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Real-Time PCR Method for Detection of *Salmonella* spp. in Environmental Samples

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ABSTRACT The methods currently used for detecting *Salmonella* in environmental samples require 2 days to produce results and have limited sensitivity. Here, we describe the development and validation of a real-time PCR *Salmonella* screening method that produces results in 18 to 24 h. Primers and probes specific to the gene *invA*, group D, and *Salmonella enterica* serovar Enteritidis organisms were designed and evaluated for inclusivity and exclusivity using a panel of 329 *Salmonella* isolates representing 126 serovars and 22 non-*Salmonella* organisms. The *invA*- and group D-specific sets identified all the isolates accurately. The PCR method had 100% inclusivity and detected 1 to 2 copies of *Salmonella* DNA per reaction. Primers specific for *Salmonella*-differentiating fragment 1 (Sdf-1) in conjunction with the group D set had 100% inclusivity for 32 *S.* Enteritidis isolates and 100% exclusivity for the 297 non-Enteritidis *Salmonella* isolates. Single-laboratory validation performed on 1,741 environmental samples demonstrated that the PCR method detected 55% more positives than the Vitek immunodiagnostic assay system (VIDAS) method. The PCR results correlated well with the culture results, and the method did not report any false-negative results. The receiver operating characteristic (ROC) analysis documented excellent agreement between the results from the culture and PCR methods (area under the curve, 0.90; 95% confidence interval of 0.76 to 1.0) confirming the validity of the PCR method.

IMPORTANCE This validated PCR method detects 55% more positives for *Salmonella* in half the time required for the reference method, VIDAS. The validated PCR method will help to strengthen public health efforts through rapid screening of *Salmonella* spp. in environmental samples.

KEYWORDS *S.* Enteritidis, environmental samples, *Salmonella*, validation of PCR method, real-time PCR

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Salmonella is one of the major causes of foodborne illnesses, with the U.S. Centers for Disease Control and Prevention (CDC) estimating that ~1.2 million cases of salmonellosis occur annually in the United States alone. *Salmonella* infections cause severe illness in infants, the elderly, and immunocompromised individuals. Most infections in humans result from eating contaminated food or drinking contaminated water. Sources of *Salmonella* infections include foods of animal origin, dairy products, pet food, fresh produce, and foods contaminated during processing. Moreover, *Salmonella* outbreaks following the consumption of eggs contaminated with *Salmonella enterica* serovar Enteritidis are relatively common (1).

The expeditious detection of *Salmonella* species in food and environmental samples requires rapid, efficient, and validated methods. A number of conventional and real-time PCR methods have been reported for the detection of *Salmonella* in naturally or artificially contaminated food samples (2–9) and fecal samples (10). A few validation

studies have been reported for food samples that were spiked with *Salmonella* (8, 9, 11, 12, 42), but no validation studies have been reported for environmental samples. The primer sets that have been used for the amplification of *Salmonella* DNA by different methods differed in their detection limits, lacked disclosure of the internal amplification control (IAC) DNA sequence used (8, 9, 11–14), or did not include a wide range of epidemiologically important isolates. In addition, the matrices and levels of contaminating competing organisms present in each environmental sample appeared to be unique, making the validation of methods for screening environmental samples a challenge.

The presence of high concentrations of PCR inhibitors in samples (15, 16) and the lack of adequate validation studies appear to be major factors underlying the lack of use of rapid PCR methods for routine screening. Hence, most public health organizations and commercial food-processing firms use an immunological screening method, the Vitek immunodiagnostic assay system (VIDAS), for detecting *Salmonella* in environmental samples and report presumptive results after 48 h (17). Since the detection of *Salmonella* by VIDAS requires thousands of target organisms in test samples (17), the probability of missing potentially positive samples is relatively high. A recent study reported that VIDAS failed to detect the presence of *Salmonella* in a number of spiked samples, although the *Salmonella* sp. used to spike the samples was in fact isolated from those samples (18). Notably, nonspiked VIDAS-negative samples are not usually tested by the culture method. Furthermore, *Salmonella* isolates were not recovered from a number of VIDAS-positive samples, because the VIDAS reagent appeared to react with O-antigen epitopes of *Enterobacter cloacae*, *Escherichia coli*, etc., present in the samples (19). Together, these data indicate the need for the development of alternate rapid, sensitive, and reliable screening methods.

Several PCR methods, both conventional and real time, have been reported for the identification of the O groups A, B, C1, C2, D, and E (20, 21, 41) and *Salmonella enterica* serovars, such as Enteritidis, Typhimurium, Typhi, Paratyphi A, and Paratyphi B (20, 22–24). A multiplex bead-based suspension array method has also been reported for the determination of common O groups of *Salmonella* isolates (25). All these methods require *Salmonella* isolates as the starting material for such identification. At present, there is no rapid molecular method for the direct screening of *Salmonella* or the *S.* Enteritidis serovar in environmental samples.

This study reports the development of a real-time PCR method that detected 126 epidemiologically important serovars belonging to all subspecies of *Salmonella enterica*. An IAC was included in the PCR assays to detect the presence of PCR inhibitors and reduce false-negative results (15, 16). The method reported herein was designed to be selective and did not detect organisms that were cross-reactive in antibody-based assays. The results obtained from screening 1,741 environmental samples were compared with those obtained with the reference method, VIDAS (17, 26). All samples that were positive based on either screening method were followed up using the culture method, as described in the *Bacteriological Analytical Manual* (27). The results demonstrate that the new PCR method described here is sensitive, specific, and reliable for screening environmental samples in less than half the time required for the standard VIDAS method. This method could be a valuable tool for the detection of *S.* Enteritidis directly in environmental samples collected from poultry farms.

