

LABORATORY INFORMATION BULLETIN

Detection of *Salmonella* in Alfalfa Sprout Spent Irrigation Water**by Rapid Immunoassays**

BY

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Moffett Center, Summit-Argo, IL² National Center for Food Safety and Technology, Illinois Institute of Technology,
Summit-Argo, IL**ABSTRACT**

Consumption of raw sprouts has been associated with numerous outbreaks of salmonellosis. Sprout producers have been advised by FDA to include microbiological testing of spent irrigation water during production as part of an overall strategy to enhance the safety of sprouts (Fed. Reg. 64[207]: 57893-57902). Two rapid immunoassays, the Assurance Gold *Salmonella* EIA and the *Salmonella* VIP, were compared with the standard method in the Bacteriological Analytical Manual (BAM) for detection of *Salmonella* in artificially inoculated spent irrigation water from sprouts. Spent irrigation water from sprouts grown from four lots of alfalfa seed was artificially inoculated with three outbreak strains of *Salmonella* and tested in four trials. Inoculation levels ranged from 0.6 – 3.6 CFU/ml spent irrigation water. Three enrichment and isolation procedures were followed as specified by each method. In both rapid immunoassays, detection was successful for 100% (66/66) of inoculated samples tested. No false positive results were observed in either rapid immunoassay for 6 uninoculated control samples tested. In the BAM method, detection was successful for 82% (49/60) of inoculated samples tested, with no false positive results. The increased efficiency of the rapid assay methods for recovery of *Salmonella* may have been related to the greater selectivity of their respective enrichment procedures as compared to the BAM method.

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INTRODUCTION

Raw sprouts have become a common source of foodborne illness (13). The causative agents of recent outbreaks have been *Escherichia coli* O157:H7 and most commonly, *Salmonella*. Microbiological surveys of sprouts have shown the presence of other microbial pathogens as well, including *Listeria monocytogenes*, *Staphylococcus*, *Bacillus cereus* and *Aeromonas* (3). During the sprouting process, the natural microbial populations present on the seeds increase to high levels in the sprouts (1, 10, 12). If microbial pathogens are present in the population, they also can develop to high levels (1, 4, 5, 7). Because sprouts are often eaten uncooked, the risk of foodborne illness is substantial, particularly in young children, the elderly, and immunocompromised persons.

Recommendations for reducing the pathogenic potential of sprouted seeds have been issued (8). The Food and Drug Administration has published guidance documents for sprout producers to enhance the safety of the product and has advised testing of each production batch for both *Salmonella* and *E. coli* O157:H7 (14). During production, water is sprayed over the sprouting seeds. FDA has recommended that the spent irrigation water, i.e., the water that has flowed through the sprouts and is collected as run-off from the sprouting equipment, be used as a sample in analysis instead of the sprouts themselves. As it passes through the sprouting seeds, the irrigation water collects microorganisms and provides a representative sample of the microbial population, including pathogens, in the entire batch.

Rapid assays have been increasingly used in food microbiological analysis, and many have been granted official status after collaborative testing through AOAC International. Several rapid immunoassays have been suggested in the sprout testing guidance (14). A comparison of rapid immunoassays for detection of *E. coli* O157:H7 in spent irrigation water from sprouts has been conducted (15). This report compares two rapid assays, the Assurance Gold *Salmonella* EIA and VIP *Salmonella*, with standard methodology found in the *FDA Bacteriological Analytical Manual* (BAM; 2) for detection of *Salmonella* in spent irrigation water.

MATERIALS AND METHODS

Production of sprouts and spent irrigation water

Sprouts were grown from seed in glass jars. Alfalfa seeds (25 g) were added to each jar and soaked in 500 ml sterile tap water for 3 h, then drained and incubated in the dark at 25° C. The sprouting seeds were rinsed once per day by swirling in 500 ml sterile tap water for 10 – 15 sec. After 48 – 72 h of sprouting, the rinse water was collected for analysis (spent irrigation water). Four alfalfa seed lots (A, B, C, D) were sprouted and used in 4 different trials. For two trials (lots A and B), water from duplicate jars of sprouts was combined for testing (1000 ml total). For the other two trials (lots C and D), water from 5 replicate jars of sprouts was combined for testing (2500 ml total).

