Detection of Live *Salmonella* sp. Cells in Produce by a TaqMan-Based Quantitative Reverse Transcriptase Real-Time PCR Targeting *invA* mRNA[∇][†]

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Salmonella enterica contamination in foods is a significant concern for public health. When DNA detection methods are used for analysis of foods, one of the major concerns is false-positive results from the detection of dead cells. To circumvent this crucial issue, a TaqMan quantitative real-time RT-PCR (qRT-PCR) assay with an RNA internal control was developed. *invA* RNA standards were used to determine the detection limit of this assay as well as to determine *invA* mRNA levels in mid-exponential-, late-exponential-, and stationary-phase cells. This assay has a detection limit of 40 copies of *invA* mRNA per reaction. The levels of *invA* mRNA in mid-exponential-, late-exponential-, and stationary-phase *S. enterica* cells was approximately 1 copy per 3 CFU, 1 copy per CFU, and 4 copies per 10^3 CFU, respectively. Spinach, tomatoes, jalapeno peppers, and serrano peppers were artificially contaminated with four different *Salmonella* serovars at levels of 10^5 and less than 10 CFU. These foods were analyzed with qRT-PCR and with the FDA's *Bacteriological Analytical Manual Salmonella* culture method (W. A. Andrews and T. S. Hammack, in G. J. Jackson et al., ed., Bacteriological analytical manual online, http://www.cfsan.fda.gov/~ebam/bam-5.html, 2007). Comparable results were obtained by both methods. Only live *Salmonella* cells could be detected by this qRT-PCR assay, thus avoiding the dangers of false-positive results from nonviable cells. False negatives (inhibition of the PCR) were also ruled out through the use of an RNA internal control. This assay allows for the fast and accurate detection of viable *Salmonella* spp. in spinach, tomatoes, and in both jalapeno and serrano peppers.

Salmonella enterica contamination in various foods is a significant public health concern, domestically and internationally (22, 29, 37). *Salmonella* infects millions of people every year, accounting for an estimated 9.7%, 25.6%, and 30.6% of illnesses, hospitalizations, and deaths, respectively, of the total U.S. food-borne diseases caused by known food-borne pathogens (29). Consumption of fresh fruits and produce increased almost 50% between 1970 and 1994 (38). Fresh produce is vulnerable to contamination during the entire "farm-to-fork" process and may have contributed to increases in food-borne outbreaks (1), including recent *Salmonella* outbreaks involving jalapeno peppers (http://www.fda.gov/oc/opacom/hottopics /tomatoes.html).

A standard and accepted method for the detection of *Salmonella* in foods is based on a traditional microbiological method described in Chapter 5 of the *Bacteriological Analytical Manual* (BAM) (2, 17). It consists of a series of steps including nonselective preenrichment, selective enrichment, and selective/differential plating, followed by biochemical and serological confirmation. This method is labor-intensive and requires a minimum of 5 days for complete analysis (18). Hence, the need

exists for the development of faster culture-independent screening and detection methods for this pathogen in produce.

In recent years, a plethora of new molecular methods based on *Salmonella* DNA detection (e.g., *invA* gene) either by conventional or real-time PCR have been developed (23, 27, 41). Real-time PCR (quantitative PCR [qPCR]) is faster and more sensitive than conventional PCR and provides real-time data, avoiding the use of gels as with conventional PCR (39). However, the main drawback with PCR methods is the potential detection of nonviable cells. Bacterial DNA is more stable than bacterial RNA and can persist in a sample long after the target organism has died, potentially leading to the production of false-positive results (8). If an mRNA target is chosen correctly, then it may be more suitable than DNA as a cell viability marker due to its inherent instability. The half-life of most bacterial mRNAs has been reported to range from 0.5 to 50 min (36). This implies that as long as bacteria are viable, they produce some mRNA molecules (e.g., *invA* mRNA, which codes for invasion protein InvA [12]). In *Salmonella enterica* Typhimurium grown in Luria-Bertani (LB) broth, *invA* mRNA was detected throughout the growth curve (24). Furthermore, several studies have shown that the *invA* gene and its mRNA are good candidates for detection of *Salmonella* spp. in environmental samples by qPCR (5, 26, 30, 31, 35, 41), quantitative reverse transcriptase real-time PCR (qRT-PCR) (10), or conventional reverse transcriptase PCR (RT-PCR) (21).

