Video Article

An Allelotyping PCR for Identifying *Salmonella enterica* serovars Enteritidis, Hadar, Heidelberg, and Typhimurium

John J. Maurer[1], Margie D. Lee[1], Ying Cheng[1], Adriana Pedroso[1]

[1]Department of Population Health, University of Georgia

Correspondence to: John J. Maurer at jmaurer@uga.edu

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Abstract

Current commercial PCRs tests for identifying *Salmonella* target genes unique to this genus. However, there are two species, six subspecies, and over 2,500 different *Salmonella* serovars, and not all are equal in their significance to public health. For example, finding *S. enterica subspecies* IIIa Arizona on a table egg layer farm is insignificant compared to the isolation of *S. enterica* subspecies I serovar Enteritidis, the leading cause of salmonellosis linked to the consumption of table eggs. Serovars are identified based on antigenic differences in lipopolysaccharide (LPS)(O antigen) and flagellin (H1 and H2 antigens). These antigenic differences are the outward appearance of the diversity of genes and gene alleles associated with this phenotype.

We have developed an allelotyping, multiplex PCR that keys on genetic differences between four major *S. enterica* subspecies I serovars found in poultry and associated with significant human disease in the US. The PCR primer pairs were targeted to key genes or sequences unique to a specific *Salmonella* serovar and designed to produce an amplicon with size specific for that gene or allele. *Salmonella* serovar is assigned to an isolate based on the combination of PCR test results for specific LPS and flagellin gene alleles. The multiplex PCRs described in this article are specific for the detection of *S. enterica* subspecies I serovars Enteritidis, Hadar, Heidelberg, and Typhimurium.

Here we demonstrate how to use the multiplex PCRs to identify serovar for a *Salmonella* isolate.

Video Link

The video component of this article can be found at http://www.jove.com/video/3130/

Protocol

1. Preparation of PCR Template

Note: In order to minimize PCR carryover contamination, it is important to keep several important key steps in the PCR physically-separate from one another: 1) template (DNA) preparation, 2) PCR setup, and 3) gel electrophoresis. It is also recommended to use barrier tips in order to prevent culture or reagent contamination of pipetters.

1. Inoculate 1 ml of Luria Bertani broth or other complex medium (Brain Heart Infusion, Superbroth, etc.) with *Salmonella* isolate from a single isolated colony. Incubate culture overnight at 37°C.
2. Transfer 1 ml to a sterile, 1.5 ml capacity microfuge tube, centrifuge the bacterial cell suspension at 4,500 xg for ˜ 2 min. to pellet cells.
3. Decant supernatant, resuspend the cell pellet in 1 ml of 100% ethanol and set at room temperature for 10 min. Pellet cells as before (4,500 xg, 2 min.), remove the supernatant and resuspend cells in 1 ml PBS.
4. Centrifuge cells (4,500 xg, 2 min.), discard supernatant and resuspend cells in 1 ml of sterile dH_2O.
5. Dilute the final cell suspension 1/20 in dH_2O (5 µl/95 µl dH_2O) and store at -20°C. The shelf-life of the whole cells as PCR template at -20°C is about one month.

2. PCR Setup

Note: Preparation and dispensing of the PCR reaction mix (template, buffers, oligonucletoideprimers, nucleotides and Taq DNA polymerase) needs to be performed in a physically separate room dedicated and preferably done in a PCR/UV workstation.

Note: The PCR set up room must also be self-contained with a designated -20°C freezer for storage of PCR reagents (buffers, oligonucleotide primers, and nucleotides), enzymes and template, dedicated pipetter set for PCR setup, disposable latex gloves, laboratory coat, barrier tips,

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microtiter plates, glass capillaries, microfuge tubes, and 10% bleach for decontaminating surfaces. Use a dedicated pipetter set (P10, P100, and P1000) to dispense PCR reagents. This pipette set must never leave the set-up workstation.

