RESEARCH REPORT
PORK SAFETY

Title: Quantization of Salmonella in transport and lairage to assess interventions for reduction of cross-contamination in pigs. - **NPB #06-086**

Investigator: DL Hank Harris; Co-PIs: I.

Institution: Iowa State University

Co-Investigators: Harris, J. Dickson, S. Gaul, B. Bosworth; Student: Lori Feldmann

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II. Industry Summary:

The sensitivity of the quantitative polymerase chain reaction assay (qPCR) was shown to be 10,000 *Salmonella* colony forming units of bacteria per gram of fecal matter; this means that this rapid and specific test is a useful tool for the industry. The study found a trend toward reduced, but not statistically significant, amount of *Salmonella* in lairage pens from standard hygiene practices of washing pens between groups of swine. This reduction in lairage pens may reduce transmission between groups and could result in a reduction of contaminated carcasses. It also allows one to determine if on farm and transport sanitation methods are reducing *Salmonella* fecal incidence at lairage pens. The lack of significance is partially due to the low number of positive pens in this study before washing. Due to the low numbers found in this study, samples were not collected from transport as they would have not lead to significant differences between various treatments.

III. Scientific Abstract:

Salmonella cross-contamination in pigs during transport and lairage has been shown to increase levels of *Salmonella* in tissues at slaughter. The objective of this study was to determine if a qPCR, recently developed in our laboratory, could be used to rapidly determine the amount of *Salmonella* present in lairage areas. Primer set StnF2, Stn-111 was tested for specificity with eight genera of *Enterobacteriaceae* and 19 *Salmonella* isolates. The primer set was specific for all of the *Salmonella* serotypes and did not cross-react with any of the *Enterobacteriaceae*. Sensitivity was determined by spiking *Salmonella*-free fecal samples with ten fold dilutions of *Salmonella* from 10^8 cfu's down to 10^1 and performing qPCR. qPCR detected *Salmonella* as low as 10^4 cfu's in feces, which is lower than what is consistently infectious in swine. To determine the difference between total *Salmonella* present and the amount of viable *Salmonella* in the samples, qPCR results were compared to most probable number (MPN) results of spiked fecal samples. Comparison of qPCR and MPN revealed that viable *Salmonella* in the spiked fecal samples was within one log of the total amount of *Salmonella* in the samples. Samples from pens at slaughter plants showed a reduction in positive results after treatments, however, this trend was not significant. The most likely reason this study did not find a reduction that was significant as the number of *Salmonella* positive pens and samples was very low before any treatment was done. These results suggest that *Salmonella* is a rare event in hogs slaughtered in the sampled plants. Due to the low numbers found in this study, samples were not collected from transport trailers as they would have not lead to significant differences between various treatments.

IV. Introduction:

Hurd, McKean and co-workers have shown conclusively that *Salmonella* cross-contamination of pigs in transport vehicles and in lairage occurs prior to slaughter resulting in higher levels of the organism in tissues at slaughter [1-5]. Cray and co-workers workers have clearly demonstrated that *Salmonella* acutely infects pigs and within a few hours after exposure the organism disseminates throughout the body organs and tissues [6]. Acute *Salmonella* infection has been shown to occur in market weight pigs after rooting in a contaminated environment at doses comparable to those commonly seen in lairage [1]. Although *Salmonella* may rapidly invade the body of pigs, a surprising recent finding by our laboratory indicates that at least 10^5 logs of *Salmonella* are required to infect a pig consistently[7]. Recent work in Ireland confirms this observation [8, 9]. It is well established that *Salmonella* may be frequently isolated from the environment of both transport vehicles and lairage [2, 8-13]. However, qualitative assessment of the frequency of isolation does not necessarily correlate with the occurrence of *Salmonella* in tissues collected at slaughter or in pork products [14]. Recently, Schmidt, et al. concluded the following based on qualitative data, "This study indicates that cleaning and disinfecting effectively reduces the amount of culturable *S. enterica* in lairage pens, but the ability of cleaned and disinfected pens to reduce the prevalence of *S. enterica* in market-weight pigs remains inconclusive" [14].

Thus, information is lacking regarding the quantitation of *Salmonella* in the environment of transport vehicles and lairage and the ability of interventions to lower these levels below the minimal infective dose. This affords the opportunity to assess interventions by quantitative methodologies for the reduction of *Salmonella* in the environment for the prevention of acute infection of the organism prior to slaughter.

