International Journal of Poultry Science 8 (2): 156-160, 2009
ISSN 1682-8356

Allelotyping PCR for Detection and Screening of *Salmonella enterica* Serovar *Enteritidis* and *Typhimurium*

S. Essam Soliman[1], C. Kilpatrick[2], S. Mohamed Ahmed[3], M. Eman Abouelhassan[1], R. Nimmanapelli[1] and P.G. Reddy[1]
[1]Department of Pathobiology, CVMNAH, Tuskegee University, USA
[2]Alabama State Veterinary Diagnostic Laboratory
[3]School of Veterinary Medicine, Suez Canal University

Abstract: Classical *Salmonella* sero-typing is an expensive and time consuming process that requires implementing a battery of O and H antisera to detect 2541 different *Salmonella enterica* serovars. During this study a rapid multiplex Polymerase Chain Reaction (PCR) scheme was developed to screen for the prevalent *Salmonella enterica* serovar *Enteritidis* and *Typhimurium*. By analyzing the nucleotide sequences of the genes for O-antigen biosynthesis including wba operon and the central variable regions of the H1 and H2 flagellin genes in *Salmonella*, designated PCR primers for two multiplex PCR reactions were used to detect and differentiate *Salmonella* serogroups A/D1, B, C1, C2, or E1; H1 antigen types l, g, m, r or z_{10} and H2 antigen complexes, l: 1,2; 1,5; 1,6; 1,7 or ll: e, n, x; e,n,z_{15}. Through the detection of these antigen gene allele combinations, the study was able to distinguish among *Salmonella enterica* serovars *Enteritidis* and *Typhimurium*. The assays were useful in identifying *Salmonella* with O and H antigen gene alleles representing ten distinct serovars. While the H2 multiplex could discriminate between unrelated H2 antigens, the PCR could not discern differences within the antigen complexes, 1,2; 1,5; 1,6; 1,7 or e, n, x; e,n,z_{15}, requiring a final confirmatory PCR test in the final serovar reporting of *Salmonella enterica*.

Key words: Alleles, *Salmonella enterica*, PCR, O antigen, H antigen

INTRODUCTION

Epidemiological measures have been implemented to reduce the source(s) of infection. Because food sources are recognized as important reservoirs of *Salmonella* (Henson, 1997; Lynch *et al*., 2006); The United States Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) implemented an "in plant" Hazard Analysis and Critical Control Point (HACCP) program to reduce the prevalence of foodborne pathogen contamination in meats, eggs and milk. Although in-plant HACCP programs have been successful, further reductions in *Salmonella* contamination may require application of a risk reduction strategy to the farm environment. A more achievable goal may be to mitigate those *S. enterica* serovars that are most frequently associated with severe human illness. Producers may also need to accurately identify the source of *Salmonella* within a specific setting, in order to identify the points where an intervention (Sanchez *et al*., 2002) may be effective. Such an approach would require knowing whether these serovars are present on the farm. Also, determining the appropriate *Salmonella enterica* serovar is a necessary first step in any epidemiological investigation of foodborne outbreaks; followed then by strain typing, using molecular based methods including Pulsed-field Gel Electrophoresis (PFGE) (Gerner-Smidt *et al*., 2006) or amplified fragment length polymorphism that is needed to match patient strain to source (Lindstedt *et al*., 2000). There are currently 2,541 *Salmonella enterica* serovars recognized based on antigenic differences in the Lipopolysaccharide (LPS) O-antigen and phase 1 (H1) and phase 2 (H2) flagellar antigens, *Salmonella* Surveillance Annual Summary (2005). *Salmonella* can be further separated into monophasic and biphasic based on whether they express only one (H1) or both flagellar antigens (H1 and H2). The antigenic formula 4,5,12 (O): l (H1): 1,2 (H2) is the biphasic *Salmonella enterica* serovar *Typhimurium* and 1,9,12 (O):g,m (H1):- (no H2) identifies the monophasic *Salmonella enterica* serovar *Enteritidis*. Worldwide, two serovars, *Enteritidis* and *Typhimurium* are responsible for 79% of reported cases of salmonellosis, (WHO Global *Salmonella* Survey, Progress Report, 2005). *Salmonella* serotyping is based on the identification of the variable O and H antigens. Because the antigenic composition of the O, H1 and H2 antigens are a reflection of their unique DNA sequence alleles; (Joys, 1985; Samuel and Reeves, 2003). PCR and similar nucleotide-based methods have made it possible to accelerate the identification of serotypes based upon the identification of unique genes or gene arrangements (Curd *et al*., 1998; Maurer *et al*. (1999).