As stated by Fey et al. (10), quantification of RNA targets is a problematic issue in bacteria that can be resolved by designing appropriate RNA standards. The use of DNA standards (PCR products or plasmid or genomic DNA) will result in a

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broad overestimation of the RNA target molecules, largely due to different reaction efficiencies between RT-PCR and PCRs (33). Several specific RNA standards have been developed by in vitro transcription (10, 16). These reports make use of specific RNA standards that are very attractive for accurate quantification of low-level mRNAs (e.g., *invA* mRNA). In particular, the *invA* gene represents a good candidate for *Salmonella* detection as it is present in all pathogenic serovars described to date (6, 34). The product of this gene is essential for the organism's ability to invade mammalian cells and subsequently cause disease (13, 14).

In order to address the crucial issue of detecting viable versus nonviable *Salmonella* cells, a TaqMan qRT-PCR assay targeting *invA* mRNA was developed. A foreign internal RNA control was also incorporated into the assay with the aim of detecting potential inhibitors present in the matrices analyzed. This assay allowed for fast and accurate detection of viable *S. enterica* cells in four matrices (tomato, spinach, jalapeno peppers, and serrano peppers). *Salmonella* sp. detection in foods is usually achieved after food samples are preenriched overnight at 37°C (9). Consequently, the method described herein is intended as an initial screening of 24-h preenrichments for the presence of *Salmonella* in produce. In turn, this method will dramatically decrease the time and effort required during standard microbiological testing since only positive preenrichment samples will be processed.

MATERIALS AND METHODS

Bacterial strains and media. Five strains belonging to four *S. enterica* serovars (serovar Enteritidis strain SE5, serovar Javiana strain 7N, serovar Newport 1240H, and serovar Saintpaul strains 1358 H and 50258), were employed in this study for artificial contamination of produce. These four strains are from the culture collection of the Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Division of Microbiology. Inclusivity of the qPCR assay for *Salmonella* sp. *invA* was demonstrated with 96 *Salmonella* serotypes (110 strains) from FDA's collection (see Table S1 in the supplemental material), and exclusivity was demonstrated with 32 non-*Salmonella* species (48 strains) from very closely related genera (Table 1). Inclusivity is the capability of the assay to detect the intended pathogen target in a wide range of strains belonging to the same bacterial species (i.e., *Salmonella* strains only), and exclusivity is the lack of signal or positive reaction with closely related non-*Salmonella* strains. Only strain SE5 was used to generate the DNA and RNA standards. Strains were grown overnight in LB medium at 35°C with shaking (250 rpm).

Preparation of *Salmonella* inocula. For artificial contamination, overnight cultures (10^9 CFU/ml) of each of the *Salmonella* serovars tested were diluted 100-fold and inoculated into 100 ml of LB medium at 35°C with shaking (250 rpm) for 3 h (optical density at 600 nm [OD_{600}] of 0.5) when cells reached mid-exponential growth phase. Bacteria were harvested (3,090 × *g* for 10 min) and washed twice with 10 ml of Butterfield's phosphate buffer (BPB). *Salmonella* suspensions were serially diluted (10-fold) in BPB. Dilutions containing approximately 10^5 CFU/ml and less than 10 CFU/ml were used for spiking studies. CFU counts were determined by aerobic plate count as described in the BAM (3).

Sample processing and artificial contamination. Bagged spinach, tomatoes, jalapeno, and serrano peppers (see Table 3 for a list) were obtained from local supermarkets in College Park, MD. These matrices were processed as indicated in the BAM (2). On day 1, three different 25-g portions of each food were blended individually with 225 ml of lactose broth (Difco, BD, Sparks, MD) at 10,000 to 12,000 rpm for 2 min (Waring Variable Speed Laboratory Blender; Torrington, CT). The three 25-g portions were designated A for uninoculated, B for high-level (10^5 CFU/ml) inoculation, and C for low-level inoculation (1 to 10 CFU/ml). The corresponding portions (B and C) were inoculated as indicated with *S.* Enteritidis strain SE5 and incubated for 1 h at room temperature. Portion A remained uninoculated. Subsequently, pH was measured in all three portions, and when the pH values differed from 6.8 ± 0.2, the portions were supplemented with 1 N NaOH. These three portions were later incubated at 35 ± 2°C for 24 ± 2 h. Taken together, these steps comprised the preenrichment step.

TABLE 1. Organisms employed to asses the exclusivity of the *invA* qPCR TaqMan assay for *Salmonella* sp. detection[a]

