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Development and Evaluation of a Multiplex Real-Time Polymerase Chain Reaction Procedure to Clinically Type Prevalent *Salmonella enterica* Serovars

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A multiplex real-time polymerase chain reaction procedure was developed to identify the most prevalent clinical isolates of *Salmonella enterica* subsp. *enterica*. Genes from the *rfb*, *fliC*, *fljB*, and *viaB* groups that encode the O, H, and Vi antigens were used to design 15 primer pairs and TaqMan probes specific for the genes *rfb*J, *wzx*, *fli*C, *fljB*, *wcd*B, the sdf-l sequence, and *invA*, which was used as an internal amplification control. The primers and probes were variously combined into six sets. The first round of reactions used two of these sets to detect *Salmonella* O:4, O:9, O:7, O:8, and O:3,10 serogroups. Once the serogroups were identified, the results of a second round of reactions that used primers and probes for the flagellar antigen l genes, 1,2; e,h; g,m; d; e,n,x; and z_{10}, and the Vi gene were used to identify individual serovars. The procedure was standardized using 18 *Salmonella* reference strains and other enterobacteria. The procedure's reliability and sensitivity was evaluated using 267 randomly chosen serotyped *Salmonella* clinical isolates. The procedure had a sensitivity of 95.5% and was 100% specific. Thus, our technique is a quick, sensitive, reliable, and specific means of identifying *S. enterica* serovars and can be used in conjunction with traditional serotyping. Other primer and probe combinations could be used to increase the number of identifiable serovars. *(J Mol Diagn 2010, 12:220–225; DOI: 10.2353/jmoldx.2010.090036)*

Information about the geographic distribution of *Salmonella* serovars is necessary for the epidemiological surveillance of salmonellosis[1] (*http://www.who.int/salmsurv/general/documents/GSS_STRATEGICPLAN2006_10.pdf*; accessed April 27, 2009). Typhimurium, Enteritidis, Typhi, Braenderup, Newport, Saintpaul, and Hadar represent 82.9% of the prevalent serovars isolated from clinical samples in Colombia, and these serovars are also prevalent in other regions of the world.[1,2] Presently, *S. enterica* serotyping uses the Kauffman-White scheme, which involves first performing agglutination reactions between specific antibodies and 46 O-surface polysaccharides to identify the serogroup, and then, within a serogroup, identifying the corresponding serovar(s) according to the results of agglutination reactions involving 119 phase 1 and 2 H flagellar antigens, and the Vi capsular antigen[2–4] and the corresponding antibodies. This method of serotyping has limited application, however, owing to the need to produce antiserums, its variable quality, and its high cost. Additionally, about 5–8% of the isolates can only be partially typed or not typed at all owing to the presence of a capsule, a rough phenotype, or a lack of flagella.[3–5]

During the past decade, molecular methods that use polymerase chain reaction (PCR) amplification of *S. enterica* DNA sequences to determine serogroups[6,7] and serovars have been developed.[8–10] Multiplex PCR has also been used to identify serovars in clinical samples and food.[11–15] The *SefA* gene, the IE1L and IE1R insertion elements, and the Sdf-I sequence have been used to identify *S. enterica* serovar Enteritidis in different matrixes.[14–16] Other published PCR gene targets have included 1) the *rfb* genes that encode the glycosyl synthase and transferase enzymes of O-polysaccharide biosynthesis[17–19]; 2) the *rfbJ* and *abe* genes that encode the enzymes involved in the synthesis of abequose (a sugar present in the O:4 and O:8 serogroups[20]; 3) the *wzx* gene that encodes a flippase[21]; 4) the *fliC* and *fliB* genes that encode the proteins of the phase 1 and 2 H

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antigens that make up part of the variable *Salmonella* flagellar domain and are expressed one at a time owing to the variation mechanism of the flagellar phase that is regulated by the *flj*BA operon[22,23]; 5) the Vi antigen, which is encoded by a number of genes found in the ViaB locus, including *wcdB*, which is specific for the Typhi serovar.[24]

Nevertheless, the aforementioned PCR studies have not used an algorithm derived from the KW scheme, which would dictate the order of the PCR reactions so that the serogroup would be identified first and then the individual serovars. Such a system could be used to readily detect the serovars isolated most frequently from clinical samples. The aim of current work was to develop multiplex real-time PCR (MRT-PCR) assays, using specific primers and TaqMan probes that are to be used in conjunction with the aforementioned algorithm, to identify the more prevalent clinical *S. enterica* isolates.