Pigs frequently harbor *Salmonella* subclinically allowing the organism to be transmitted amongst pigs prior to slaughter [1, 4, 15, 16]. Transmission of *Salmonella* from pigs with subclinical infections to naïve pigs during transportation and lairage has been proposed to be a major source of *Salmonella* introduction into the food chain [4, 5]. Events immediately prior to slaughter have been shown to correlate with an increased rate of *Salmonella* isolation from pig carcasses [5, 15] and from pork products [17-19].

Dorr (2005) has shown that the level of *Salmonella* increased in pigs from nursery to slaughter by 6.4% to 56.7%, 5% to 65%, and 0% to 50% for 3 farms investigated respectively [20]. Furthermore, Rostagno et al. have shown that most cross-contamination occurs in lairage rather than in transport vehicles [2]. Several countries employ national surveillance programs for monitoring *Salmonella* on pig farms [21]. The Danish *Salmonella* Control Program, centered on serologic monitoring using the Danish mix-ELISA to categorize farms for subsequent intervention measures, has been credited with a downward trend in *Salmonella* occurrence since its implementation in 1993 [21-23]. In the U.S., on farm studies have consistently shown that a majority of farms are exposed to *Salmonella* [24]. Pigs from farms with high amounts of exposure to *Salmonella* (from other pigs and animals, feed, the environment) are a source of *Salmonella* for the transport and lairage environment as well to pigs from other farms [3]. Studies in Denmark found a very good correlation between herd serology and positive bacteriologic findings in the cecal contents and carcass [25]. There was also an increasing risk of carcass samples being positive as the seroprevalence increased. A 2000 study of 37 U.S. farms producing breeding stock suggested that prevalence of *Salmonella* as determined by culture of pooled pen fecal samples generally agreed with Danish mix-ELISA seroprevalence [26].

The Danes have dramatically lowered the level of *Salmonella* in tissues, on carcasses, and in fresh pork by monitoring herds and categorizing each based upon *Salmonella* seroprevalence and applying interventions [21]. Several of the interventions involve ration modification in the form of utilizing meal feed rather than pelleted feed [27-30], addition of organic acid to the feed [31-33], and the use of fermented liquid diets [34, 35]. Although avirulent live *Salmonella* vaccines are not approved for use in Denmark, they are available in the U.S. [36-41]. Another U.S. study found that pigs reared in hoop buildings (deeply bedded with straw) had a low *Salmonella* seroprevalence [42].

Morrow has recently called attention to the need for studies to determine if current interventions used for

transport vehicles are adequate for reducing infectious diseases in pigs [43]. With regard specifically to *Salmonella*, the few studies that have been conducted would indicate that the level of *Salmonella* in cleaned and disinfected transport vehicles is below that necessary to cause acute infection in pigs [10]. Furthermore, the reported level of environmental contamination of lairage appears to be at or below the level of *Salmonella* required to cause acute infection [9]. Thus, it would appear that the routine sanitation currently in place for transport vehicles and lairage may be adequate in most instances if properly carried out. Rostagno, et al. recently showed that maintaining pigs on transport vehicles rather than in a highly contaminated lairage environment prior to slaughter reduced the level of acute infection [44].

We propose to ascertain if a quantitative real time polymerase chain reaction (qPCR) assay recently developed in our laboratory could be used to rapidly determine whether the minimal infective dose (MID) of *Salmonella* is present in transport vehicles and lairage. Results from this qPCR assay could be available within 6-8 hours of collection and be used to improve sanitation procedures as needed to maintain levels at or below the MID for acute infection. In earlier studies, the qPCR assay was successfully tested for primer specificity for *Salmonella* detection, Figure 1. The detection level was determined to be 10^4 cfu per gram of fecal material, an amount that is below the amount needed to constantly infect a pig. In this study, the qPCR assay was applied to field samples collected at a lairage.

V. Objectives:

Evaluate a rapid quantitative real time PCR assay for rapid detection of Salmonella in environmental samples collected from transport vehicles and lairage.

Determine the effectiveness of cleaning, disinfection and heat for reduction of Salmonella in transport vehicles.

Determine the effectiveness of cleaning and disinfection for reduction of Salmonella in lairage.

Determine the antimicrobial sensitivity of Salmonella isolated from transport and lairage.

