to be in seed lot X (90%), followed by seed lots Y (70%) and Z (20%), respectively. The BAM results indicated that assay of the water samples (18 positives) was somewhat more efficient in detecting positives than was assay of the sprouts samples (15 positives). This was perhaps due to reduction or dilution of interfering substances and/or competing microorganisms that may mask the presence of typical *Salmonella* colonies on confirmatory plates. These data support previous work (11–13), which showed that irrigation water can be a good indicator of the contamination state of sprouts.

Of the 3 rapid assays, the GENE-TRAK results (36 positives) most closely matched those of the official BAM method (33 positives), followed by the Gold EIA (41 positives) and, finally, the BAX (13 positives). There were 3 discrepant results between the GENE-TRAK and BAM assays: seed lot X replicate 3 sprouts, and seed lot Z replicate 1, both sprouts and water. It is likely that the seed lot X discrepant sprouts represented a false negative by the BAM, because the water sample from that same replicate was positive by the BAM. The seed lot Z replicate 1 discrepant was also most likely false negatives by the BAM, because all 3

rapid assays yielded positive results from those samples, and *Salmonella* colonies were actually confirmed from the Gold EIA enrichments of those samples. It is unlikely that subsamples from the same jar of sprouts would be differentially contaminated. Therefore, although the BAM method detected 33 samples as positive, it is likely that the true number of positives among the samples was 36. The GENE-TRAK assay produced positive results for all of these 36 samples.

For 5 of the samples, the Gold EIA produced results that could be interpreted as false positives, as judged by their discrepancies with the BAM official method as well as with the other rapid assays: seed lot Y replicate 4 sprouts; seed lot Y replicate 8 sprouts and water; seed lot Y replicate 9 sprouts; and seed lot Z replicate 9 water. However, there were no confirmed positives that the Gold EIA tested as negative. False-positive results from the Gold EIA were also noted in a previous study of the growth of *Salmonella* during sprouting (11).

Reflecting the results obtained from the inoculated samples (Table 2), the performance of the BAX assay was poor for naturally contaminated samples, as it detected *Salmonella* in <50% of samples shown to be positive by the BAM official method. The pair-wise comparison of results using the McNemar test showed that, for both sprouts and water samples, the BAX method results were significantly different ($p \leq 0.05$) from each of the other methods, but there were no significant differences between the Gold EIA, GENE-TRAK, and BAM method results.

Conclusions

Sprouts and sprout irrigation water are challenging matrices for *Salmonella* detection because of the presence of high populations of indigenous microorganisms. The rapid assays studied here, as well as the BAM official method, varied in their sensitivity and specificity of detection. The field of microbiological methods is a dynamic one, with new developments appearing annually. For example, both the GENE-TRAK and BAX assays have been reformatted, and these next generation systems have been introduced recently into the market (17, 18). It would be useful for method developers to include sprouts and sprout irrigation water in their testing panels, not only for the challenges they would offer to the assays, but also in light of the guidance provided to the sprout industry for pathogen testing (3).

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Exhibit 1051
Mullins Decl. Appendix G-2
(Stewart from OUP)

Comparison of Assurance Gold Salmonella EIA, BAX for Screening/Salmonella, and GENE-TRAK Salmonella DLP Rapid Assays for Detection of *Salmonella* in Alfalfa Sprouts and Sprout Irrigation Water

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The Assurance Gold Salmonella EIA, BAX for Screening/Salmonella, and GENE-TRAK Salmonella DLP rapid assays were compared with official cultural methods described in the *Bacteriological Analytical Manual* (BAM) for analysis of alfalfa sprouts and sprout irrigation water for the presence of *Salmonella*. The lower limits of detection of 4 serovars of *Salmonella* cells (*S. tennessee*, *S. muenchen*, *S. mbandanka*, and *S. cubana*) in pure culture were determined as approximately log₁₀ 2, 5, and 6 for the BAX, GENE-TRAK, and Gold EIA, respectively. Despite its low detection limit, the BAX did not perform as well as the other assays in analyzing contaminated sprouts and sprout irrigation water. For 4 different lots of sprouts and sprout irrigation water samples inoculated with the 4 serovars at low [1–2 colony forming units (CFU/g)] and high (68–180 CFU/g) levels, the BAX detected *Salmonella* in 58/64 (90.6%) of the samples, compared with 64/64 (100%) by the GENE-TRAK, Gold EIA, and BAM methods. Assay performance was also compared for analysis of naturally contaminated sprouts and sprout irrigation water with 3 lots of alfalfa sprouted seeds associated with different salmonellosis outbreaks. Positive assay results for the naturally contaminated samples were Gold EIA 41, GENE-TRAK 36, BAM 33, and BAX 13.