Organism	No. of strains tested
Vibrio parahaemolyticus	4[b]
Vibrio vulnificus	1
Escherichia coli	9[c]
Enterobacter cloacae	1
Enterobacter aerogenes (ATCC 13048)	1
Cronobacter sakazakii (formerly *E. sakazakii*)	1
Yersinia enterocolitica	1
Yersinia pseudotuberculosis	1
Hafnia alvei	2
Morganella morganii	1
Edwardsiella tarda	1
Klebsiella pneumoniae	1
Proteus vulgaris	1
Pseudomonas aeruginosa	1
Serratia marcesans	1
Aeromonas hydrophila	1
Citrobacter freundii	1
Citrobacter koseri (ATCC 27028)	1
Staphylococcus aureus	1
Streptococcus faecalis	1
Bacillus subtilis	1
Bacillus cereus	1
Listeria monocytogenes	1
Listeria innocua	1
Shigella sonnei	2
Shigella flexneri	2
Shigella boydii	2
Shigella dysenteriae	2
Achromobacter spp.	1
Providencia stuartii (ATCC 33672)	1
Proteus mirabilis	1
Proteus hauseri (deposited as *P. vulgaris*) (ATCC 13315)	1
Total	48

[a] Exclusivity was demonstrated by the absence of positive signal for all strains tested; i.e., all *invA* qPCR results were negative.
[b] *V. parahaemolyticus* pandemic strains VpHY145 and JYKVP6 and non-pandemic strains 428/00 and 357–99 (15).
[c] Five *E. coli* classes (virotypes) that cause diarrheal diseases were included: strain 10009 (enterotoxigenic); strains 10010, 10015, 10016, 10017, and 10012 (enteroinvasive); strain 10023 (enterohemorrhagic); strain 10035 (enteropathogenic), and strain ATM395 (enteroaggregative).

After 24 h (day 2), 1 ml was taken from each sample (A, B, and C). Two volumes (2 ml) of RNA Protect reagent (Qiagen, Valencia, CA) was added to each sample and processed as indicated by the manufacturer. This reagent is employed for RNA stabilization and protection in order to avoid the activity of endogenous RNases that can degrade RNA during the storage and extraction procedures. Samples were stored at −70°C until processed for RNA extraction.

Food samples were then analyzed as described in the BAM (2) and as depicted in Fig. 1 in Hammack et al. (18). Briefly, 0.1 ml from the preenrichment culture was added to 10 ml of Rappaport-Vassiliadis (RV) medium, and 1 ml was added to 10 ml of tetrathionate (TT) broth (Difco) and incubated for 24 ± 2 h at 42 ± 0.2°C and 43 ± 0.2°C, respectively (except for tomato samples, where TT broth was incubated at 35 ± 2°C). After 24 h (day 3), tubes were vortexed, and 10-μl portions from TT and RV broths were streaked onto bismuth sulfite, xylose lysine desoxycholate, and Hektoen enteric agars. Plates were incubated at 35 ± 2°C for 24 ± 2 h. On day 4, plates were examined for the presence of typical *Salmonella* colonies. Typical colonies were confirmed as *Salmonella* as specified in the BAM (2).

Nucleic acid extraction. DNA and RNA extractions were performed with the DNeasy and RNeasy Mini Kits, respectively, as recommended by the manufacturer (Qiagen). For RNA extraction, the lysis was performed with 100 μl of lysozyme (1 mg/ml) for 5 min at room temperature. DNase I (Qiagen) treatment was performed for 30 min at room temperature.

TABLE 2. Primers and probes used in this study

Function and target	Primer or probe	Sequence (5′→3′)[a]	Position on gene[b]	Product size (bp)	Accession no.[c]	Reference or source[d]
Generation of standards, *invA*	invA-T7-F2	<u>TAATACGACTCACTATAGGG</u>AACAGTGCTCGTTTACG	1	885	DQ644631	10
	invA-T7-R1	GCAGAGTTCCCATTGAAATGGTC	865			10
PCR						
invA	invA_176F	CAACGTTTCCTGCGGTACTGT	175	116	DQ644631	4
	invA_291R	CCCGAACGTGGCGATAATT	291			4
	invA_Tx_208	TX-CTCTTTCGTCTGGCATTATCGATCAGTACCA-BHQ2	207			4
myIC	dd-myIC-f	CTAACCTTCGTGATGAGCAATCG	1	198	FJ357008	CFSAN/ORS
	dd-myIC-r	GATCAGCTACGTGAGGTCCTAC	198			CFSAN/ORS
	dd-myIC-Cy5	Cy5-AGCTAGTCGATGCACTCCAGTCCTCCT-Iowa BlackRQ-Sp	62			CFSAN/ORS

[a] The sequence corresponding to the T7 promoter is underlined. TX, Texas Red.
[b] Positions on genes are given according to the accession numbers.
[c] Accession number at the NCBI (http://www.ncbi.nih.gov/).
[d] ORS, Office of Regulatory Science.