Determine the serotype of Salmonella present in transport and lairage.

VI. Materials and Methods:

Extraction Procedure for Test and Pen samples for qPCR Assay:
200 µl of each sample were extracted according to the "Protocol for Isolation of DNA from Stool for Pathogen Detection" found in the QIAamp DNA Stool Mini Kit Handbook (Qiagen 2001). DNA was stored at -20°C until qPCR was performed.

qPCR:
All qPCR reactions were performed in an iCycler (BioRad, Hercules, CA). QuantiTect Probe PCR Master Mix was used in 25 uL reactions according to manufacturer's instructions, with each primer at a final concentration of 400nM and probe at a final concentration of 200nM (Qiagen, Valencia, CA). All samples were run in triplicate. Cycling conditions were as follows: Step 1: 15 minutes- 95ºC, 1 repeat; Step 2: 15 seconds- 94ºC, 60 seconds- 60ºC, 45 repeats; Step 3: 10 minutes- 72ºC, 1 repeat; Step 4: infinite hold- 4ºC. A run was only considered valid when it had a correlation coefficient value between 0.990 and 1 and an efficiency value between 90% and 110%. A sample was considered positive if two or more of the triplicate wells were positive.

Testing of the Sponge Collection Procedures:
Hydrasponge with 10ml buffered peptone water (Biotrace, Inc.) were tested to determine the sensitivity and accuracy of the collection system. The sponge was used to collect samples of a dilution series of known concentrations from a concrete floor, similar to the ones found in the holding pens of an abattoir. Samples with

10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 log cfu were applied to a clean concert floor. A 10 cm by 10 cm area was delineated by a plastic mask which came with the hydrosponge. The area inside the mask was swabbed with the sponge and placed into the collection bag. The samples were extracted and quantified by qPCR.

Field Samples from Slaughter Plants:
In Slaughter plant A, samples were collected from nine holding pens by employees according to established protocols. The pens had five samples collected immediately after the hogs were moved out of the pen (before wash BW), five samples collected immediately after the pen was hosed out (after wash AW), and five samples immediately after the pen was treated with a sanitizing agent (after sanitation AS). This resulted in 15 samples per pen. Samples from slaughter plant B from ten holding pens were collected. As this facility does not routinely disinfect lairage pens between pig groups, samples were only collected before and after wash. This resulted in 10 samples per pen. The samples were placed on ice and shipped over night to the laboratory. The fluid was collected from the sponge and placed into a sterile 15 ml conical tube and placed in a -80^0 C freezer until the samples were extracted for qPCR. qPCR was used to determine the amount of *Salmonella* present in these samples.

VII. Results:

Testing of the Sponge Collection Procedures:
The samples showed that the sponge collection followed by qPCR were able to reliably detect 10^4 cfu and greater from the concrete floor and results are shown in Figure 3. Samples that contained 10^1, 10^2 and 10^3 log cfu and applied to the floor were not detected using qPCR.

Field Samples from Slaughter Plants
In slaughter plant A, of the nine pens tested, 3 were positive before washing, one pen remained positive after washing and all pens became negative after subsequent sanitation. On a sample basis, 3 samples were positive before wash, one was positive after wash, and none of the 45 samples were positive after sanitation. In slaughter plant B, of the ten pens tested, 2 were positive before washing and one pen remained positive after washing. On a sample basis, 7 samples were positive before wash and one of the fifty samples was positive after washing.

VII. Discussion:

The sensitivity of the qPCR assay was shown to be 10^4 (Figure 2). While more pen sampling is needed to validate these results and increase significance, these data suggest that currently used procedures at slaughter plant A reduce *Salmonella* contaminated feces transmission between groups at lairage. This reduction in transmission between groups could result in a reduction of contaminated carcasses and it also allows one to determine if on farm and transport methods are reducing *Salmonella* fecal incidence at lairage pens. The lack of significance is partially due to the low number of positive pens in this study before washing. Due to the low numbers of *Salmonella* found in this study, samples were not further characterized nor collected from transport as they would have not lead to significant differences among various treatments.