RESULTS

Limits of detection. The sensitivity of the PCR method was determined using purified positive-control DNA from *S.* Enteritidis at concentrations ranging from 1 to 10^5 copies per reaction in quadruplicate reactions. The results from a representative assay (Table 1) demonstrated that the *invA* primer-probe set detected the presence of a single copy of *Salmonella* genomic DNA with a probability of 50%, whereas two or more copies of the genome were detectable with a probability of 100%. The assay was equally sensitive for detecting *Salmonella* group D and *Salmonella* Enteritidis targets. The logarithm of the number of *Salmonella* DNA copies in a sample correlated ex-

TABLE 1 Sensitivity of real-time PCR[a]

No. of DNA copies	*invA* C_T value[b]				Mean ± SD *invA* C_T value
	Expt 1	Expt 2	Expt 3	Expt 4	
10^5	22.48	23.29	23.14	23.16	23.02 ± 0.37
10^4	26.63	26.74	26.77	26.90	26.76 ± 0.11
10^3	29.74	29.79	29.56	29.50	29.65 ± 0.14
10^2	33.31	33.06	32.35	32.99	32.93 ± 0.41
10	36.29	35.45	36.11	36.46	35.83 ± 0.44
2	37.73	36.53	36.27	36.33	36.72 ± 0.69
1	38.26	Neg	Neg	38.70	38.48 ± 0.31

[a]The lower limit of detection by the PCR method was determined by testing various concentrations of *Salmonella* Enteritidis DNA, ranging from 1 to 10^5 copies per reaction. The assay was performed in quadruplicate.

[b]The C_T values were obtained from the 4 assays and represent the number of PCR cycles at which the fluorescence generated crossed the minimum threshold set for the assay. Neg, negative (no C_T value was displayed).

tremely well with the *invA* threshold cycle (C_T) value that represented the DNA copy number in the PCR method (Pearson correlation $r = -0.99$). The correlation was linear over the entire range of DNA concentrations analyzed (from a single copy to 10^5 copies), suggesting that the PCR method can be used to quantify the number of *Salmonella* cells present in samples.

Validation of the PCR method. The exclusivity of the primers and probes used in the multiplex PCR was confirmed by testing 10^5 copies of DNA from each of the 22 non-*Salmonella* target organisms (Table 2). The inclusivity of the primers and probes used was first confirmed by testing a panel of 12 control *Salmonella* organisms and then extended to testing a random panel of 329 *Salmonella* isolates obtained from food and environmental samples. The results demonstrated that the *Salmonella*-specific *invA* primer-probe set identified all 329 *Salmonella* isolates that contained 126 serovars

TABLE 2 Exclusivity of PCR primers and probes was confirmed by testing 10^5 copies of purified DNA from each of the 22 non-*Salmonella* target organisms in the multiplex real-time PCR

Control organism	Result by PCR target[a]			
	invA	*prt*	Sdf-1	IAC
Escherichia coli (ATCC 8759)	Neg	Neg	Neg	Pos
E. coli (ATCC 43888)	Neg	Neg	Neg	Pos
E. coli (ATCC 43895)	Neg	Neg	Neg	Pos
E. coli (ATCC 6209)	Neg	Neg	Neg	Pos
E. coli (ATCC 6210)	Neg	Neg	Neg	Pos
Shigella flexneri (ATCC 29903)	Neg	Neg	Neg	Pos
Cronobacter sakazakii (ATCC 12868)	Neg	Neg	Neg	Pos
Enterobacter aerogenes (ATCC 13048)	Neg	Neg	Neg	Pos
Enterobacter cloacae (lab isolate)	Neg	Neg	Neg	Pos
Citrobacter freundii (lab isolate)	Neg	Neg	Neg	Pos
Klebsiella pneumoniae (ATCC 13883)	Neg	Neg	Neg	Pos
Proteus hauseri (ATCC 13315)	Neg	Neg	Neg	Pos
Pseudomonas aeruginosa (ATCC 9027)	Neg	Neg	Neg	Pos
Pantoea sp. (lab isolate)	Neg	Neg	Neg	Pos
Vibrio cholerae (ATCC 35971)	Neg	Neg	Neg	Pos
Yersinia enterocolitica (ATCC 23715)	Neg	Neg	Neg	Pos
Acinetobacter sp. (lab isolate)	Neg	Neg	Neg	Pos
Bacillus subtilis (ATCC 6633)	Neg	Neg	Neg	Pos
Staphylococcus aureus (ATCC 6538)	Neg	Neg	Neg	Pos
Rhodococcus equi (ATCC 6939)	Neg	Neg	Neg	Pos
Listeria monocytogenes (ATCC 19115)	Neg	Neg	Neg	Pos
Listeria innocua (ATCC 33090)	Neg	Neg	Neg	Pos
No-template control	Neg	Neg	Neg	Pos
S. Enteritidis (ATCC 13076)	Pos	Pos	Pos	Pos

[a]The results shown here were obtained after 40 cycles of PCR. Pos, positive (displayed a C_T value in the PCR); Neg, negative (displayed no C_T value in the PCR).