Note: Obtain molecular biology or PCR grade dH_2O and aliquot 1 ml into 1.5 ml capacity centrifuge tubes. Dispense aliquoted dH_2O after each use to avoid possible cross-contamination. A similar approach is recommended with the other PCR reagents.

Note: Decontaminate the work area before use with UV illumination and/or wiping down surfaces with 10% bleach. Wear laboratory coat and latex gloves in performing PCR setup. Minimize back and forth traffic from PCR set-up to areas where the PCR reactions are incubated in the thermocylcer and PCR products (amplicons) are separated on agarose gels. If you go back into the PCR set-up area, dispose of the pair of latex gloves you are currently wearing and put on a new pair of gloves.

1. Make a master primer stock in dH_2o to a concentration of $5x10^{-4}$ M. Dilute the primers 1/20 in dH_2O (5 µl/95 µl dH_2o; 25 µM). The oligonucleotide sequence for each flagellin typing primer is described in Table 1.
2. Retrieve PCR reagents and template from -20°C freezer, and allow reagents and samples to thaw out on ice. Leave the Taq DNA polymerase in freezer until needed.
3. Dispense reagents for allelotyping PCR reaction mix as described in Table 1.
4. Dispense 9µl of the PCR reaction mix into each of 10 round bottom wells in a sterile, 96-well, polystyrene microtiter plate, starting at the top of the column (e.g. A1) and moving down the column to the top of the next.
5. Add 1 µl of dH_2O to the 9 µl of PCR mix (no DNA control) to the first well (A1),0.5µl of the positive control templates (i/g,m typing primers- Typhimurium and Enteritidis; r/z10 typing primers- Heidelberg and Hadar; and 1,2/e,n,x typing primers- Typhimurium and Hadar) to 9 µl of the PCR mix in the 2nd well (B1), and 1 µl of *Escherichia coli* K12 DH5α to the 9 ml of PCR mix in the third well (C1).
6. For each additional well (D1-H1; A2, B2), add 1 µl of the thawed sample template to the 9 µl of the PCR reaction mix. For the i/g, m allelotyping PCR, *S. enterica serovar* Typhimurium (i) and Enteritidis (g,m) serve as positive controls, and *Salmonella serovars* Heidelberg (r) and Hadar (z10) are the positive controls for the r/z_{10} allelotyping multiplex PCR.
7. Place 10 µl capacity glass capillaries into designated holders for the Idaho Technology Rapidcycler (8).
8. once secured in the holder, load each glass capillary with the PCR reaction mix by touching the capillary to the bottom wells of the microtiter plate. Tilt the holder to allow the liquid to move down the tube and provide space between the ends and the PCR mix before heat sealing the glass tubes with a butane torch to seal both ends of the capillaries.
9. Place the capillary tubes and holder into the Idaho Technology Rapidcycler and use thermocycler programs described in Table 1 for the different allelotyping primers to run the PCR.
10. Proceed to gel electrophoresis step when the thermocycler program is completed.

3. Gel Electrophoresis

Note: Wear a different lab coat designated for use in electrophoresis lab and a new pair of disposable latex gloves.

Note: Wear UV protective eye goggles or face shield and always were gloves when handling ethidium bromide, especially agarose gels.

