The Association between Cleaning and Disinfection of Lairage Pens and the Prevalence of *Salmonella enterica* in Swine at Harvest

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ABSTRACT

A series of four field trials were conducted to evaluate the ability of a cleaning and disinfection procedure in swine lairage pens to reduce the prevalence of *Salmonella enterica* in slaughtered pigs. A cleaning and disinfection procedure was applied to lairage pens at a large Midwest abattoir. Each trial consisted of a cleaned (alkaline chloride detergent) and disinfected (H2O2 plus peracetic acid sanitizer) pen (treated) and a control pen, each holding 90 to 95 pigs for 2 to 3 h before slaughter. Ileocecal lymph nodes, cecal contents, and rectal contents were collected from 45 pigs from each study pen at harvest and cultured for *S. enterica*. In all trials, cleaning and disinfection reduced the prevalence of *S. enterica*-positive floor swabs in the treated pen (P < 0.05). However, the postharvest prevalence of *S. enterica*-positive pigs varied between trials. In trial 1, there was no significant difference in the prevalence of *S. enterica* in pigs between treatment and control groups. In trials 2 and 3, the prevalence of *S. enterica* was higher in pigs from treated pens versus pigs from control pens (91% versus 40%, P < 0.0001, and 91% versus 24%, P < 0.0001, respectively). In trial 4, the prevalence of *S. enterica* was lower in pigs from treated pens compared with pigs from control pens (5% versus 42%, P < 0.0001). This study indicates that cleaning and disinfection 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.

Several studies report a disparity between prevalence of *Salmonella enterica*-infected pigs on the farm compared with penmates slaughtered at the abattoir (5, 14, 15, 17). This disparity suggests that infection of swine with *S. enterica* might occur rapidly during transport from farm to abattoir, while holding pigs in facilities at collection points or lairage pens at the abattoir, or at both times. Furthermore, recent publications have indicated that market-weight pigs can become infected with *S. enterica* Typhimurium after 30 min exposure to a contaminated pen environment (13).

Improving hygiene or sanitation remains a primary recommendation as a method of reducing pig exposure to *S. enterica* in the preharvest production environment (5, 12, 24). Published studies about improved farm or lairage hygiene have reported a reduction in the prevalence of *S. enterica* in pigs (10, 21). Hygiene, however, represents a broad category of practices, from cleaning and disinfection to all-in and all-out management to biosecurity measures. When these practices are implemented simultaneously, the effect of any single practice cannot be evaluated fully.

Given the current emphasis placed on identifying interventions to reduce the amount of *S. enterica* found in pork and the lack of conclusive results from previous studies, a series of field trials were designed to evaluate the ability of a cleaning and disinfection procedure in swine lairage pens to reduce the prevalence of *S. enterica* in slaughtered pigs, the rational of the study being that reducing the quantity of *S. enterica* in the swine lairage pen available to rapidly infect swine would reduce the prevalence of *S. enterica*-positive pigs at slaughter. The null hypothesis was that pigs held in cleaned and disinfected lairage pens would have the same prevalence of culturable *S. enterica* in lymph node, cecal, and fecal samples compared with pigs held in an uncleaned lairage pen. The alternate hypothesis was that pigs held in cleaned and disinfected lairage pens would have a different prevalence of culturable *S. enterica* in lymph node, cecal, and fecal samples compared with pigs held in a dirty lairage pen.

MATERIALS AND METHODS

Abattoir facilities. All trials were conducted at a commercial abattoir in the Midwest region of the United States. The annual plant kill is around 4.5 million hogs (17,000 daily) sourced from 2,500 farms in four states. Lairage floors and walls were concrete. Nipple waterers were present in study pens. Study pens ranged from 94 to 135 m². All alleyways and scales leading to the study pens were rinsed with high-pressure cold water prior to entry of study pigs. Alleyways leading from study pens to the kill area were also rinsed immediately before study pigs entered them. Study pigs were killed immediately following the morning break to allow time for ante-mortem veterinary inspection and minimum study exposure times. The trials occurred on Tuesdays at 3-week intervals (except for a 2-week interval between trials 3 and 4) from March to May 2003.

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Study pigs. Study pigs were from one multisite production system, which marketed more than 20,000 pigs annually. Pigs arrived in a single trailer load to the abattoir between 8:00 and 8:30 a.m. and were unloaded into a rinsed sorting pen. Randomization occurred by alternating 5 to 10 pigs from the sorting pen onto two scales for weighing. After weighing, the pigs were moved to the treated (cleaned and disinfected) or control pen. Time from unloading to placement into the study pens was 20 to 30 min in trials 1, 2, and 4. Unexpected plant downtime occurred during trial 3, forcing a 2-h holding time in the sorting pen. Holding times in study pens prior to slaughter ranged from 2 to 3 h for all trials.