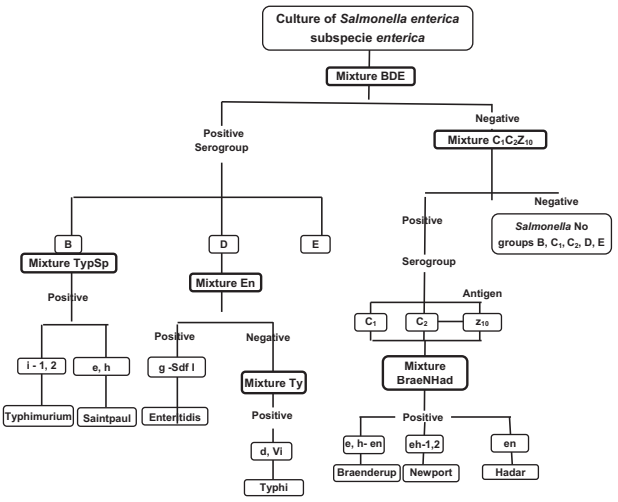

Figure 1. The algorithm derived from the Kaufmann-White scheme, which dictates the order of the MRT-PCR reactions so that the *S. enterica* subsp. *enterica* serogroups are first identified followed by the identification of serotypes.

Materials and Methods

Strains

Eighteen *Salmonella* serovar reference strains were used to standardize the MRT-PCR procedure (*S*. Typhimurium ATCC 14028, *S*. Typhi NTCC 9001, *S*. Enteritidis CDC 64, *S*. Weltevreden INS 210/2004, *S*. Dublin INS-Canada 4, *S*. Braenderup CDC H9812, *S.* Hadar CDC 06-07, *S*. Newport INS-Canada F5-5, *S*. Heidelberg CDC 16, *S*. Saintpaul CDC 108, *S*. Gallinarum ISP-278-74, *S*. Mbandaka World Health Organization 5,7, *S.* Muenchen CDC 1966, *S*. Kentucky CDC 2865-56, *S.* Agona CDC 006/111-19-92, *S.* Anatum CDC 49 287 b2k, *S*. Javiana CDC 160, *S.* Derby CDC 002); 262 serotyped *Salmonella* isolates and 5 non-*Salmonella* enterobacteria isolates were blindly and randomly chosen to evaluate the procedure. These bacterial isolates belong to the collection of the Microbiology Group at the Instituto Nacional de Salud, Colombia. All isolates were stored at −70°C in 20% skim milk (Difco, Becton Dickinson, Franklin Lakes, NJ).

DNA Extraction

The bacterial strains were subcultured in brain heart infusion broth (Difco, Becton Dickson) for 18 hours at 36°C. The bacteria of the cultures were lysed at 98°C for 10 minutes in the infusion broth and then centrifuged at 20,442 × *g* for 5 minutes to isolate the bacterial DNA from the supernatants. The DNA concentration used for MRT-PCR assays was 1 ng/μl.

