Differential ex vivo responses of primary leukocytes from turkey pedigree lines to Salmonella Heidelberg

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ABSTRACT Escalating product recalls as a consequence of Salmonella-contaminated poultry products have resulted in detrimental economic impacts in the poultry industry. One potential long-term alternative method to Salmonella prevention is genetic selection to improve innate resistance. This study evaluated the ex vivo effects of Salmonella Heidelberg (SH) on phagocytic and bactericidal leukocyte function in turkeys from six pedigree lines (A–F). Day-of-hatch poults (n = 48) were placed and raised in cages (2 birds/gender/genetic line/cage) to 35 d when heterophils and peripheral blood mononuclear cells (PBMCs) were extracted from males and females of each line. Cells were used in phagocytic and bactericidal assays to determine the ex vivo effects of SH on turkey leukocyte activity. Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS Institute Inc.) with differences considered significant at P ≤ 0.05 and data reported as LS Means with SEM. Although genetic line had no significant effect on phagocytosis of SH by heterophils and PBMCs, cumulatively, female cells exhibited higher phagocytosis potential than those from males. The main effect of gender was significant on bactericidal activity of PBMCs when incubated at a 1:10 and 1:100 PBMC to SH ratio. Genetic line also had a significant effect on bactericidal activity of PBMCs with cells from line F exhibiting the best activity. These results suggest that gender had a marked cumulative effect on phagocytosis of SH by heterophils and PBMCs while both genetic line and gender had a prominent effect on bacterial killing of SH by turkey PBMCs. Once able to determine genetic markers associated with these immune responses to Salmonella, genetic selection for increased resistance may become feasible in turkeys.

Key words: turkey, Salmonella Heidelberg, phagocytosis, heterophils, leukocytes

INTRODUCTION

Salmonella enterica, subspecies enterica, are Gram-negative bacterial pathogens consisting of more than 2,500 different serovars, of which only a finite number is associated with poultry. The most commonly isolated serovars in poultry include S. Enteritidis, S. Typhimurium, S. Heidelberg, and S. Kentucky, all of which are non-host-adapted and can cause infections in a variety of animal hosts and humans (Desin et al., 2013). The inflammatory response induced by S. Typhimurium and S. Enteritidis often restricts infection to the gastrointestinal tract, where it may establish a carrier state and become a potential source of poultry-product contamination resulting in foodborne illness (Cheng et al., 2013). Salmonellosis is one of the most prevalent infectious foodborne diseases in the world (McCarthy et al., 2009; WHO, 2013) with S. Heidelberg being among the most prevalent serovar isolated from both chicken and turkey carcasses (Logue and Nde, 2007). An increasing number of Salmonella-contaminated poultry products have resulted in widespread, often voluntary product recalls and detrimental economic impacts (Tarr, 2011).

Biosecurity measures are functional in a Salmonella prevention plan, but alone they do not negate the risk of contamination. Currently, researchers are investigating the use of vaccines and dietary direct-fed microbials to mitigate Salmonella (Grimes et al., 2008; Desin et al., 2013). One potential long-term and cost-effective alternative to conventional methods of Salmonella prevention is the selection for genetic resistance. Previous studies have focused on the genetics and genomics of resistance to Salmonella in chickens; however, few trials have focused on these aspects in turkey breeding flocks. The initial stage in such studies is to identify the phenotypic traits of interest and determine that a genetic basis actually exists for those particular traits. Estimated heritability of parameters of Salmonella response as well as differences observed between genetic lines of chickens suggests a partial genetic control of most response phenotypes. This implies that genetic
selection to improve resistance to *Salmonella* carrier state is feasible in chickens (Cheng et al., 2013). Some studies suggest that particular major histocompatibility complex (MHC) haplotypes and polymorphisms may serve as markers for genetic resistance to *S. Enteritidis* in young chicks (Liu et al., 2002). Studies of genetically distinct chicken lines suggest a strong relationship between heterophils and resistance to systemic *S. Enteritidis* (Swaggerty et al., 2005). With the avian heterophil being a critical component of host defense against bacterial infections, this knowledge would be essential in determining factors to consider when analyzing traits for genetic selection of *Salmonella* resistance.

