RESEARCH REPORT

PORK SAFETY

Title: A Rapid, Specific Test for *Salmonella* Subtypes - **NPB #99-136**

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Date Received: 8/8/2000

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 the O antigens of Salmonella lipopolysaccharide. Forty-eight isolates obtained from porcine feces or lymph nodes following challenge with *Salmonella enterica* serovar Typhimurium or *Salmonella choleraesuis* were tested using the PCR-ELISA procedure. DNA from all isolates were amplified using the PCR procedure for selected serovars and amplified products were visualized on agarose gel electrophoresis, as well as subjected to the ELISA procedure. Those isolates that were identified as positive with the PCR-ELISA with Salmonella primers for serogroup B had a mean absorbance reading of 2.14 +/- 0.57. Negative controls and non-Salmonella bacteria had a mean absorbance reading of 0.27 +/- 0.15. Those isolates that were identified as positive by the PCR-ELISA assay with Salmonella primers for serogroup C1 had a mean absorbance reading of 2.75 +/-0.59. Negative controls and non-Salmonella bacteria had a mean absorbance reading of 0.86 +/- 0.32. Results of the ELISA procedure were verified by agarose gel electrophoresis. All isolates were identified by biochemical and phenotypic characteristics. Of the 48 isolates evaluated, 36 isolates were serovar B, 2 isolates were serovar C1, and 10 were neither serovar A, B, C1, C2 or D. Results of this study indicate this PCR-ELISA procedure appears to be a rapid and accurate method for serogrouping Salmonella isolates.

INTRODUCTION

Salmonella are important foodborne pathogens that are responsible for serious cases of foodborne illness. Salmonella may be transmitted by a wide variety of agricultural products and processed foods. Several serovars of this bacterium occur, with varying degrees of relevance to human and animal health. Identification of pathogenic microorganisms, including Salmonella, is important for the surveillance, prevention, and control of foodborne diseases. An accurate and rapid procedure for identification of

Salmonella is needed to identify sources, reservoirs, and transfer through the food chain of these foodborne pathogens.

Current available screening tests only provide presumptive identification of Salmonella as a group without identification of serovars. Negative results are considered definitive, but positive results must be confirmed by conventional methods and serology. DNA-based assays have been used for identification of pathogens because these methods rely on the nucleic acid composition of the bacterium instead of their phenotypic expression of factors that may be variable under culture conditions. Therefore, DNA-based tests may be a better method for identification of food borne pathogens.

The concept of targeting gene sequences that encode for species specificity is promising. In Salmonella, the *rfb* gene clusters are responsible for biosynthesis of the 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 organism for detection of Salmonella serovars (Luk, et al., 1993). In this study, a PCR-ELISA procedure was developed to identify Salmonella serovars A, B, C1, C2 and D.

OBJECTIVES

1. To develop a PCR-ELISA detection system for identification and differentiation of Salmonella serovars.

2. To evaluate the PCR-ELISA based identification system for its ability to identify Salmonella variants from swine samples, comparing results from PCR-ELISA against established microbiological procedures for Salmonella detection and identification.

MATERIALS AND METHODS

Bacterial Strains: Eight American Type Culture Collection (ATCC, Rockville, MD) strains and one marker strain, *S. typhimurium* 798(4232) N+ (NADC, USDA, Ames, IA) were used as control organisms in this study. *Salmonella typhimurium* 798(4232) N+ (NADC) contained a nalidixic acid resistance marker. The *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. montevido* ATCC 8387. These strains represent the following serovars: A, B, C1, D1, C2, E1, G2, and C. 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.

Animals: A total of 36 pigs, weaned at 18 days of age, were divided into six groups of equal number and housed in independent rooms with separate environmental and waste handling systems. A 4 x 4 nursery pen and an 8 x 8 finisher pen were located in each room. One group of 12 pigs was challenged with *S. typhimurium* 798(4232) N+ (NADC) (2 x 10^9 colony forming units). Another group of 12 pigs was challenged with *S. choleraesuis* ATCC 13312 (2 x10^9 CFU) and the third group of 12 pigs was not challenged (controls). Each of the challenged pigs was challenged both nasally (2 ml) and orally (3 ml) with *S. typhimurium* or *S. choleraesuis*.

