Preharvest Salmonella Detection for Evaluation of Fresh Ground Poultry Product Contamination

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ABSTRACT

Salmonella is an important economic and public health concern for the poultry industry. Fresh ground product has been linked with multiple salmonellosis outbreaks in humans. Exposure can be controlled by proper handling and preparation by consumers; however, the industry desires to minimize carriage levels in the final product. A substantial obstacle in reducing product contamination stems from limitations in diagnostic methodologies. Detection of Salmonella contamination currently requires extended incubation periods, and by the time test results are available, the fresh product has reached retail shelves. The goal of this study was to develop a preharvest diagnostic protocol for the evaluation of ground product contamination. The turkey processing plant where this research was conducted had previously established Salmonella screening (BAX system) of ground product, thus providing an opportunity for preharvest sample comparison. Drag swabs were collected from live-haul trailers entering the processing plant over a 12-month period. The swabs were added to modified buffered peptone water and incubated at 40°C. After incubation for 6 h or overnight, samples were tested for the presence of Salmonella with the DNAlabe assay and related to ground turkey samples from corresponding lots. The linear relationship for the percentage of Salmonella-positive live-haul trailers was significant for both the 6-h (slope = 1.02, R² = 0.96, and P < 0.0001) and overnight (slope = 0.35, R² = 0.93, and P = 0.0015) incubations, with the percentage of Salmonella-positive ground turkey samples. These data indicate that preharvest screening provides a meaningful evaluation of product contamination. Additionally, the 6-h incubation protocol is rapid enough to allow for product mitigation and could potentially aid in the reduction of future salmonellosis outbreaks.

Salmonella has become an important economic and public safety concern for the commercial poultry industry. Contamination of poultry products has been linked to multiple human Salmonella outbreaks in the United States (6–8, 11, 12). Testing for Salmonella in raw poultry is routinely conducted in processing plants; however, the prolonged turnaround time required for testing does not allow for mitigation of product contamination. If a preharvest Salmonella detection protocol could be developed to evaluate contamination prior to product leaving the processing plant, it could potentially decrease the incidence of foodborne salmonellosis and the associated economic impact.

Studies have been conducted to investigate the spread of Salmonella in live poultry, but Salmonella carriage during rearing and grow out has not been definitively linked with product contamination (9, 10, 14). The lack of a clear link may be the result of intermittent Salmonella shedding (9, 10, 14, 19). Salmonella is thought to be shed in fecal material in response to stress (14, 15). Specifically, transport to the processing plant has been indicated as a factor that can increase shedding and product contamination (9, 10, 14). Salmonella shed during transport can then, presumably, be carried in on carcasses and spread throughout the plant. Therefore, rapid identification of shedding flocks as they arrive at the processing plant may provide an opportunity to initiate pathogen mitigation steps to aid in the reduction of product contamination.

Poultry can be processed and transported to retail markets in as little as 24 h; therefore, testing protocols must provide results during this time frame to allow for mitigation of Salmonella. Although isolation and identification using traditional bacteriology methods are routinely used for detection, this process can require greater than 96 h to complete and has limited sensitivity (1, 3). PCR assays have been used successfully for Salmonella detection; however, most protocols require a lengthy overnight enrichment and an additional 3 to 4 h for DNA isolation and detection (3, 16). Similar to PCR, isothermal amplification targets a DNA sequence specific to Salmonella but does not require DNA isolation or thermocycling for detection. Instead, this type of amplification relies on specialized enzymes that operate under static temperature conditions to denature and elongate DNA.
The robust nature and the increased sensitivity makes these assays interesting candidates for preharvest screening (5). The DNAble assay, which incorporates the isothermal amplification methodology, recently received AOAC International performance testing certification for poultry drag swab samples (4). Preliminary studies conducted in our laboratory also indicated good sensitivity with shortened enrichment times, thus providing a potential tool for rapid determination of Salmonella in fecal material.