Design of primers and standards for qPCR. All primers and probes (Table 2) employed in this study were purchased from IDT (Coralville, IA). The target for qPCR and qRT-PCR was the *invA* gene of *S. enterica* serovar Enteritidis SE5 and its corresponding mRNA. Primers and probes for the TaqMan assay were designed using Primer Express, version 1.5, using *invA* gene sequences available at NCBI (Applied Biosystems, Carlsbad, CA) (4). To enable an accurate quantification of the RNA targets for the establishment of the detection limit of the assay, RNA standards for *invA* were generated. The DNA standards for *invA* (865 bp) were generated as previously described (10), except using 60°C as the PCR annealing temperature. Briefly, PCR products generated with primers invA-T7-F2 and invA-T7-R1 were purified with a MinElute PCR Purification Kit (Qiagen) and subsequently transcribed in vitro with T7 polymerase using the Riboprobe System-T7 following the manufacturer's instructions (Promega, Madison, WI). This was followed by digestion with DNase I, purification using RNeasy (Qiagen), and a second DNase I digestion on the purification column. The transcripts were then analyzed by agarose gel electrophoresis (1% gels containing 0.65% formaldehyde) and quantified using a RiboGreen quantitation kit (Invitrogen, Carlsbad, CA). DNA standards were also quantified using a PicoGreen quantification kit (Invitrogen). Both measurements were conducted in a NanoDrop ND-3300 Fluorospectrometer (Thermo Fisher Scientific Inc, Waltham, MA). A second primer set (Table 2) also targeting the *invA* gene but with target sequences located within the sequences recognized by the first set was used to conduct the real-time quantification assays.

Calculation of copy numbers. The numbers of copies of the qPCR and qRT-PCR standards were calculated by assuming average molecular masses of 680 Da for 1 nucleotide of double-stranded DNA and 340 Da for 1 nucleotide of single-stranded RNA. The calculation was done with the following equation: copies per nanogram = $(NL \times 10^{-9})/(n \times mw)$, were n is the length of the standard in base pairs or nucleotides, mw is the molecular weight per nucleotide, and NL is the Avogadro constant (6.02×10^{23} molecules per mol).

Exogenous internal control. An exogenous RNA internal control (myIC) was incorporated into the qRT-PCR assay. The myIC RNA was generated and processed as described above for *invA* RNA standards using as a template a modified pZErO-2 plasmid (Invitrogen) containing an inserted myIC sequence. This sequence is a synthetic construct that does not match any currently available sequence in the GenBank database (http://www.ncbi.nlm.nih.gov/). Modified pZErO-2 containing myIC sequence, primers, and probes for qPCR analysis employed for in vitro transcription were kindly provided by Deanne Deer (FDA/CFSAN) (Table 2). The myIC RNA was serially diluted to a final concentration that gave a positive signal between cycles 15.5 to 16 when the *invA* RNA standards were used for testing. myIC RNA was tested for robustness in qRT-PCR multiplexing reactions. Here, robustness is taken to be a reflection of the performance of the internal control across several reaction conditions (e.g., spinach samples).

***invA* mRNA detection in live and dead *Salmonella* cells.** In order to determine the feasibility of the use of *invA* mRNA as a marker of viability for *Salmonella* cells, dead (autoclaved) and live cells were spiked into 25-g bagged spinach portions. These portions were processed as indicated above (see "Sample processing and artificial contamination" above). In this case, we again employed

three 25-g portions, except that portion C was inoculated with 1 ml of autoclaved *Salmonella* strain SE5 cells (dead cells). One ml of an inoculum containing 10^6 CFU/ml was added to portion B (live cells), while portion A remained uninoculated (*Salmonella* negative control). The procedure for preparing *Salmonella* cells was the same as described above (see "Preparation of *Salmonella* inocula" above), except that the *Salmonella* strain SE5 suspension was diluted 100-fold with BPB to obtain a final concentration of 10^6 CFU/ml rather than 10^5 CFU/ml (total volume, 100 ml). This suspension was separated into two 50-ml portions, one of which was autoclaved for 15 min at 121°C (portion C; dead cells) while the other remained without treatment (portion B; live cells). After 24 h, one ml was taken from each of the three preenrichment portions (A, B, and C) for RNA extraction. RNA extraction was conducted as described above. *invA* qRT-PCR was performed for each RNA sample extracted from each portion by triplicate.