TABLE 3 Summary of *Salmonella* isolates tested for the inclusivity PCR[a]

O group	No. of isolates tested	No. of serovars per O group
35	1	1
39	2	1
51	3	1
61	3	2
B	47	14
C1	61	21
C2	35	10
D1	54	10
D2	3	2
E1	40	8
E2	2	1
E4	9	2
F	4	3
G1	3	3
G2	4	4
H	4	4
I	14	8
J	1	1
K	2	1
L	3	2
M	8	6
N	2	2
O	1	1
P	5	2
Q	2	2
R	1	1
S	2	2
T	2	2
U	4	2
V	2	2
W	1	1
X	2	2
Y	1	1
Z	1	1
Total	329	126

[a]The panel included 126 serovars from 34 O groups. Fifty-seven isolates were from the D1 and D2 O groups, of which 32 were *S.* Enteritidis.

belonging to all subspecies of *S. enterica* (Table 3). Similarly, the group D-specific primers and probe (*prt*) identified all 57 group D isolates in the panel (contained 12 distinct serovars of D1 and D2 groups) and did not react with the remaining 272 isolates that were not group D. The *tyv* primer and probe set gave results similar to those for the *prt* primer-probe set, thus establishing that either the *prt* or *tyv* primer-probe set could be used for identifying group D serovars. Notably, both the *prt*- and *tyv*-specific reagent sets recognized all serovars that contained the antigenic factor O-9, including those isolates that were identified as the antigenic formula and listed under group D by the WHO Collaborating Centre for Reference and Research on *Salmonella* (28). The *S.* Enteritidis-specific Sdf-1 primer-probe set recognized all 32 *S.* Enteritidis serovars included in the panel. However, the Sdf-1 primer-probe set reacted with one out of 297 non-Enteritidis *Salmonella* isolates, thus exhibiting an exclusivity of 99.7% for non-Enteritidis *Salmonella* serovars.

PCR, VIDAS, and culture results of environmental samples. A total of 1,741 environmental samples collected from 14 different food manufacturing companies were screened by PCR and VIDAS assays, and culture analyses were performed on all samples that were positive with either the VIDAS or PCR. Of 1,741 samples tested, the reference method, VIDAS, detected *Salmonella* in 12 samples, PCR detected *Salmonella* in 11 samples, and the culture method found *Salmonella* in 9 samples (Table 4). None of the environmental samples tested showed an inhibitory effect in PCR, as evidenced by the PCR results of the IAC. Of the 12 VIDAS-positive samples, *Salmonella* was found

TABLE 4 Comparison of results obtained from the PCR, VIDAS, and culture methods[a]

Source (no. of samples)	PCR result or C_T value	VIDAS result	Culture result[b]
Cheese product plant (132)	22.49	Negative	Positive
	38.13	Negative	Positive
	37.79	Negative	Negative
Cheese product company (140)	25.75	Negative	Positive
	26.31	Positive	Positive
	27.25	Positive	Positive
	28.84	Positive	Positive
	31.26	Positive	Positive
	34.08	Negative	Positive
	34.40	Negative	Positive
	Negative	Positive	Negative
Whey powder company (154)	All negative	7 positives	7 of 7 negative
6 food product companies (686)	36.64	All negative	1 of 1 negative
3 dairy product companies (309)	All negative	All negative	Not performed
2 food and cheese companies (320)	All negative	All negative	Not performed
Total (1,741)	11 positive	12 positive	9 positive

[a]A total of 1,741 environmental samples from 14 food-processing facilities were screened by PCR and VIDAS methods. Culture analyses were performed for a total of 19 samples that were positive according to the PCR or VIDAS method. *Salmonella* isolates were obtained from 9 of 11 PCR-positive samples and 4 of 12 VIDAS-positive samples. VIDAS failed to detect *Salmonella* in 5 culture-positive samples.
[b]Culture analyses were performed for all samples that were positive with PCR or VIDAS screening.

by the culture method in only 4 samples. In contrast, *Salmonella* was isolated from 9 of 11 PCR-positive samples. Notably, the VIDAS method failed to detect the presence of *Salmonella* in 5 samples that were positive by the culture method. Most public health and regulatory organizations perform culture analyses on all presumptive positives found by the screening method, and no culture analysis is generally performed on samples that were reported as negative by the screening method. In summary, the PCR method is sensitive and specific, detected 55% more culture-positive samples than the VIDAS method, and reported no false negatives. There was an excellent correlation between the culture method and the PCR method.

The ROC analysis of the agreement of the PCR method with the culture method produced an AUC of 0.90, with a 95% confidence interval between 0.76 and 1.0 (Fig. 1A). The Cohen's kappa of this agreement (29) was 0.79 $\pm$ 0.13 (standard error of the mean [SEM]), and the empirical probability, as described in Materials and Methods, of

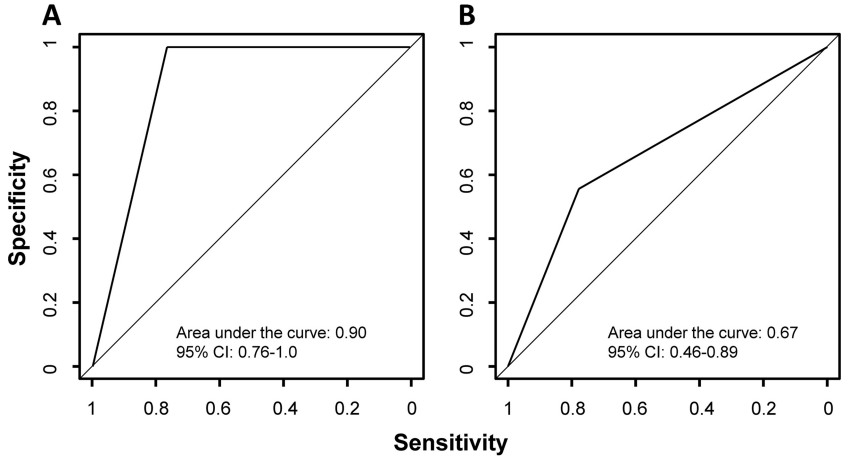

FIG 1 (A) ROC analysis of the agreement between the PCR method and culture method (gold standard). The AUC of 0.90 signifies excellent agreement between the two methods. (B) ROC analysis of the agreement between the VIDAS method and culture method (gold standard). The AUC of 0.67 signifies modest agreement between the two methods, whereas the confidence interval (CI) indicates high signal-to-noise ratio.