MATERIALS AND METHODS

Bacterial strains: The *Salmonella enterica* isolates used in this study were from human, poultry, livestock and wildlife Beltran *et al*. (1991); Swamy *et al*. (1996)

and serotyped by the National Veterinary Service Laboratory using classical methods (Table 2). The isolates were used to test the specificity of PCRs specific for O, H1 and H2 alleles. Additional *Salmonella* isolates of unknown serovars were obtained from some poultry farms in Alabama (Liu *et al*., 2002). The commercial chicken broiler house environment and chicken carcasses were sampled for *Salmonella*. Serotyping was done using standard serological typing procedures for *Salmonella* O, H1 and H2 antigens.

PCR primer design: From comparative analysis of the wba operon for *Salmonella* serogroups A/D1, B, C1, C2 and E1 (Brown *et al*., 1992; Verma and Reeves, 1989), serogroup-specific gene (s) (National Center for Biotechnology Information (NCBI) were identified for PCR primer design. Similarly, an alignment within the central variable region (Joys, 1985; Vanegas and Joys, 1995), of fliC (H1) and fljB (H2) alleles were identified, using the DNA analysis software AlignPlus® version 3.0 (Scientific and Educational Software), candidate sequences to differentiate *Salmonella* with the H1 flagellin antigens l, g,m, r, z_{10} and the H2 flagellin antigen complexes 1,2, 1,5, 1,6, 1,7 and e,n,x, e,n,z_{15} alleles. These gene sequences were analyzed, using the GeneRunner® DNA analysis software and identified suitable primer sets that were compatible in a single multiplex PCR reaction and designed to produce an amplicon with size unique for the sequence (s) targeted by a specific primer set, (Table 1).

Multiplex allelotyping PCR for *Salmonella* O, H1 and H2 antigen genes and differentiating *S. enterica* serovars *Enteritidis* and *Typhimurium*: The O-antigen multiplex PCR was designed to detect serogroup A/D1, B, C1, C2, or E1 specific genes or alleles. The O-antigen multiplex PCR was performed using the Cepheid smart cycler, using smart cycler PCR tubes. Each reaction had a final concentration of 1.5 mM $MgCl_2$, 50 mM Tris, pH 8.3, 0.25 mg/ml bovine serum albumin, 0.5 μM primer, 0.2 mM deoxynucleotides, 0.5 units of Taq DNA polymerase and 1 μl of whole cell template. The PCR was performed with pre-amplification heating (D'Aquilla *et al*., 1991). The program parameters for PCR include an initial five min incubation at 85°C, to mix the two PCR reaction mixes, followed by 30 cycles of denaturation (94°C for 5 min), annealing (55°C for 1 min) and extension (72°C for 1 min). Amplicons were separated on 1.5% agarose gel with Tris-acetate-EDTA buffer (Sambrook *et al*., 1989) and ethidium bromide (0.2 μg/ml) at 100 V. The 100-bp ladder was used as a Molecular Weight (MW) standard for determining the MW of the PCR products. Various *Salmonella enterica* serovars belonging to serogroups A/D1, B, C1, C2, E1 were used in the PCR to test the specificity of the primer sets.