The large number of foodborne illnesses linked to raw vegetable sprouts (1) led in 1999 to industry recommendations from the National Advisory Committee for the Microbiological Criteria for Foods and from the U.S. Food and Drug Administration (FDA) which were aimed at increasing the safety of sprouts (2, 3). Among the recommendations was the testing of every sprout production batch for the pres-

ence of *Salmonella* and *Escherichia coli* O157:H7, the 2 primary infective agents associated with sprout consumption.

Production of sprouts involves seed germination and sprouting in warm, moist conditions that encourage rapid growth of indigenous bacteria to levels as high as 10⁹ colony forming units (CFU)/g (4, 5). If pathogenic bacteria are present, they also are likely to undergo rapid growth (6–12). The sprouts are periodically irrigated during production, and the irrigation water collects bacteria, including pathogens, as it passes through the sprouts. In fact, this “spent” irrigation water was shown to contain bacterial populations at levels within approximately 1 log of the populations in the sprouts themselves (13). Pathogens are similarly distributed to the spent irrigation water, as shown in sprouting studies of both artificially inoculated seeds (12, 13) and naturally contaminated seeds associated with salmonellosis outbreaks (11).

In its Guidance to Industry (3), FDA has recommended that spent irrigation water be used as an analytical sample in preference to the sprouts themselves, because it is easy to sample and contains bacterial populations similar to those present in the sprouts. Sampling of spent irrigation water is recommended as early as 48 h from the start of production, when bacterial populations, including pathogens, tend to reach maximum levels (11–13). Rapid assays as specified (3) may be used for testing, allowing quick turnover of results within the normal production schedule and before the release of product. In this way, distribution of contaminated product may be prevented.

The rapid immunochemical-based assays suggested for testing spent irrigation water samples are the Assurance Gold Salmonella EIA, the Visual Immunoprecipitate (VIP) Assay for Salmonella, the VIP for EHEC, and the Reveal for *E. coli* O157:H7 (3). We compared nucleic acid-based assays for testing both sprouts and spent irrigation water, specifically, the BAX for Screening/Salmonella and the GENE-TRAK Salmonella DLP Assay. These 2 assays were compared with the Assurance Gold Salmonella EIA and official methods based on conventional culture for detection of *Salmonella* in both artificially inoculated and naturally contaminated samples.

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Table 1. Lower limits of detection^a of 4 *Salmonella* serovars in pure culture by the Assurance Gold *Salmonella* EIA, BAX for Screening/*Salmonella*, and GENE-TRAK *Salmonella* DLP assays

<i>Salmonella</i> serovar	Detection by assay ^b					
	Assurance Gold EIA		BAX		GENE-TRAK	
	log ₁₀ CFU	Result	log ₁₀ CFU	Result	log ₁₀ CFU	Result
<i>S. tennessee</i>	6.43	+	TNP	TNP	6.38	+
	5.43	–	5.38	+	5.38	+
	4.43	–	4.38	+	4.38	–
	3.43	–	3.38	+	3.38	–
	2.43	–	2.38	+	2.38	–
	1.43	–	1.38	–	1.38	–
	TNP	TNP	0.38	–	TNP	TNP
<i>S. muenchen</i>	6.48	+	TNP	TNP	6.34	+
	5.48	–	5.15	+	5.34	+
	4.48	–	4.15	+	4.34	–
	3.48	–	3.15	+	3.34	–
	2.48	–	2.15	+	2.34	–
	1.48	–	1.15	–	1.34	–
	TNP	TNP	0.15	–	TNP	TNP
<i>S. mbandanka</i>	6.40	+	TNP	TNP	6.34	+
	5.40	–	5.36	+	5.34	+
	4.40	–	4.36	+	4.34	–
	3.40	–	3.36	+	3.34	–
	2.40	–	2.36	+	2.34	–
	1.40	–	1.36	–	1.34	–
	TNP	TNP	0.36	–	TNP	TNP
<i>S. cubana</i>	6.40	+	TNP	TNP	6.18	+
	5.40	–	5.36	+	5.18	+
	4.40	–	4.36	+	4.18	–
	3.40	–	3.36	+	3.18	–
	2.40	–	2.36	+	2.18	–
	1.40	–	1.36	–	1.18	–
	TNP	TNP	0.36	–	TNP	TNP

^a Determinations of lower detection limits of the assays were made once for each serovar.
^b + = Positive assay result; – = negative assay result; TNP = testing not performed at the corresponding log₁₀ CFU level.