One-step qRT-PCR and data analysis. The qRT-PCRs were carried out using a SuperScript III Platinum One-Step Quantitative RT-PCR System kit according to the specifications of the manufacturer (Invitrogen). This kit consists of SuperScript III RT/Platinum *Taq* Mix, 2× Reaction Mix (a buffer containing a 0.4 mM concentration of each deoxynucleoside triphosphate, and 6 mM MgSO$_4$), 50 mM magnesium sulfate (MgSO$_4$), and ROX (6-carboxy-X-rhodamine) reference dye (25 μM). Reaction mixtures were scaled down to a final volume of 20 μl. Additional MgCl$_2$ was added to the master mix to a final concentration per tube reaction mix of 5 mM. Final concentrations of primers in the qRT-PCR mix were 200 nM and 100 nM for *invA* and myIC, respectively. Both probes were added to a final concentration of 150 nM. qRT-PCR and data analysis were performed on a Rotor-Gene 3000 (Corbett) qPCR machine. qRT-PCR conditions were as follows: an initial cycle of 15 min at 50°C for the generation of the cDNAs; a second cycle of 2 min at 95°C to activate the hot-start *Taq* polymerase; and 40 cycles of denaturation at 95°C for 15 s, primer annealing at 60°C for 15 s (the acquisition of both dyes Cy5 and Texas Red were performed at the end of this cycle), and extension at 72°C for 15 s. In each reverse transcription reaction mixture, the same RNA samples were not supplemented with reverse transcriptase to detect DNA contamination. If the differences between the threshold cycle (C_T) values for qRT-PCR and qPCR were greater than 4 cycles, the result was considered positive since differences in 3.1 to 3.6 cycles between samples were mostly due to differences in concentration of approximately 10-fold (if efficiency is between 90 to 110%) (http://www.stratagene.com/techtoolbox/calc/qpcr_slope_eff.aspx). For C_T values that dipped below this range, a new DNase I treatment was performed. Subsequently, if similar results were obtained below the range reported, the analyzed sample was considered negative for the presence of live *Salmonella* cells. To generate a calibration curve, the serially diluted RNA standard (100 pg to 0.001 fg) was quantified in each qRT-PCR run. The calibration curves were generated by the Rotor-Gene software, version 6.1. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the background or threshold (C_T value). Reaction efficiency was calculated using the slope of the resultant calibration curve (i.e., efficiency = $10^{-1/slope} - 1$). An efficiency of 1.0 indicated a product doubling in each cycle. The number of *invA* mRNA copies was determined using the calibration curve generated by the Rotor-Gene software.

RESULTS

Evaluation of the qPCR and qRT-PCR method. The DNA standards generated by PCR from genomic DNA and the RNA standards generated by in vitro transcription of PCR products were evaluated by both qPCR and qRT-PCR. PCR primers specific for the *invA* gene (invA_176F and invA_291R) were used (Table 2). Linear calibration curves with a correlation coefficient (R^2) of ≥0.99 and linear ranges of ≥8 orders of magnitude for both *invA* DNA and RNA were obtained (see Fig. S1 in the supplemental material). This corresponds to detection limits of 10 and 40 copies for *invA* DNA and RNA, respectively. The efficiency of the qPCR and qRT-PCR ranged from 0.93 to 0.99 and from 0.90 to 0.96, respectively. For the purposes of this study, only qRT-PCR was employed from this point forward (e.g., detection of live cells). In all qRT-PCR assays, the same RNA samples were amplified by PCR to test for DNA contamination. Most of the samples resulted in no amplification, and in some samples a very high C_T value was obtained, indicative of the presence of small amounts of DNA contamination (e.g., in the standards containing 10^9 and 10^8 *invA* RNA copies per reaction mixture, the contaminating DNA was estimated to be 10^3 to 10^2, respectively). These data indicated that DNA contamination was negligible throughout this study.

Specificity of the *invA* qPCR TaqMan assay. Supplemental Table S1 shows a panel of 110 *Salmonella* strains (96 serotypes) used to test for inclusivity of this assay (*invA* qPCR) using the primer set invA_176F and invA_291R. *Salmonella* sp. strains analyzed encompassed five of the six *S. enterica* subspecies (*S. enterica* subsp. I, 89 serovars; *S. enterica* subsp. II, 3; *S. enterica* subsp. IIIa, 1; *S. enterica* subsp. IIIb, 1; and *S. enterica* subsp. IV, 1) and one *Salmonella bongori* strain. These strains represented a broad spectrum of *Salmonella* serovars, including many of the serotypes most frequently isolated in United States from 1968 to 1998, according to the CDC (7, 29). All *Salmonella* strains were identified correctly (100% inclusivity). The 48 non-*Salmonella* strains employed in the exclusivity tests did not give any positive signal (Table 1). These strains were chosen for exclusivity testing because many are close phylogenetic kin to the *Salmonella* and are known to associate with the food supply.