Primer and TaqMan Probe Design

For this study, 15 sets of primer pairs and TaqMan probes were designed. The sequences of the *rfb*, *fliC*, *fljB*, *viaB* genes and the *invA* gene, and the Sdf-I sequence were obtained from GenBank, National Center for Biotechnology Information (*http://www.ncbi.nlm.nih.gov/GenBank*; accessed May 2, 2007). Multiple alignments using ClustalW (EMBL-EBI; *http://www.ebi.ac.uk/clustalw/index.html*; accessed May 2, 2007) were performed, and the primer sequences and the TaqMan probes specific for the Typhimurium, Saintpaul, Enteritidis, Typhi, Braenderup, Newport, and Hadar serovars were designed using the Beacon Designer v7.0 (PREMIER Biosoft International) software. The specificities of the primers and probes was evaluated *in silico* using the Blastn (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*; accessed May 2007) and ClustalW algorithms, and the sequences that might form dimers and secondary structures were assessed using OligoAnalyzer software (*http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/*; accessed May-August, 2007). The probes were labeled at their 5′ ends with one of the reporter fluorophores, FAM (6-carboxyfluorescein), Cal Fluor Red-560 (CFR560), Cal Fluor Orange-610 (CFO610), or Quasar-670, and at their 3′ ends with a Black Hole Quencher sequestering molecule. The primers and the probes were synthesized by Biosearch Technologies, Inc. Novato, CA. As a strategy to identify the serovars, an algorithm was developed based on the KW scheme, which involves first identifying the O antigens of the serogroup and then, within a serogroup, identifying the serovars (Figure 1). Primers and probes for the O:4; O:9; O:7; O:8; and O:3,10 serogroups, and the phase I; 1,2; e,h; g,m; d; e,n,x; and z_{10} flagellar antigens, were distributed among six mixtures named: BDE, C1C2z10, TypSp, En, Ty, and BraeNHad. Each mixture also included the *inv*A primers and probe set as an internal amplification control.[25] Table 1 shows the compositions of the mixtures.

MRT-PCR Amplification Conditions

To standardize the MRT-PCR assays, the DNA of the *Salmonella* reference strains were used, and the primer and TaqMan probe sets were first individually tested and then tested in mixtures.[25,26] All reactions were done in 20 μl of the master mix, 1× DyNAmo Probe qPCR (Finnzymes, Finland). The optimum concentrations of the