The H1-1 multiplex PCR was used to identify isolates with antigens l or g, m; while the H1-2 multiplex PCR was designed to detect isolates with antigens r or z_{10}. Finally, the H2 multiplex PCR was created to differentiate isolates with either H2 antigen complexes 1,2; 1,5; 1,6; 1,7; or e,n,x; e,n,z_{15}. In order to identify the H1 and H2 alleles, capillary PCR reaction was performed to amplify the alleles of fliC and fljB by three multiplex PCRs with the Rapidycler™ hot-air thermocycler (Wittwer *et al*., 1989) in 10-μl capacity capillary tubes. The 10-μl PCR mix for the fliC l and g,m multiplex consisted of 2.0 mM $MgCl_2$, 50 mM Tris (pH 8.3), 0.25 mg/ml bovine serum albumin, 0.5 μM of each primer, 0.2 mM deoxynucleotides, 5% DMSO, 1.0 units of Taq DNA polymerase and 1 μl whole cell template. For fliC r and z_{10} multiplex, 3.0 mM $MgCl_2$ and 1.0 μM of each primer were used for each reaction. For the amplification of the H2 alleles, the fljB multiplex consisted of 3.75 mM $MgCl_2$, 62.5 mM Tris, pH 8.3, 0.31 mg/ml bovine serum albumin, 0.5 μM of each primer, 0.2 mM deoxynucleotides, 5% DMSO, 1.0 units of Taq DNA polymerase and 1 μl whole cell template in a 10 μl volume. The program parameters for the hot-air thermocycler were an initial heating step of 94°C for 1 min; 94°C for 1 sec, 55°C for 1 sec and 72°C for 20 sec with a slope of 2.0 for 40 cycles and a final extension at 72°C for 4 min. Amplicons were detected. The specificity of the PCR detection was tested against various *Salmonella* serovars possessing the relevant fliC and fljB alleles, Table 2. Escherichia coli LE392 served as a negative control.

RESULTS AND DISCUSSION

PCR differentiation of *Salmonella enterica* serovars *Enteritidis* and *Typhimurium*: Multiplex PCRs targeted to the O, H1 and H2 alleles associated with two *Salmonella enterica* serovars *Enteritidis* and *Typhimurium*. Specific PCR primers to identify specific *Salmonella* serogroups, H1 and H2 alleles were designed based on the divergence of the glycosyl synthase genes, the unique linkage between two genes for a specific O-antigen of *Salmonella*, or allele-specific sequences within the hypervariable region of H1 and H2 antigen genes. In the primer design, a unique amplicon size was selected in order to facilitate development of a multiplex PCR (Table 1). The abilities of the multiplex PCR to identify the serogroups correctly was evaluated for 200 *Salmonella* isolates representing ten different serotypes which belonged to one of the six major serogroups, A, B, C1, C2, D1 and E1. With the exception of serogroups A and D1, which produce the same size amplicons (Kappa = 0.98), the multiplex PCR accurately distinguished *salmonellae* belonging to serogroups B, C1, C2 and E1 (Kappa = 1.00) (Table 2). The inability to distinguish serogroups A and D1 is due to the high degree of nucleotide sequence homology between the prt (paratose synthase) genes (Verma and Reeves, 1989). The fliC multiplex PCRs successfully detected

Table 1: Primers used for multiplex PCR to detect and differentiate *Salmonella enterica* serovars

Target gene		Nucleotide sequence		Expected Size (bp)
O-antigen multiplex	abe_1 (B)	Forward	GGCTTCCGGCTTTATTGG	561
		Reverse	TCTCTTATCTGTTCGCCTGTTG	
	wbaD-manC (C1)	Forward	ATTTGCCCAGTTCGGTTTG	341
		Reverse	CCATAACCGACTTCCATTTCC	
	abe_2 (C2)	Forward	CGTCCTATAACCGAGCCAAC	397
		Reverse	CTGCTTTATCCCTCTCACCG	
	prt (A/D1)	Forward	ATGGGAGCGTTTGGGTTC	624
		Reverse	CGCCTCTCCACTACCAACTTC	
	wzx – wzy (E1)	Forward	GATAGCAACGTTCGGAAATTC	281
		Reverse	CCCAATAGCAATAAACCAAGC	
H1-1 multiplex	fliC (l)	Forward	AACGAAATCAACAACAACCTGC	508
		Reverse	TAGCCATCTTTACCAGTTCCC	
	fliC (g,m)	Forward	GCAGCAGCACCGGATAAAG	309
		Reverse	CATTAACATCCGTCGCGCTAG	
H1-2 multiplex	fliC (r)	Forward	CCTGCTATTACTGGTGATC	169
		Reverse	GTTGAAGGGAAGCCAGCAG	
	fliC (z_{10})	Forward	GCACTGGCGTTACTCAATCTC	363
		Reverse	GCATCAGCAATACCACTCGC	
H2 multiplex	fljB (l: 1,2; 1,5; 1,6; 1,7)	Forward	AGAAAGCGTATGATGTGAAA	294
		Reverse	ATTGTGGTTTTAGTTGCGCC	
	fljB (ll: e,n,x; e,n,z_{15})	Forward	TAACTGGCGATACATTGACTG	152
		Reverse	TAGCACCGAATGATACAGCC	