1. Prepare 100 ml molten 1.5% (w/v) molecular biology grade agarose in 1xTAE (or 1X TBE) electrophoresis buffer (7) by repeatedly heating the agarose suspension in a microwave set at 20% power for 2-5 min intervals with periodic visual inspection. Set the molten agarose in 60°C water bath until it equilibrates to the temperature of the water bath.
2. Set up the gel mold with comb before pouring the molten agarose. Choose a gel comb with sufficient teeth to produce enough wells for controls, samples, and at least 3 molecular weight standards for their placements at the ends and the middle of the agarose gel.
3. Add 2 µl of ethidium bromide (10 mg/ml) to 100 ml of molten 1.5 % (w/v) agarose in 1xTAE (or TBE), gently swirl bottle to mix, avoid producing bubbles, and gently pour the contents of the bottle into the middle of the gel mold for 15 by 10 cm agarose gel. Use the edge of tissue paper or paper towel to remove any bubbles in the agarose gel.
4. Let the gel mold set at room temperature until agarose solidifies (˜30-45 min). Transfer the gel tray with gel & comb to submersible, horizontal electrophoresis chamber containing 1X TAE (or 1X TBE) electrophoresis buffer. Gently remove the comb from the agarose gel.
5. Check placement of the gel tray relative to the cathode or positive pole of the electrophoresis chamber to be sure this pole is at the bottom of the gel. Note: Remember the negatively charged DNA migrates towards the cathode (i.e., "run to red").
6. Premix 200 µl of DNA molecular weight markers (0.25 µg/µl) with 40 µl of 6X DNA Loading Dye. Load 4.8 µl of the premixed DNA Molecular Weight Marker XIV to the first, middle and last wells.
7. Load each of the PCR samples starting with well 2 and advancing to each subsequent well, moving from left to right. Avoid loading the sample in any of the wells containing the molecular weight standard.
8. To load samples contained in each glass capillary:
    1. Remove each capillary from its holder and etch both ends of the capillary with a glass cutter.
    2. Place thumb and forefinger of both hands on either side of the capillary marked by the glass cutter and snap the capillary.
    3. Place the capillary dispenser on the end opposite of the PCR reaction mix and slowly turn the plunger clockwise until the liquid is at the very bottom of the tube.
    4. Place the capillary into the well to be loaded and slowly turn the plunger clockwise to dispense the Ficoll-weighted sample into the agarose well. Be careful not to puncture the well with the capillary or introduce an air bubble into the well by turning too much past when the last of the liquid leaves the capillary.
9. Once all the samples and standards are loaded, set the constant voltage of the power supply to 90 V (9 volts/cm agarose gel).
10. Discontinue electrophoresis once the yellow dye (Taurazine) front is 1 cm from the bottom of the gel.
11. Once electrophoresis is completed, transfer the gel to a UV transilluminator to visualize DNA fragments and molecular weight standard. Capture image on Polaroid film using a Polaroid camera with orange glass filter (settings: 2 x, and 4.5 y) or as a digital image using a CCD camera and print image with a thermal printer.
12. Place the capillary holders in 10% bleach for 15-30 min. Rinse 3 times with dH_2O and place back in PCR setup room to air dry.

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4. Representative Multiplex PCR Results

Results for allelotyping PCR of *Salmonella* isolates 1-10 are shown in Figure 1 (lanes 3-12) and interpreted as follows. An O allele designation is given to *Salmonella* isolate based on amplicon (PCR product) matching in size to the PCR control and as predicted for the O allele primer sets used (3): B- 560bp, C1- 340bp, C2- 400 bp, D1- 620 bp, and E1- 280 bp. For isolates tested with the O allelotyping PCR (Fig. 1A), we assigned O alleles B (lanes 10-13), C1 (lanes 7-9), C2 (lanes 4, 5), D1 (lane 3), and E1 (lane 6) to the ten *Salmonella* isolates tested in lanes 3-12. These same *Salmonella* isolates were tested, in the same order as Fig. 1A, for H1 alleles i; g,m; r; and z10 (Fig. 1B,C). Again, a H1 allele is assigned to each isolate dependent on the presence of an amplicon matching in size to the PCR control and as predicted for the 4 H1 allele primer sets used (3): i- 510 bp (Fig. 1B, lane 2); g,m- 310 bp (Fig. 1B, lane 2); r- 170 bp (Fig. 1C, lane 2); and z10- 360 bp (Fig. 1C, lane 2). H1 alleles were assigned to one of the ten *Salmonella* isolates: i - isolates 3 and 8 (Fig. 1B, lanes 5 & 10); g,m- isolates 1 and 5 (Fig. 1B, lanes 3 & 7), r to isolates 6 and 9 (Fig. 1C, lanes 8 & 11); and z10 to isolates 2, 7, and 10 (Fig. 1C, lanes 4, 9, &12). *Salmonella* isolate 4 was negative for the four H1 alleles tested. Finally, *Salmonella* isolates were tested by PCR for H2 alleles associated with the 1,2; 1,5; 1,6; 1,7 antigen complex and e,n,x; e,n,z15 antigen complex. The primer sets for the H2 1,2; 1,5; 1,6; 1,7 complex and H2 e,n,x; e,n,z15 complex produce 290 bp and 150 bp amplicons, respectively (3). The primer sets cannot distinguish specific H2 alleles within either H2 antigen complex to assign a definitive allele type, ex. 1,2, to an isolate (3). *Salmonella* isolates 4, 6, 8-10 were positive for H2 alleles associated with H2 1,2; 1,5; 1,6; 1,7 antigen complex, while isolates 2 and 7 were positive for H2 alleles associated with e,n,x; e,n,z15 antigen complex (data not shown). While *Salmonella* isolates 1, 3, and 5 were negative for the two H2 antigen complexes, only isolates 1 and 5 were negative for H2 flagellin, as determined using a generic H2 flagellin PCR (1) (data not show). These results are summarized in Table 2 along with the presumptive *Salmonella serovar* assigned to each isolate.

