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Research Paper

Evaluation of a Multiplex PCR Assay for the Identification of *Salmonella* Serovars Enteritidis and Typhimurium Using Retail and Abattoir Samples

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ABSTRACT

A multiplex PCR was developed to identify the two most common serovars of *Salmonella* causing foodborne illness in Canada, namely, serovars Enteritidis and Typhimurium. The PCR was designed to amplify DNA fragments from four *Salmonella* genes, namely, *invA* gene (211-bp fragment), *iroB* gene (309-bp fragment), Typhimurium STM 4497 (523-bp fragment), and Enteritidis SE147228 (612-bp fragment). In addition, a 1,026-bp ribosomal DNA (rDNA) fragment universally present in bacterial species was included in the assay as an internal control fragment. The detection rate of the PCR was 100% among *Salmonella* Enteritidis ($n = 92$) and *Salmonella* Typhimurium ($n = 33$) isolates. All tested *Salmonella* isolates ($n = 194$) were successfully identified based on the amplification of at least one *Salmonella*-specific DNA fragment. None of the four *Salmonella* DNA amplicons were detected in any of the non-*Salmonella* isolates ($n = 126$), indicating an exclusivity rate of 100%. When applied to crude extracts of 2,001 field isolates of *Salmonella* obtained during the course of a national microbiological baseline study in broiler chickens and chicken products sampled from abattoir and retail outlets, 163 isolates, or 8.1%, tested positive for *Salmonella* Enteritidis and another 80 isolates, or 4.0%, tested as *Salmonella* Typhimurium. All isolates identified by serological testing as *Salmonella* Enteritidis in the microbiological study were also identified by using the multiplex PCR. The new test can be used to identify or confirm pure isolates of the two serovars and is also amenable for integration into existing culture procedures for accurate detection of *Salmonella* colonies.

Key words: Enteritidis; Identification; PCR; *Salmonella*; Serovars; Typhimurium

Foodborne salmonellosis has far-reaching health and economic impacts; the genus *Salmonella* is responsible for the highest burden of infections contracted from food owing to its high prevalence, high rate of hospitalization, and number of deaths *(5)*. *Salmonella* serovars Enteritidis and Typhimurium are the two most common and dominant serovars responsible for foodborne illnesses in Canada *(32, 38)*. They are jointly responsible for nearly 80% of all human *Salmonella* infections globally *(30, 38),* and their detection and control will alleviate the significant costs in illnesses and hospitalizations associated with *Salmonella (5, 23)*. Culture methods for detecting *Salmonella* are well established and are routinely used for food testing, clinical diagnosis, and surveillance *(2, 8)*. Despite the advancement in the culture of *Salmonella* as a result of decades of productive research work, the isolation procedures currently in use are not optimal: false-positive and false-negative colonies are common. Both *Citrobacter* and *Proteus* spp. are commonly misidentified as *Salmonella* because of similar colonial features on selective xylose lysine desoxycholate and xylose lysine Tergitol 4 agars *(31)*. Furthermore, atypical *Salmonella* organisms that do not produce H_2S, hitherto an important biochemical marker, or ferment lactose are increasingly being reported in food safety and food animal investigations *(25, 37)*. Because of the nonoptimal test sensitivity of culture procedures, underreporting of *Salmonella* contamination is probably common because bacterial growth showing uncharacteristic properties on selective media will be disregarded by diagnostic bacteriology laboratories. Instructively, efforts to recover *Salmonella* from food sources consumed by patients during a foodborne outbreak, as required during food safety investigations, often prove abortive. On the other hand, reduced specificity of culture methods means more analysis will be needed to rule out any non-*Salmonella* that, nonetheless, may exhibit some *Salmonella*-like properties (e.g., H_2S production); more extensive analysis leads to delayed reporting of test results.