Experimental

Bacteria and Media

Sprout-associated outbreak *Salmonella* serovars of *S. tennessee*, *S. muenchen*, *S. mbandanka*, and *S. cubana* were cultured at 35°C for 18–24 h in brain heart infusion (BHI) broth, unless otherwise indicated. Cell numbers were determined after plating onto plate count agar (PCA) and incubating the plates at 35°C for 18–24 h. Culture media were obtained from Difco Laboratories (Detroit, MI) and Becton Dickinson Microbiology Systems (Sparks, MD), unless otherwise indicated.

Alfalfa Seed Sprouting

Sprouts were grown from alfalfa seeds in sterile glass jars. Alfalfa seeds were added to each jar, soaked in 500 mL sterile tap water for 3 h, and then drained, capped, and incubated in the dark at 25°C. The sprouting seeds were rinsed once each day by swirling in 500 mL sterile tap water. Sprouting was allowed to occur for 2–3 days; sprouts were then stored at 4°C for up to 3 days until needed. On day of use, the sprouts were rinsed once more with sterile tap water. Both sprouts and final irrigation water were sampled for use in the rapid detection assay comparisons.

Table 2. Detection of inoculated *Salmonella* in sprouts and sprout irrigation water by BAM, Gold EIA, BAX, and GENE-TRAK assays

Seed lot	Serovar ^a	Sample ^b	Inoculation level ^c	Detection by assay ^d			
				BAM	Gold EIA	BAX	GENE-TRAK
A	T	Sprouts	High	+	+	+	+
			Low	+	+	—	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	—	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	—	+
		Water	High	+	+	+	+
			Low	+	+	—	+
	C	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
B	T	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	—	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	C	Sprouts	High	+	+	+	+
			Low	+	+	—	+
		Water	High	+	+	+	+
			Low	+	+	+	+
C	T	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+

Table 2. (continued)

Seed lot	Serovar ^a	Sample ^b	Inoculation level ^c	Detection by assay ^d			
				BAM	Gold EIA	BAX	GENE-TRAK
D	C	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	T	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mu	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	Mb	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
	C	Sprouts	High	+	+	+	+
			Low	+	+	+	+
		Water	High	+	+	+	+
			Low	+	+	+	+
Totals, sprouts: No. positives / 32 tested				32	32	27	32
Totals, water: No. positives / 32 tested				32	32	31	32
Totals, all samples: No. positives / 64 tested (%)				64/64 (100)	64/64 (100)	58/64 (90.6)	64/64 (100)

^a T = *S. tennessee*; Mu = *S. muenchen*; Mb = *S. mbandanka*; C = *S. cubana*.
^b A single test was performed for each sample at each inoculation level.
^c High, 68–180 CFU/g; low, 1–2 CFU/g.
^d + = Positive assay result; – = negative assay result.

Sample Preparation

Sprouts (25 g) and irrigation water (25 mL) samples were added to 225 mL appropriate enrichment broth, as specified by the assay protocol. The sprouts were homogenized for 2 min in sterile bags using a Stomacher Model 400 blender (Tekmar Co., Cincinnati, OH) set at medium speed. Irrigation water samples were swirled to mix. The mixtures were allowed to stand at ambient temperature for 60 min, and were then adjusted to pH 6.8 ± 0.2.

Assay Protocols

(a) Assurance Gold Salmonella ELA.—The package instructions indicating “Raw Foods” was followed, as specified by the manufacturer (BioControl Systems, Inc., Bellevue, WA). The enrichment protocol was, as follows: sample pre-enrichment in 225 mL buffered peptone water supplemented with novobiocin (BPW + n) at 18 µg/mL for 22–26 h at 35°C; selective enrichment by transfer of 0.1 mL BPW + n pre-enrichment to 10 mL Rappaport-Vassiliadis (RV) broth

and 1.0 mL pre-enrichment to 10 mL tetrathionate (TT) broth, with incubation at 42°C for 5–8 h; and post-enrichment by transfer and combination of 0.5 mL RV and 1 mL TT into a single tube containing 10 mL prewarmed trypticase soy broth supplemented with novobiocin (TSB + n) at 10 µg/mL with incubation for 16–20 h at 42°C. The post-enrichments were extracted and the assay was performed according to package instructions, using reagents as specified and supplied by the manufacturer. An OTC 40 Manual EIA Plate Reader (Organon Teknika Corp., Durham, NC) was used for color development determination in the microtiter plate wells and for calculation of the cut-off value.