Lairage treatment. Cleaning and disinfection of the treated pen involved a five-step procedure: (i) high-pressure cold water rinse; (ii) application of alkaline chloride detergent diluted to 1.2% concentration (pH 12.3, 2.5% available chlorine) with a minimum contact time of 10 min; (iii) high-pressure cold water rinse; (iv) application of hydrogen peroxide (6.9%), peroxyacetic acid (4.4%), and octanoic acid (3.3%) sanitizer diluted to 3.1% concentration with a minimum contact time of 10 min; and (v) high-pressure cold water rinse. Products were applied with separate 4-gallon backpack-style sprayers. After cleaning and disinfection, no visible debris remained on pen floors or walls. The control pen was moistened with cold water to approximate the same moisture level as the treated pen before study pigs entered the pen.

Sample size. A total of 40 floor samples per pen and 45 sets of pig tissue samples per treatment were collected, processed, and cultured in each trial. With 80% power, this study was able to detect a 28% difference in prevalence in pen samples and a 22% difference in prevalence in pig samples as statistically significant ($\alpha = 0.05$).

Pen sample collection. Prior to cleaning and disinfection, floor samples were collected from the control and treated pens. The sampling procedure used a sterilized 100-cm$^2$ gauze pad (Johnson & Johnson, Arlington, Tex.) held at arm’s length, which was dropped and allowed to “flutter” to the floor. Forty samples were randomly distributed throughout the control and treated pens. Contact time with the floor ranged from 5 to 9 min before gauze pads were collected with sterilized tweezers into sterilized bags (Nasco, Fort Atkinson, WIs.). After cleaning and disinfection, the sampling procedure was repeated in the treated pen. All samples were transported in a cooler to the National Animal Disease Center (Ames, Iowa) for culture. Samples were immediately processed on arrival at the National Animal Disease Center.

Pen sample culture procedure. Buffered peptone water (BPW, 25 ml; Remel, Lenexa, Kans.) was added to each sample bag containing pen swabs. Samples were stomached for 30 s at 230 rpm (Stomacher 400 Circulator, Seward Ltd., London, UK). For preenrichment, 10 ml of solution was added to a bag containing 90 ml of tetrathionate broth (TET; Difco/Becton Dickenson, Sparks, Md.), and 10 ml of solution was added to a bag containing 90 ml of BPW. TET and BPW were incubated at 37°C for 24 h. After incubation, 0.1 ml from TET and BPW bags was transferred into 9.9 ml of Rappaport-Vassiliadis enrichment broth (RV1; Difco/Becton Dickenson) containing 0.001 $\mu g$ Novobiocin (Sigma Chemical Co., St Louis, Mo.) and incubated at 42°C for 24 h. A second preenrichment step followed, as 0.1 ml from RV1 was transferred into another 9.9 ml of Rappaport-Vassiliadis enrichment broth (RV2) and incubated at 42°C for 24 h. After the second preenrichment (i.e., on day 3 postcollection), samples were tested for the presence of $S. enterica$ antigen by antigen capture enzyme-linked immunosorbent assay (ELISA; Assurance Gold EIA Salmonella, BioControl Systems, Bellevue, Wash.). Samples were considered positive if the optical density was equal to or greater than 0.40 (400 nm, PersonAllAB automated microplate analyzer; BioChem ImmunoSystems, Allentown, Pa.). Positive samples were streaked onto xylose lysine tergitol agar plates (XLT4; Difco/Becton Dickinson) and brilliant green sulphapyridine (BGS) agar plates (Oxoid Ltd., Hampshire, England) and incubated at 37°C for 24 h. Suspect $S. enterica$ colonies were selected and streaked onto Rombach agar (CHROMagar, Paris, France) for confirmation and incubated at 37°C for 24 h.

Pig sample collection. Viscera from 45 of the 90 to 95 pigs in each study pen were collected at slaughter. From each pig, approximately 1 g of ileocaecal lymph node was collected by sterile equipment into sterilized bags. Approximately 10 g of contents from the cecum and 10 g of distal colon and rectum contents were collected with a scissors by cutting a small hole in the viscera and milking the contents into a sterile bag. All samples were transported in a cooler to the National Animal Disease Center for culture. Samples were processed 18 h postcollection.