The goal of this study was to develop a preharvest diagnostic protocol for the evaluation of ground product contamination. Previous studies have indicated a link between Salmonella fecal shedding during transport and product contamination (9, 10, 14). To investigate this link, the DNAble assay was identified as a potential tool for detection of Salmonella in fecal material on live-haul trailers entering the processing plant. If a successful testing protocol can be developed that provides a rapid evaluation of product contamination, it could lead to significant reductions in consumer exposure. Implementation of such a protocol would also aid in reducing the economic impact of a product recall.

**MATERIALS AND METHODS**

*Salmonella cultures.* Salmonella enterica subsp. enterica serovars Hadar, Heidelberg, Saintpaul, and Typhimurium from commercial poultry grow-out operations were kindly provided by Mr. Marion Morgan and Dr. Neil Pumford at Northwest Arkansas Veterinary Services LLC (Bentonville, AR). Salmonella enterica subsp. enterica serovar Enteritidis (ATCC BAA-1587, Manassas, VA) was included as an experimental control and for growth comparison. All cultures were grown overnight in buffered peptone water (BPW; Oxoid, Thermo Fisher Scientific, Inc., Waltham, MA) and on Columbia blood agar plates containing 5% sheep’s blood (Remel, Thermo Fisher Scientific, Inc., Waltham, MA) and on Columbia blood agar plates containing 5% sheep’s blood (Remel, Thermo Fisher Scientific, Inc.) at 37°C. Blood plates were examined to ensure no contamination was present. Sterile glycerol was added to cultures in BPW (1:2) and frozen at –80°C in 1.5-ml aliquots. After 1 week, an aliquot of each isolate was thawed and serially diluted (1:10). Then 10 μl from each dilution (10⁻¹ to 10⁻⁶) was plated on Columbia blood agar in quaduplicate. After overnight incubation at 37°C, the colonies were counted at the dilution that had at least 10 colonies and no more than 30 colonies. The number of CFU per milliliter was determined by calculating the average number of colonies per replicate and accounting for the dilution factor and volume plated.

*Salmonella growth kinetics.* An aliquot from each isolate of frozen Salmonella stock was allowed to thaw at room temperature and then diluted to 2.0 × 10⁶ CFU/ml in modified BPW containing DNAble supplement (mBPW; EnviroLogix Inc., Portland, ME). Cultures were added to a 96-well plate (200 μl per well) and incubated at 37 or 40°C for 20 h. Optical density (OD) was measured by using a SpectraMax 340PC384 with SoftMaxPro Version 5.4.5 software (Molecular Devices LLC., Sunnyvale, CA) each hour at 600 nm.

*Sensitivity.* An aliquot from each isolate of frozen Salmonella stock was allowed to thaw at room temperature, diluted to 2.0 × 10⁶ CFU/ml, and the concentration was reevaluated, as described previously to ensure accuracy. During overnight incubation of plates (16 to 18 h), the diluted 2.0 × 10⁶ CFU/ml cultures were stored at 4°C. When the concentrations were confirmed, cultures were serially diluted (1:10) to 2.0 × 10⁶ CFU/ml — 2.0 × 10¹ CFU/ml in tubes (17 by 100 mm; BD Falcon, Bedford, MA) with mBPW. Tubes containing 10 or 9 ml of mBPW with 1 g of feces collected from turkeys were incubated at 40°C. Samples (125 μl) were removed at 6 h and overnight (20 to 24 h) for Salmonella detection. The experiments were repeated three times to confirm results.