As a consequence of the labor-intensive nature of the culture method, a bacteriology laboratory has to balance increased costs and reporting delays with reduced test accuracy; extensive subculturing of every questionable isolate is not realistic. To address the need of accurately identifying *Salmonella* in a submitted sample, a high-

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TABLE 1. Salmonella *isolates tested by multiplex PCR (group 1)*[a]

Salmonella serogroup	No. of isolates	Multiplex PCR results				*Salmonella* serovar or source
		invA	*iroB*	STM 4497	SE147228	
A	3	+	+	−	−	Paratyphi A ($n = 2$), Kiel
	1	+	+	−	+	Nitra
B	33	+	+	+	−	Typhimurium and variants
	13	+	+	−	−	Saintpaul, Indiana, Agona, Heidelberg, Kiambu, Schwarzengrund, Abortusequi, Tumodi, and variants or undescribed serovars ($n = 5$)
C1	10	+	+	−	−	Braenderup, Mbadanka, Infantis, Cholerasuis, Tennessee, Montevideo, Thompson, and variants ($n = 3$)
C2	1	+	+	−	−	Hadar
C3	4	+	+	−	−	Kentucky ($n = 3$) and variant
D1	92	+	+	−	+	Enteritidis
	9	+	+	−	−	Biovar Gallinarum ($n = 2$), biovar Pullorum ($n = 4$), serovars Berta, Dublin, and Panama
	2	+	−	−	−	Biovars Gallinarum and Pullorum
D2	2	+	+	−	−	Ouakam
	1	+	−	−	−	Hillingdon
E	4	+	+	−	−	Orion, Give, Uganda, and Seftenberg
O	1	+	+	−	−	
Arizonae	1	+	+	−	−	
Unknown/rough	1	+	+	−	+	
	8	+	+	−	−	
Untested	6	+	+	−	−	Collection ($n = 2$), biosolid expts ($n = 4$)
	2	+	−	−	−	Biosolid expt
Total	194					

[a] *Salmonella* isolates ($n = 194$) were tested for inclusivity analysis. +, positive; −, negative.

throughput method with good accuracy that can help screen a large number of colonies and be seamlessly and effectively integrated into a culture-based testing procedure is required.

Following colony isolation and identification of the organism as *Salmonella,* further characterization of the isolate is needed in support of any required regulatory action (e.g., to comply with food safety investigation guidelines). It is necessary to determine the serovar designation of the isolate because a number of *Salmonella* organisms do not constitute a food safety risk (e.g., *Salmonella* serovars Gallinarum and Sofia). To avoid or reduce delay, it is desirable to generate as much reliable information in a timely manner from either a single test or an integrated laboratory testing procedure. Full characterization of an isolate of *Salmonella* within a diagnostically reasonable period of time is challenging because of the diversity and complexity inherent in the genus that includes as many as 2,600 serovars. The previous alphabetic designation (e.g., serogroups A, B, C, and D) has now largely been abandoned because there were not enough letters to designate the groups *(19)*. The supplementation of alphabets with numbers as a means of accommodating all tested isolates remained problematic because mobile genetic elements, such as plasmids and phages, influenced the antigenicity of the isolates and therefore the serological test results, requiring regular changes to a designation. Alteration in the surface lipopolysaccharide, such as loss of O antigen, can translate to an inability to achieve serological characterization or serovar designation. The solution to the complexity of *Salmonella* characterization appears to lie in the application of DNA-based methods, building on the seminal use of DNA hybridization to reclassify the large number of isolates into only two species, which led to a remarkable simplification of the taxonomy *(7, 12)*.

The main goal of the study was to develop a rapid PCR method that could be integrated into a culture-based procedure so that colonies are not missed owing to overgrowth by other bacteria, which is a common occurrence. In addition, we sought to simultaneously determine whether a colony belonged to serovar Enteritidis or Typhimurium using a single assay. We were able to apply the test to a large number of field isolates obtained during a national microbiological baseline study.

MATERIALS AND METHODS

Bacterial isolates. Three groups of bacterial isolates were tested during this study. Group 1 consisted of 194 *Salmonella* isolates retrieved from the Canadian Food Inspection Agency Microbiology Culture Bank (Table 1). A total of 33 serovars were represented in group 1 and included the most common causes of foodborne outbreaks (Table 1). Some isolates in group 1 ($n = 13$) were successfully characterized antigenically as belonging to serogroup B or C but were not given a serovar designation either because they represented variants of existing serovars or have not previously been recognized. Another subset were termed rough isolates ($n = 9$) and could not be typed serologically. The group also included *Salmonella* isolates obtained from a biosolid experiment but have not been serotyped. Group 2 consisted of 126 non-*Salmonella* isolates retrieved from the same Canadian Food Inspection Agency inventory (Table 2). Group 3 consisted of

TABLE 2. *Bacterial organisms belonging to genera other than* Salmonella *tested by PCR (group 2)*[a]

