RESEARCH REPORT
PORK SAFETY

Title: A Rapid and Specific Test for *Salmonella* Serovars with Particular Reference to *Salmonella choleraesuis* serovar C1 - **NPB # 01-107**

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ABSTRACT

A polymerase chain reaction based enzyme linked immunosorbent assay (PCR-ELISA) was developed to identify Salmonella serovars A, B, C1, C2, and D. Primers were selected from the *rfb* gene cluster, which is responsible for biosynthesis of O antigens of Salmonella lipopolysaccharide. Salmonella isolates (n=203) were tested using the PCR-ELISA procedure. Of those Salmonella isolates, 156 isolates had been serogrouped previously. These isolates were used to determine the sensitivity and specificity of this PCR-ELISA procedure. DNA from all isolates was amplified using the PCR procedure for selected serovars and amplified products were visualized on agarose gels, as well as subjected to the ELISA procedure. The sensitivity of this procedure to correctly identify Salmonella serovars was 92% and the specificity was 99%. Eighty-eight percent of serovar D/A, 91% of serovar B, 89% of serovar C1, and 100% of serovar C2 were identified correctly with this procedure. Of the remaining 47 isolates that were not serogrouped, 5 were identified as serovar B, 7 as serovar C1, 1 isolate as serovar D/A and 34 isolates were neither B, C1, C2 or D/A. Results of this study indicate that the PCR-ELISA procedure is a rapid and accurate method for serogrouping Salmonella isolates. Utilization of the PCR-ELISA procedure for Salmonella serogrouping would aide the swine industry in identification, surveillance, prevention and control of Salmonella on the farm.

INTRODUCTION

Salmonella is one of the most important foodborne pathogens in the world. In the United States, salmonellosis accounts for 60% of all bacterial disease outbreaks, with an estimated 2 million cases occurring annually (Bean et al., 1996). Salmonella are transmitted by a wide variety of agricultural products and processed foods including pork. Identification of pathogenic microorganisms, including salmonella, is important for surveillance, prevention, and control of foodborne diseases (CAST, 1994). DNA-based assay systems have been developed and used for identification of pathogens because these methods rely on the nucleic acid composition of the bacterium instead of phenotypic expression of factors that may be variable under culture

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conditions. Therefore, DNA-based tests may be a better method for identification of foodborne pathogens particularly Salmonella species.

The concept of targeting gene sequences that encode for species specificity is promising. In Salmonella, *rfb* gene clusters are responsible for biosynthesis of O antigens of Salmonella lipopolysaccharide (Wyk and Reeves, 1989; Verma and Reeves, 1989). This *rfb* gene cluster has been targeted as a molecular marker for the detection of Salmonella serovars (Luk, et al., 1993). In this study, a PCR-ELISA procedure was developed to identify Salmonella serovars B, C1, C2 and D/A based on differences in the *rfb* gene cluster between Salmonella species.

OBJECTIVES

1. Continue development of a PCR-ELISA detection system for identification and differentiation of salmonella subgroups with emphasis on serovar C1.

2. Evaluate this PCR-ELISA system for its ability to identify salmonella subgroups from farm and pork samples.

MATERIALS & METHODS

Bacterial Strains: Eight American Type Culture Collection (ATCC, Rockville, MD) strains were used as control organisms. *Salmonella* ATCC strains included: *S. paratyphi A* (ATCC 11511), *S. typhimurium* (ATCC 14028), *S. choleraesuis* (ATCC 13312), *S. enteritidis* (ATCC 13076), *S. newport* (ATCC 6962), *S. anatum* (ATCC 9270), *S. worthington* (ATCC 9607) and *S. montevideo* (ATCC 8387). These strains represent the following serovars: A, B, C1, D1, C2, E1, G2, and C. In addition, 10 strains from the *Enterobacteriaceae* group were included to determine potential cross-reactivity. These bacteria included: *P. aeruginosa* (ATCC 35554), *P. aeruginosa* (ATCC 35659), *P. mirabilis* (ATCC 35659), *K. pneumoniae* (ATCC 13883), *E. coli* (ATCC 25922), *E. coli* (ATCC 31619), *E. coli* (ATCC 12014), *E. cloacae* (ATCC 961), *S. marcescens* (ATCC 13380), and *S. flexneri* (ATCC 12022).