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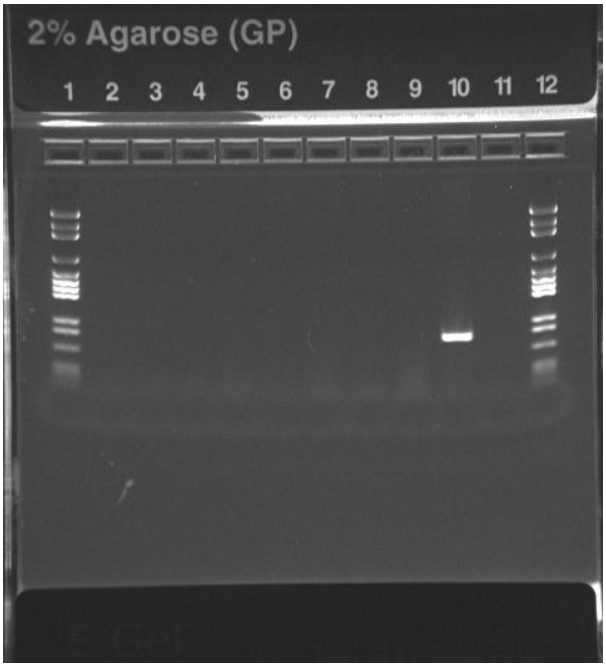
Primer Specificity



Lane 1: pGEM DNA ladder (Promega)
Lane 2: *Enterobacter*
Lane 3: *E. coli*
Lane 4: *Klebsiella*
Lane 5: *Proteus*
Lane 6: *Pseudomonas*
Lane 7: *Serratia*
Lane 8: *Shigella*
Lane 9: *Yersinia*
Lane 10: Positive control
Lane 11: Negative control
Lane 12: pGEM DNA ladder (Promega)

Figure 1: BioRad Geldock image of standard PCR using primer set StnF2, Stn-111 tested against *Enterobacteriaceae.*