Only recently has research been published concerning factors related to the feasibility of genetic selection for *Salmonella* resistance in turkeys. Genovese and colleagues (2006) measured functional differences in heterophils isolated from a commercial turkey line and those isolated from wild-type Rio Grande turkeys. Results of this study suggested immunological advantages and disadvantages between genetic lines. Therefore, there is a need for further research on the differences and similarities between the innate immune response of commercial turkey lines and wild-type turkeys to improve genetic resistance and decrease pathogen contamination in commercial turkey lines (Genovese et al., 2006). A more practical alternative would be to develop genetic resistance to *Salmonella* at the pedigree level in order to ultimately improve resistance at the commercial flock level, thereby diminishing the risk of *Salmonella* contamination and reducing its consequential economic impact. Therefore, the objective of this study was to determine the ex-vivo effects of *S. Heidelberg* (*SH*) on phagocytic and bacterial killing of *SH* by heterophils and peripheral blood mononuclear cells (PBMCs) in six turkey pedigree lines with diverse breeding goals for commercial and reproductive traits.

### MATERIALS AND METHODS

#### Experimental Birds and Housing

This project was approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. In this study, 48 5-week-old turkey poults of six different pedigree lines (represented by genetic lines A, B, C, D, E, and F in Table 1) were obtained from Aviagen Turkeys (Lewisburg, WV). Poults were separated by genetic line and gender at the hatchery. Each genetic line was wing-banded with a distinct color (blue, pink, purple, orange, green, and yellow, respectively) for identification purposes. Birds were placed in cages (2 birds/gender/genetic line/cage 0.2 m²) housed in the Virginia Tech Litton-Reaves BSL2 Animal Research Facility and provided ad libitum water and non-medicated starter feed meeting primary breeder recommendations in mash form.

#### Bacterial Culture

For this experiment, a field isolate of *SH* was used in phagocytic and bactericidal assays. The isolate was obtained from a suspect bird (from a commercial poultry grow-out operation) via cloacal swab in buffered peptone water, and confirmed by PCR following colony selection. For the assays, culture enrichment was performed in tetraphionate broth base containing iodine-potassium iodide solution. Using XLT4 agar, a single colony of *SH* was isolated and used to prepare a pure culture in Luri-Bertani (LB) broth for 18–24 h. Serial dilutions were performed to obtain 1 × 10⁷ colony forming units/mL (CFU/mL). Concentration was confirmed by plating onto XLT4, incubating at 37°C for 24 h, and performing colony counts. This culture was prepared 18 to 24 h prior to use in the phagocytic and bactericidal assays.

#### Heterophil and PBMC Functional Assays

In order to determine the phagocytic and bactericidal activity of turkey heterophils and PBMCs among genders and across genetic lines, blood was collected from four birds per gender, per genetic line (eight birds per genetic line) from a total of 48 birds.

### Table 1. Aviagen parental (purebred) genetic lines used in this study.

<table>
<thead>
<tr>
<th>Genetic line</th>
<th>Gender</th>
<th>Size</th>
<th>Selection Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Male</td>
<td>Weight</td>
<td>FCR¹, and Livability</td>
</tr>
<tr>
<td>B</td>
<td>Female</td>
<td>Very Heavy</td>
<td>Weight, FCR¹, and Eggs²</td>
</tr>
<tr>
<td>C</td>
<td>Female</td>
<td>Heavy</td>
<td>Weight, FCR¹, and Eggs²</td>
</tr>
<tr>
<td>D</td>
<td>Female</td>
<td>Heavy</td>
<td>Weight, Eggs², and Hatchability</td>
</tr>
<tr>
<td>E</td>
<td>Male</td>
<td>Legs</td>
<td>FCR², Leg Health, and Livability</td>
</tr>
<tr>
<td>F</td>
<td>Female</td>
<td>Small</td>
<td>Eggs² and Hatchability</td>
</tr>
</tbody>
</table>

¹FCR: Feed conversion ratio.
²Genetic selection for reproductive traits (i.e., number of eggs set in 24 weeks of lay as well as hatch of settable eggs for the same 24-week period).
injected via the brachial vein. Five hours post-injection, the birds were euthanized by cervical dislocation, and \( \sim 30 \text{ mL} \) of blood per bird were collected through cardiac puncture immediately following euthanasia and centrifuged at \( 50 \times g \) for 10 min at low deceleration. For the density-based separation of cell populations, 15 mL of serum and buffy coat were collected and layered on top of approximately 6 mL of Histopaque-1077 (Sigma-Aldrich, St. Louis, MO), which was overlaid on 9 mL of Histopaque-1119 (Sigma). The cells were centrifuged at \( 250 \times g \) for 60 min (deceleration = 1) and the interphases were collected for PBMCs and heterophils. The collected cells were diluted in twice the volume of incomplete DMEM media, followed by pelleting at \( 250 \times g \) for 10 min and washing with incomplete DMEM. The cells were re-suspended with 2 to 3 mL of media and their concentrations determined by counting using Trypan blue exclusion. The working number of heterophils was adjusted to \( 6 \times 10^5 \text{ cells/well} \) (in 24-well plate) and that of PBMCs to \( 1 \times 10^7 \text{ cells/mL} \) in a 100-mm dish, which were incubated overnight at \( 39^\circ C \) with 5% \( \text{CO}_2 \).