Salmonella isolates serogrouped previously at USDA-APHIS (n=100), Ames IA and at Washington State University (n=56), Pullman, WA and an additional 47 salmonella isolates from Washington State University that were not serogrouped were evaluated by this PCR-ELISA procedure to identify Salmonella serovars A, B, C1, C2 and D. All isolates were stored at -80°C until the PCR-ELISA analysis was performed. All Salmonella strains were first streaked on XLD agar (BBL, Becton Dickinson and Company, Cockeysville, MD) and one colony was selected for DNA isolation using the InstaGene matrix (Bio-Rad, Melville, NY) following manufacturers directions.

Polymerase Chain Reaction: Primers for the PCR were selected based on *rfb* gene clusters specific for *Salmonella* serovars B, C2, and D as reported by Luk et al. (1993, 1997). The primer for *Salmonella* serovar C1 was selected based on the sequence of Salmonella *enterica* group C1 *rfb* gene cluster as described by Lee et al. (1992). Sequences for primer pairs are listed in Table 1.

Primers were purchased commercially (IDT, Coralville, IA) and were synthesized with the 5' end labeled with biotin. DNA amplification was performed following the protocol of Luk et al. (1993, 1997) with modifications. Each PCR reaction contained: 10 µl of DNA, 0.2 µg of each biotin-labeled primer, 200 µM of each dATP, dCTP, and dGTP, 150 µM of dTTP and 50 µM digoxigenin-11'-dUTP (Boehringer Mannheim, Indianapolis, ID), 5 U of *Taq* polymerase (Promega, San Diego, CA), 5 µl 10X magnesium-free thermophilic buffer [500 m*M* KCl, 100 m*M* Tris-HCl (pH 9.0), and 1% Triton X-100], 4 µl of 25 m*M* $MgCl_2$, and sterile H_20 to bring the total volume to 50 µl. Following an initial denaturation step at 94.5^0C for 5 min, parameters for the thermocycler (iCycler, Bio-Rad, Hercules, CA) were set at 95^0 C for 30 sec, 57^0C for 30 sec, and 72^0C for 30 sec. DNA was amplified for thirty cycles, followed by an extension step of 72^0 C for 150 sec.

Detection of PCR products by gel electrophoresis and photography of agarose gels was as described by Gillespie et al. (1997). Amplified products were electrophoresed in 2% agarose with TBE buffer (0.9 *M* Tris base, 0.09 *M* boric acid, 2.5 m*M* EDTA; pH 8.3). Gels were run at 150 V for 2 h and stained with ethidium bromide (1.0µg/ml; Sigma Chemical Co., St. Louis, MO). The DNA was visualized by transillumination (Fotodyne Inc., Heartland, WI) and then photographed (type 55 Polaroid film; Polaroid Corp., Cambridge, MA).

Enzyme Linked Immunosorbent Assay: To detect PCR products by ELISA, the protocol described by Luk et al. (1997) was used. Microtiter plates (96-well plates; Costar, Corning Incorporated, Corning, NY) were coated with streptavidin (0.5 µg/100 µl / well; Sigma) overnight at 4^0C. After coating, plates were washed three times with 200µl PBS, pH 7.4. Unsaturated binding sites were blocked with 200 µl of 1% (wt/vol) bovine serum albumin (Sigma) in PBS, pH 7.4 for 1 h at 37^0C and then washed three times with 200 µl PBS, pH 7.4. Samples of the diluted PCR product (10 µl sample and 90 µl PBS, pH 7.4) were added in triplicate to streptavidin-coated wells and incubated at 37^0C for 1 h. After three washes with 200 µl PBS (pH 7.4), 100µl of a 1:2000 dilution of anti-digoxigenin Fab-alkaline phosphate conjugate (Boehringer Mannheim) were added to each well and incubated at 37^0C for 1 h. After washing four times with 0.05% Tween 20 in PBS (pH 7.4), 100 µl of substrate solution (*p*-nitrophenylphosphate; Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added and color was developed at 37^0C for 30 min. ELISA absorbency was measured at 405 nm with a BIO-TEK microplate reader (BIO-TEK Instruments, Inc, Winooski, VT).