Following bacterial challenge, pigs were monitored for shedding of *Salmonella* by collection of fecal swabs at 4, 7, 11, 18, 36, and 140 days after challenge. Additional fecal samples were taken from the 12 pigs challenged with *S. choleraesuis* at 21 days after challenge. Tonsil, spleen, and colonic lymph node tissue were taken from one pig postmortem at 15 days post challenge, and from one pig sacrificed from each group (6 pigs) at 173 days post challenge, prior to market. Fecal samples were obtained by insertion of a sterile, non-absorbable Dacron polyester swab (Fisher Scientific, Pittsburgh, PA). Swabs were placed in individual sterile test tubes and transported on ice to the laboratory. Tissue samples were obtained postmortem via sterile surgical removal of the tonsils, spleen, and colonic lymph nodes. Samples were placed in sterile test tubes and transported on ice to the laboratory. All samples were analyzed for *Salmonella* using established microbiological procedures for detection and identification of S*almonella*.

Microbiological Identification: The possibility of low recovery numbers and heavy contamination of porcine feces by competing non-salmonellae made direct plating unfeasible, therefore, samples were first enriched to enhance detection. Swabs were placed in stomacher bags (Fisher Scientific) containing 90 ml tetrathionate broth (Difco Laboratories, Detroit, MI), sealed and incubated at 42°C for 18-24 h. Tissue samples were minced and homogenized in 1 ml of 0.9 % saline, 90 ml tetrathionate broth added and incubated at 42°C for 18-24 h. After incubation, each culture was plated on xylose-lysine-tergitol-4 medium (XLT4) (Difco) with 50ug nalidixic acid/ml added, XLT4 without nalidixic acid, and brilliant green medium (Difco). Plates were incubated at 37°C for 24 h.

Black or black-centered colonies with pink outer shells on XLT4 + nalidixic acid within 24 h were considered presumptive for the original *S. typhimurium* challenge strain. As the *S. choleraesuis* culture used for the challenge did not decarboxylate lysine or produce H_2S, yellow colonies on XLT4 were selected for further tests. If no suitable colonies were found on XLT4 + nalidixic acid or XLT4 media, pink colonies on brilliant green medium within 24 h were considered presumptive for *Salmonella* and were selected for further testing. Confirmation of *Salmonella* was based on biochemical reactions of each isolate using the following: triple sugar iron agar (TSI) (Difco), lysine iron agar (LIA) (Difco), and API 20E system (Bio-Mérieux Vitek Inc., Hazelwood, MO). All isolates confirmed as *Salmonella* were stored at -80°C until the PCR-ELISA analysis was performed.

Polymerase Chain Reaction: Primers for the PCR were selected based on the *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). The sequence for the primers were as follows:

serovar B:
rfb(B) F, 5`-GAGAATATGTAATTGTCAG-3`
rfb(B) R, 5`-GTAACCGTTTCAGTAGTTC-3`

serovar C2:
rfb(C2) F 5`-ATGCTTGATGTGAATAAG-3`
rfb(C2) R 5`-CTAATCGAGTCAAGAAAG-3`

serovar C1:
rfb(C1) F 5`-AAGTGTGTTTGATTGTTGG-3`
rfb (C1)R 5`-GTAACCGTTTCAGTAGTTC-3`

serovar D:
rfb(D)F, 5`-AGTCACGACTTACATCCTAC-3`
rfb(D)R, 5`-ACCTGCTATATCAGCACAAC-3`

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 (Ericomp, San Diego CA or 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 absorbance was measured at 405 nm with a BIO-TEK microplate reader (BIO-TEK Instruments, Inc, Winooski, VT).

RESULTS

Eight ATCC *Salmonella* 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 distinctive 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 (Fig.1). For serovar B, a DNA fragment was detected at approximately 851 base pairs with *S. typhimurium* ATCC 14028 (Fig.1). For serovar C2, a DNA fragment was observed at approximately 795 base pairs with *S. newport* ATCC 6962 (Fig.1). For serovar C1, a DNA fragment was observed at approximately 781 base pairs with S. *choleraesuis* ATCC 13312 and 410 base pairs with *S. enteritidis* (Fig.1). *Salmonella anatum* ATCC 9270, *S. worthington* ATCC 9607 and *S. montevido* ATCC 8387 were tested with primers for serovar B, C1, C2, and D; however, no DNA fragments were seen with these S*almonella* species and these primers (data not shown).