Evaluation of the RNA internal control. An exogenous RNA internal control, designated myIC, that could be used to measured false-negative PCR results was generated by in vitro transcription. This myIC RNA molecule was generated from a pZErO-2 plasmid (Invitrogen) containing a DNA sequence (myIC DNA) that can be used as an internal control in DNA TaqMan assays (D. Deer, unpublished data). This plasmid also contains a T7 promoter close to the myIC sequence that allowed its in vitro transcription. The primers and probe (labeled with Cy5) employed for the amplification of myIC by TaqMan assay are described in Table 2. myIC was evaluated combined with the *invA* RNA qRT-PCR as a duplex TaqMan PCR assay to assess its performance across several reaction conditions. Figure 1 shows the result of the amplification by the duplex assay (*invA* qRT-PCR) of RNA samples extracted from 1 ml of preenrichments of bagged spinach portions (25 g) that were artificially contaminated with different levels of *Salmonella* (2 CFU to 10^5 CFU). All samples tested were positive for *invA* mRNA, and bagged spinach preenrichments did not inhibit the PCR. The internal control (myIC) showed approximately the same C_T values for all samples analyzed.

***invA* mRNA quantification in pure cultures of *Salmonella*.** The presence of specific *invA* mRNA was determined in *Salmonella* strain SE5 mid-exponential-phase cells (OD of 0.418), late-exponential-phase cells (OD of 1.3), and stationary-phase cells (overnight culture). The numbers of *invA* mRNA molecules per CFU averaged 0.4, 1, and 0.004 copies/CFU in mid-exponential-, late-exponential-, and stationary-phase cells, respectively. It is important to note that nonhost environments are often stressful habitats for *Salmonella* and could seriously impair the biological activity of the *Salmonella* cells, potentially resulting in depressed transcription and hence a low copy number of mRNA molecules. Under such conditions, the efficiency of an assay based on the detection of mRNA as a sign of cell viability could be limited. Thus, a preenrichment step is essential to allow *Salmonella* present in the sample to increase in biomass and express de novo *invA* mRNA that will allow for its subsequent detection using this method. Accordingly, our results showed that *invA* mRNA was present in large enough quantities to be detected by the TaqMan assay, pointing to its reliability as a target for qRT-PCR detection.

***invA* mRNA detection in bagged spinach preenrichments previously spiked with live and dead *Salmonella* cells.** Twenty-four-hour sample preenrichments previously spiked with live and dead cells were analyzed for the presence of *invA* mRNA by qRT-PCR. The bagged spinach preenrichment, previously spiked with live *Salmonella* cells, was the only sample to result in a positive reaction by *invA* qRT-PCR (data not shown). These findings provided indisputable evidence that *invA* mRNA is a good target for detection of live *Salmonella* in this produce commodity.

Application of the qRT-PCR duplex assay to *Salmonella* detection in different produce commodities. In order to assess the performance of the qRT-PCR detection assay developed for this study, four different produce commodities were artificially contaminated with five different *S. enterica* strains (four serovars) at levels of 10^6 and less than 10 CFU/25 g (Table 3). After 24 h, the preenrichments were used for detection of *Salmonella* using both the qRT-PCR assay and the BAM *Salmonella* culture method (18). Uninoculated samples from each commodity were used as negative controls. RNA was extracted from the preenrichments and used for *invA* qRT-PCR amplification in triplicate. All artificially contaminated samples were positive for *Salmonella* using both qRT-PCR and BAM methodologies (Table 3). Higher levels of inoculation were also positive for *Salmonella* by both methods (results not shown). Only lower inoculation levels are shown in order to highlight the power of this technique. *Salmonella* levels as low as 2 CFU/25 g were detected after preenrichment (24 ± 2 h). Absence of RT-PCR inhibitors was demonstrated by amplification of the RNA internal control since the RNA control would not have been amplified had there been PCR inhibitors present in the foods (Fig. 1B).
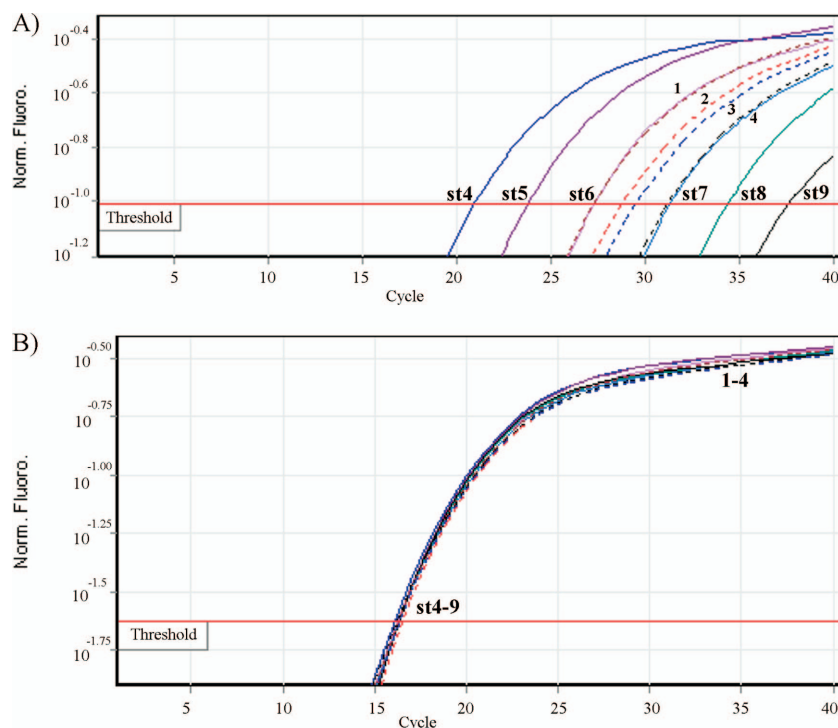
FIG. 1. Robustness of the RNA internal control amplification in multiplexing reactions (*invA* qRT-PCR). Multiplex qRT-PCRs of *invA* RNA and the RNA internal control in bagged spinach samples artificially contaminated with different levels of *Salmonella* were performed. Solid lines are the *invA* RNA standards (st) that are identified in terms of the number of *invA* RNA copies per reaction mixture: st4, 1 pg (4×10^6); st5, 100 fg (4×10^5); st6, 10 fg (4×10^4); st7, 1 fg (4×10^3); st8, 0.1 fg (4×10^2); and st9, 0.01 fg (40). Dotted lines are samples inoculated with different levels of *Salmonella* as follows: strain SE5 at 10^5 CFU/25 g (line 1) and 8 CFU/25 g (line 2); strain Javiana 7N at 10^5 CFU/25 g (line 3) and 2 CFU/25 g (line 4). (A) qRT-PCR results for the *invA* RNA (ROX channel) amplification. (B) qRT-PCR results for the RNA internal control (Cy5 channel) included in the same RNA samples. Norm fluoro, normalized fluorescence.