this level of agreement being reached by chance was 0.0013. These results establish statistically robust (significant) agreement between the new PCR method and the culture method, which has been used as the gold standard for this comparison (Fig. 1A). Moreover, the same ROC analysis of the agreement between the VIDAS results and the culture method results yielded an AUC of 0.67, with a 95% confidence interval between 0.46 and 0.89 (Fig. 1B). The Cohen's kappa for the VIDAS versus culture agreement could not be clearly established, owing to a very high likelihood that the agreement was produced by chance. The empirical *P* value for this agreement being reached by pure chance was 0.9, as described in Materials and Methods. Taken together, these observations suggest that the new PCR method significantly outperforms the reference VIDAS screening method for detecting *Salmonella* in environmental samples.

DISCUSSION

The PCR results for 329 *Salmonella* isolates and the 22 non-*Salmonella* environmental organisms confirmed the specificities of the *invA*, *prt*, *tyv*, and Sdf-1 primer-probe sets used in the assay. The *invA* reagent detected all 6 subspecies of *Salmonella enterica*. The majority of the *Salmonella* spp. isolated from the environmental samples belonged to the subspecies *enterica*, and only a few isolates were from the other 5 subspecies of *S. enterica*. The *prt* and *tyv* target-specific reagents detected all the 57 group D-positive isolates (12 distinct serovars) that shared the antigenic factor O-9, whereas the other 272 isolates of 114 serovars from the 32 O groups were negative, thus confirming the absence of the shared antigenic factor O-9 in those isolates. The Sdf-1-specific set recognized all *S.* Enteritidis isolates, as expected. Surprisingly, this reagent also detected a non-Enteritidis *Salmonella* isolate from a thyme sample. But, this isolate did not contain the O-9-specific sequence that was common to all group D serovars and has been identified as *S. enterica* serovar Montevideo of group C1. This result demonstrated that the Sdf-1-specific reagent, if used as a stand-alone PCR reagent, possibly reacts with non-Enteritidis *Salmonella* serovars that contained the Sdf-1 target sequence and thus might not be a reliable reagent for the identification of *S.* Enteritidis.

Analysis of genomic sequences from *S.* Montevideo isolates indicated that *S.* Montevideo isolates exhibited sequence identity of 99% or higher, covering 98 to 100% of the genomic sequence, whereas 98 to 99% sequence identity was shared with other serovars of the C1 group that covered a region of 93 to 95%. The segment of the genome that showed greater similarity decreased to 90 or 91% with isolates belonging to group B. The data from the Sequence Read Archive (SRA) of the Sdf-1-positive *S.* Montevideo isolate showed the presence of a stretch of 382 nucleotides (accession no. SRR1220746) that covered the entire Sdf-1 region of 333 nucleotides of reference *S.* Enteritidis with 98% identity (Fig. 2). Thus, it is not surprising that the Sdf-1 reagent reacted positively with this isolate, even though the group D-specific PCR reagent did not react. In addition to sequence similarity with Sdf-1, the isolate also exhibited sequence similarity to Sdf-3, Sdf-4, Sdf-5, Sdf-6, and Sdf-8 sequences (data not shown). The sequences (from the SRA) of several other *S.* Montevideo isolates examined did not have any significant similarity with Sdf-1 or other *Salmonella* differential fragment sequences.

The mechanism involved in the acquisition of the Sdf-1 sequence by non-Enteritidis *Salmonella* serovars is not known. We are currently investigating the possibility that genetic elements from *S.* Enteritidis were horizontally transferred to non-Enteritidis *Salmonella* serovars via phage infection. Plasmid-mediated lateral transmission of genetic elements, like the O-antigen biosynthesis genes, has been reported (30, 31). Thus, an approach using the O group-specific reagent together with a serovar-specific Sdf-1 PCR reagent would be more reliable for identifying the *S.* Enteritidis serovar than existing methods that use only one of the targets common to most *S.* Enteritidis strains.