Using the PCR-ELISA assay, we initially evaluated 5 ATCC strains including *S. paratyphi* ATCC 11511, *S. typhimurium* ATCC 14028*, S. choleraesuis* ATCC 13312*, S. enteritidis* ATCC 13076 and *S. newport* ATCC 6962. Results were as follows: *Salmonella* serovar B primers detected *S. typhimurium* ATCC 14028; Salmonella serovar C2 primers detected *S. newport* ATCC 6962; *Salmonella* serovar D primers detected *S. paratyphi* ATCC 1151 and *S. enteritidis* ATCC 13076. Salmonella serovar C1 primers detected *S. choleraesuis* ATCC 13312*,* and *S. enteritidis* ATCC 13076. Primers for serovar D also detected serovar A as was reported by Luk et al. (1993). Cross reactivity between serovar primers C1 and serogroup D were seen in both the agarose gel and ELISA data. The PCR-ELISA method is specific for the selected serovars of Salmonella with limited cross-reactivity between the four serovars. Salmonella serovar A reacted with serovar D primers and serovar D reacted with serovar C1 primers*.*

To evaluate the PCR-ELISA based identification system for its ability to identify Salmonella variants and to compare results against established microbiological procedures for Salmonella detection and identification, a challenge with *S. typhimurium* or *S. choleraesuis* was conducted. Fecal shedding of *S. typhimurium* was detected at 4 days after challenge and continued with decreasing frequency until 36 days after challenge (Fig. 2). This pattern agrees with results of past challenge models. No *S. typhimurium* isolates were found at 140 days after challenge or from tissue samples taken from the 6 pigs sacrificed at 173 days after challenge. No fecal shedding of *S. choleraesuis* was detected throughout the duration of the study. Upon death at 15 days after challenge of a pig in the *S. choleraesuis* challenge group, necropsy was performed and tonsil, spleen and colonic lymph node samples were taken. No fecal shedding had been detected prior to death, however, upon examination of tissue samples, *S. choleraesuis* was recovered from the lymph nodes. No *S. choleraesuis* was detected in tissue samples from sacrificed pigs at 173 days after challenge. In the control group, one of 12 pigs showed *S. typhimurium* in a fecal specimen at 4 days past challenge. This is possibly due to cross contamination by staff on the day of challenge.

Forty-eight isolates from porcine feces or lymph nodes following challenge with *S. typhimurium* 798(4232) (NADC) or *S. choleraesuis* ATCC 13312 were evaluated. Results of the PCR-ELISA procedure indicated that 36 isolates belonged to serogroup B, 2 isolates belonged to serogroup C1 and 10 did not belong to serogroups B, C1, C2 or D. Those isolates that were identified as positive with the PCR-ELISA with Salmonella primers for serogroup B had a mean absorbance reading of 2.14 +/- 0.57 and those of the negative controls and non-Salmonella bacteria had a mean absorbance reading of 0.27 +/- 0.15 (Fig 3). The non-Salmonella isolates had a mean

absorbance reading of 0.29 +/- 0.16 and the negative controls had a mean absorbance of 0.24 +/- 0.12 with the same primer group. Those isolates that were identified as positive with the PCR-ELISA with Salmonella primers for serogroup C1, had a mean absorbance reading of 2.75 +/-0.59 and those of the negative controls and non-Salmonella bacteria had a mean absorbance reading of 0.86 +/- 0.32 (Fig 4). The non-Salmonella isolates had a mean absorbance reading of 0.83 +/- 0.19 and the negative controls had a mean absorbance of 1.08 +/- 0.60 with the same primer group. In addition, the 1:10 dilution of the PCR product used for the ELISA sample was also electrophoresed in a 2% agarose gel. All isolates that had positive ELISA reading showed DNA fragments of the appropriate size on the agarose gel (Figs. 5 and 6). Those isolates that had negative ELISA readings showed no fragments on agarose gels (Figs. 5 and 6).

Results of this study indicate that primers for Salmonella serovars B, C2 and D are specific and cross-reactivity does not occur, with the exception of serogroup A cross-reacting with serogroup D. This PCR-ELISA procedure correctly identified 36 isolates as *Salmonella* serogroup B, and 2 isolates as Salmonella serogroup C1 when compared to conventional biochemical testing. Results of the *Salmonella* serovar C1 primer are promising with cross-reactivity occurring only with the D serovar of Salmonella. However, with the limited number of *S. choleraesuis* isolates evaluated, further evaluation of this primer is needed.

SUMMARY

- A PCR-ELISA detection system for identification and differentiation of Salmonella serovars B, C1, C2, and D was developed and evaluated.
- PCR-ELISA results using primers for Salmonella serovar B agreed 100% with phenotypic and biochemical identification.
- PCR-ELISA results using primers for Salmonella serovar C1 are promising, however, additional isolates are needed for further evaluation.
- Isolation of DNA, the PCR procedure, gel analysis and the ELISA procedure can be completed in one day.
- *S. typhimurium* was shed in porcine feces up to 36 days after challenge.
- *S. choleraesuis* was detected in one of 12 pigs challenged and only in tissue samples after necropsy.
- Additional studies need to be conducted with *S. choleraesuis* to develop the in vivo challenge model and to provide additional isolates for evaluation by the PCR-ELISA detection system.