Determination of Sensitivity and Specificity: Sensitivity and specificity of PCR-ELISA was determined using the following formulas (Martin, 1984):

Sensitivity % = true positive samples/(true positive samples + false negative samples) x 100.

Specificity % = true negative samples/(true negative samples + false positive samples) x 100.

RESULTS

Eight *Salmonella* ATCC strains were evaluated with the PCR method outlined above, followed by gel electrophoresis of the PCR product. Agarose gel electrophoresis of the PCR product resulted in a distinct fragment for each serovar. For serovar A and D, a DNA fragment was

observed at approximately 703 base pairs with *S. paratyphi A* ATCC 1151 and *S. enteritidis* ATCC 13076. For serovar B, a DNA fragment was detected at approximately 851 base pairs with *S. typhimurium* ATCC 14028. For serovar C2, a DNA fragment was observed at approximately 795 base pairs with *S. newport* ATCC 6962. For serovar C1, a DNA fragment was observed at approximately 781 base pairs with S. *choleraesuis* ATCC 13312 and *S. montevideo* ATCC 8387. *Salmonella anatum* ATCC 9270 (serovar E1) and *S. worthington* ATCC 9607 (serovar G2) were tested with primers for serovar B, C1, C2, and D; however, no DNA fragments were seen. (Table 2, Figure 1).
Ten strains from the *Enterobacteriaceae* group were included to determine potential cross-reactivity. No cross-reactivity was observed with any of the primer pairs for specific serovars with any of the 10 strains tested. The PCR-ELISA results were confirmed by gel electrophoresis (Table 2).

Salmonella (n=100) isolated from bovine and swine samples submitted to USDA-APHIS (Ames, IA) from various states and serogrouped at USDA-APHIS by the method described by Ewing (1986) were evaluated by PCR-ELISA to determine sensitivity and specificity of the procedure. A sensitivity of 96% and a specificity of 98% was determined for the PCR-ELISA to identify Salmonella serovars C1, C2 and D previously serogrouped by USDA-APHIS. This procedure correctly identified 18 of 20 isolates as serovar D/A, 2 isolates had repeated (4X) negative reactions with primers specific for serovar D/A (Table 3). Twenty-four of 25 isolates were identified correctly as serovar C1, one isolate had repeated (4X) negative reactions with primers specific for serovar C1 (Table 3). Two isolates were positive for serovar C1, but were typed previously as serovar E (Table 3). The PCR-ELISA correctly identified all 25 Salmonella serovar C2 isolates (Table 3). The remaining isolates belonged to serovars E (n=20), G2 (n=5) and K (n=5). No PCR amplified fragments or positive ELISA readings were observed with these isolates and primers specific for serovars B, C1, C2 or D/A, except for 2 serovar E isolates reacting with the C1 primer pair (Table 3).