Genus	Frequency
Achromobacter	1
Acinetobacter	2
Actinobacillus	1
Aeromonas	1
Alcaligenes	2
Bacillus	5
Bacteriodes	1
Branhamella	1
Burkholderia	1
Campylobacter	2
Citrobacter	5
Corynebacterium	2
Enterobacter	6
Enterococcus	5
Escherichia	10
Hafnia	1
Klebsiella	11
Lactobacillus	1
Listeria	5
Micrococcus	3
Moraxella	2
Morganella	1
Pasteurella	3
Proteus	10
Providencia	1
Pseudomonas	3
Rhodococcus	2
Serratia	2
Sphingobacterium	1
Staphylococcus	11
Stenotrophomonas	1
Steptococcus	20
Yersinia	3
Total	126

[a] Non-*Salmonella* isolates ($n = 126$) were tested for exclusivity analysis. Frequency is the number of species, strains, or isolates tested in each bacterial genus.

2,001 field isolates of *Salmonella* spp. obtained during the 2012 to 2013 National Microbiology Baseline Study in Broiler Chicken conducted in abattoir and retail outlets across Canada. The design and results of the study are to be included in a separate publication (D.L., personal communication).

DNA template for PCR. Purified DNA and crude bacterial extracts were prepared and used in the PCR, as described in the following. At first, DNA was purified from overnight cultures of bacterial isolates belonging to inclusivity and exclusivity groups ($n = 320$, Tables 1 and 2) by means of an automated extraction procedure (Promega Maxwell, Promega Corporation, Madison WI) and used as a template in the PCR. Subsequently, crude bacteria extracts were prepared from all the bacterial isolates used in this study (group 1 = 194, group 2 = 126, and group 3 = 2,001) by placing a bacterial colony, from a streaked BHI agar plate on which a bacterial isolate was grown overnight, into a 1.5-ml Eppendorf tube containing 50 μl of water and then boiling in a hot water bath for 10 min. Contents of the tube were spun at 12,000 rpm for 2 min; the supernatant was recovered and used as a crude bacterial DNA preparation in the multiplex PCR.

***Salmonella* serotyping.** Serotyping of *Salmonella* isolates was performed at the OIE Salmonella Reference Centre, Public Health Agency of Canada, Guelph, Ontario, Canada, as part of the routine testing and characterization procedure for *Salmonella* isolates obtained by federal laboratories. The serotyping results for group 3 isolates were provided after the completion of the multiplex PCR (see test details in the following).

Markers and design of multiplex PCR for *Salmonella* Enteritidis and Typhimurium. Four DNA fragments representing portions of previously described *Salmonella* genes were targeted by the multiplex PCR (Table 3). The genes, with amplicon sizes in parentheses, are *invA* gene (211 bp), *iroB* gene (309 bp), *Salmonella* Typhimurium STM 4497 (523 bp), and *Salmonella* Enteritidis SE147228 (612 bp). In addition, a fifth fragment (1,026 bp) from the 16S rDNA, which is common among all bacteria, was included. The *invA* gene, which is responsible for entry of *Salmonella* into epithelial cells *(18),* is present in both species of *Salmonella* and appears to be a reliable marker for the genus *(10)*. The *iroB* gene has been shown to be present in all *Salmonella enterica* organisms, including those of food safety importance, as well as species infecting poultry, but which do not constitute food safety hazards *(6)*. The gene has not been detected in *Salmonella bongori,* which is more common in cold-blooded animals and rarely infects humans, nor was it detected in non-*Salmonella* bacterial species that are of food safety importance, such as *Escherichia coli (29), Listeria (29),* and *Yersinia (6)*. *STM 4497* is a gene coding for a cytoplasmic protein whose function is yet to be described but is known to be specific for *Salmonella* Typhimurium *(9, 35)*. The 612-bp *Salmonella* Enteritidis–specific fragment was designed to include the *Salmonella* difference I locus, Sdf I (293 bp), that was previously described by Agron et al. (*1)* (accession AF370707.1). The presence of the *Salmonella* difference I sequence was investigated among a group of high-resolution *Salmonella* Enteritidis genomes that we generated and deposited into GenBank (accession CP009083-CP009093 and CP011942) *(27)*. By means of the Basic Local Alignment Search Tool analysis of genomes in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi), we confirmed that the sequence was uniquely present in the chromosome of *Salmonella* Enteritidis but not in other *Salmonella* serovars nor in other tested bacteria. To develop an appropriate-sized *Salmonella* Enteritidis–specific fragment for inclusion in a multiplex PCR, primer sequences anterior and posterior to the described sequence that would yield a fragment $\geq$600 bp were sought. To identify an appropriate internal positive control for the multiplex PCR, a 16S rDNA sequence was targeted using a previously described forward universal primer, E334F *(4, 33),* and a newly developed reverse primer, 295526R, to generate a panbacteria DNA amplicon of a suitable size (approximately 1 kb) for optimal resolution of the amplicons in the multiplex PCR; this allowed evaluation of the presence of a sufficient amount of input bacteria DNA in the assay. Available universal primers, which were designed for taxonomic and phylogenetic analyses, typically target smaller DNA fragments (i.e., $<$650 bp) *(33)*. To develop the 295526R primer (Table 3), rDNA sequences of a gram-negative bacteria, namely *Salmonella enterica* serovar Enteritidis, were compared with those found in a gram-positive bacteria, namely *Listeria monocytogenes,* by using genome sequences developed in our laboratory *(27*; I.G.M., unpublished data). A homologous region was identified in the 16S rDNA that would allow amplification of a DNA fragment approximately 1 kb