[1]Indicates the unique genes or the junctions between the two genes used for designing PCR primers. () = antigen (s) detected.

Table 2: Comparison of multiplex PCR to serotyping for identifying *Salmonella* O alleles

Antigenic Formula				O muliplex PCR					PCR				multiplex PCR	
			S. *enterica*						l/g,m multiplex PCR		r/z_{10} multiplex PCR			
O	H1	H2	Serovars	B	C1	C2	D1	E1					1,2	e,n,x
A	a	1,5	Paratyphi A	0	0	0	1	0	0	0	0	0	1	0
B	b	1,2	Paratyphi B	1	0	0	0	0	0	0	0	0	1	0
B	l	1,2	*Typhimurium*	74	0	0	0	0	74	0	0	0	74	74
B	r	1,2	Heidelberg	24	0	0	0	0	0	0	24	0	24	0
B	z	1,7	Indiana	2	0	0	0	0	0	0	0	0	2	0
C1	c	1,5	Paratyphi C	0	1	0	0	0	0	0	0	0	1	0
C1	r	1,5	Infantis	0	2	0	0	0	0	0	2	0	2	0
C2	l	z_6	Kentucky	0	0	24	0	0	24	0	0	0	0	0
C2	z_{10}	e,n,x	Hadar	0	0	10	0	0	0	0	0	10	0	10
D1	g,m	-	*Enteritidis*	0	0	0	20	0	0	20	0	0	0	0
D1	-	-	Gallinarum	0	0	0	4	0	0	4	0	0	0	0
E1	e,h	1,5	Muenster	0	0	0	0	2	0	0	0	0	2	0

Table 3: Allelotyping PCR for the identification of *Salmonella enterica* serovars *Enteritidis* and *Typhimurium*

O-multiplex	H1-multiplexes [(1)]	H2-multiplex	Serovars	Sensitivity	Specificity
B	l	l[(2)]	*Typhimurium*	1.00	1.00
A/D1	g,m	-[(3)]	*Enteritidis*	1.00	0.96

[(1)]Identifies H1 alleles l; g,m; r; or z_{10}, [(2)] Covers H2 alleles 1,2; 1,5; 1,6 and 1,7, [(3)] PCR negative for H2-multiplex.

the H1, l, r, or z10, alleles and no amplicons were produced for serovars with other H1, flagellins (Kappa = 1.00), (Table 2). However, the fliC g,m primer set produced the same size amplicon only for salmonellae that possessed both the g and m, or g alone, or either epitope, g or m, in combination with other serotype-specific epitopes, or non-motile salmonellae that possess the fliC g,m allele Li *et al*. (1993) and therefore it did not have the specificity of the other H1 primer sets (Kappa = 0.58 vs. 1.00) (Table 2). To complement our PCR-based H allelotyping, a fljB multiplex PCR was designed to detect the H2 antigen alleles by targeting conserved regions within fljB alleles encoding the antigen complexes l: 1,2; 1,5; 1,6; 1,7 or ll: e,n,x; e,n,z15 and producing unique size amplicons, Table 1. The expected size amplicons were produced for only those

Salmonella enterica serovars belonging to H2 antigen complexes I: 1,2; 1,5; 1,6: 1,7 and. II: e,n,x; e,n,z15. The H2 multiplex PCR however could not distinguish H2 1,2 allele (Kappa = 0.75) or e,n,x (Kappa = 0.54) among the different H2 alleles within each antigen complex; indistinguishable amplicons were produced for *Salmonella* isolates bearing 1,2 vs 1,5; 1,6; or 1,7 (Table 2).