(b) BAX for Screening/Salmonella.—The enrichment protocol, as specified by the manufacturer (Qualicon, Inc., Wilmington, DE), was, as follows: sample pre-enrichment in 225 mL lactose broth for 22–26 h at 35°C; post-enrichment by transfer of 10 µL pre-enrichment to 500 µL BHI broth and incubate for 3 h at 35°C. The cells were then lysed, and the DNA was amplified by polymerase chain reaction (PCR), using re-

Table 3. Detection of *Salmonella* in sprouts and sprout irrigation water from outbreak-associated seeds by BAM, Gold EIA, BAX, and GENE-TRAK assays

Seed lot	Replicate	Sample	Detection by assay ^a			
			BAM	Gold EIA	BAX	GENE-TRAK
X	1	Sprouts	+	+	+	+
		Water	+	+	—	+
	2	Sprouts	+	+	—	+
		Water	+	+	—	+
	3	Sprouts	—	+	—	+
		Water	+	+	—	+
	4	Sprouts	+	+	—	+
		Water	+	+	+	+
	5	Sprouts	+	+	—	+
		Water	+	+	—	+
	6	Sprouts	+	+	+	+
		Water	+	+	—	+
	7	Sprouts	+	+	—	+
		Water	+	+	—	+
	8	Sprouts	+	+	—	+
		Water	+	+	—	+
	9	Sprouts	—	—	—	—
		Water	—	—	—	—
	10	Sprouts	+	+	—	+
		Water	+	+	—	+
Y	1	Sprouts	—	—	—	—
		Water	—	—	—	—
	2	Sprouts	+	+	—	+
		Water	+	+	+	+
	3	Sprouts	—	—	—	—
		Water	—	—	—	—
	4	Sprouts	—	+	—	—
		Water	+	+	—	+
	5	Sprouts	+	+	—	+
		Water	+	+	—	+
	6	Sprouts	+	+	—	+
		Water	+	+	—	+
	7	Sprouts	+	+	+	+
		Water	+	+	+	+
	8	Sprouts	—	+	—	—
		Water	—	+	—	—
	9	Sprouts	—	+	—	—
		Water	+	+	+	+
	10	Sprouts	+	+	+	+
		Water	+	+	+	+
Z	1	Sprouts	—	+	+	+
		Water	—	+	+	+
	2	Sprouts	+	+	+	+
		Water	+	+	+	+

Table 3. (continued)

Seed lot	Replicate	Sample	Detection by assay ^a			
			BAM	Gold EIA	BAX	GENE-TRAK
	3	Sprouts	—	—	—	—
		Water	—	—	—	—
	4	Sprouts	—	—	—	—
		Water	—	—	—	—
	5	Sprouts	—	—	—	—
		Water	—	—	—	—
	6	Sprouts	—	—	—	—
		Water	—	—	—	—
	7	Sprouts	—	—	—	—
		Water	—	—	—	—
	8	Sprouts	—	—	—	—
		Water	—	—	—	—
	9	Sprouts	—	—	—	—
		Water	—	+	—	—
	10	Sprouts	+	+	—	+
		Water	+	+	—	+
Totals, sprouts: No. positives / 30 tested			15	20	6	17
Totals, water: No. positives / 30 tested			18	21	7	19
Totals, all samples: No. positives / 60 tested (%)			33 (55)	41 (68)	13 (22)	36 (60)

^a + = Positive assay result; — = negative assay result.

agents supplied by the manufacturer, as specified by the package instructions and User's Guide (14). Amplification was performed in a GeneAmp 2400 PCR thermal cycler (Applied Biosystems, Foster City, CA) using the BAX-70 program (14), as follows: 2 min at 94°C, followed by 38 cycles of 15 s at 94°C, 3 min at 70°C, and finally cooling to 25°C. The amplified products were loaded onto precast 2% SeaKem Gold agarose gels containing ethidium bromide (BioWhittaker Molecular Applications, Rockland, ME), electrophoresed in 0.5X Tris-borate-EDTA (TBE) buffer at 180 V for 25 min (14), and visualized by UV transillumination. Results were interpreted based on the presence of a 200 base pair (bp) internal control band and an 800 bp target band for each sample. The presence of the target band, regardless of the control band, indicated a positive result for *Salmonella*. Lack of the target band with a positive control band indicated a negative result, and absence of both bands indicated an indeterminate result.