Table 1. TaqMan Probes and Oligonucleotide Primers Used

Mixture name	Designation	Sequence	Amplicon position	Gene/NCBI access no.
BDE	rfbJ-Tq	5′-FAM-TCTCTTATCTGTTCGCCTGTTGT-3′	305	*rfb*J
	rfbJ-F	5′-GCATTTACCACATCATCTAC-3′	275	AE008792
	rfbJ-R	5′-GCGATTAGAGCATGTATATG-3′	424	
	O:9.wzx-Tq	5′-CFO560-CTTATTATTGCCGCTTCCTCTTGG-3′	37	*Wzx*
	O:9.wzx-F	5′-GTTGGTTAGAATTCCAAGAC-3′	15	M65054
	O:9.wzx-R	5′-AAAGAGTAAATACAGCGTATG-3′	154	
	O:3,10.wzx-Tq	5′-CFR610-AATCTAGCCATTCGTTGCTGACT-3′	1411	*wzx*
	O:3,10.wzx-F	5′-CATGGTTCTTTATAAGCATATTC-3′	1328	X60665
	O:3,10.wzx-R	5′-TACTCATACACACTTGTATAAAG-3′	1435	
C1C2Z10	O:7.wzx-Tq	5′-FAM-ACATAAGCACAGTCACAACCTGG-3′	835	*wzx*
	O:7.wzx-F	5′-TTCTTAATTTAAGCTACGTCAC-3′	764	M84642
	O:7.wzx-R	5′-CACTAGCTGTAATAGCATTAAC-3′	865	
	O:8.abe-Tq	5′-CFO560-CTCGGCACTCCAACCTAATCG-3′	834	*abe*
	O:8.abe-F	5′-AGAAACGCATAGTAGTAGTAAG-3′	720	*X61917*
	O:8.abe-R	5′-TTTCACACCCTTCTCAATAG-3′	858	
	fliC.Z10-Tq	5′-CFR610-CACTACCGTCGCAGCTTCTG-3′	936	fliCZ10
	fliC.Z10-F	5′-ATCAAGTAGTGTTCAGGATG-3′	864	AY353472
	fliC.Z10-R	5′-ACCATTCTTATCAGTGTACG-3′	963	
TypSP	fliC.i-Tq	5′-CFO560-ACTCTTGCTGGCGGTGCGACTTCC-3′	772	*fli*Ci
	fliC.i-F	5′-GTTGATAAGACGAACGGTGAGG-3′	748	D13689
	fliC.i-R	5′-CTGCTGTCAATGCGGCTTTAG-3′	875	
	fljB.1,2-Tq	5′-FAM-CGCCAGCCGCAAGGGTTACTGTAC-3′	782	*flj*B1,2
	fljB.1,2-F	5′-TGTTACTATTGGTGGCTTTACTGG-3′	708	AF045151
	fljB.1,2-R	5′-CAGCAGGCATTGTGGTCTTAG-3′	809	
	fliC.eh-Tq	5′-CFR610-TACCGTCTACGCCACCAAGT-3′	1129	*fli*Ceh
	fliC.eh-F	5′-TAATGTAACCACTTATACTGATTC-3′	1056	AY649703
	fliC.eh-R	5′-TTACCGTCGATAGTAACAAC-3′	1157	
Ty	fliC.d-Tq	5′-FAM-CACCGCCTGTTCTGAAGTTATGT-3′	1219	*fli*Cd
	fliC.d-F	5′-TCTGAAGTTGTTACTGCTAC-3′	1141	L21912
	fliC.d-R	5′-TTATCTGTATTAACCTCTTTAAGC-3′	1244	
	wcdB-Tq	5′-CFO560-CTCCAACTGCCACATTATAGACCT-3′	799	wcdB
	wcdB-F	5′-GATTCAGGCCAATCTATTATC-3′	723	D14156
	wcdB-R	5′-AAGCTCATTTAACGAAGTTC-3′	822	
En	fliC.g-Tq	5′-CFR610-CCTGAACAGACAACTCACGCAC-3′	295	*fli*Cg,m
	fliC.g-F	5′-TAACGACGGCATTTCTATTG-3′	204	AY649709
	fliC.g-R	5′-GATCGGAATCAGAGTTAGTC-3′	325	
	Sdf.1-Tq	5′-FAM-AGTAAATCAGCCTGTTGTTGCTC-3′	239	Sdf-1
	Sdf.1-F	5′-CTCAGATTCAGGGAGTATATC-3′	208	AF370707
	Sdf.1-R	5′-TTCGTTCTTCTGGTACTTAC-3′	317	
BraeNHad*	fljB.enx-Tq	5′-CFO560-AGCACCGAATGATACAGCCC-3′	795	*fli*Cenx
	fljB.enx-F	5′-TGTAAGTGGTTATACCGATG-3′	702	AY353305
	fljB.enx-R	5′-CCTGTAACAGTAGATTTAGTTG-3′	821	
Internal control†	invA-Tq	5′-Quasar670-TACTGCTCGTAATTCGCCGC-3′	1773	InvA
	invA-F	5′-TAACCTTGTGGAGCATATTC-3′	1692	M90846
	invA-R	5′-GAATAACATCCTCAACTTCAG-3′	1804	

*The BraeNHad mixture contained the primers and TaqMan probe for the *fliC.eh*, *fljB.enx*, and *fljB.1,2* genes.
†Each mixture included the primers and the TaqMan probe for *invA* amplification.
Tq, TaqMan.