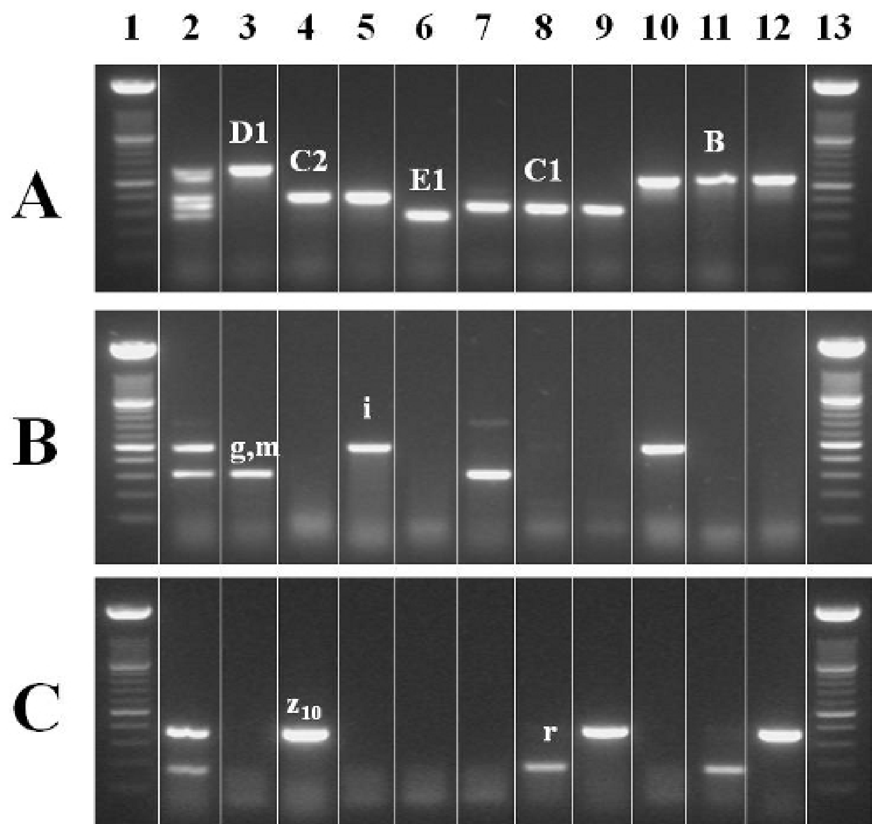

Figure 1. Multiplex PCR for Identifying Specific O and H Alleles Associated with *S. enterica* Serovars Enteritidis, Hadar, Heidelberg, and Typhimurium. (A) O Allele Multiplex PCR. (B,C) H1 Allele Multiplex PCR for Identifying Flagellin Alleles: i/g,m (B) and r/z10 (C). Lanes 1and 13: 100 bp ladder (Promega; Madison, WI); lane 2: multiplex PCR controls for O alleles B, C1, C2, D1, and E (A), H1 alleles i/g,m (B), and H1 alleles r/z10 (C); and lanes 3-12: *Salmonella* isolates 1-10 (Table 2).