TABLE 3. *List of primers used to develop the* Salmonella *multiplex PCR assay for the identification of* Salmonella *species and for distinguishing* Salmonella *serovars Enteritidis and Typhimurium*

Primer name	Sequences (5′→3′)	Reference(s)	Size (bp)	Specificity
STM4497 F	GGAATCAATGCCCGCCAATG	*9, 35*	523	*Salmonella* Typhimurium
STM4497 R	CGTGCTTGAATACCGCCTGTC	*9, 35*		
iroB F	GAACGTACACCTGATCGCAAG	*35*	309	*Salmonella* genus
iroB R	GCACCCTGGCCAAAGACTATC	*35*		
invA F	ATCAGTACCAGTCGTCTTATCTTGAT	*35*	211	*Salmonella* genus
invA R	TCTGTTTACCGGGCATACCAT	*35*		
SE1472298-2 F	CTTGGAGAGCTGCGCTAAAG	This study	612	*Salmonella* Enteritidis
SE1472298-2 R	TAAGGCACCTCTCAACACTG	This study		
*E334*F	CCAGACTCCTACGGGAGGCAG	*33*	1,026	Universal primer
*295526*R	ACGATTACTAGCGATTCCG	This study		
PepT-Forward	GTTTGCCATATTGCTGCGAGGC	*17*	Variable	*Salmonella* Nitra (150 bp), Enteritidis
PepT-Reverse	GCGCTATCTCGGCGGCTG	*17*	(150–2,300)	(2,061 bp), and Typhimurium (2,300 bp)

downstream of the previously described E334F sequence. Evaluation of bacterial genome sequences from 12 different species in GenBank confirmed the presence of both primer sequences separated by about 1 kb (data not shown). Multiplex PCR amplification was carried out in a 50-μl volume containing bacterial template (1.5 μl of crude extract or 5 ng of purified DNA), a mixture of five pairs of primers (0.1 to 0.15 μM; Table 3), *Taq* DNA polymerase (1.5 U), deoxynucleoside triphosphate mix (300 μM), and $MgCl_2$ (2 mM; Thermo Fisher Scientific, Mississauga, Ontario, Canada). The thermocycling profile consisted of an initial denaturing step at 94°C for 5 min, followed by 35 cycles of denaturation for 1 min at 94°C, annealing at 62°C for 2 min, and extension at 72°C for 2 min. At the end of cycling, a final extension step was carried out at 72°C for 10 min. Following PCR, DNA amplicons were separated by electrophoresis through a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light; gel images were captured and stored electronically as TIFF files. A previously developed PCR method *(17)* targeting the peptidase T gene (*pepT*) was used to differentiate between serovars *Salmonella* Enteritidis and Nitra (see primer information in Table 3). PCR amplification of the *pepT* target was performed with the following cycling conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 5 min.

Statistical analysis. To evaluate test repeatability, results of independent testing of groups 1 and 2 isolates by three analysts were compiled and analyzed for interrater agreement by using a statistical software package (MedCalc Software bvba, Ostend, Belgium). Agreement was tested by estimating kappa values from pairing all three analysts with a procedure that discounts those agreements attributable to chance alone *(3)*. Strength of agreement was rated by using the scale provided by Landis and Koch *(21)* and Everritt *(15)* as follows: 0.21 to 0.40 = fair; 0.41 to 0.60 = moderate; 0.61 to 0.80 = substantial; and 0.81 to 1.00 = almost perfect.