Pig sample culture procedure. Ten grams of cecal contents was divided and placed into each of two sterile bags—one bag contained 90 ml of TET and one bag contained 90 ml of BPW—then incubated at 37°C for 24 h. The same procedure was repeated with 10 g of fecal (distal colon and rectum) contents. Ileocaecal lymph nodes were rinsed in ethyl alcohol, flamed, then placed in sterilized filter bags. Samples were smashed with a rubber mallet. Phosphate-buffered saline (25 ml; Remel) was added, and samples were then stomached for 30 s at 230 rpm. Ten milliliters of the stomached solution was added to a bag containing 90 ml of TET and a bag containing 90 ml of BPW and incubated at 37°C for 24 h. After incubation, 0.1 ml from TET and BPW bags was transferred into 9.9 ml of RV1 containing 0.001 $\mu g$ of Novobiocin and incubated at 42°C for 24 h. A second preenrichment step followed, as 0.1 ml from RV1 was transferred into another 9.9 ml of RV2 and incubated at 42°C for 24 h. BPW samples were tested for the presence of $S. enterica$ by antigen capture ELISA (Assurance Gold EIA Salmonella, BioControl Systems). Samples were considered positive if the optical density was equal to or greater than 0.40 (400 nm). Samples determined positive were streaked onto XLT4 and BGS agar plates. All TET samples were streaked onto XLT4 and BGS agar plates. Plates were incubated at 37°C for 24 h. Suspect $S. enterica$ colonies were selected and streaked onto Rombach agar for confirmation and incubated at 37°C for 24 h.

Analysis. Statistical analysis was performed by SAS software (version 8.2, SAS Institute Inc., Cary, N.C.). For each pen, prevalence of $S. enterica$ was the number of positive $S. enterica$ swabs divided by the number of total pen swabs. A series of contingency tables ($2 \times 2$) were constructed between before and after, before and control, and after and control pens, and a two-tailed Fisher’s exact test was used to determine associations between pen prevalence and pen treatment. Pig prevalence was determined by the number of positive tissue samples divided by the total number of tissue samples collected for each tissue type. Any positive pig prevalence was calculated by the number of pigs with at least one tissue type positive divided by the total number of pigs sampled. Contingency tables ($2 \times 2$) were constructed between treated and control pigs, and a two-tailed Fisher’s exact test was used to determine associations between pig prevalence and pen treatment.

The data were modeled by an extension of the generalized linear model as described by Wolfinger and O’Connell (29) and
TABLE 1. Frequency count of S. enterica-±positive samples in control pens, treated pens before cleaning and disinfection, and treated pens after cleaning and disinfection

<table>
<thead>
<tr>
<th>Trial</th>
<th>Control Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13/40 (33) A</td>
<td>8/40 (20) A</td>
</tr>
<tr>
<td>2</td>
<td>29/40 (73) A</td>
<td>40/40 (100) B</td>
</tr>
<tr>
<td>3</td>
<td>37/40 (93) A</td>
<td>40/40 (100) A</td>
</tr>
<tr>
<td>4</td>
<td>40/40 (100) A</td>
<td>34/40 (85) B</td>
</tr>
</tbody>
</table>

* Within each row, results without a common letter differ significantly (P < 0.05).

Odds ratios (OR) were not available (NA) when the number of ±positive samples was zero in at least one cell.

implemented by the GLIMMIX macro in SAS. The outcome variable was dichotomous (i.e., S. enterica-±positive [yes/no]). Explanatory variables included treatment (fixed effect) and trial (random effect) and a treatment × trial interaction term (random effect). If the interaction term was significant, the trials were treated as separate events; Fisher’s exact test was used as the test for statistical association. The measure of association used was the odds ratio, and 95% Wald confidence intervals are presented.

The odds ratio (OR) is a ratio of the odds of a S. enterica-±positive pig from a treated pen compared with the odds of a S. enterica-±positive pig from a control pen. For example, for lymph node samples in trial 1, the odds of a sample being S. enterica-±positive to S. enterica-±negative were 2.37 in the treated pen and 3.39 in the control pen; therefore, OR = 0.7. Odds ratios are a measure of the intensity of the relationship between a risk factor (cleaning and disinfection) and an outcome (S. enterica infection). OR = 1 indicates no relationship between the factor and the outcome; OR < 1 indicates the factor is associated with a lower incidence of the outcome; OR > 1 indicates the factor is associated with a higher incidence of the outcome.

RESULTS

S. enterica was identified in control and before-treatment study pens in all four trials, with prevalence of positive swabs ranging from 20 to 100% (Table 1). The prevalence of S. enterica-±positive swabs in the after-treatment pens ranged from 0 to 15%, a significant difference in prevalence from the before-treatment pens in all trials (Table 1).

The treatment × trial interaction term was significant in the GLIMMIX model, suggesting some unmeasured difference between trials; therefore, trials are presented separately.