primers in each reaction mixture were: BDE: rfbJ, 600 nmol/L; O:9.wzx, 100 nmol/L; O:3,10.wzx, 600 nmol/L; invA, 200 nmol/L; C1C2-Z_{10}: O:7.wzx, 600 nmol/L; O:8 rfbJ, 100 nmol/L; fliC.z_{10}, 500 nmol/L; invA, 200 nmol/L; TypSp: fliC.i, 500 nmol/L; fljB.1,2, 400 nmol/L; fliC.e,h, 100 nmol/L; invA, 200 nmol/L; Ty: fliC.d, 500 nmol/L; wcdB, 100 nmol/L; invA, 200 nmol/L; En: Sdf.1, 300 nmol/L; fliC.g, 300 nmol/L; invA 400 nmol/L; BraeNHad: fliC.eh, 400 nmol/L; fliC.enx, 100 nmol/L; fljB.1,2, 600 nmol/L; invA, 200 nmol/L. Each reaction mixture also contained 100 nmol/L corresponding TaqMan probe (Biosearch Technologies, Inc.) and 1 ng/μl of the bacterial DNA template.

All reactions used the same amplification conditions and were performed in a CDF-3240 Chromo 4 four-color real-time apparatus (Bio-Rad, Hercules, CA) with the following cycles: first cycle, 95°C for 15 minutes; 35 cycles, 94°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The fluorescence of a mixture was recorded after the extension step for each reaction cycle. The results were analyzed using Opticon Monitor software (version 3.1.32, Bio-Rad). Each MRT-PCR run included the following samples: 1) a positive control, ie, DNA from a known serovar; 2) a negative control, ie, a reaction mixture minus the bacterial DNA template and the internal amplification control (*invA*); and 3) DNA samples from the serovars that were to be typed.

The thermocycler wells were selected including the samples, the internal control of amplification, the positive and negative controls in the arithmetic scale, and then in the logarithmic scale. The threshold fluorescence level was taken to be just the sum of the fluorescence gener-

ated by the negative control, by samples containing intact probes, by the samples used for the control curves, and by positive samples just before the start of amplification. The threshold cycle value (Ct) was defined as the cycle for which the fluorescence was overlapped the threshold level.

The reproducibility of the technique was determined using duplicate runs of the positive control that were performed on different days. The standard deviation was used as the dispersion measurement for the Ct, with an acceptance criterion of 2 SD.

Data Analysis

The Epi-info v.6.0 software (Centers for Disease Control and Prevention, Atlanta, GA) was used to calculate the sensitivities and the specificities of the MRT-PCR reactions.

Results

The sequences of the genes that encode the O:4 (*rfb*J), O:9 (*wzx*), O:7 (*wzx*), O:8 (*abe*), and O:3,10 (*wzx*) serogroups are dissimilar, which allowed us to use them to design unique primers and TaqMan probes. Conversely, the sequences of the genes that encode the G complex antigens (g,m; g,p; g,m,t) and for the flagellar phase 1 complex antigens (1,2; 1,5; 1,6) are about 95–98% identical, which precluded designing unique primers and TaqMan probes for those genes. As alternatives, we prepared primers and TaqMan probes for the Sdf-I chromosomal sequence, as described by Agron and colleagues,[16] and the *fli*C.g gene to identify the Enteritidis and Dublin serovars.[22] The sequences of the EN complex genes were 98% identical so that a single set of primers and probe was used to identify both the e,n,x and e,n,z_{15} antigens (Table 1). The positive amplification curves OR were similar to those of the positive controls in that Ct values of less than 25 cycles were found.

The sets of primer pairs and probe for the reference strains were tested, and each was shown to be specific for its reference strain. Once the specificities had been established, the MRT-PCR conditions for the mixtures were optimized. The serovars were identified using the six MRT-PCR mixtures according to the order diagrammed

Table 2. Agreement Between the Results of the Serotyping and the MRT-PCR Runs for the Test (Randomly and Blindly Chosen) Cases