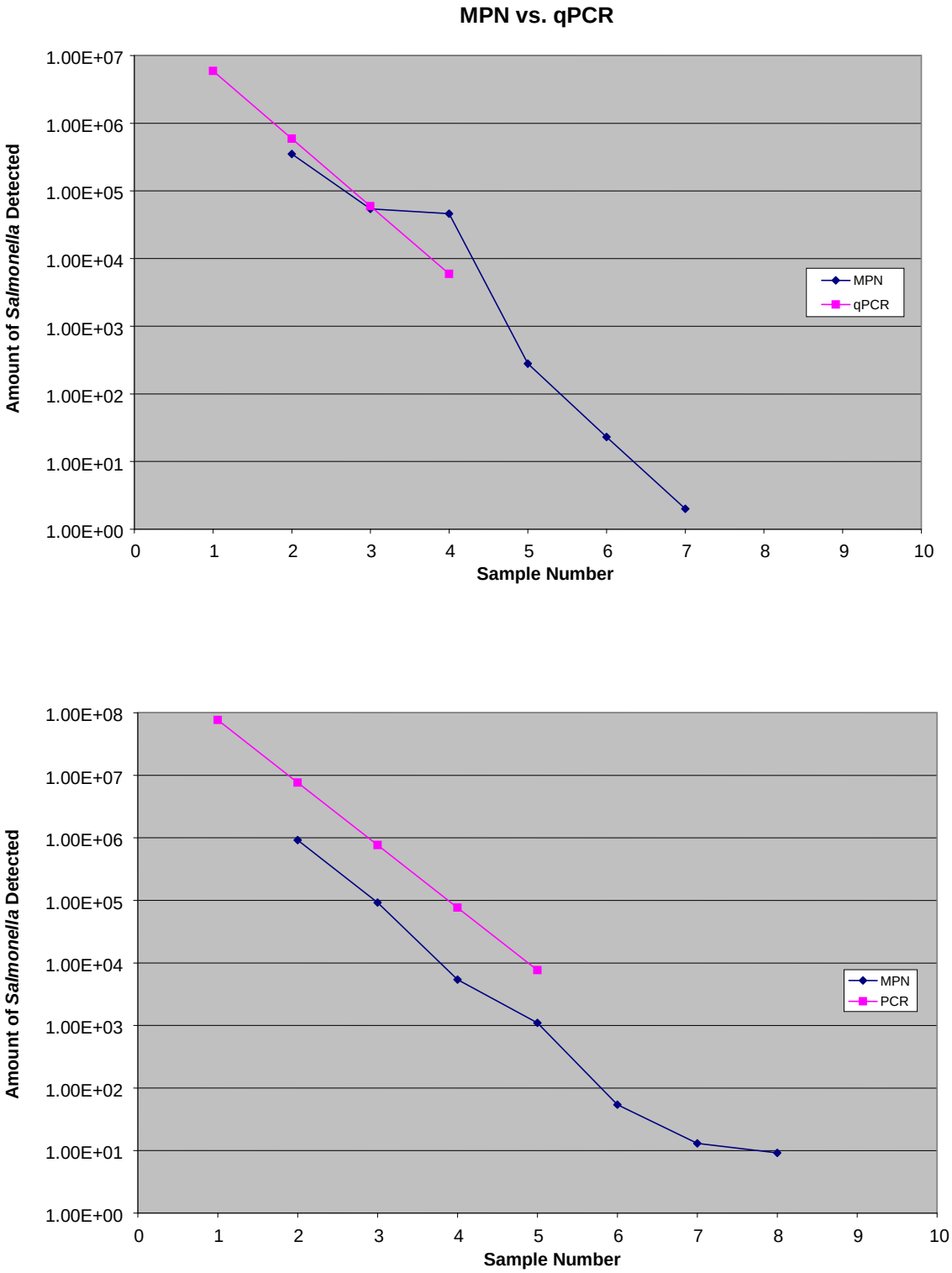


Figure 2.

Comparison of most probable number (MPN) and qPCR results. The MPN was not determined higher than log 10^6. qPCR results are not reliable below log 10^4. Top and bottom graphs represent data obtained from 2 separate runs.

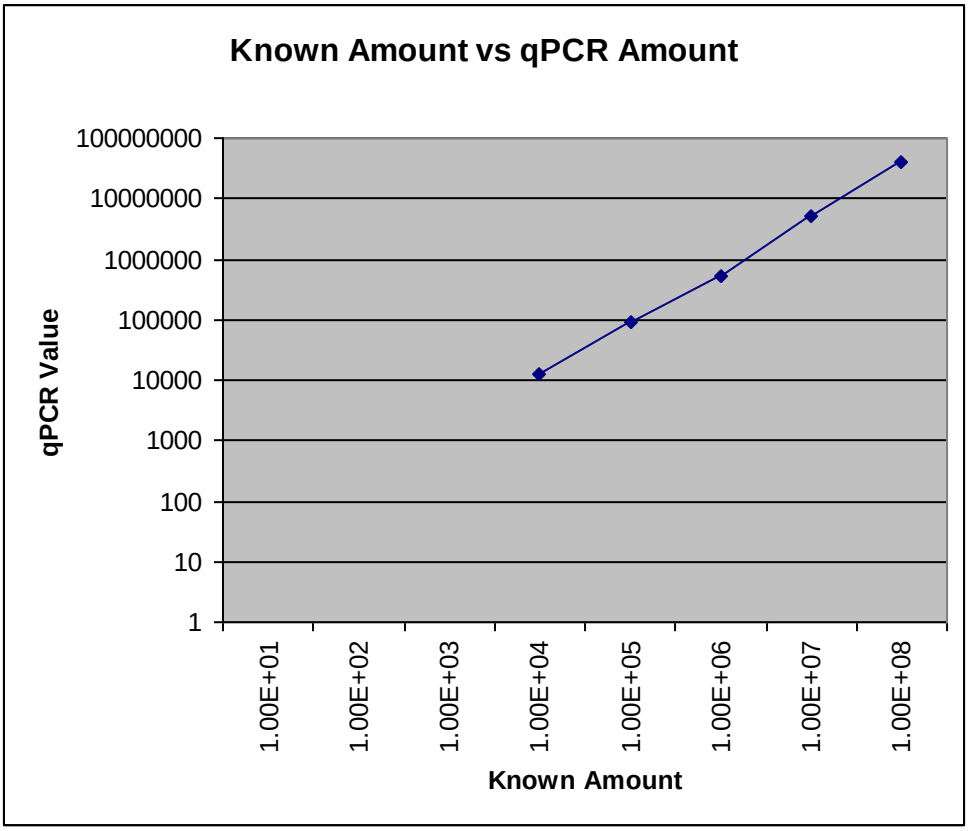


Figure 3. Comparison of samples with known amount of *Salmonella* (cfu) added and the quantification, by qPCR, of the samples when collect by hydrosponge from a concrete floor. qPCR did not detect samples below 10^4 cfus.