(c) *GENE-TRAK Salmonella DLP assay*.—The package instructions indicating “All Other Food Products” was used, as specified by the manufacturer (GENE-TRAK Systems Corp., Hopkinton, MA). The enrichment protocol was, as follows: sample pre-enrichment in 225 mL lactose broth for 22–26 h at 35°C; selective enrichment by transfer of 0.1 mL pre-enrichment broth to 10 mL RV broth and 1.0 mL pre-enrichment broth to 10 mL TT broth, with incubation at 42°C for 22–26 h; and post-enrichment by transfer of 1.0 mL RV and 1.0 mL TT each into separate tubes containing 10 mL

GN enrichment broth (EM Science, Gibbstown, NJ), with incubation for 6 h at 35°C. The cells were then lysed, and the nucleic acid hybridization reaction was performed, using reagents and dipsticks supplied by the manufacturer, as specified by the package instructions. A GENE-TRAK photometer was used for color development determination and for calculation of the cut-off value.

(d) *Salmonella official methodology*.—Official methods by conventional culture, as specified in the *Bacteriological Analytical Manual* (BAM; 15), were used as standard methods with which rapid detection assays were compared. Samples were pre-enriched in lactose broth for 22–26 h at 35°C. The selective enrichment procedure for “Foods with a High Microbial Load” was used, as follows: 0.1 mL lactose broth pre-enrichment was transferred to 10 mL RV broth and another 1.0 mL was transferred to TT broth, with incubation at 42°C for 22–26 h. The RV and TT selective enrichments were then streaked to xylose lysine desoxycholate (XLD), bismuth sulfite (BS), and Hektoen enteric (HE) agar plates. After incubation of the plates at 35°C for 24–48 h, the presence of typical colonies was recorded. If necessary, colony presence was confirmed in tubes of triple sugar iron agar and lysine iron agar. This procedure was considered sufficient for *Salmonella* confirmation because, taken together, the steps would exclude isolation of all genera of the *Enterobacteriaceae* except *Salmonella* and *Edwardsiella*. Any *Edwardsiella* colonies that may have been present would not have been mistaken morpho-

logically for those of *Salmonella* serovars used as artificial inoculants or present as natural contaminants of the seeds.

Determination of Assay Lower Detection Limits

The 4 *Salmonella* serovars were cultured to mid-exponential phase in TSB + n, BHI, and GN broths. Serial 10-fold dilutions of each were prepared for determining the lower detection limits of *Salmonella* in pure culture in each of the rapid assays. Various dilutions of the cultures were placed on ice until used directly in the assay and tested according to package instructions. The appropriate final enrichment broths for the assays were used as diluents, i.e., TSB + n for the Assurance Gold EIA; BHI broth for the BAX for Screening/*Salmonella*; GN broth for the GENE-TRAK *Salmonella* DLP. Cell numbers were determined from these dilutions by plating onto PCA as described above.

Testing of Artificially Inoculated Samples

Sprouts were produced from 4 different alfalfa seed lots, A, B, C, and D, obtained from commercial suppliers. For each lot, seeds (100 g) were added to glass jars (2 L capacity), sprouted, and stored as described above. On the day of testing, sprouts (25 g) and irrigation water (25 mL) samples were prepared as described above in 225 mL of the various pre-enrichment broths.

The 4 *Salmonella* serovars were each cultured, serial 10-fold dilutions were made in Butterfield's phosphate buffer (BPB; 15), and cell concentrations were determined after plating on PCA. Appropriate dilutions (0.1 mL) of each serovar were added to the pre-enrichment broths to achieve high- and low-level inoculations of ca $\log_{10}$ 2 and 1, respectively. Uninoculated control samples of the sprouts and irrigation waters were also prepared. The enrichment protocols and detection assays were performed as described above for the control and artificially inoculated samples. All assays were initiated on the same day using portions of the same inoculated samples.