Bacterial strains

Sprout outbreak-associated strains *S. Cubana*, *S. Muenchen*, and *S. Tennessee* were grown for 18 – 24 h in Brain Heart Infusion broth at 35°C. Cultures were diluted in Butterfield's Phosphate Buffer to approximately 100 CFU/ml for use in inoculations, and plated on Plate Count Agar by the spread plate method for enumeration.

Inoculation and enrichment of spent irrigation water

Two enrichment procedures were performed; one, according to the rapid assay package instructions as specified for "Raw Foods," and the other according to standard methodology in the BAM. For seed lot A and B trials, spent irrigation water (25 ml) was distributed into each of 16 flasks containing 225 ml Buffered Peptone Water supplemented with novobiocin at 18 µg/ml (BPW+n) according to rapid assay instructions and 16 flasks containing 225 ml Lactose broth according to the BAM method. The broths were swirled to mix thoroughly, allowed to stand 60 min at ambient temperature, then swirled and adjusted to pH 6.8 ± 0.2 . Each *Salmonella* strain was inoculated into 5 replicate flasks of each pre-enrichment medium at a concentration approximating 1 CFU/ml. One flask of each pre-enrichment medium was not inoculated for use as a control. All pre-enrichment flasks were incubated for 18 – 26 h at 35°C.

For seed lot C and D trials, in addition to the above procedure, large volume testing of spent irrigation water was performed as recommended in the Guidance document (14); i.e., 375 ml spent irrigation water was distributed into each of 4 flasks containing 3375 ml BPW+n. The above procedure was followed for inoculation (*S. Muenchen* only) and incubation of flasks.

Selective and post-enrichments

Three selective enrichments were performed, as specified by each method. For the Assurance Gold Salmonella EIA, 0.1 ml of the BPW+n enrichment was transferred to a tube containing 10 ml Rappaport-Vassiliadis (RV) broth and another 1 ml was transferred to a tube containing 10 ml Tetrathionate (TT) broth, and the tubes were incubated 5 – 8 h in a 42°C water bath. For the Salmonella VIP, the BPW+n enrichments were transferred to RV and TT broths as indicated above, and the tubes were incubated 18 – 24 h in a 42°C water bath. For the BAM method, selective enrichment was performed as specified for "Raw flesh foods, highly contaminated foods and animal feeds; i.e., 0.1 ml of the Lactose broth enrichment was transferred to a tube containing 10 ml RV broth and another 1 ml was transferred to a tube containing 10 ml TT broth, and the tubes were incubated 22 - 26 h in a 42°C water bath.

Post-enrichments were specified for two methods and performed according to rapid assay instructions. For the Assurance Gold Salmonella EIA, 0.5 ml RV and 1 ml TT selective enrichments were transferred and combined into a single tube containing 10 ml pre-warmed Trypticase Soy broth supplemented with novobiocin at 10 µg/ml (TSB+n), and the tube was incubated for 16 – 20 h in a 42°C water bath. For the Salmonella VIP, 0.5 ml RV and 0.5 ml TT selective enrichments were transferred and combined into a single tube containing 10 ml pre-warmed TSB+n supplemented with 2,4-dinitrophenol at 0.1 µg/ml (TSB+DNP+n) and the tube was incubated for 5 – 8 h in a 42°C water bath. A post-enrichment procedure is not specified in the BAM method.

Rapid assays

For both Assurance Gold Salmonella EIA and Salmonella VIP, the respective post-enrichments were extracted and the assays were performed according to the package instructions. Both rapid assays are products of BioControl Systems, Inc., Bellevue, WA. The EIA is a colorimetric microtiter plate assay, and results may be read either visually or instrumentally. We used an OTC 40 Manual EIA Plate Reader (Organon Teknika Corp., Durham, NC) for reading of the microtiter plate wells and calculation of the cut-off value. The VIP is a one-step assay based on immunoprecipitate formation and visual interpretation of formation of a colored band.