All *S.* Enteritidis isolates tested in this study were positive with the *prt*-, *tyv*-, and Sdf-1-specific reagents, as expected. All other serovars of group D were positive with both of the *prt* and *tyv* PCR reagents but not with the Sdf-1 reagent. Thus, the new method is more reliable than current methods: the *protE6*-specific PCR reagent recog-

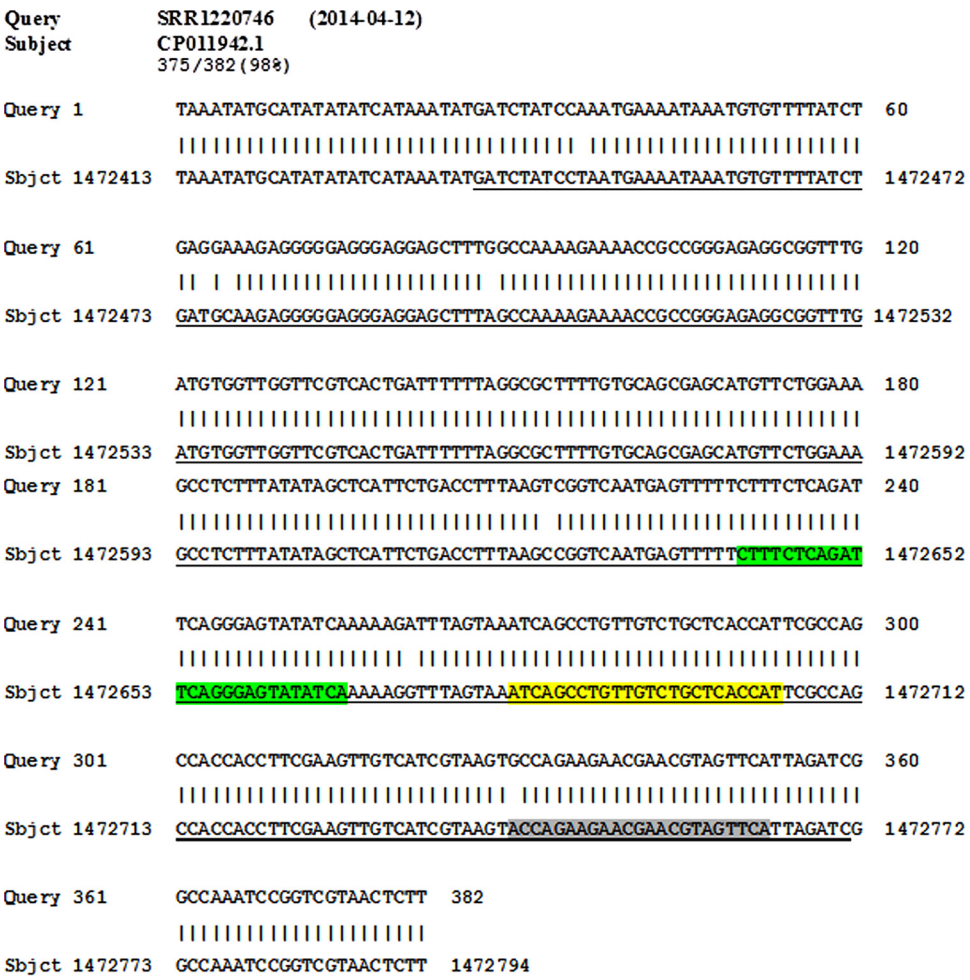

FIG 2 Similarity between the nucleotide sequence of an *S.* Montevideo isolate and the Sdf-1 sequence. The Sdf-1 sequences (333 nucleotides) for *S.* Enteritidis strain OLF-00D989 87-1 (accession no. CP011942.1) and an *S.* Montevideo isolate (accession no. SRR1220746) were aligned to show the similarity. The sequence of Sdf-1 of *S.* Enteritidis is underlined. The Sdf-1 PCR primer and probe sequences used in the PCR are highlighted: the forward primer sequence is in green, the probe sequence is in yellow, and the reverse primer sequence is in gray. Sbjct, subject.

nized several phage types of *S.* Enteritidis but not all (9, 32), and the Sdf-1-specific reagent (33, 34) would have identified the *S.* Montevideo isolate as *S.* Enteritidis.

The environmental swab samples were collected from different places and various production environments that included surfaces associated with food preparation, kitchen floor, enrobing rooms, and packing and storage areas. Although the VIDAS method has been validated using a large panel of food matrices after artificially spiking them with *Salmonella*, a similar validation has not been done for environmental samples that contain varied matrices and a variety of competing organisms. The PCR results of environmental samples demonstrated an excellent correlation with the culture method. In addition to our observations, a recent study has shown the VIDAS method could not detect the presence of *Salmonella* in certain produce samples that were spiked with *Salmonella* (false-negative results), even though the *Salmonella* used for spiking was isolated from those samples using the culture method (18). The PCR method could be the alternative and reliable method for screening such samples. The PCR method is highly sensitive, detecting the presence of as few as 40 *Salmonella* cells/ml (preenrichment) with a probability of 50%, which increased to 100% for 80 or more cells. On the other hand, the VIDAS method did not detect the presence of *Salmonella* in 5 samples that were PCR positive, and *Salmonella* isolates were obtained from the enrichments that were used for the VIDAS assay. The above-described results

clearly demonstrated that secondary enrichments from the above-mentioned 5 VIDAS-negative samples contained *Salmonella* cells, although fewer cells than were required for detection by VIDAS. Alternatively, the *Salmonella* cells present in those enrichments did not have a sufficient number of antigenic epitopes required for detection by the VIDAS method; this possibility was unlikely because the same *Salmonella* serovar was isolated from both VIDAS-positive and -negative samples. Based on the C_T values obtained from the PCR assays, it appears that there was no direct relationship between the number of *Salmonella* cells present in the preenrichments and the negative VIDAS results (18). These results suggest that the diversity of matrices and the composition and levels of competing organisms present in different environmental samples could have influenced the outcome of the VIDAS results. The fact that the PCR method detected 55% more *Salmonella* positives than VIDAS confirmed the higher sensitivity of the PCR method. In addition, PCR did not yield any false-negative results, unlike the VIDAS method. The results from the PCR screening of 1,741 environmental samples showed an excellent correlation with the culture method, thus confirming the reliability and validity of the PCR method for screening environmental samples for detecting *Salmonella*. In conclusion, our PCR-based approach detected 55% more positives in half the time required for the VIDAS method, and thus the PCR method has great potential to strengthen public health initiatives through expeditious detection of *Salmonella* spp. and *S.* Enteritidis in environmental samples.