Serogroup	Serotyping		No.*	MRT-PCR		Agreement (%)
	Serovar	Antigenic formula		Result	No.*	
B	Typhimurium	1,4,[5],12:i:1,2	36	B; i; 1,2	36	100
	Saintpaul	1,4,[5],12:e,h:1,2	29	B; e,h; 1,2	29	100
	Agona	1,4,5,12:f,g,s:[1,2]	1	B; -;-	1	100
	Derby	1,4,5,12:f,g:[1,2]	1	B; -; -	1	100
	Paratyphi B	1,4,5,12:b:1,2	2	B;-;1,2	2	100
	Sandiego	4,5,12:e,h:enz$_{15}$	1	B; e,h; -	1	100
C_1	Braenderup	6,7,14:e,h:e,n,z$_{15}$	26	C_1; e,h; n,Z_{15}	26	100
	Infantis	6,7:r:1,5	1	C_1; -; -	1	100
	Virchow	6,7:r:1,2	1	C_1; -; 1,2	1	100
	Isangi	6,7,14:d:1,5	2	C_1; -; -	2	100
	Livingstone	6,7,14:d:l,w	1	C_1; -; -	1	100
	Montevideo	6,7,14:g,m[p],s:[1,2,7]	2	C_1; -; -	2	100
	Mbandaka	6,7,14:z$_{10}$:e,n,z$_{15}$	3	C_1; z$_{10;}$ e,n,z$_{15}$	3	100
	Virchow	6,7:r:1,2	1	C_1; -; 1,2	1	100
C_2	Newport	6,8,20:e,h:1,2	28	C_2; e,h; 1,2	27	96.4
	Hadar	6,8:z$_{10}$:e,n,x	28	C_2; z$_{10;}$ e,n,x	26	92.8
	Kentucky	6,8:i:z$_6$	3	C_2; -; -	3	100
	Bovismorbificans	6,8,20:r,[i]:1,5	1	C_2; -; -	1	100
	Corvalis	8,20:z$_4$,z$_{23}$:z$_6$	1	C_2; -; -	1	100
	Kottbus	6,8:e,h:1,5	1	C_2; e,h; -	1	100
	Manhattan	6,8:d:1,5	2	C_2; d; -	2	100
	Muenchen	6,8:d:1,2	1	C_2; -; 1,2	1	100
D	Typhi	9,12,[Vi]:d:-	27	D; Vi; d:-	25	92.6
	Enteritidis	1,9,12:g,m:-	26	D; g; m; Sdf-I	25	96.1
	Grupo D	1,9,12:-:-	3	D; -; -	0	0
	Dublin	1,9,12,[Vi]:g,p:-	28	D; g; -	27	96.4
	Javiana	1,9,12:l,z$_{28}$:1,5	2	D; -; -	0	0
	Panama	1,9,12:l,v:1,5	2	D; -; -	2	100
E	Muenster	3,10:e,h:1,5	1	E; -; -	1	100
L	Grupo L	21:-:-	1	-; -; -	1	100
Other Genus	*A. hydrophila*	N/A	1	Negative	1	100
	Citrobacter freundii	N/A	1	Negative	1	100
	Escherichia coli	N/A	1	Negative	1	100
	K. pneumoniae	N/A	1	Negative	1	100
	Shigella sonnei	N/A	1	Negative	1	100
Total			267		255	95.4

*Number of isolate serovars of interest.
N/A, not applicable.

in Figure 1. First, a serogroup was assigned using the data obtained from the BDE and $C_1C_2Z_{10}$ MRT-PCR reactions, and depending on the result, the mixtures that would identify the serovar antigens was selected and tested. Using this strategy, only two rounds of MRT-PCR reactions were needed to identify the Typhimurium, Saintpaul, Enteritidis, Typhi, Braenderup, Newport, and Hadar serovars.

The serotyping and MRT-PCR results were in agreement for 255 of the 267 isolates (95.5%). Table 2 shows the percentage agreement between the serotyping and MRT-PCR results for the individual serovars. Overall, MRT-PCR had a sensitivity of 95.5%, and a specificity of 100%. Discrepancies between the serotyping and MRT-PCR results were found for 12 isolates, the serogroup was not identified for 9 isolates, and the serovar was not identified for three isolates. The discrepancies are related to technical errors that occurred while handling the samples ($n = 3$) and misinterpretations of the amplification curves ($n = 9$).