PCR Master Mix			**Thermocycler (Rapidcycler)**		
Reagents	**Composition/ concentration**	**Amount**		**Parameters**	**Expected Size**
H1 i/g,m					
dH_2O		63 µl	Program 1	94°C for 1 min	
10X PCR buffer	20 mM $MgCl_2$	10 µl	Program 2	94°C for 1 s	
	500 mMTris pH8			55°C for 1 s	
	2.5 mg/ml BSA			72°C for 20 s	

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	5% Ficoll 400			For 40 cycles	
	10 mMTartrazine			Slope = 2.0	
Primer i (f)*	AACGAAATCAACAACAACCTGC	2 µl	Program 3	72°C for 4 min	510 bp
Primer i (r)*	TAGCCATCTTTACCAGTTCCC	2 µl			
Primer g,m (f)*	GCAGCAGCACCGGATAAAG	2 µl			310 bp
Primer g,m (r)*	CATTAACATCCGTCGCCTTAG	2 µl			
DMSO		5 µl			
dNTP	10 mM	2 µl			
Taq	5 units/µl	2 µl			
		90 µl			
H1 r/z_{10}					
dH_2O		55 µl	Program 1	94°C for 1 min	
10X PCR buffer	30 mM $MgCl_2$	10 µl	Program 2	94°C for 1 s	
	500 mMTris pH8			55°C for 1 s	
	2.5 mg/ml BSA			72°C for 20 s	
	5% Ficoll 400			For 40 cycles	
	10 mMTartrazine			Slope = 2.0	
Primer r (f)*	CCTGCTATTACTGGTGATC	4 µl	Program 3	72°C for 4 min	170 bp
Primer r (r)*	GTTGAAGGGAAGCCAGCAG	4 µl			
Primer z_{10} (f)*	GCACTGGCGTTACTCAATCTC	4 µl			360 bp
Primer z_{10} (r)*	GCATCAGCAATACCACTCGC	4 µl			
DMSO		5 µl			
dNTP	10 mM	2 µl			
Taq	5 units/µl	2 µl			
		90 µl			
PCR Master Mix			**Thermocycler (Rapidcycler)**		
Reagents	**Composition/ concentration**	**Amount**		**Parameters**	**Expected Size**
H2 1,2/e,n,x					
dH_2O		63.6 µl	Program 1	94°C for 1 min	
10X PCR buffer	20 mM $MgCl_2$	10 µl	Program 2	94°C for 1 s	
	500 mMTris pH8			55°C for 1 s	
	2.5 mg/ml BSA			72°C for 20 s	
	5% Ficoll 400			For 40 cycles	
	10 mMTartrazine			Slope = 2.0	
Primer 1,2 (f)*	AGAAAGCGTATGATGTCGAA	2 µl	Program 3	72°C for 4 min	290 bp
Primer 1,2 (r)*	ATTGTGGTTTTAGTTGCGCC	2 µl			
Primer e,n,x (f)*	TAACTGGCGATACATTGACTG	2 µl			150 bp
Primer e,n,x (r)1	TAGCACCGAATGATACAGCC	2 µl			
DMSO		5 µl			
dNTP	10 mM	2 µl			
Taq	5 units/µl	2 µl			
		90 µl			
Generic H2					

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dH_2O		72 µl	Program 1	94°C for 1 min	
10X PCR buffer	30 mM $MgCl_2$	10 µl	Program 2	94°C for 10 s	
	500 mMTris pH8			45°C for 10 s	
	2.5 mg/ml BSA			72°C for 35 s	
	5% Ficoll 400			For 40 cycles	
	10 mMTartrazine			Slope = 2.0	
Primer *fljB* (f) *	CAAGTAATCAACACTAACAGTC	2 µl	Program 3	72°C for 4 min	1,500 bp
Primer *fljB* (r) *	TTAACGTAACAGAGACAGCAC	2 µl			
dNTP	10 mM	2 µl			
Taq	5 units/µl	2 µl			
		90 µl			

Table 1. Protocol for *Salmonella* flagellinallelotyping PCR: composition, conditions, and expected results.