Comparison of multiplex PCR allelotyping of O, H1 and H2 genes with conventional serotyping in differentiating *S. enterica* serovars *Enteritidis* and *Typhimurium*: Validation of the allelotyping method is important for its integration with conventional *Salmonella* culture and typing methods used in diagnostic and food microbiology (Herrera-Leon *et al*., 2007). Therefore the allelotyping multiplex PCR was assessed against the standard conventional *Salmonella* serotyping method in identifying *Salmonella* O, H1 and H2 antigens for ten different serovars of salmonellae isolated mainly from chicken carcasses and poultry environments (Tables 2 and 3).

An initial multiplex PCR is performed to determine which O antigen allele that an isolate possesses and a serogroup designation is given or unknown, based on PCR results. If the isolate possesses O alleles for serogroups B, C2, or A/D1, then a 2nd allelotyping PCR is done to determine the presence of H2 alleles: l; g,m; r; or z_{10}. Based on the results of this 2nd allelotyping PCR, an H1 allele type can be given an isolate as either being l; g,m; r; z_{10} or unknown, if no amplicons with the expected size for the H1 allelotyping PCR are produced. If both O and H1 allelotyping PCR detects O and H1 alleles associated with *Salmonella enterica* serovars *Enteritidis* and *Typhimurium*, then a 3rd final H2 allelotyping PCR is performed to further differentiate the isolate to serovar level. The expansion of O-antigen PCR to detect serogroups C1 and E1, affords a laboratory the opportunity to detect other *Salmonella enterica* serovars, as the antigenic formula for O, H1 and H2 antigens defines the serovar. Also identifing monophasic *Salmonella enterica* serovars by including a generic *Salmonella* fljB (H2) PCR test (Dauga *et al*., 1998). The limitations with our multiplex PCR are that it cannot distinguish among serogroup/serovar variants that arise due to phage conversion and the resulting chemical/antigenic alteration of the somatic O antigen (*Salmonella* Surveillance Annual Summary, 2005) or subtle point mutations in H2 antigen gene, fljB responsible for loss of flagellar expression observed in some *S. enterica* serovar *Typhimurium* strains (Zamperini *et al*., 2007). Also, the allelotyping primers for H1 g,m allele identifies those H1 alleles bearing g or m in any possible combination, Table 2, therefore H1 multiplex would not be able to discern serogroup D1, *S. enterica* serovars *Enteritidis* [H1: g,m] [g,m,q].

REFERENCES

Beltran, P., S.A. Plock, N.H. Smith, T.S. Whittam, D.C. Old and R.K. Selander, 1991. Reference collection of strains of the *Salmonella typhimurium* complex from natural populations. J. Gen. Microbiol., 137: 601-606.

Brown, P.K., L.K. Romana and P.R. Reeves, 1992. Molecular analysis of the rfb gene cluster of *Salmonella* serovar Muenchen (strain M67): the genetic basis of the polymorphism between groups C2 and B. Mol Microbiol., 6: 1385-1394.

Curd, H., D. Liu and P.R. Reeves, 1998. Relationships among the O-antigen gene clusters of *Salmonella enterica* groups B, D1, D2 and D3. J. Bacteriol., 180: 1002-1007.

Dauga, C., A. Zabrovskaia and P.A.D. Grimont, 1998 Restriction fragment length polymorphism analysis of some flagellin genes of *Salmonella enteric*. J. Clin. Microbiol., 36: 2835-2843.

D'Aquilla, R.T., L.J. Bechtel, J.A. Videler, J.J. Eron, P. Gorczyca and J.C. Kaplan, 1991. Maximizing sensitivity and specificity of PCR by pre-amplification heating. Nucl. Acids Res., 19: 3749.

Gerner-Smidt, P., K. Hise, J. Kincaid, S. Hunter, S. Rolando, E. Hyytia-Trees, E.M. Ribot, B. Swaminathan and PulseNet, Taskforce, 2006. PulseNet USA: a five-year update. Foodborne Pathog Dis., 3: 9-19.