MATERIALS AND METHODS

Control organisms. A *Salmonella* control panel and a non-*Salmonella* (negative) control panel containing common environmental organisms were used for confirming the inclusivity and exclusivity of the PCR. The *Salmonella* panel included 12 reference organisms, i.e., *Salmonella bongori* (ATCC 43975); 6 serovars of *S. enterica* subsp. *enterica*: Paratyphi A, group A (ATCC 9281), Typhimurium, group B (ATCC 13311), Javiana, group D (ATCC 10721), Enteritidis, group D (ATCC 13076), Gaminara, group I (ATCC 8324), and Cerro, group K (ATCC 10723), and 5 reference organisms from other subspecies of *S. enterica*, i.e., *S. enterica* subsp. *salamae* (ATCC 15786), *S. enterica* subsp. *arizonae* IIIa 35:-:x,z15 (CFSAN014667), *S. enterica* subsp. *diarizonae*, IIIb 47:i:z_{53}:z_{57} (ATCC 12325), *S. enterica* subsp. *houtenae*, IV 43:z4,z23:- (CFSAN011677), and *S. enterica* subsp. *indica*, VI 1,6,14,25:a:e,n,z15 (CFSAN015097). The DNA extracts from 12 control organisms of a *Salmonella* panel were prepared from 1.0 ml of overnight culture using InstaGene matrix, according to the manufacturer's instructions (catalog no. 732-6030; Bio-Rad); briefly, 1.0 ml of each bacterial suspension was pelleted by centrifugation at 14,000 $\times$ *g* for 2 min in an Eppendorf microcentrifuge, and each pellet was mixed thoroughly with 200 μl of InstaGene matrix by vigorous agitation at high speed on a vortex mixer for 10 s and incubated for 15 to 30 min in an Eppendorf thermomixer at 56°C. The samples were removed from the thermomixer, agitated at high speed on a vortex mixer for a few seconds, and placed in a heat block at 100°C for 10 min. The samples were removed from the heat block, agitated on a vortex mixer for 10 s, and centrifuged at 14,000 $\times$ *g* for 2 min. The supernatants containing the extracted DNA were diluted 1:1,000 and used in the PCR for confirming the inclusivity of *Salmonella*-specific PCR primers and probes. The non-*Salmonella* control panel contained 22 non-*Salmonella* organisms, including 18 ATCC reference organisms and 4 laboratory isolates (Table 2). Purified DNA from the non-*Salmonella* control organisms and a positive-control *Salmonella* (serovar Enteritidis) strain (ATCC 13076) was prepared from 1.0-ml overnight cultures of bacteria using the DNeasy blood and tissue kit (catalog no. 69504; Qiagen, Inc., USA) and an automated QIAcube instrument. The concentration of purified DNA was determined with the Qubit double-stranded DNA (dsDNA) BR assay kit (catalog no. Q32850), according to the protocol provided by the manufacturer. Copies of genomic DNA present in the positive-control *S.* Enteritidis organism and all non-*Salmonella* environmental control organisms were determined using the online URI Genomics & Sequencing Center copy number calculator for double-stranded DNA. A total of 10^5 copies of the purified DNA from each non-*Salmonella* organism were used for confirming the exclusivity of PCR primers and probes.

***Salmonella* isolates used for PCR method validation.** For validation of the PCR method, a panel of 329 *Salmonella* isolates, confirmed by pulsed-field gel electrophoresis at the FDA Northeast Regional Laboratory (NRL) and with conventional serology performed at the FDA Arkansas Regional Laboratory (ARL), were randomly chosen from the NRL *Salmonella* culture bank and tested for the inclusivity of the PCR assay. The panel consisted of 126 distinct serovars belonging to 34 O groups that were commonly found in food or the environment. The CDC-recommended taxonomical nomenclature was used to identify species, subspecies, and serovars in this report (35).

Isolates from frozen stocks were first plated on selective agar medium, and a single typical colony was picked and grown in 4.0 ml of Luria-Bertani medium overnight at 37°C in a shaker incubator. DNA was extracted from 1.0 ml of culture, according to the procedure described above for the preparation of DNA from the control *Salmonella* panel, with 200 μl of InstaGene matrix. DNA extracts were stored at −20°C until use. DNA samples from isolates were diluted 1:1,000 prior to use in PCR.

Processing of environmental samples. A total of 1,741 environmental sponge swab samples collected by the FDA during the surveillance inspections of 14 different food manufacturing companies

(from 2015 to 2017) were used for this study. The swab samples were analyzed within 24 h of receipt. To each of the swab/sponge samples, lactose broth or modified buffered peptone water was added in a Whirl-Pak bag, and samples were incubated for 24 ± 2.0 h at 35°C, according to the procedure described in the *Bacteriological Analytical Manual* (27) and AOAC official methods 2009.03 (17). One-tenth of a milliliter of primary enrichment from each sample was transferred into 10 ml of SX2 broth and incubated at 42 ± 1°C for an additional 24 ± 2.0 h; aliquots from the secondary enrichments were used for the VIDAS. One milliliter of preenrichment from each sample was used for the extraction of DNA for the PCR screening.