**Phagocytosis Assay**

Following incubation, approximately \( 5 \times 10^5 \) heterophils/well or \( 6 \times 10^5 \) PBMCs/well were incubated with a 1:10 cell:SH ratio (diluted number of SH \( 10^9 \text{ CFU/mL} \)). The mixture of cells and SH was incubated at \( 39^\circ C \) for 1 h, followed by the addition of ice-cold PBS to stop phagocytosis. The cells were then stained using Diff-Quick staining solution (Thermo Fisher Scientific, Wilmington, DE) and the total number of heterophils showing phagocytosis out of the total number of heterophils was counted using a phase contrast microscope (40× to 100×). The number of phagocytic cells was calculated as a percent of the total number of cells.

**Bactericidal Assay**

To examine resistance and susceptibility to *Salmonella* sp. in the six different genetic lines of turkeys, we measured survival ratio of SH cultured with PBMCs. Isolated PBMCs in 100-mm plates were washed and non-adherent cells were removed after the cells were incubated 24 h. The adherent PBMCs were harvested by trypsinization and the number of cells was counted. Approximately \( 4 \times 10^6 \) cells were transferred to a 1.5-mL microcentrifuge tube, followed by the addition of either 1:10 or 1:100 diluted number of SH \( (10^9 \text{ CFU/mL}) \). The bacteria were incubated either in medium alone or medium with antibiotics (50 \( \mu \text{g/mL} \) of kanamycin and gentamicin) as negative and positive controls, respectively. The mixture of PBMCs and SH was incubated at \( 39^\circ C \) for 1 h. Bacteria were then pelleted by centrifugation for 2 min at \( 16,244 \times g \). The bacterial pellets were re-suspended, diluted 1:5,000 with peptone water, and plated onto XLT4 agar plates. The number of colonies was counted 24 h post-incubation \( (39^\circ C) \). The number of colonies was multiplied by the dilution factor and then divided by the total volume of culture plated to achieve the number of CFU per mL of culture. The number of SH remaining post-incubation with bactericidal PBMCs was analyzed.

**Statistical Analysis**

The data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS). Differences between genetic lines and genders were tested using Student’s \( t \)-test following ANOVA with significance reported at \( P \leq 0.05 \). Results are reported as Least Square Means (LS means) with standard errors of the mean (SEM).

**RESULTS**

**Phagocytosis Assay**

Genetic line had no significant effect on phagocytosis of SH by heterophils. Cumulative gender-based phagocytosis of SH by heterophils illustrated that females had a significantly higher percentage phagocytosis than males \( (P = 0.0053) \) (Figure 1-A). Similarly, the main effect of genetic line had no significant impact on phagocytosis of SH by PBMCs. Cumulative gender-based phagocytosis of SH by PBMCs illustrated females having a significantly higher percentage phagocytosis than males \( (P = 0.027) \) (Figure 1-B). The numbers of bacteria counted per cell were not significantly different.

![Figure 1. Cumulative effect of turkey gender on phagocytosis of *S. Heidelberg* by heterophils (A) and PMBCs (B). Data were analyzed as LS Means + SEM (n = 4/gender/line). Bars lacking a standard letter (a-b) differ significantly. There was a significant effect of gender on percent phagocytosis of *S. Heidelberg* by heterophils (P = 0.0053) and PMBCs (P = 0.027).](image-url)
TURKEY LEUKOCYTE RESPONSE TO SALMONELLA HEIDELBERG

**Figure 2.** Effect of genetic line on bacterial killing by PBMCs from female and male turkey poults incubated at a 1:10 cells to S. Heidelberg ratio. Data were analyzed as LS Means + SEM (n = 4; bars lacking a standard letter (a-c) differ significantly. There was a significant effect of genetic line (A-F) on the killing of S. Heidelberg by turkey PBMCs (P = 0.0052 for female and P = 0.0149 for male poults).

**Bactericidal Assay**

When incubated at a 1:10 PBMC to SH ratio, within genetic line, female lines B and D had the highest while lines A and F exhibited the lowest bacterial counts when compared to other genetic lines (P = 0.0052) (Figure 2). Within sex, specifically male cells, lines A, B, and D conveyed the highest while lines C and F had the lowest bacterial counts (P = 0.0149) (Figure 2). Furthermore, significant differences were found when analyzing the cumulative effect of gender on bactericidal activity of PBMCs (Figure 3) where female cells had higher bactericidal activity than male cells at 1:100 (P = 0.035) and 1:10 (P = 0.043) cells to SH ratios.