*The working stock concentration of PCR oligonucleotide primers is 25 µM

Isolate	O Allele					H1 Allele				H2 Allele			Serovar
	B	**C1**	**C2**	**D1**	**E**	**i**	**g,m**	**r**	**z10**	**1,2; 1,5; 1,6; 1,7**	**e,n,x; e,n,z15**	**Generic[1]**	
1	-	-	-	+	-	-	+	-	-	-	-	-	**Enteritidis**
2	-	-	+	-	-	-	-	-	+	-	+	+	**Hadar/ Istanbul[3]**
3	-	-	+	-	-	+	-	-	-	-	-	+	Kentucky
4	-	-	-	-	+	-	-	-	-	+	-	+	Unknown
5	-	+	-	-	-	-	+	-	-	-	-	-	Montevideo
6	-	+	-	-	-	-	-	+	-	+	-	+	Infantis or Virchow[2]
7	-	+	-	-	-	-	-	-	+	-	+	+	Mbandaka
8	+	-	-	-	-	+	-	-	-	+	-	+	**Typhimurium**
9	+	-	-	-	-	-	-	+	-	+	-	+	**Heidelberg**
10	+	-	-	-	-	-	-	-	+	+	-	+	Haifa

Table 2. Allelotyping PCR Results (Fig. 1) and *Salmonella* Serovar Designation Based on Identification of O, H1, and H2 Alleles

> [1]H2 PCR produces a 1.5 kb amplicon for all *Salmonella* possessing H2 flagellin, regardless of H2 allele (1). This PCR is used to distinguish monophasic from the biphasic *Salmonella*.
[2]H2 multiplex PCR cannot distinguish among the different alleles for the H2 1,2; 1,5; 1,6; 1,7 antigen complex (3).
[3]Phage conversion of *S. enterica* serovar Hadar alters LPS O antigen to produce serovar Istanbul. The O allelotyping PCR cannot discern these subtle genetic/antigenic changes.

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Serovar[1]	O Allele					H1 Allele				H2 Allele		
	B	C1	C2	D1	E	i	g,m	r	z10	1,2; 1,5; 1,6; 1,7	e,n,x; e,n,z15	Generic[2]
California	+	-	-	-	-	-	+	-	-	-	-	-
Typhimurium	+	-	-	-	-	+	-	-	-	+	-	+
Heidelberg	+	-	-	-	-	-	-	+	-	+	-	+
Haifa	+	-	-	-	-	-	-	-	+	+	-	+
Montevideo	-	+	-	-	-	-	+	-	-	+	-	+
Othmarschen	-	+	-	-	-	-	+	-	-	-	-	-
Athinai	-	+	-	-	-	+	-	-	-	-	+	+
Papuana	-	+	-	-	-	-	-	+	-	-	+	+
Mbandaka	-	+	-	-	-	-	-	-	+	-	+	+
Chincol	-	-	+	-	-	-	+	-	-	-	+	+
Kentucky	-	-	+	-	-	+	-	-	-	-	-	+
Hadar/ Istanbul[3]	-	-	+	-	-	-	-	-	+	-	+	+
Enteritidis	-	-	-	+	-	-	+	-	-	-	-	-
Seremban	-	-	-	+	-	+	-	-	-	+	-	+
Campinense	-	-	-	+	-	-	-	+	-	-	+	+
Lome	-	-	-	+	-	-	-	+	-	-	-	+
Portland	-	-	-	+	-	-	-	-	+	+	-	+
Ruanda	-	-	-	+	-	-	-	-	+	-	+	+
Treguier	-	-	-	+	-	-	-	-	+	-	-	+
Simi	-	-	-	-	+	-	-	+	-	-	+	+
Weltevreden	-	-	-	-	+	-	-	+	-	-	-	+
Kristianstad	-	-	-	-	+	-	-	-	+	-	+	+
Biafra	-	-	-	-	+	-	-	-	+	-	-	+