Results from pig samples are summarized in Table 2. In trial 1, no statistically significant difference in S. enterica prevalence between treated and control pigs existed in any collected tissues (P = 0.16 to 1.00). In trials 2 and 3, a significantly higher prevalence of S. enterica-±positive samples in fecal, cecal, or any samples occurred in pigs from the treated pen compared with the pigs from the control pen (P < 0.0001, OR > 1). In trial 2, S. enterica prevalence in lymph node tissues did not differ between treatment and control pigs (P = 1.00). However in trial 3, the prevalence of S. enterica-±positive lymph nodes was significantly higher in the treated group (P = 0.001). In trial 4, the treated pigs had a significantly lower prevalence of S. enterica in fecal, cecal, or any samples compared with control pigs (P < 0.003 to 0.0001, OR < 1). The prevalence of S. enterica in lymph node tissues did not vary between treatment and control pigs (P = 0.49). Odds ratios for the association between treatment and the prevalence of S. enterica in pig tissues in each trial are shown in Table 2.

DISCUSSION

Despite our success in demonstrating a reduction in S. enterica recovery from pen floors, we could not consistently reduce the prevalence of S. enterica in pigs. The results from pigs in the four trials were inconsistent, with all possible outcomes occurring; no difference in prevalence (null hypothesis), treated group with higher prevalence, and control group with higher prevalence. We had

TABLE 2. S. enterica-±positive samples in cecal, fecal, ileocecal lymph node (LN), and any samples from pigs held in treated and control pens*

<table>
<thead>
<tr>
<th>Trial</th>
<th>Sample</th>
<th>Treated (%)</th>
<th>Control (%)</th>
<th>P</th>
<th>OR*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cecal</td>
<td>0/39 (0)</td>
<td>3/42 (7)</td>
<td>0.24</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>2/21 (10)</td>
<td>0/31 (0)</td>
<td>0.16</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>2/39 (5)</td>
<td>3/42 (7)</td>
<td>1.00</td>
<td>0.7</td>
<td>0.11–4.45</td>
</tr>
<tr>
<td></td>
<td>Any sample</td>
<td>3/39 (8)</td>
<td>6/42 (14)</td>
<td>0.48</td>
<td>0.5</td>
<td>0.12–2.15</td>
</tr>
<tr>
<td>2</td>
<td>Cecal</td>
<td>31/45 (69)</td>
<td>3/45 (7)</td>
<td>&lt;0.0001</td>
<td>31</td>
<td>8.2–117.3</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>33/38 (87)</td>
<td>3/33 (9)</td>
<td>&lt;0.0001</td>
<td>66</td>
<td>14.5–300</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>15/44 (34)</td>
<td>16/45 (36)</td>
<td>1.00</td>
<td>0.94</td>
<td>0.39–2.24</td>
</tr>
<tr>
<td></td>
<td>Any sample</td>
<td>41/45 (91)</td>
<td>18/45 (40)</td>
<td>&lt;0.0001</td>
<td>15.4</td>
<td>4.7–50.4</td>
</tr>
<tr>
<td>3</td>
<td>Cecal</td>
<td>38/45 (84)</td>
<td>10/45 (22)</td>
<td>&lt;0.0001</td>
<td>19</td>
<td>6.5–55.4</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>18/36 (50)</td>
<td>2/30 (7)</td>
<td>&lt;0.0001</td>
<td>14</td>
<td>2.9–67.7</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>10/45 (22)</td>
<td>0/45 (0)</td>
<td>0.001</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Any sample</td>
<td>41/45 (91)</td>
<td>11/45 (24)</td>
<td>&lt;0.0001</td>
<td>31.7</td>
<td>9.2–108.5</td>
</tr>
<tr>
<td>4</td>
<td>Cecal</td>
<td>2/44 (5)</td>
<td>12/45 (27)</td>
<td>0.007</td>
<td>0.13</td>
<td>0.3–0.62</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>0/36 (0)</td>
<td>10/43 (23)</td>
<td>0.0015</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>0/44 (0)</td>
<td>2/45 (4)</td>
<td>0.49</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Any sample</td>
<td>2/44 (5)</td>
<td>19/45 (42)</td>
<td>&lt;0.0001</td>
<td>0.065</td>
<td>0.01–0.3</td>
</tr>
</tbody>
</table>

* Odds ratios (OR) were not available (NA) when the number of S. enterica-±positive samples was zero in at least one cell.
anticipated that cleaning and disinfection would be associated with no effect or a protective effect, as found in trials 1 and 4. Previous lairage field trials have reported results consistent with trial 1: no difference in the prevalence of S. enterica—positive pigs in treated and control pens (6, 9) or the inability to prevent contamination of pigs from Salmonella-free and serumegative herds when held in treated pens (27). On-farm field trials have reported a negative association (i.e., cleaning decreased S. enterica prevalence in slaughtered pigs) (19, 20) or a combination of increased and decreased prevalence of S. enterica in pigs (18). Observational studies examining associations between on-farm or truck and trailer cleaning and disinfecting procedures and S. enterica in pigs at slaughter have found no association (1–4, 8, 22) or a positive association (12, 25, 28).