Testing of Naturally Contaminated Samples

Sprouts were produced from 3 different alfalfa seed lots, X, Y, and Z, implicated in 3 different salmonellosis outbreaks. For each lot, seeds (200 g) were added to each of 10 glass jars (2 L capacity), sprouted, and stored as described above. On the day of testing, sprouts (25 g) and irrigation water (25 mL) samples from each of the 10 replicate jars were prepared as described above in 225 mL of the various pre-enrichment broths. The enrichment protocols and detection assays were performed as described above. All assays were initiated on the same day using portions of the same samples from the outbreak-associated lots. A pair-wise comparison of results was performed using the McNemar test (16) to compare *Salmonella* detection percentages of the 4 methods for analysis of sprouts and rinse water from the naturally contaminated seed lots.

Results and Discussion

For purposes of discussion, the names of the various assays are abbreviated, as follows: Assurance Gold *Salmonella* EIA,

Gold EIA; BAX for Screening/*Salmonella*, BAX; GENE-TRAK *Salmonella* DLP assay, GENE-TRAK; and *Salmonella* official methodology, BAM.

Lower Detection Limits of Assays

The rapid assays were compared for lower limits of cell detection of 4 outbreak *Salmonella* serovars grown in pure culture (Table 1). The number of cells tested in each assay was computed based on assay sample volumes and plate counting results from individual cultures. Although assays were performed on separate days using separate cell cultures, results could be compared because the 4 serovars grew to similar population levels each time. Each assay demonstrated a consistent lower detection limit at approximately the same $\log_{10}$ CFU level for all 4 *Salmonella* serovars.

Of the 3 rapid assays compared, the BAX demonstrated the lowest limit of detection, approximately $\log_{10}$ 2 CFU. The lower detection limits of the other 2 assays exceeded the BAX by 3–4 log levels. In practical terms, these data indicate that, under the best conditions (i.e., pure cultures in enrichment media prescribed by the assay manufacturers), at least 10^2 CFU are needed for BAX to register a positive result, and at least 10^5 and 10^6 CFU are needed by the GENE-TRAK and Gold EIA, respectively. These comparative results were expected. Detection by the BAX assay is based on exponential amplification of specific DNA regions, of which only a few molecular copies are necessary, theoretically, to obtain a positive signal. The GENE-TRAK had a lower detection limit than the Gold EIA by about 1 log. It is difficult to speculate on the reasons for the differences in detection limit of the GENE-TRAK and Gold EIA assays. The GENE-TRAK assay detects the presence of ribosomal RNA molecules through hybridization to 2 DNA probes: a capture probe bound to a dipstick, and an enzyme-labeled DNA probe, which catalyzes color formation upon addition of a chromogenic substrate. The Gold EIA uses a sandwich format to detect cellular antigens through antibody binding and a chromogenic enzyme-substrate reporter system. The number of target molecules present, probe affinity, and efficiency of the assay format all play a role in influencing lower detection limits.

Assay Performance: Inoculated Sprouts and Irrigation Water Samples

The Gold EIA, BAX, and GENE-TRAK assays were compared with the BAM method for their ability to show as positive the sprouts and irrigation water samples that had been artificially inoculated with *Salmonella* (Table 2). Four different lots of alfalfa seeds were sprouted, and the sprouts and water samples were inoculated at high (68–180) and low (1–2) levels with each of the 4 *Salmonella* serovars. In all, 64 inoculated samples were tested by each assay. Uninoculated control samples from each of the 4 lots were negative by all assays.

The BAM official methodology identified all 64 samples as positive, as did the Gold EIA and the GENE-TRAK assays. The BAX identified 58 out of 64 (90.6%) samples as positive, and showed a higher percentage of detection of positives among water samples (31 of 32, or 96.9%) than the sprout

samples (27 of 32, or 84.4%). This result was surprising, in light of the superior detection limit exhibited by the BAX compared with the other assays (Table 1). At least 2 contributing factors may explain the inferior performance of the BAX: (1) The BAX protocol calls for a short enrichment in nonselective media, whereas the other assays have more extensive enrichment procedures that include selective media. Nonselective enrichment is likely to be less successful in increasing the numbers of *Salmonella* against the high microbial load that is naturally present in sprouts. Therefore, the BAX enrichment protocol could result in fewer *Salmonella* cells in the assay sample volume. Given the detection limit of the BAX (Table 1), the low cell number might not be a problem, except for contributing factor (2): PCR inhibitors that may be present in a sample can limit the efficiency of DNA amplification and compromise the sensitivity of the reaction. Because of the simple BAX enrichment scheme, these PCR inhibitors may not be diluted sufficiently in the reaction. The lower detection rate in the sprouts (84.4%) relative to the irrigation water (96.9%) may also be explained by these 2 contributing factors.