VIDAS screening. One-half of a milliliter of secondary enrichment from each sample was transferred to VIDAS strips, and VIDAS was performed according to the Easy SLM method (17).

PCR screening. DNA was extracted from 1.0 ml of preenrichment samples, with 200 μl of InstaGene matrix, according to the manufacturer's instructions described for the extraction of DNA from bacterial cultures (catalog no. 732-6030; Bio-Rad). Five microliters of the extracted DNA, undiluted (representing 25.0 μl of the preenrichment sample), was used in the real-time PCR.

Primers and TaqMan probes. PCR primer pairs and TaqMan probes specific for the gene *invA* of *Salmonella* spp. (36), the paratose synthase (*prt*) gene, and the tyvelose epimerase (*tyv*) gene of group D and group A *Salmonella*, the *Salmonella*-differentiating fragment 1 (Sdf-1) sequence of *S.* Enteritidis, and the IAC DNA were designed using the online primer design software of Integrated DNA Technologies, Inc., USA (http://www.idtdna.com/calc/analyzer). IDT synthesized a gene construct of 139 bp of IAC DNA as G block DNA: ATGATTACGAATTCACTGATCTGACTTCACTCCTGTCTACTATACAGATATAACGCTGAC TCGTCCATACAATAAGACACCTTGATGTAGTCTGATAGTGGTCAGTGACGAGTGTCATCTAGAGTCGAATTCACT GCAA. The IAC served as the reagent activity control to monitor the presence of PCR inhibitors in samples. The target sequence of *invA* was chosen from a conserved region shared by *Salmonella bongori* and the subspecies of *S. enterica* (36). A cell surface polysaccharide present in O groups A, B, and D is a polymer made of a repeating unit of four sugars, of which three form the backbone mannosyl-rhamnosyl-galactose common to all three O groups, whereas the fourth sugar is a dideoxyhexose linked to a mannose residue, which is specific to each O group (37). The gene *rfbS* is responsible for the synthesis of CDP-paratose, the fourth sugar of the group A epitope (O-2), whereas the *rfbE* is responsible for converting CDP-paratose to CDP-tyvelose of the group D epitope (O-9) (37). All group A members contain *rfbE* and were derived from group D by a single frameshift mutation in the *rfbE* sequence (37). Thus, both group A and group D serovars contain *rfbS* and *rfbE* sequences, whereas *rfbE* is nonfunctional in group A. Thus, either the *prt* or *tyv* PCR primer-probe set could be used for the identification of groups A and D. No group A serovar was found among the 1,700 *Salmonella* isolates recovered from food and environmental samples in our laboratory during the past 18 years or among the 10,000 isolates that were sequenced by the FDA from 2012 (http://www.ncbi.nlm.nih.gov/projects/pathogens). Essentially all PCR-positive environmental and food samples are likely to be of the group D serovar. For each gene target, four sets of primer-probe sequences were evaluated for secondary structures and dimer-forming potential with other primers and probes using the Oligo Analyzer application software program of IDT. Thus, a set of 12 sequences for each target was first tested against 36 primer sequences specific to other three targets in a chessboard-type analysis. Then, four primer-probe sequence sets exhibiting the least heterodimer-forming potential were selected for use in multiplex PCR and were synthesized by IDT (Table 5). The stock solutions (100 μM) of primers and probes were prepared according to IDT's instructions. The optimal concentrations of primers and probes, annealing and elongation temperatures, and the specificity of the reactions were initially tested for each individual primer-probe set and then confirmed in the multiplex reaction. The real-time PCR assay described herein has been optimized for detecting three *Salmonella* target genes, *invA*, *prt*, and *tyv*, as well as Sdf-1 in a single reaction.

Multiplex real-time PCR. The optimal PCR conditions were configured for the SmartCycler II platform in 25-μl reaction mixtures. The method is easily adaptable to the Fast 7500 (Applied Biosystems). USB VeriQuest Fast Probe quantitative PCR (qPCR) master mix with no reference dye (product no. 75685; Affymetrix, Inc., USA) was used for the assays. Master mix reagents from GE, Eurogentec, and Qiagen were also tested and gave comparable results. The optimal conditions for performing the multiplex assay were first determined using DNA templates from a positive-control *S.* Enteritidis and 22 non-*Salmonella* target organisms. The concentrations of primers and probes used in PCR were as follows: 200 nM *invA* forward and reverse primers and 30 nM probe; 250 nM *prt* forward and reverse primers and 200 nM probe (or 300 nM *tyv* forward primer, 200 nM *tyv* reverse primer, and 200 nM probe); 250 nM Sdf-1 forward primer, 300 nM reverse primer, and 40 nM probe; 200 nM IAC forward and reverse primers and 100 nM probe. The PCR master mix was prepared from the reaction components that were thawed and kept on ice. A known quantity of IAC DNA (100 to 200 copies) was added to provide a C_T value of 32 to 34. The PCR master mix was supplemented with 0.10 U of VeriQuest *Taq* DNA polymerase. Master mix (20 μl) was distributed to the required number of SmartCycler reaction tubes for each run, and the caps were loosely closed. To the no-template control (NTC) tubes, 5.0 μl of molecular biology-grade water was added, and the caps were closed tightly. To the sample-containing tubes, 5.0 μl of DNA extract from samples was added and the caps closed tightly. Finally, 5.0 μl of positive control containing 10^3 or 10^4 DNA copies, giving a C_T value of 29 to 26, was added to positive-control tubes. The tubes were briefly centrifuged to bring the reaction mixtures to the bottom of the tubes. The tubes were then placed in thermal cyclers and subjected to a two-step PCR protocol. The protocol consisted of a 180-s hold at 95°C, followed by 40 cycles of 10 s at 94°C and 30 s at 60°C. The fluorescence generated in each reaction was recorded at the extension step of each cycle. The PCR run took ~46 min.