**Fecal cultures.** Turkey feces was plated onto Columbia blood agar (incubated in 7.5% CO₂), MacConkey, MacConkey with sorbitol and xylose lysine Tergitol 4 (Remel, Thermo Fisher Scientific, Inc.) plates and incubated overnight at 37°C. Morphologically distinct colonies were subcultured onto Columbia blood agar and again incubated overnight (7.5% CO₂ at 37°C). Pure isolates from plates were identified by using Sensititre veterinary identification plates and SWIN software (Thermo Fisher Scientific, Inc.). Feces was also plated on Campy CVA (cefoxoperazone, vancomycin, and amphotericin B) agar (Remel, Thermo Fisher Scientific, Inc.), placed in an airtight bag with a microaerophilic sachet, and incubated 48 h at 42°C. Plates were examined for the presence of Campylobacter (spiral morphological appearance at 1,000× magnification and gram-negative staining). A 1-g aliquot of fecal material was also transferred to a sterile 9-ml tube of selenite broth (Remel, Thermo Fisher Scientific, Inc.) and allowed to incubate overnight at 37°C. Selenite samples were then subcultured on Hektoen agar and HardyChrom (Hardy Diagnostics, Santa Maria, CA) and incubated overnight at 37°C. Plates were examined for the presence of morphologically characteristic Salmonella colonies (black colonies).

**Preharvest sample collection.** Live-haul trailers were in transit for 5 to 7 h prior to reaching the processing plant. Upon arrival (1 to 3 h before processing), swabs (Hydraflock, Puritan Medical Products Co., LLC, Guilford, ME) were dragged along the back of the trailers through fecal droppings on the floor surface. Individual swabs containing approximately 1 g of feces were then placed in a tube (17 by 100 mm) containing 9 ml of mBPW. A total of 220 swabs (1 swab per trailer) were collected over a 12-month period (2 to 11 trailers per lot; median = 8 trailers per lot). The swabs in mBPW were stored on ice for up to 3 h during collection and then incubated at 40°C. Samples (125 μl) were removed at 6 h and overnight (20 to 24 h) for Salmonella detection.

**Preharvest Salmonella detection.** Samples were prepared for DNAble (EnviroLogix, Inc.) analysis, according to the manufacturer’s directions. In brief, DNA was extracted by adding 25 ml of MB2 and 125 ml of enriched sample to 1.5-ml tubes and incubating for 15 min at 98°C. Extracted DNA (5 μl) was then transferred to corresponding RB1 tubes. A multichannel pipette was used to transfer 50 μl of RB1, containing extracted DNA, to the lyophilized master mix tube strips and then placed in the Axxin fluorescent strip reader (Axxin Inc., Richmond, Victoria, Australia). Samples were determined to be positive or negative for Salmonella by the DNAble software provided with the reader.

**Postharvest Salmonella detection.** Ground turkey (breast meat, drumsticks, fillets, thighs, and wings) samples were collected at the grinder head every hour by the quality assurance technician. Samples were tracked by lot, which corresponded with the flock and day of processing. Lots were composed of at least 98% meat harvested from one flock and up to 2% meat from other flocks. A total of 436 samples were collected for this study (6 to 37 samples per lot; median = 16 per lot). The samples were transported on ice to the processing plant’s pathogen detection laboratory and stored at 4°C for up to 13 h. Samples were then tested according to the BAX system original PCR protocol (DuPont Nutrition and Health, Wilmington, DE), which is an AOAC International–certified and
U.S. Department of Agriculture–Food Safety and Inspection Service–adopted method for detection of Salmonella in raw poultry samples (17, 18). Briefly, 25 g from each sample was added to 225 ml of BPW in a stomacher bag. Samples were then stomached on medium speed for 1 min and incubated for 20 to 24 h at 34 to 36°C. Following incubation, 5 μl of BPW was added to 200 μl of BAX lysis buffer and lysed according to the manufacturer’s directions. Samples were cooled to 4°C, and 50 μl of lysate was transferred to PCR tubes containing the BAX PCR tablets. Tubes were transferred to the thermocycler, and Salmonella detection was then conducted according to the manufacturer’s directions.

Statistics. Data analysis was conducted in JMP Pro 10 (SAS Institute Inc., Cary, NC). Growth curves were modeled with a repeated measures factorial one-way analysis of variance design to determine interactions between incubation temperature and time (n = 16). When interactions (time × temperature) were significant, a post hoc slice analysis, including Bonferroni correction, was used to compare incubation temperatures for each time point. Regression analyses were conducted to determine the relationships between the percent positive live-haul trailer samples, 6 h or overnight sample incubations, with the percent positive ground turkey samples by lot (n = 28). A mixed model design was used to account for clusters of observations within flocks. Residual plots were inspected to assess model fit for the regression analyses (i.e., errors were normally distributed with constant variance).