An additional 56 Salmonella isolates previously serogrouped by Dr. Dale Hancock at Washington State University were evaluated. A sensitivity of 88% and a specificity of 99% were determined for the PCR-ELISA to identify Salmonella serovars B, C1, C1 and D/A. This procedure correctly identified 12 of 14 isolates as serovar D/A, two isolates had repeated (4X) negative reactions with primers specific for serovar D/A (Table 4). Twenty-one of 23 isolates were identified correctly as serovar B, two isolates had repeated (4X) negative reactions. One isolate was positive for serovar B, but was previously serogrouped as serovar C1 (Table 4). Nine of 12 isolates were identified correctly as serovar C1, three isolates had repeated (4X) negative reactions (Table 4). The PCR-ELISA correctly identified all 7 Salmonella serovar C2 isolates (Table 4). All Salmonella isolates that were identified incorrectly by PCR-ELISA are currently being re-serotyped to ensure that the previous serogrouping was correct.

Forty-seven Salmonella isolates of unknown serogroup obtained from Washington State University were tested with this procedure. Of the 47 Salmonella isolates, 5 were identified as serovar B, 7 as serovar C1, 1 as serovar D/A (Table 5). The remaining 34 Salmonella isolates tested negative for serovars B, C1, C2 and D/A (Table 5).

Mean absorbance for all 203 isolates using the PCR-ELISA procedure are presented in Figure 2. Mean absorbance of all isolates that were identified as positive by PCR-ELISA with Salmonella primers for serovar B was 3.22 +/- 1.10. Negative controls and non-Salmonella bacteria had a mean absorbance of 0.13 +/- 0.07. Those isolates that were identified as positive by PCR-ELISA assay with Salmonella primers for serovar C1 had a mean absorbance of 3.07 +/-0.80. Negative controls and non-Salmonella bacteria had a mean absorbance of 1.17 +/- 0.68. Those isolates that were identified as positive by PCR-ELISA assay with Salmonella primers for serovar C2 had a mean absorbance of 2.40+/-0.82. Negative controls and non-Salmonella bacteria had a mean absorbance of 0.15 +/- 0.11. Those isolates that were identified as positive by PCR-ELISA assay with Salmonella primers for serovar D/A had a mean absorbance of 1.97 +/-0.69. Negative controls and non-Salmonella bacteria had a mean absorbance of 0.21 +/- 0.17.

All PCR DNA amplified products were evaluated by both 2% agarose gel electrophoresis and the ELISA protocol. Either method can be utilized to detect PCR DNA amplified products. A total of 72 samples can be loaded onto a 2% agarose gel and subjected to electrophoresis at 100 volts for 2 hours. A total of 29 samples in triplicate can be loaded onto one ELISA plate. The entire ELISA procedure takes approximately 5 hours to complete and 4 plates of 29 samples each (total of 116 samples) can be run simultaneously. The PCR procedure with detection by 2% agarose gel is just as efficient, if not more efficient, than the ELISA detection method for determining Salmonella serovars. The ELISA method involves biotin labeled primers, digoxigenin labeled dUTP's, and a microplate reader capable of reading absorbance at 405 nm.

Results of this study indicate that primers for Salmonella serovars B, C1, C2 and D are specific and cross-reactivity does not occur, except for serovar A cross-reacting with serovar D. This PCR-ELISA procedure correctly identified 116 of 126 Salmonella isolates previously serogrouped. This resulted in an overall sensitivity of 92% and a specificity of 99%.

SUMMARY

- A PCR-ELISA detection system for identification and differentiation of Salmonella serovars B, C1, C2, and D/A was developed and evaluated.
- No cross-reactivity was observed with members of the *Enterobacteriaceae* family and specific primers for Salmonella serovars B, C1, C2 or D.
- Sensitivity of the PCR-ELISA method was 92% and the specificity was 99%.
- 88% of serovar D/A, 91% of serovar B, 89% of serovar C1, and 100% of serovar C2 were identified correctly with this procedure.
- Isolation of DNA, PCR procedure, gel analysis and the ELISA procedure can be completed in one day.