Each lot of master mix we prepared was subjected to quality control to ensure that each reaction mixture contained 100 to 200 copies of IAC DNA and that a single copy of *Salmonella* genomic DNA

TABLE 5 List of primer and probe sequences used in the multiplex PCR[a]

Name	No. of bases	Position in genome	Sequence (5′ to 3′)[b]
Primers			
invA forward	20	2924483–2924506	AGCGTACTGGAAAGGGAAAG
invA reverse	24	2924598–2924579	ATACCGCCAATAAAGTTCACAAAG
prt forward	22	2175083–2175104	AGCTCCATAGAAATGCTCCAAT
prt reverse	22	2175213–2175192	GAACATCACTGCCACCAAATAC
tyv forward	25	2174357–2174376	ACTAAGTATATGCCTGATAGCTGTT
tyv reverse	20	2174454–2174462	GCCGTACTGCCTCAAGTAAA
Sdf-1 forward	26	1472650–1472675	CTTTCTCAGATTCAGGGAGTATATCA
Sdf-1 reverse	23	1472772–1472750	TGAACTACGTTCGTTCTTCTGGT
IAC forward	27	16–42	CTGATCTGACTTCACTCCTGTCTACTA
IAC reverse	24	116–93	GACACTCGTCACTGACCACTATCA
Probes			
invA (+)	26	2924529–2924554	6FAM/CGTCACCTTTGATAAACTTCATCGCA–BHQ1
prt (−)	26	2175150–2175125	Cy3/CCGCCGCCATTATAGATAAAGTTTGT–BHQ2
tyv (−)	25	2174454–2174430	Cy3/TGCAGGTCAAGTGGCAATGACTACA–BHQ2
Sdf-1 (+)	24	1472690–1472713	TexRdXN/ATCAGCCTGTTGTCTGCTCACCAT–BHQ2
IAC (+)	28	54–81	Cy5/CGCTGACTCGTCCATACAATAAGACACC–BHQ2

[a]The sequences, lengths, and locations of primers and probes are shown within the *Salmonella enterica* serovar Enteritidis genome (strain SA20082034, accession no. CP007425.2). The sequence, length and location of the IAC primers and probe are shown on the IAC gene. The lengths of the PCR products generated by the primer pairs were as follows: *invA*, 115 nucleotides; IAC, 100 nucleotides; *prt*, 130 nucleotides; *tyv*, 130 nucleotides; and Sdf-1, 123 nucleotides.

[b]6FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1; TexRd, Texas Red.

could be detected. Duplicate reactions of positive and negative controls were included with each run. An increase in the C_T value of the IAC target in a sample compared with the C_T of NTC indicated the presence of PCR inhibitors in that sample. The IAC could yield a negative result if a sample contained $\geq 10^4$ copies of the positive target as a consequence of one or more reagents of the PCR mixture becoming depleted sooner than the C_T value of IAC reached the threshold set for the assay. A sample that yielded a positive C_T value for at least one of the targets was considered a valid test. Samples showing inhibition of IAC in the absence of a positive target in the assay were required to be reprocessed and retested.

Culture analysis. The secondary enrichments from all VIDAS- and PCR-positive samples were plated onto xylose lysine deoxycholate (XLD), Hektoen enteric (HE), and bismuth sulfite (BS) agar plates and incubated for 24 h at 35 ± 2°C (27). Typical and atypical control *Salmonella* cultures (ATCC 8324 and ATCC 29934) were included as positive controls. Colonies showing typical/atypical morphology were inoculated on triple sugar iron/lysine iron agar (TSI/LIA) slants, and the cultures showing characteristic reactions were identified biochemically using the Vitek 2 GN card and AOAC Official Methods of Analysis method 2011.17 and confirmed with serological tests.

Statistical analysis. All statistical analyses were performed in R (38), except for the Cohen's kappa calculation, which was performed using an online calculator (http://vassarstats.net/kappa.html). The correlation between the number of DNA copies (and thus the number of *Salmonella* cells present in each sample) and the PCR signal (InvA C_T) was established with the Pearson correlation coefficient (38).

The agreement of the results of the PCR and VIDAS methods with the microbial culture method (gold standard) was assessed as follows. (i) Agreement between the PCR method and culture method and between the VIDAS method and the culture method was assessed with receiver operating characteristic (ROC) analysis. The level of agreement was enumerated as the area under the curve (AUC) (39). (ii) The level of agreement was calculated using unweighted Cohen's kappa (29). (iii) The probability of chance agreement was enumerated with an empirical statistics model as follows. The number of agreements (positive-positive or negative-negative) was calculated for each comparison (PCR versus culture, and VIDAS versus culture). The number of agreements in each comparison represented the agreement score for that comparison. The sets of results from the test method and the gold standard method were randomized and randomly matched. The agreement score was calculated for this random match. This procedure was repeated 10,000 times and produced a distribution of 10,000 chance agreement scores for each comparison. The distribution of the agreement scores was compared to the real agreement score for each comparison, and the probability of chance agreement was enumerated as the fraction of trials that reached the agreement score that was equal or higher than the real agreement score from the experiment.

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