RESULTS
Salmonella growth kinetics. All of the Salmonella serovars began log phase growth between 6 and 12 h of incubation regardless of temperature (37 and 40°C). Interactions (time × temperature) for all five serovars (Salmonella Enteritidis: P < 0.0001; Salmonella Hadar: P < 0.0001; Salmonella Heidelberg: P < 0.0001; Salmonella Saintpaul: P < 0.0001; and Salmonella Typhimurium: P < 0.0001) were significant. Between 12 and 17 h, Salmonella Enteritidis (Fig. 1A) had significantly higher OD values at 37°C compared with 40°C (P ≤ 0.0025). During log phase, Salmonella Hadar (Fig. 1B) and Salmonella Heidelberg (Fig. 1C) had significantly higher (P ≤ 0.0025) OD values at 40°C compared with 37°C. Beginning at 8 h and continuing through at least 15 h of incubation, Salmonella Saintpaul (Fig. 1D) and Salmonella Typhimurium (Fig. 1E) had significantly higher (P ≤ 0.0025) OD values at 40°C compared with 37°C.

Sensitivity. Following 6-h and overnight incubations in mBPW, the DNAble assay consistently detected all five Salmonella isolates at all concentrations tested. To determine if the sample matrix had an impact on sensitivity, the experiment was repeated with the addition of feces collected from turkeys. For these samples, the assay again consistently detected all five Salmonella isolates at all concentrations, regardless of incubation time. The feces was cultured, and isolates were identified to confirm the presence of competitive flora (three Escherichia coli isolates, one Klebsiella pneumonia subsp. pneumonia isolate, two Enterococcus faecalis isolates, and one Enterococcus faecium isolate). Cultures were negative for both Campylobacter spp. and Salmonella spp.

Salmonella contamination. The percentage of Salmonella-positive samples from live-haul trailers and ground turkey are summarized in Table 1. Overall, 15 lots (n = 28) of ground turkey were determined to have Salmonella contamination with the BAX system (i.e., at least one ground turkey sample in the lot tested positive). Of these lots, 53.3% (8 lots) and 86.7% (13 lots) could be identified as Salmonella positive (i.e., at least one trailer in the lot tested positive) with the 6 h and overnight preharvest testing protocol, respectively. For the 13 lots with no ground product contamination, 100% (13 lots) with 6 h and 69.2% (9 lots) with overnight protocol could be identified as Salmonella negative (i.e., all trailers in the lot tested negative). When the percentage of Salmonella-positive live-haul trailers was compared with the percentage of ground turkey samples by lot, the linear relationship for both the 6 h (Fig. 2A; y intercept = 7.41, 95% confidence interval [0.62 to 14.19]; slope = 1.02, 95% confidence interval [0.63 to 1.41]; $R^2 = 0.96, P < 0.0001$) and overnight (Fig. 2B; y intercept = 5.01, 95% confidence interval [−4.12 to 14.14]; slope = 0.35, 95% confidence interval [0.15 to 0.55]; $R^2 = 0.93; P = 0.0015$) incubations were significant.

DISCUSSION
A recent report from the Centers for Disease Control and Prevention indicates 10% of Salmonella outbreaks in the United States are linked with poultry meat products (7), including raw ground chicken and turkey. Although the risk of illness caused by these products is relatively low, the economic and public health concerns are substantial. In an effort to alleviate these concerns, considerable research has been conducted to investigate Salmonella infections in poultry and determine ways to reduce Salmonella contamination during harvest. Fecal material is believed to be the primary source of poultry product contamination. During transport to the processing plant, Salmonella may be shed in the feces (9, 10, 13, 14). Fecal material can then contribute to the spread of Salmonella throughout the plant (2, 10, 14). Detection of contamination is limited because most testing protocols require at least 24 h; thus, contaminated product reaches retail shelves before a determination can be made. The objective of this study was to develop a rapid method for preharvest evaluation of poultry product contamination.