Confirmation

Regardless of rapid assay results, confirmation of the presence of *Salmonella* was performed. All post-enrichment tubes were streaked to Xylose Lysine Desoxycholate (XLD), Hektoen Enteric (HE), and Bismuth Sulfite (BS) agar plates. For the BAM method, all selective enrichment tubes (RV and TT broths) were separately streaked to XLD, HE and BS plates. After incubation of the plates at 35°C for 24 h, randomly selected colonies having morphology typical of *Salmonella* were transferred to Triple Sugar Iron and Lysine Iron agars for confirmation of the presence of *Salmonella* (2). Table 1 compares the various procedural steps in the Assurance Gold Salmonella EIA, the Salmonella VIP and the BAM methods.

Other microbiological analyses

Total aerobic mesophiles (total counts) in spent irrigation water were enumerated after preparing serial dilutions in Butterfield's Phosphate Buffer, plating onto Plate Count Agar by the spread plate method, and incubation of plates at 30°C for 24 – 48 h. Presumptive coliforms in spent irrigation water were determined in a 3-tube most probable number series in Lauryl Tryptose broth by noting gas production after incubation of inoculated tubes for 48 h at 35°C (6).

RESULTS AND DISCUSSION

Table 2 summarizes all of the results of the spent irrigation water analyses for the four alfalfa seed lots. High populations of indigenous microorganisms in sprouts have been noted previously (1, 9, 10, 11, 12), and this report shows that the spent irrigation water also harbors high levels of total counts and presumptive coliforms. A previous study reported total counts and coliforms in spent irrigation water from $1.5 \times 10^6 - 8.0 \times 10^6$ CFU/ml and $7.8 \times 10^5 - 2.7 \times 10^6$ CFU/ml, respectively (15). It is possible that the differences between the results may have been caused by different seed lots, or most likely, by the different sprout production methods used.

Both the Assurance Gold Salmonella EIA and the Salmonella VIP detected *Salmonella* in 100% of the inoculated samples, for all outbreak strains, for all alfalfa seed lots, at both 25- and 375-ml sampling volumes. In these samples, the *Salmonella* inoculation levels varied from 0.6 – 3.6 CFU/ml spent irrigation water. None of the uninoculated control samples were positive in the rapid assays. The Assurance Gold Salmonella EIA was easier to interpret than the Salmonella VIP because the instrument reading and cut-off value calculation provided an objective discrimination between positive and negative samples. The Salmonella VIP was often difficult to interpret because of smearing of the colored bands, and in the case of the negative control samples, the appearance of faintly discernible colored bands, which required subjective interpretation of results.

The BAM method detected *Salmonella* in all inoculated samples of lots B, C and D, but in only 4 of 15 inoculated samples of lot A. The natural microbial flora present in lot A may have inhibited the growth of the inoculated *Salmonella* or perhaps obscured recognition of typical colonies on confirmation plates.

It is possible that the different enrichment procedures (Table 1) were responsible for the increased efficiency of detection and confirmation by the rapid assays compared to the BAM method. Inclusion of novobiocin in the pre-enrichment and the additional post-enrichment steps most likely provided greater selectivity to the rapid assay methods in comparison to the BAM method. In the case of the high microbial populations naturally present in spent irrigation water from sprout production, the additional selectivity may have allowed increased rates of recovery of *Salmonella*. Additional studies are needed to explore this finding.