RESULTS

PCR inclusivity. When tested with the multiplex PCR, all 194 *Salmonella* isolates in group 1 generated the 211-bp fragment of the *invA* gene, while the *iroB* gene fragment (309 bp) was detected in 192 isolates. One of three isolates belonging to *Salmonella* serovar Gallinarum and the only *Salmonella* serovar Hillingdon isolate both failed to generate the *iroB* gene fragment. Representative results of the multiplex PCR are illustrated in Figure 1. All 92 isolates of *Salmonella* Enteritidis tested as part of group 1 isolates (Table 1) contained the three expected fragments, namely *invA*, *iroB,* and a *Salmonella* Enteritidis–specific band (612 bp; Fig. 1). In the same vein, all 33 isolates identified as *Salmonella* Typhimurium or variants in group 1 had the *invA, iroB,* and the *Salmonella* Typhimurium–specific band (STM 4497; 523 bp; Fig. 1). All of the isolates ($n = 13$) with no serovar designation were successfully characterized antigenically and belonged to serogroups B ($n = 9$), C_1 ($n = 3$), and C_3 ($n = 1$) and may represent new variants of existing serovars or previously unrecognized serovars. Four of the nine serogroup B isolates tested as *Salmonella* Typhimurium, while the remaining five isolates and all four group C isolates could only be identified as *Salmonella*. In contrast, one of the nine isolates with unknown serogroup designation, presumably because of the loss of O antigen and consequently classified as rough, tested as *Salmonella* Enteritidis in the multiplex PCR. None of the rough *Salmonella* isolates tested as Typhimurium. The inclusivity of the multiplex PCR, for all known *Salmonella* isolates, was 100% ($n = 197$). The sensitivity of the multiplex PCR test to designate *Salmonella* Enteritidis ($n = 96$) and *Salmonella* Typhimurium ($n = 27$) was 100%. A single isolate of *Salmonella* serovar Nitra in group 1 was indistinguishable from *Salmonella* Enteritidis, generating three *Salmonella* PCR amplicons (data not shown). The accuracy of the multiple PCR to identify *Salmonella* Enteritidis isolates in this study was 99%. PCR amplification of the *pepT* gene allowed for the differentiation of our single *Salmonella* Nitra isolate from all Enteritidis isolates, as depicted in Figure 2, using a representative collection ($n = 17$). Although the *Salmonella* Nitra isolate yielded a 150-bp amplicon, all *Salmonella* Enteritidis isolates showed a 2,061-bp fragment. In contrast, a control *Salmonella* Typhimurium isolate gave a 2,300-bp fragment for the same *pepT* gene. These observations indicate the robustness of the multiplex PCR.