DISCUSSION

Numerous molecular assays have been developed for the detection of *Salmonella* in different matrices (8, 10, 23, 28, 32). Some of these are based on conventional PCR, and some others are based on qPCR technology, whether using SYBR green I or molecular probes (e.g., TaqMan). The main drawback of these methodologies is that they rely on the detection of DNA (8). DNA from dead cells can be detected, but FDA cannot take regulatory action unless the cells are shown to be viable. Thus, detection of DNA is not enough, and the FDA must obtain an isolate to take regulatory action. However, fulfillment of

this requirement takes approximately 1 week and is somewhat laborious. This problem may be circumvented with the RNA detection technology described in this study (10, 16).

A novel qRT-PCR assay was developed that uses specific primers for the detection of *invA* mRNA (product of *invA* gene) of *Salmonella* spp. with TaqMan probes. This assay also includes an RNA internal control to detect potential PCR inhibitors that may be present in food samples. We also determined the number of *invA* mRNA molecules per CFU in different *Salmonella* strain SE5 growth stages and found that at least 1 copy of *invA* mRNA was present in every CFU in

TABLE 3. *S. enterica* detection by *invA* RNA qRT-PCR and BAM culture method in artificially contaminated produce commodities

Food sample	Strain	Inoculation level (CFU/25 g)	*Salmonella* sp. detection by the indicated method		DNA detection by qPCR (C_T)[a,b]
			qRT-PCR (C_T)[a]	BAM	
Bagged spinach	*S.* Enteritidis SE5	8	+ (27.6)	+	+ (37.9)
	S. Javiana 7N	2	+ (28.4)	+	−
Tomatoes	*S.* Enteritidis SE5	4	+ (28.7)	+	+ (37.3)
	S. Javiana 7N	4	+ (30.1)	+	−
Jalapeno peppers	*S.* Saintpaul 1358 H	10	+ (33.5)	+	−
	S. Newport 1240H	3	+ (34.2)	+	−
Serrano peppers	*S.* Saintpaul 50258	5	+ (33.9)	+	−
	S. Newport 1240H	3	+ (35.0)	+	−

[a] C_T values are given where the fluorescence signal was higher than the background.
[b] The same RNA samples were amplified by qPCR to test for DNA contamination.

exponential growth. These levels of expression per CFU are similar to what was reported for *S. enterica* serovar Typhimurium strain ATCC 14028 (a maximum of 10 copies per CFU at exponential growth) (10). In our case, considering RNA losses that can occur during the RNA extraction procedure and DNase I treatment, we can estimate that the number of *invA* mRNA molecules per cell could be between 1 and 10 copies. Thus, a total population of approximately 10^8 CFU/ml would produce approximately 10^8 to 10^9 copies of *invA* mRNA/ml. The volume from each of the analyzed samples was 1 ml, and, after RNA extraction, the amount added to each reaction mixture was equivalent to 28 μl of the initial volume. *invA* mRNA amounts contained within this 28 μl were approximately equivalent to 10^6 copies, a value well above the detection limit of the qRT-PCR, which was 40 *invA* mRNA copies. Therefore, the selected specific RNA could be used as molecular targets for either identification and/or determination of metabolic activity of *Salmonella* cells in foods as well as environmental samples using the developed qRT-PCR method described herein.