Table 3.*Salmonella enterica* Serovars Identified by Allelotyping PCR

[1]The serovars highlighted in bold are the ones more commonly encountered by diagnostic or food microbiology laboratory.
[2]H2 PCR produces a 1.5 kb amplicon for all *Salmonella* possessing H2 flagellin, regardless of H2 allele (1). This PCR is used to distinguish monophasic from the biphasic *Salmonella*.
[3]Phage conversion of *S. enterica* serovar Hadar alters LPS O antigen to produce serovar Istanbul. The O allelotyping PCR cannot discern these subtle genetic/antigenic changes.

Discussion

Most commercially available PCR tests for the detection of *Salmonella* targets genes unique to the genus (ex. *invA*). However, not all *Salmonella* are equal in their ability to cause disease in humans or prevalence in animal populations. Early recognition of antigenic differences in *Salmonella* LPS O-antigen and flagellinH1 and H2 antigens has lead to the differentiation of *Salmonella* into over 2,500 serovars based on these antigenic differences. There are 46 distinct O antigens and 86 distinct flagellin (H) antigens identified in the genus *Salmonella*. *Salmonella* may possess one (H1) or two different (H1&H2) flagellins. The different combination of O, H1, and H2 antigens account for the 2,500 *Salmonella* serovars recognized today. A serovar is assigned based on the antigenic formula derived from the identification of the individual O and H antigens. The antigenic formula is written as O: H1: H2; and where "-", refers to the absence of H1 or H2 flagellin and "NT" (non-typeable) for *Salmonella* negative for the O-antigen. For example, the serovar Typhimurium is assigned to a *S. enterica* isolate with the antigenic formula B: i: 1,2 while Enteritidis is given to an isolate with the formula D1: g,m: -. This antigenic variation in the *Salmonella* population is the outward manifestation of differences at the nucleotide level that can therefore be easily exploited by PCR.

We have identified the genetic differences associated with specific O and H alleles to develop an allelotyping PCR for identifying *S. enterica* serovars: Enteritidis, Hadar, Heidelberg, and Typhimurium (3). The correct assignment and reporting of *Salmonella* serovar requires testing for all alleles associated with O: H1: H2 antigenic formulas for Enteritidis (D1: g,m: -), Hadar (C2: z10: e,n,x), Heidelberg (B: r: 1,2), and Typhimurium (B: i: 1,2). Without knowledge of O allele or antigen, the finding of the H1 g,m allele alone does not necessarily identify the *Salmonella* isolate as Enteritidis as other *Salmonella* serovars: Agona, California, and Montevideo, also possess this same allele. While the allelotyping PCR was originally designed to detect the fore mentioned *Salmonella* serovars, this test can identify an additional 19 serovars (Table 3). Our allelotyping PCR was originally designed to detect O and H alleles. In practice, it has become more practical and cost effective for us to identify

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the O-antigen by slide agglutination test, using O-specific antisera, rather than performing the O allelotyping PCR when working with isolated *Salmonella* cultures. However, we do recommend using the O allelotyping PCR if your lab uses this PCR as a screen (2) or for typing *Salmonella* isolates submitted on FTA cards (6), and include a *Salmonella*-specific PCR with the first screen of samples (4). Limit the allelotyping PCRs to only those samples that are identified as *Salmonella* by PCR or biochemical/antigenic tests.