Henson, S., 1997. Estimating the incidence of food-borne *Salmonella* and the effectiveness of alternative control measures using the Delphi method. Int. J. Food Microbiol., 35: 195-204.

Herrera-Leon, S., R. Ramiro, M. Arroyo, R. Diez, M.A. Usera and M.A. Echeita, 2007. Blind comparison of traditional serotyping with three multiplex PCRs for the identification of *Salmonella* serotypes. Res. Microbiol., 158: 122-127.

Joys, T.M., 1985. The covalent structure of the phase-1 flagellar filament protein of ***Salmonella*** *typhimurium* and its comparison with other flagellins. J. Biol. Chem., 260: 15758-15761.

Li, J., N.H. Smith, K. Nelson, P.B. Crichton, D.C. Old, T.S. Whittam and R.K. Selander, 1993. Evolutionary origin and radiation of the avian-adapted non-motile salmonellae. J. Med. Microbiol., 38: 129-139.

Lindstedt, B.A., E. Heir, T. Vardund and G. Kapperud, 2000. Fluorescent amplified-fragment length polymorphism genotyping of *Salmonella enterica* subsp. *enterica* serovars and comparison with pulsed-field gel electrophoresis typing. J. Clin. Microbiol., 38: 1623-1627.

Liu, T., K. Liljebjelke, E. Bartlett, C. Hofacre, S. Sanchez and J.J. Maurer, 2002. Application of nested polymerase chain reaction to detection of *Salmonella* in poultry environment. J. Food Prot., 65: 1227-1232.

Lynch, M., J. Painter, R. Woodruff and C. Braden, 2006. Surveillance for foodborne-disease outbreaks-United States, 1998-2002. Morbid Mortal Weekly Rep.,1-34.

Maurer, J.J., D. Schmidt, P. Petrosko, S. Sanchez, L. Bolton and M.D. Lee, 1999. Development of primers to O-antigen biosynthesis genes for specific detection of Escherichia coli O157 by PCR. Appl. Environ. Microbiol., 65: 2954-2960.

Salmonella Surveillance Annual Summary, 2005. http://www.cdc.gov/ncidod/dbmd/phlisdata/salmtab/2005/*Salmonella*AnnualSummary2005.pdf

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Eds. Molecular Cloning: a Laboratory Manual. 2. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Samuel, G. and P. Reeves, 2003. Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. Carbohydr Res., 338: 2503-2519.

Sanchez, S., C.L. Hofacre, M.D. Lee, J.J. Maurer and M.P. Doyle, 2002. Animal sources of salmonellosis in humans. J. Am. Vet. Med. Assoc., 221 :492-497.

Swamy, S.C., H.M. Barnhart, M.D. Lee and D.W. Dreesen, 1996. Virulence determinants invA and spvC in salmonellae isolated from poultry products, wastewater and human sources. Appl. Environ. Microbiol., 62: 3768-3771.

Vanegas, R.A. and T.M. Joys, 1995. Molecular analyses of the phase-2 antigen complex 1, 2 of *Salmonella* sp. J. Bacteriol., 177: 3863-3864.

Verma, N.K. and P.R. Reeves, 1989. Identification and sequence of rfbS and rfbE, which determine antigenic specificity of group A and group D salmonellae. J. Bacteriol., 171: 5694-5701.

WHO Global *Salmonella* Survey, Progress Report, 2005. http://www.who.int/salmsurv/links/GSSProgressReport2005.pdf.

Wittwer, C.T., G.C. Fillmore and D.R. Hillyard, 1989. Automated polymerase chain reaction capillary tubes with hot air. Nucl. Acids Res., 17: 4353-4357.

Zamperini, K., V. Soni, D. Waltman, S. Sanchez, E.C. Theriault, J. Bray and J.J. Maurer, 2007. Molecular characterization reveals *Salmonella enterica* serovar 4,[5],12:i:- from poultry is a variant *Typhimurium* serovar. Avian Dis., 51: 958-964.