It is important to note, however, that in non-host environments, *Salmonella* persists most likely in a starved and highly stressed state. As an example, we found that in stationary phase 4 out of 1,000 cells will actually contain *invA* mRNA, indicating that this mRNA in a non-host environment was negligible and most likely would go undetected using this methodology. However, the addition of a requisite preenrichment step in the culture medium substantially increases cell number and subsequent mRNA production accordingly. Thus, a preenrichment culture provides an essential preliminary step in the application of this assay to the reliable detection of *Salmonella invA* mRNA from the surface of various produce commodities.

It has become increasingly evident that there is a need for internal controls for PCR to rule out the presence of PCR inhibitors that can cause false-negative results from *Salmonella*-positive samples (19, 20). For this reason, we developed and included an exogenous RNA internal control. The inclusion of this internal control did not affect either the amplification or the detection limit of the qRT-PCR assay.

Numerous studies, performed to establish the use of RNA as proof of viability, employed heating (either autoclaving or boiling) of the cells (11). Some reports suggested that *invA* mRNA degrades rapidly in *S.* Typhimurium ATCC 14028 from 10 copies to less than 1 copy per CFU after 50 h of incubation in drinking and pond water (10). Also, Jacobsen and Hoben (21) showed that the spiking of soil manure with *Salmonella* cells followed by pasteurizing the samples at 65°C for 30 min resulted in a failure to detect *invA* mRNA by RT-PCR. However, *invA* DNA was still detectable, indicating that mRNA was being degraded rapidly. We performed a similar experiment where we spiked dead and live *Salmonella* cells into bagged spinach and tested the respective preenrichments for the presence of *invA* mRNA. Only the bagged spinach preenrichment, spiked with live cells, gave a positive signal by the qRT-PCR assay reported here. This clearly showed that the qRT-PCR assay, based on *invA* mRNA detection, has great potential to be used as a viability marker for *Salmonella* cells in food commodities.

This study reports a qRT-PCR TaqMan assay that allowed

the fast and accurate detection of viable *Salmonella* cells in spinach, tomato, jalapeno peppers, and serrano peppers. The assay performed comparably to the traditional BAM *Salmonella* culture. It also is noteworthy that the method reported here performed optimally relative to several other PCR-based methods that have been recently shown to detect *Salmonella* using *invA* targets (5, 9, 25, 26, 28). DNA-based methods have the intrinsic limitation of detecting DNA from both dead and live cells, making the outcome suspect (5). Moreover, conventional PCR, while providing a target amplicon with relative ease and little expense, is incapable of revealing identity (i.e., primary sequence structure), thus failing to ensure the nature of the PCR product (25, 34). In addition, the use of a TaqMan assay had several advantages over the use of the SYBR green I assay, including greater sensitivity and greater sequence-specific hybridization that allowed for verification of PCR product identity (10, 21, 40). Our method also revealed substantial promise for detecting specific serovars of *S. enterica*—a claim unable to be guaranteed by previously reported *invA*-based methodologies (25, 27). Ready-to-use mixtures, such as the one used in this study, facilitate the performance of the assay while being both straightforward and reproducible.

It is noteworthy that, rather than performing replicates of several inoculations using the same strain, we opted to spike the same four produce matrices presented in Table 3 with two different *Salmonella* serovars, thereby offering a more powerful approach than simply repeating the experiment with the same strain multiple times. After all, the ultimate goal of the assay is to detect numerous disparate serovars, not simply *S.* Enteritidis alone, for example. Thus, increasing the biocomplexity of the test provided a more rigorous challenge to the capability of the method to detect *Salmonella* in general. Here, a move toward this end was taken by including a total of four different serovars comprising five distinct strains across the four produce types tested.

In conclusion, we have developed a method that has the potential to be used as an initial screening of the preenrichment cultures and as a preliminary proof of viability for *Salmonella* without precluding the need for the BAM method, which is necessary to yield a physical isolate for regulatory reasons. This assay will reduce the overall time and resources expended in the laboratory since only positive samples containing viable cells will be processed after the preenrichment step. Expenses associated with the assay were comparable to other previously reported methods at around $7 per sample, making the assay widely affordable for routine analysis. Despite this relative ease of performance and cost, it is important that future studies assess the use of this assay in additional food matrices. Also, in order for this method to be applied extensively, collaborative studies should be conducted to assess the interlaboratory reproducibility of this much-needed RNA-based assay.

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