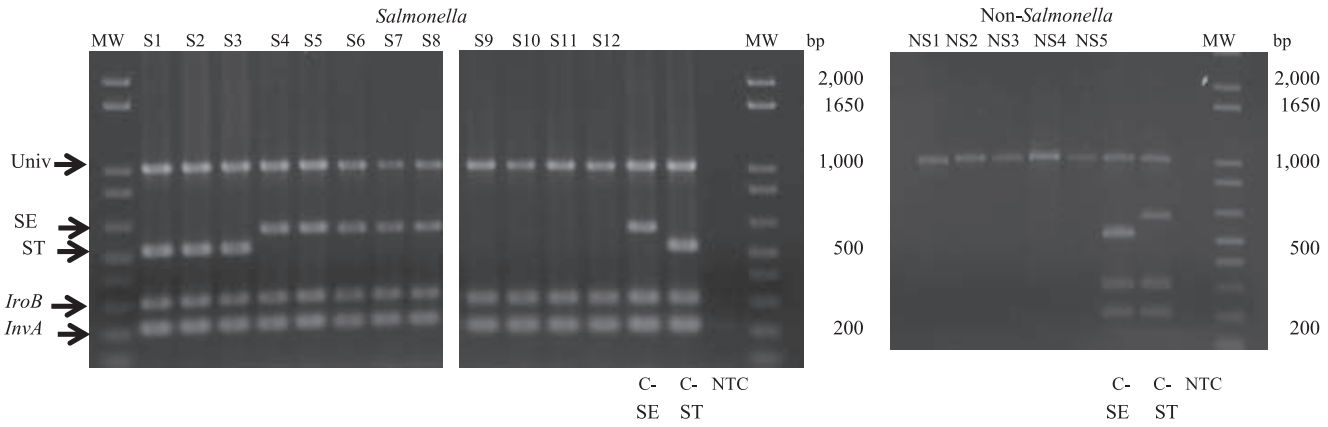

FIGURE 1. *Multiplex PCR for* Salmonella *Enteritidis and Typhimurium assay showing amplification of* Salmonella *DNA fragments in bacterial DNA extracts. Multiplex PCR showing amplification of* Salmonella *DNA fragments from crude DNA extracts. Agarose electrophoresis of DNA fragments amplified from crude extracts of* Salmonella *(S1-S12) and non-*Salmonella *(NS1-NS5) organisms by using the multiplex PCR for* Salmonella *Enteritidis and Typhimurium, as described under "Materials and Methods." Fragment sizes of the respective genes include* invA *gene (211 bp),* iroB *gene (309 bp),* Salmonella *Typhimurium STM 4497,* Salmonella *Typhimurium (523 bp),* Salmonella *Enteritidis SE147228,* Salmonella *Enteritidis (612 bp), and the 16S rDNA (1,026 bp). Control wells contained amplified products from* Salmonella *Enteritidis (C-SE),* Salmonella *Typhimurium (C-ST), or water (NTC). MW represents the molecular weight ladder.*

PCR exclusivity. The *Salmonella*-specific fragments were not amplified by the multiplex PCR in any of the 126 non-*Salmonella* isolates. Thus, the exclusivity rate of the multiplex PCR for the genus *Salmonella* in this study was 100%.

Quality assurance. The pair of primers designed to universally identify bacteria species was used to generate a 1,026-bp fragment in all the isolates tested (i.e., inclusivity and exclusivity groups, $n = 320$; Fig. 1), and this eventually served as a positive control to discount inadequate DNA as the cause of a nil amplification and avoid unnecessary retesting (see rationale discussed later).

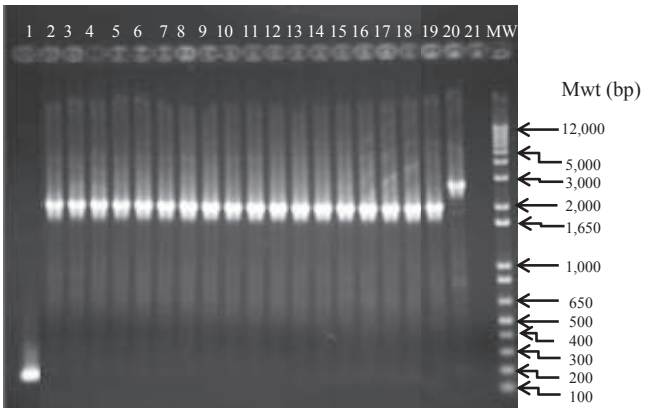

FIGURE 2. *PCR assay for distinguishing* Salmonella *Enteritidis from* Salmonella *Nitra. The* pepT *gene was amplified by using primers described under "Materials and Methods" (also see Table 3), resulting in the amplification of a 150-bp fragment in* Salmonella *Nitra (lane 1) compared with a 2,061-bp fragment in all* Salmonella *Enteritidis isolates tested (lanes 2 to 19). The* Salmonella *Typhimurium isolate gave a 2,300-bp fragment for the same* pepT *gene (lane 20). Lane 21 is the negative template control (water), while MW represents the molecular weight ladder.*

Test agreement. Kappa estimates to evaluate agreement of the results between any pair of analysts was 1.0 (i.e., almost perfect or perfect).

Application of test identifying *Salmonella* serovars Typhimurium and Enteritidis on *Salmonella* isolates during a national study conducted in broiler chicken. Of a total of 2,001 *Salmonella* isolates recovered from broiler chicken caeca, whole chicken carcasses, and parts collected from the abattoir and retail outlets and tested by the multiplex PCR for *Salmonella* Enteritidis and Typhimurium, 163 isolates, or 8.1%, were found to belong to *Salmonella* serovar Enteritidis, while 81 isolates, or 4.0%, tested as *Salmonella* serovar Typhimurium. All 163 *Salmonella* Enteritidis isolates were also successfully tested by a newly developed single nucleotide polymorphism–PCR assay *(27)* and were assigned to seven different clades (D.O., unpublished data), further confirming their identity as *Salmonella* Enteritidis isolates. The agreement between the multiplex PCR results in this study and serovar designations carried out in a reference laboratory was 100% for *Salmonella* Enteritidis ($n = 38$) and 95% for *Salmonella* Typhimurium ($n = 21$).