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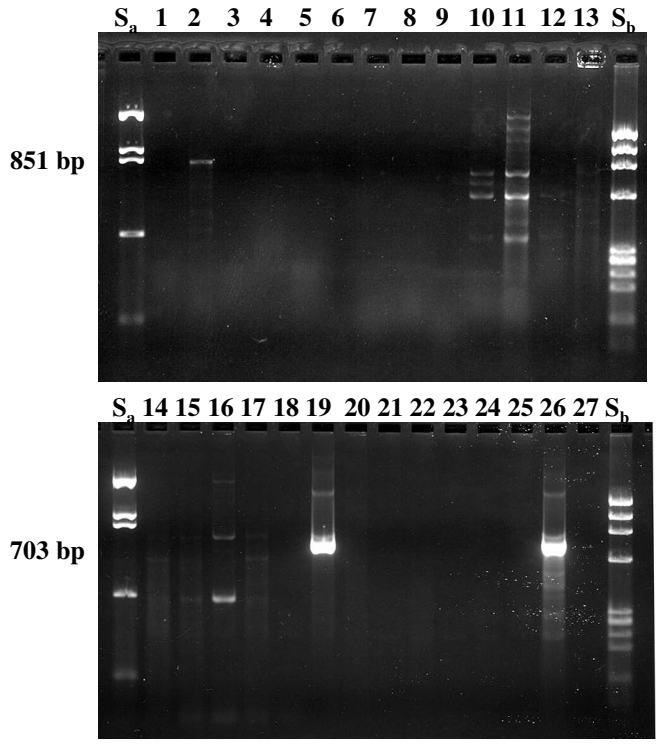

Figure 1. S_a pBR322 DNA-*BstN* I digest, lanes: 1,10,19, *S. paratyphi A*, 2,11,20, *S. typhimurium*, 3,12,21, *S. choleraesuis*, 4,13,22, *S. newport*, 5,14,23, *S. anatum*, 6,15,24, *S. worthington* , 7,16,25, *S. montevido*, 8,17,26, *S enteritidis*, 9,18,27, negative control, S_b PhiX174 RF DNA *Hae* III digest. Primers: lanes 1-*9 rfb* (B), 10-18 *rfb* (C2), 19-27 *rfb* (D).

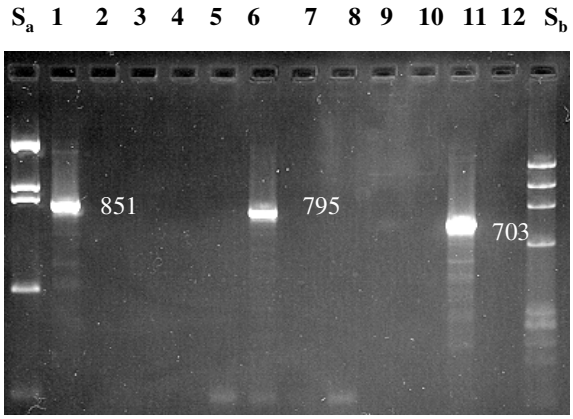

Figure 2. S_a pBR322 DNA-*BstN* I digest, lanes: 1, 5, 9, *S. typhimurium*, 2, 6, 10, *S. newport*, 3, 7, 11, *S. enteritidis*, 4,8,12, negative control, S_b PhiX174 RF DNA *Hae* III digest. Primers: lanes1-4 *rfb* (B), 5-8 *rfb* (C2), 9-12 *rfb* (D).

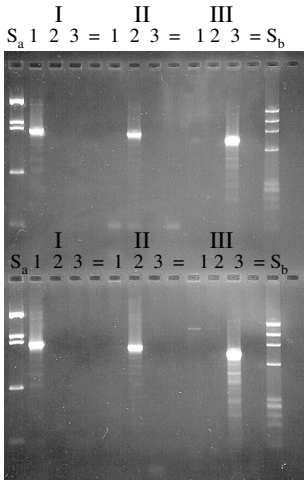

Figure 3. Panel A, S_a pBR322 DNA-*BstN* I digest, 1 *S. typhimurium*, 2 *S. newport,* 3 *S. enteritidis*, = negative control, S_b PhiX174 RF DNA *Hae* III digest. Primers: I Group B, II Group C2,III Group D.
Panel B, S_a pBR322 DNA-*BstN* I digest, 1 *S. typhimurium*, 2 *S. newport,* 3 *S. enteritidis,* = negative control, S_b PhiX174 RF DNA *Hae* III digest. Biotin labeled Primers: I Group B, II Group C2, III Group D.