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TABLE 1: Oligonucleotide primers used for *Salmonella* PCR-ELISA.

primers	Oligonucleotide Sequence (5′ to 3′) Reference	Serogroup	Base pairs
rfb(B)-F	GAGAATATGTAATTGTCAG	B	851
	Luk et al. (1993)		
rfb(B)-R	GTAACCGTTTCAGTAGTTC		
rfb(C2)-F	ATGCTTGATGTGAATAAG	C2	795
	Luk et al. (1993)		
rfb(C2)-R	CTAATCGAGTCAAGAAAG		
rfb(C1)-F	GGCGCTGATTTAACAGGTG	C1	781
	Lee et al. (1992)		
rfb(C1)-R	CATAAGCACAGTCACAACCTGG		
rfb(D)-F	AGTCACGACTTACATCCTAC	D and A	703
	Luk et al. (1993)		
rfb(D)-R	ACCTGCTATATCAGCACAAC		

TABLE 2: Cross-reactivity of *Salmonella* primers.

Organism	ATCC[1] number	Serogroup	2% Agarose gel				**ELISA**			
			Primer B	Primer C1	Primer C2	Primer D/A	Primer B	Primer C1	Primer C2	Primer D/A
S. newport	6962	C2	-[2]	-	795 bp[3]	-	-	-	+[4]	-
S. enteriditis	13076	D1	-	-	-	703	-	+	-	+
S. typhimurium	14028	B	851	-	-	-	+	-	-	-
S. paratyphi A	11511	A	-	-	-	703	-	+	-	+
S. anatum	9270	E1	-	-	-	-	-	-	-	-
S. montevideo	8387	C1	-	781	-	-	-	+	-	-
S. choleraesuis	13312	C1	-	781	-	-	-	+	-	-
S. worthington	9607	G2	-	-	-	-	-	-	-	-
P. aeruginosa	35554	x[5]	-	-	-	-	-	-	-	-
P. vulgaris	13315	x	-	-	-	-	-	-	-	-
K. pneumoniae	13883	x	-	-	-	-	-	-	-	-
E. coli	25922	x	-	-	-	-	-	-	-	-
P. mirabilis	12453	x	-	-	-	-	-	-	-	-
E. cloacae	961	x	-	-	-	-	-	-	-	-
S. marcescens	13880	x	-	-	-	-	-	-	-	-
S. flexneri	12022	x	-	-	-	-	-	-	-	-

[1]American Type Culture Collection, [2]no reaction, [3]base pairs, [4]positive reaction, [5]non-*Salmonella* strains.

TABLE 3: Results of PCR-ELISA on previously identified serovars of Salmonella obtained from USDA-APHIS.

	PCR-ELISA			
Serogroup	B	C1	C2	D
D (n=20)	0	0	0	18[a]
C1 (n=25)	0	26[b]	0	0
C2 (n=25)	0	0	25	0
E (n=20)	0	0	0	0
G2 (n=5)	0	0	0	0
K (n=5)	0	0	0	0
Total (n=100)	0	26	25	18

[a] Two isolates were negative, but previously serogrouped positive.

[b] Two isolates were positive, but previously serogrouped negative; one isolate was negative, but previously serogrouped positive.

TABLE 4: Results of PCR-ELISA on previously identified serovars of Salmonella obtained from Washington State University.

	PCR-ELISA			
Serogroup	**B**	**C1**	C2	D/A
D/A (n=14)	0	0	0	12[a]
B (n=23)	22[b]	0	0	0
C1 (n=12)	0	9[c]	0	0
C2 (n=7)	0	0	7	0
Total (n=56)	22	9	7	12

[a] Two isolates were negative, but previously serogrouped positive.
[b] Two isolate were negative, but previously serogrouped positive; one isolates was positive, but previously serogrouped negative;
[c] Three isolates were negative, but previously serogrouped positive.

TABLE 5: Results of PCR-ELISA on Salmonella isolates of unknown serogroups.

Serogroup	**Number positive**
B	5
C1	7
C2	0
D/A	1
Not B, C1, C2 or D/A[a]	34
Total	47

[a] Isolates were negative when tested for Salmonella serovars B, C1, C2 and D/A.