The most likely explanation for variability of the association between cleaning and disinfection and S. enterica prevalence is that biases have distorted the outcomes of these studies. Many of the studies were case-control studies, case reports, or cross-sectional studies, so recall bias, selection bias, and uncontrolled confounding might explain differences in outcomes. We chose to conduct a series of field trials to reduce the effect of bias on our outcome. In previous field trials that found no association between S. enterica prevalence and cleaning and disinfection, several confounders remained uncontrolled.

We addressed many of these confounders in our field trials. For example, we collected pen samples to ensure that S. enterica exposure occurred. Furthermore, confounders such as variable sources of pigs and variable employees or staff performing cleaning and disinfection in each trial were eliminated by use of a single pig source and application of product by a single individual in all trials. Confounding by distance traveled from farm to abattoir or trailer contamination with S. enterica was controlled through restriction in the study design. In our study, the study population was restricted to animals arriving from a single source, in a single trailer, on a single day. Randomization and allocation of pigs to treated or control pens after arrival at the abattoir further controlled for these potential confounders. However, confounding by an unknown, and therefore unmeasured, variable might have occurred in this study.

Multicenter trials frequently experience differences in center results attributable to unknown confounders associated with the center. The four trials in this study are similar to a multicenter trial. The heterogeneity of both the study population (pigs) and the centers (lairage environment) more closely resemble how cleaning and disinfection will be performed in the modern lairage environment. It is the involvement of multiple centers in this study that enhances the generalizability of the results. Despite controlling for confounding through randomization and restriction (i.e., single source of pigs, a single abattoir, and a single person applying disinfection), an unknown confounding variable associated with the lairage environment appears to have lead to variability or imprecision in the results.

Another possible explanation for the variability of the association between cleaning and disinfection and S. enterica prevalence could be related to improper randomization of study pigs. Although pigs were randomized by small groups into either treatment or control pens, we question whether this achieved randomization of previously infected pigs between groups. For example, it has been suggested that S. enterica—positive lymph nodes might be more representative of previous or on-farm infection rather than recent exposure in the lairage environment. Also, recent data indicates clustering of S. enterica—shedding animals in finishing pens (23). In light of this, the S. enterica prevalence in lymph nodes in trial 3 could indicate a lack of randomization of study pigs (22% positive treatment pigs and 0% positive control pigs). The counterpoint to that observation is then that trials 1, 2, and 4 would appear to have achieved adequate randomization (no difference in lymph node prevalence of S. enterica between groups).

One confounder could be the population of S. enterica present on the floor before and after disinfection. S. enterica contains more than 2,400 serovars, which vary in environmental survivability, antimicrobial resistance, virulence, and pathogenicity. This diversity allows for the existence of microcosms of organisms within the environment with variable phenotypic traits. In biofilm microcosms, for example, S. enterica organisms existing close to the biofilm–solid surface interface have demonstrated increased resistance to antimicrobial agents (16) and increased virulence (7). The decreased ability of disinfectant agents to reach deep areas in biofilms (11, 26) might leave subpopulations of S. enterica in the newly cleaned environment that are more able to invade or infect susceptible animals.

The results of this study, although inconsistent, highlight the lack of knowledge about the ecology of S. enterica in the abattoir. Although cleaning and disinfection can successfully reduce or eliminate S. enterica from lairage pens, consistent and significant reduction in the levels of S. enterica recovered from pigs held in those pens was not attained. Knowledge of the mechanisms involved in survivability of recovered S. enterica strains and increased virulence of biofilm strains might explain the results in this and previous studies. The results also illustrate the need to ensure that a relevant outcome is measured during trials. If only the floor samples had been collected, we might have falsely concluded, on the basis of biological feasibility, that the prevalence in swine was likely to have decreased. By concentrating on the outcome of interest—the prevalence in pigs—this error was avoided.

This study demonstrates that simple cleaning and disinfection of lairage pens in itself is not a feasible intervention method for reducing the postharvest prevalence of S. enterica in pigs in the modern lairage environment and highlights the need for a better understanding of the ecology of S. enterica in the lairage environment.

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