The protocol described in this article has been optimized for use with the Idaho Technology Rapidcycler, a hot-air thermocycler that allows one to scale down reaction volumes and runs reaction conditions in less time than many heating block thermocyclers. To adapt this protocol for use with a heating block thermocycler may require optimizing reaction conditions empirically by lowering/raising annealing temperatures; adjusting primer concentrations; or adjusting $MgCl_2$ concentrations. For example, we have had to adapt the protocol for the MJ Research PTC-200 thermocycler as follows. Working with the same reaction volumes described in Table 1, the working stock concentrationof the i primer set was diluted to 2.5 µM, the annealing temperature was lowered from 55°C to 45°C, and incubation times at the denaturation, annealing and extension steps was lengthened to 20 s for the i/g, m allelotyping PCR. Having the appropriate positive and negative controls are essential in the initial start up and optimization for any PCR, including published protocols. We recommend acquiring known *Salmonella* serovars, specifically Anatum (E1: e,h: 1,6), Enteritidis (D1: g,m: -), Hadar (C2: z10: e,n,x), Heidelberg (B: r: 1,2), Montevideo (C1: g,m,s: 1,2,7) and Typhimurium (B: i: 1,2); and a laboratory *Escherichia coli* K12 strain (DH5α, LE393, JM109, etc.) as positive and negative controls respectively for the allelotyping PCR tests described in this article. Several of these *Salmonella* serovars will serve as either positive or negative controls, depending on which allele is tested.

Certain precautions and guidelines need to be followed while performing this and any other diagnostic PCR. First, the physical separation of template preparation, PCR set-up, and gel electrophoresis is critical in preventing possible PCR carry-over contamination and erroneous reporting of false-positives. In addition, the inclusion of appropriate controls is necessary in any routine PCR as these controls identify problems as they arise and assist in the interpretation of results (5). Most importantly, consistency is essential, especially with regards to the electrophoretic conditions for amplicon (PCR product) detection andestimation of amplicon size. To illustrate this point, we purposely suspended electrophoresis earlier than intended in Figure 1A. The molecular weight standards (lanes 1 and 13) and positive control (lane 2) are not sufficiently separated to accurately estimate sizes or observe separation of DNA fragments where there are small differences (˜50 bp) in sizes. Adequate separation of DNA fragments, especially the molecular weight standards, is essential as reporting positives is dependent on the accurate estimation of amplicon size and distinguishing "true positive" from non-specific amplicons that are sometimes observed in the daily running of any PCR (5). For example, there is a non-specific amplicon in Figure 1B, lane 7 (˜700 bp in size). Had electrophoresis been discontinued too early, when the dye front was only at the half way point in the agarose gel, there would be little differentiation between 500 bp and the 700 bp DNA fragments. Therefore, sample in lane 7 would have been erroneously reported as positive for both i and g,m alleles.

Finally, one needs to recognize the limitations associated with any test. Several of the H1/H2 allelotyping PCR do not have the discriminatory power to discern subtle genetic differences in alleles belonging to the H2 1,2; 1,5; 1,6; 1,7 antigen complex; e,n,x/e,n,z15 antigen complex and the H1 G antigen complex, which includes the g,m allele. One or two amino acid changes account for the different epitopes present in these flagellar antigens. It was therefore difficult for us to design primers that could exploit these sometimes single base pair differences in the flagellin gene sequence (3). For example, *Salmonella* serovars Dublin (D1: g,p: -) and Berta (D1: f,g,t: -) are also PCR positive with our g,mallelotyping primers. The H2 allelotyping primers cannot distinguish Typhimurium (B: i : 1,2) from Lagos (B: i: 1,5), Heidelberg (B: r: 1,2) from Bradford (B: r: 1,5), Winneba (B: r: 1,6), or Remo (B: r: 1,7), or Hadar (C2: z10: e,n,x) from Glostrup (C2: z10: e,n,z15). While the likelihood of encountering some of these serovars: Lagos, Bradford, Winneba, Remo or Glustrup is remote, it is a possibility and requires either providing disclaimer on the tests' limitation or another confirmatory test.

In conclusion, this PCR-based serotyping rapidly identifies *S. enterica* serovars Enteritidis, Hadar, Heidelberg and Typhimurium, and O, H1, and H2 alleles associated with 19 additional serovars. This assay is a rapid, cost-effective alternative to traditional microbiological approach to serotyping *Salmonella*.

Disclosures

No conflicts of interest declared.

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