Assay Performance with Naturally Contaminated Sprouts and Irrigation Water Samples

The Gold EIA, BAX, and GENE-TRAK assays also were compared with the BAM method for their ability to detect *Salmonella* in sprouts grown from seeds associated with salmonellosis outbreaks (Table 3). Ten replicate jars of sprouts were produced from each of 3 seed lots incriminated in different outbreaks, and the sprouts and irrigation water from each were assayed by the various methods. Because of the uncertainty of the distribution of *Salmonella* cells in these naturally contaminated samples, the identities of the true positives and true negatives among the samples were uncertain. Based on the number of positive replicates (sprouts or water) as confirmed by the official BAM method, the highest contamination incidence appeared to be in seed lot X (90%), followed by seed lots Y (70%) and Z (20%), respectively. The BAM results indicated that assay of the water samples (18 positives) was somewhat more efficient in detecting positives than was assay of the sprouts samples (15 positives). This was perhaps due to reduction or dilution of interfering substances and/or competing microorganisms that may mask the presence of typical *Salmonella* colonies on confirmatory plates. These data support previous work (11–13), which showed that irrigation water can be a good indicator of the contamination state of sprouts.

Of the 3 rapid assays, the GENE-TRAK results (36 positives) most closely matched those of the official BAM method (33 positives), followed by the Gold EIA (41 positives) and, finally, the BAX (13 positives). There were 3 discrepant results between the GENE-TRAK and BAM assays: seed lot X replicate 3 sprouts, and seed lot Z replicate 1, both sprouts and water. It is likely that the seed lot X discrepant sprouts represented a false negative by the BAM, because the water sample from that same replicate was positive by the BAM. The seed lot Z replicate 1 discrepant was also most likely false negatives by the BAM, because all 3

rapid assays yielded positive results from those samples, and *Salmonella* colonies were actually confirmed from the Gold EIA enrichments of those samples. It is unlikely that subsamples from the same jar of sprouts would be differentially contaminated. Therefore, although the BAM method detected 33 samples as positive, it is likely that the true number of positives among the samples was 36. The GENE-TRAK assay produced positive results for all of these 36 samples.

For 5 of the samples, the Gold EIA produced results that could be interpreted as false positives, as judged by their discrepancies with the BAM official method as well as with the other rapid assays: seed lot Y replicate 4 sprouts; seed lot Y replicate 8 sprouts and water; seed lot Y replicate 9 sprouts; and seed lot Z replicate 9 water. However, there were no confirmed positives that the Gold EIA tested as negative. False-positive results from the Gold EIA were also noted in a previous study of the growth of *Salmonella* during sprouting (11).

Reflecting the results obtained from the inoculated samples (Table 2), the performance of the BAX assay was poor for naturally contaminated samples, as it detected *Salmonella* in <50% of samples shown to be positive by the BAM official method. The pair-wise comparison of results using the McNemar test showed that, for both sprouts and water samples, the BAX method results were significantly different ($p \leq 0.05$) from each of the other methods, but there were no significant differences between the Gold EIA, GENE-TRAK, and BAM method results.

Conclusions

Sprouts and sprout irrigation water are challenging matrices for *Salmonella* detection because of the presence of high populations of indigenous microorganisms. The rapid assays studied here, as well as the BAM official method, varied in their sensitivity and specificity of detection. The field of microbiological methods is a dynamic one, with new developments appearing annually. For example, both the GENE-TRAK and BAX assays have been reformatted, and these next generation systems have been introduced recently into the market (17, 18). It would be useful for method developers to include sprouts and sprout irrigation water in their testing panels, not only for the challenges they would offer to the assays, but also in light of the guidance provided to the sprout industry for pathogen testing (3).

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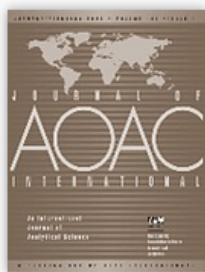
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