The growth kinetics of five Salmonella serovars of particular importance to the poultry industry were evaluated. Because the body temperature of poultry is typically between 40 to 43°C, experiments were conducted to determine the optimal incubation temperature (37°C versus 40°C) for these isolates. Interestingly, the isolate obtained from the American Type Culture Collection (Salmonella Enteritidis) grew significantly faster at 37°C, while the four isolates obtained from poultry (Salmonella Hadar, Salmonella Heidelberg, Salmonella Saintpaul, and Salmonella Typhimurium) grew significantly faster at 40°C; indicating that some isolates may be host adapted. Thus, samples were incubated at 40°C for all subsequent experiments.

The sensitivity analysis was conducted on $2 \times 10^3 - 2 \times 10^5$ CFU/ml serial dilutions for all five Salmonella isolates. Dilutions were prepared in mBPW with or without turkey feces. Following both the 6 h and overnight incubations,
the assay consistently detected *Salmonella* at the lowest dilution tested (2 × 10^4 CFU/ml, equivalent to 200 CFU/swab), regardless of sample matrix. Flora and other potential inhibitors present in the feces did not appear to have any negative impact on assay sensitivity.

For preharvest screening of live-haul trailers with the DNAble assay, samples incubated for both 6 h and overnight had significant linear relationships with ground turkey contamination as determined with the BAX system. These data further confirm previous reports indicating an association between the transport environment and product contamination (9, 10, 14). For this study, the 6-h sample testing protocol (slope = 1.02 and R^2 = 0.96) was better at predicting the percentage of *Salmonella*-positive ground turkey samples than the overnight sample testing protocol (slope = 0.35 and R^2 = 0.93). Although the sensitivity analysis of artificially spiked samples indicated similar detection capabilities for both 6-h and overnight incubations, discrepancies in sensitivity are apparent for the field samples. For example, 53.3% (8 lots) and 86.7% (13 lots) of the ground product contamination could be identified with the 6-h and overnight incubations, respectively. Thus, the detection of *Salmonella* in preharvest samples with the overnight incubation appears to be more sensitive than with the 6-h incubation. The discrepancies are likely the result of low *Salmonella* numbers or the presence of injured *Salmonella*
that may only be detected following extended incubation (i.e., overnight). Interestingly, increased sensitivity (i.e., overnight incubation) did not provide a better evaluation of the percentage of Salmonella-positive ground turkey samples. Other factors, including the incoming Salmonella load, likely play an important role in product contamination.

Preharvest sample testing protocols for the evaluation of Salmonella in ground product were investigated in this study. The data presented here indicate that preharvest screening of live-haul trailers with the DNAble assay provides a meaningful evaluation of ground turkey contamination, as determined with the BAX system. More importantly, the 6-h preharvest testing protocol is rapid enough to allow for mitigation of product contamination. These initial results are encouraging and suggest that additional studies to refine this testing strategy are warranted. Further studies should be conducted to determine cut-off thresholds for preharvest Salmonella levels (high versus low load) and improve the turnaround time of predictive testing protocols. To our knowledge, this is the first report describing this approach for evaluation of product contamination. Collection of field data for a study of this scope is difficult and should be considered in future study designs. Animal handling, time of year, weather conditions, changes to carcass processing, and coordination of data collection among labs must all be considered as these factors may have an impact on study results.

Overall, similar testing strategies could be incorporated into current quality assurance programs used in poultry processing plants and could potentially be applied to other industries (e.g., beef or pork). However, one prohibitive factor may be the cost associated with the numbers of tests necessary to evaluate preharvest contamination. In summary, preharvest screening for the presence of Salmonella can provide a meaningful evaluation of product contamination.

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