DISCUSSION

We have developed a multiplex PCR that could be used to unequivocally identify *Salmonella* based on the *invA* gene and to reliably type serovars Enteritidis and Typhimurium. The *invA* gene was detected in all *Salmonella* isolates that were so designated at the beginning of the study ($n = 194$), and it proved to be a reliable marker as suggested in previous studies *(10, 32),* while the *iroB* gene was present in all *Salmonella* isolates except two. Our choice of *Salmonella* Enteritidis–specific PCR target used in this study was done following a careful evaluation of the literature and analysis of the usefulness of other published specific sequences.

Various targets have been suggested as useful markers for the specific detection of *Salmonella* Enteritidis, many of which occur on plasmids *(20, 24, 26)* and mobile elements *(22, 39, 40),* and are often not consistently or stably present in all strains of *Salmonella* Enteritidis. Some of the identified sequences, especially those coding for phage proteins, could have significant homology with non-*Salmonella* sequences. Two chromosomal targets for fimbrial proteins identified in *Salmonella* Enteritidis, namely, SefA and SefB have also been used by many workers *(13, 34, 41),* but the Basic Local Alignment Search Tool analysis revealed that these targets are present in as many as five other serovars apart from *Salmonella* Enteritidis making them nonspecific targets. The most widely used PCR test for *Salmonella* Enteritidis is based on primers first described by Wood et al. *(42),* targeting a gene subsequently identified as the *prot6e* gene located on the 60-kb virulence plasmid and coding for a protein involved in fimbrial biosynthesis *(11).* The inability of the assay to detect all known *Salmonella* Enteritidis isolates either due to loss of the plasmid *(24, 42)* or an unpredictable modification that may involve a deletion of the target makes the assay nonideal, and a need for a more reliable test that could also serve as a confirmatory test has been raised *(36).* The need for a reliable target led us to the chromosomal encoded *Salmonella* Enteritidis–specific target SE1472298-2 identified by Argon et al. *(1),* which we modified and adapted to fit for use in our assay. The presence of the target in all of our *Salmonella* Enteritidis samples, which included isolates obtained over a period of 15 years (2000 to 2014) and belonging to different clades *(28),* suggests that the SE1472298-2 target is quite stable. The successful use of crude extract as the PCR template, as has been shown by other laboratories, permits use of the test for rapid screening of isolates and thereby facilitates effective integration into existing culture isolation procedures. By using the assay to screen colonies, atypical *Salmonella* (e.g., lactose fermenters or isolates that fail to produce H_2S) could be more readily detected and this should translate to increased recovery of *Salmonella* from food and field samples, while simultaneously identifying those that belong to *Salmonella* serovars Enteritidis and Typhimurium. The use of universal primers to detect a well-conserved bacterial sequence (16S rDNA) turned out be an important quality control step and was used to show that failure to add a template for one sample by one of three analysts participating in test repeatability assessment explained why test agreement was less than perfect (kappa = 0.97; data not shown). Once the internal control was instituted and used to demonstrate the presence of a template, test repeatability results among analysts was shown to be perfect (i.e., kappa = 1.0; see "Results" section).

The difficulty in distinguishing between *Salmonella* serovars Enteritidis and Nitra has been reported in studies using bead-based suspension arrays *(16),* PCR-based microarray *(17),* and whole genome phylogeny *(14),* leading to the suggestion that the two serovars may actually belong to the same lineage of organism *(14).* However, we were able to confirm that our single *Salmonella* Nitra isolate had a truncated *pepT* gene, as previously reported by Franklin et al. *(17),* whereas the *pepT* gene is much larger in *Salmonella* Enteritidis and even larger in *Salmonella* serovar Typhimurium. Because of the infrequent isolation of *Salmonella* Nitra in food and its lack of importance as a food safety pathogen, we have decided not to include primers that will distinguish *Salmonella* Enteritidis and Nitra in our multiplex PCR. At the same time, this study shows that the two serovars can be distinguished by using a second PCR test when deemed necessary. The new multiplex PCR described in this study is useful for the rapid identification of the two most common serovars of *Salmonella* and can be integrated into isolation procedures aimed at detecting the presence of the pathogen in food.

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