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Table 1. Comparison of *Salmonella* Enrichment Procedures in the Assurance Gold *Salmonella* EIA, the *Salmonella* VIP and the BAM Methods

Step	Assurance Gold	VIP	BAM
Pre-enrichment	BPW+n, 35°C, 18-26 h	BPW+n, 35°C, 18-26 h	Lactose broth, 35°C, 22-26 h
Selective enrichment	RV + TT, 42°C, 5-8 h	RV + TT, 42°C, 18-24 h	RV + TT, 42°C, 22-26 h
Post-enrichment	TSB+n, 42°C, 16-20 h	TSB+DNP+n, 42°C, 5-8 h	None
Isolation	XLD, BS, HE, 35°C, 24-48 h	XLD, BS, HE, 35°C, 24-48 h	XLD, BS, HE, 35°C, 24-48 h
Confirmation	TSI, LIA, 35°C, 24 h	TSI, LIA, 35°C, 24 h	TSI, LIA, 35°C, 24 h

BPW + n, Buffered peptone water with novobiocin

BS, Bismuth sulfite agar

HE, Hektoen enteric agar

LIA, Lysine iron agar

RV, Rappaport-Vassiliadis broth

TSB + n, Trypticase soy broth with novobiocin

TSB + DNP + n, Trypticase soy broth with 2,4-dinitrophenol and novobiocin

TSI, Triple sugar iron agar

TT, Tetrathionate broth

XLD, Xylose lysine desoxycholate agar

Table 2. Detection of *Salmonella* in Sprout Spent Irrigation Water by the Assurance Gold *Salmonella* EIA, the *Salmonella* VIP and the BAM Methods

Seedlot	Total Counts (CFU/ml)	Presumptive coliforms (MPN/ml)	Strain	Inoculum ^a (CFU/ml)	Method ^b				
					Assurance Gold		VIP		BAM
					Positives ^c	Confirmed ^d	Positives ^c	Confirmed ^d	Confirmed ^d
A	2.2 x 10 ⁸	7.5 x 10 ⁷	Cubana	1.0	5/5	5/5	5/5	5/5	2/5
			Muenchen	1.1	5/5	5/5	5/5	5/5	0/5
			Tennessee	0.9	5/5	5/5	5/5	5/5	2/5
			none	0	0/1	0/1	0/1	0/1	0/1
B	1.1 x 10 ⁷	1.5 x 10 ⁶	Cubana	1.7	5/5	5/5	5/5	5/5	5/5
			Muenchen	1.8	5/5	5/5	5/5	5/5	5/5
			Tennessee	0.8	5/5	5/5	5/5	5/5	5/5
			none	0	0/1	0/1	0/1	0/1	0/1
C	3.35 x 10 ⁸	2.3 x 10 ⁸	Cubana	0.8	5/5	5/5	5/5	5/5	5/5
			Muenchen	0.7	5/5	5/5	5/5	5/5	5/5
			Tennessee	0.6	5/5	5/5	5/5	5/5	5/5
			none	0	0/1	0/1	0/1	0/1	0/1
			Muenchen ^e	0.7	3/3	3/3	3/3	3/3	ND ^f
			none ^e	0	0/1	0/1	0/1	0/1	ND ^f
D	3.6 x 10 ⁸	1.5 x 10 ⁸	Cubana	1.7	5/5	5/5	5/5	5/5	5/5
			Muenchen	3.6	5/5	5/5	5/5	5/5	5/5
			Tennessee	1.8	5/5	5/5	5/5	5/5	5/5
			none	0	0/1	0/1	0/1	0/1	0/1
			Muenchen ^e	3.6	3/3	3/3	3/3	3/3	ND ^f
			none ^e	0	0/1	0/1	0/1	0/1	ND ^f
Total, inoculated				0.6 – 3.6	66/66	66/66	66/66	66/66	49/60
Total, control not inoculated				0	0/6	0/6	0/6	0/6	0/4

^a CFU *Salmonella* per ml sprout water (25 ml sprout water added to 225 ml enrichment broth, except as indicated in (^e)^b Enrichment procedures varied for each method (see next page); regardless of rapid test result (^c), confirmation (^d) was performed by identification of *Salmonella* colonies isolated on selective agars.^c No. positives / total tested^d No. confirmed / total tested^e Large volume samples (375 ml sprout water added to 3375 ml enrichment broth)^f ND, not determined