Discussion

For this study, an MRT-PCR system was developed to identify the Typhimurium, Enteritidis, Typhi, Saintpaul, Braenderup, Newport, and Hadar serovars, which are prevalent in clinical samples in Colombia and other regions of the world.[2,27] This new approach uses an algorithm, based on the Kauffman-White scheme that allows the serogroup to be identified first and then the serovar. This algorithm allows for the optimization of the composition of the primer and probe mixtures of the MRT-PCR assays.

Other studies have shown that molecular typing using PCR has certain advantages over serotyping in that only molecular typing can identify isolates that cannot be typed owing to the loss of the O antigen in rough strains, the presence of a capsule, and/or the absence of flagella.[28–30] Herrera-León and colleagues[9,10] developed an approach consisting of three rounds of multiplex PCR reactions for the identification of the six serovars (Enteritidis, Typhimurium, Hadar, 4,5,12:i:-, Ohio, Infantis) that make up 79.8% of the *Salmonella* serovars isolated from Spaniards.[31] Some of these isolates are prevalent in other regions of the world.[1] For example, in Colombia, the most common serovars isolated from Colombians are Typhimurium, Enteritidis, Typhi, Braenderup, Dublin, Newport, Saintpaul, Uganda, Anatum, and Hadar, representing 82.9% of the total isolates (Instituto Nacional de Salud, *http://www.ins.gov.co*; accessed April 28, 2007). Our method has the advantage that only two rounds of MRT-PCR reactions are needed for serovar identification (Figure 1), and the results are highly sensitive (95.5%) and specific (100%).

According to McQuiston and colleagues[22] the sequences of the *Salmonella fliC* and *fljB* genes (encoding the phase 1 and 2 flagellar antigens) are highly conserved at the 5′ and 3′ termini, whereas the sequences of the central regions are variable. However, this finding is true only for the genes that encode H antigens that are not part of the flagellar complex—the sequences of genes that encode proteins of the complex are very similar, which precludes using those sequences to identify serovars.

Echeita and colleagues[8] reported the design of a universal (sense) primer and specific (antisense) primers for each of the phase 1 complex antigens (1,2; 1,5; 1,6; and 1,7), when we examined the specificities of the primer sequences using the Blastn algorithm, however, it was determined that some of the primers could amplify unspecific DNA associated with the same complex genes of different bacterial genera. Therefore, the current study included primers and a probe for the Sdf-I sequence, as described by Agron et al,[16] as well as primers and a probe for *fliC-g* to identify Enteritidis. In doing so, we obtained 96% agreement between the serotyping and MRT-PCR results. Thus, it is necessary to design probes that target sequences that are distinct from those genes encoding the O, H, and Vi antigens for Derby, Agona, Essen and Hato serovars to distinguish these antigens from others that make up part of a complex.[8] Amplification of *invA* did not occur when enterobacteria DNAs were used as templates. Therefore, as expected, *invA* is specific for *Salmonella*.[29,30]

The fact that there was not 100% agreement between the serotyping and MRT-PCR results may be a consequence of sequence variations within the gene *wzx*, which identifies the O:9 serogroup, and within the genes *fliC* and *fljB*, as reported.[21,22,32] Because these genetic variations exist, it is possible that the primers that we designed to target these genes may have failed to do so. However, because our MRT-PCR procedure was shown to be highly sensitive (95.5% sensitivity), it has potential as a complementary or alternative technique to serotyping. By recombining the designed primers and probes to produce new mixtures, our MRT-PCR procedure, could be used to identify *Salmonella* serovars other than those used in this study.

In conclusion, the aforementioned work by ourselves and others demonstrates that MRT-PCR is a useful adjunct to traditional serotyping, because it can identify *Salmonella* serovars that the latter approach misses. Additionally, as MRT-PCR procedures for the detection of *S. enterica* in food have been reported,[12–15] it is possible that our MRT-PCR procedure might be used to identify *Salmonella* serovars in clinical samples and food.

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