**DISCUSSION**

Although phenotypes of genetic resistance to *Salmonella* have been widely studied in chickens, less research has focused on determining these factors of *Salmonella* resistance in pedigree turkey lines. In this study, ex-vivo effects of SH on leukocyte function were observed in six turkey purebred lines that have broadly different breeding goals. Genetic lines emphasizing growth tend to be more sensitive to a stressful environment and have proven to be more likely to show respiratory illness or enteric illness if a bacterial challenge is present (Aviagen Turkeys, personal communication). Similarly, research on disease susceptibility in chicken lines has demonstrated that allocation of a substantial portion of resources towards a particular demand decreases the ability of the host to respond to other needs such as immunocompetence (Siegel and Honaker, 2009).

Heterophils are the avian equivalent to the mammalian neutrophil and are the first cells to defend the host against bacterial infection. Phagocytosis is an active and receptor-mediated internalization of foreign antigens by phagocytic cells such as heterophils and PBMCs. Heterophils have the rapid ability to kill bacterial pathogens through phagocytosis, degranulation, and generation of an oxidative burst (Swaggerty et al., 2003). Bactericidal assays permit the ability to test leukocytes in order to determine their ability to kill...
a culture of live bacteria. Ultimately, phagocytic cells ingest and destroy bacteria through cell lysis. In some cases, bacteria are able to survive within the cell and multiply within macrophages. In this study, cumulative gender (across genetic lines) had a significant effect on phagocytosis, while genetic line and gender had a significant impact on bactericidal killing of SH by heterophils and PBMCs for both assays. These results were similar to findings of Redmond et al. (2011) where genetic crosses significantly affected the phagocytosis and bacterial killing, with heterophils from a broiler X Fayoumi advanced intercross line outperforming those from a broiler X Leghorn advanced intercross line. Results were also consistent with those of Swaggerty et al. (2003) suggesting that heterophil function and efficiency can be genetically transferred to progeny. Furthermore, the authors concluded that heterophil function was sex-associated and could be genetically controlled by the rooster. These outcomes correspond to our findings of gender having a significant contribution to the process of phagocytosis of SH by heterophils and PBMCs. Curiously, these results contradict findings previously reported by Genovese et al. (2006), which proposed that heterophils isolated from commercial turkeys and Rio Grande turkeys on d 4, 7, and 14 post-hatch show no significant differences in the percentage of heterophils phagocytizing S. Enteritidis. These differing results could be due to the age at which heterophils were isolated, the SH serovars used, or the fact that only one commercial line was used for comparison.

Regarding the bactericidal assay in this study, similar to the phagocytosis results, gender effects were observed where cells from female turkeys were more effective than those from males. Interestingly, genetic lines B and D demonstrated higher bacterial counts (from both females and males) than all other genetic lines. These two genetic groups were both heavy female lines with line B being selected for weight, feed conversion ratio, and eggs, and line D being selected for weight, eggs, and hatchability. It would be reasonable to suggest that this increased selection for a variety of traits combined with the particular genetics of lines B and D rendered their innate responses to SH less robust in comparison with lighter bird lines. Furthermore, genetic line F, a small bird primarily selected for eggs and hatchability, illustrated the lowest bacterial counts when incubated at a 1:10 ratio, translating in higher or more effective ex-vivo responses. Overall, these results suggest that an increased selection for weight could pull a large amount of resources from immunocompetence; therefore, resulting in increased susceptibility to SH.

The role of genetic background and potential of genetic selection as a long-term method of Salmonella prevention has been well researched in chickens (Berthelot et al., 1998; Girard-Santosuosso et al., 1998; Beaumont et al., 1999; Kaiser and Lamont, 2001; Lamont, 2010; Redmond et al., 2011; Cheng et al., 2013). Studies of genetically distinct chicken lines point to a strong relationship between innate immune cells (e.g., heterophils) and resistance to systemic Salmonella Enteritidis (Swaggerty et al., 2005). However, there is a long way ahead to confirm these results, as comparative research and literature remain limited for turkeys. As has been demonstrated in chickens, genetic selection for Salmonella resistance could be achievable in turkey pedigree lines once the genetic markers of resistance are identified.

In conclusion, these data suggest that both genetic line and gender are essential components involved in genetic resistance to Salmonella. Similarly, phagocytosis and bacterial killing of SH by heterophils and PBMCs suggested a relationship between efficiency of function and genetic line and gender. The observed gender effect could potentially be applied in selection efforts within breeding programs, especially as more pathogen and host genomic information pertaining to immune responses becomes available (Ricke et al., 2013; Dalloul
et al., 2014). To our knowledge, this study is the first to evaluate differences in leukocyte function in purebred turkey lines. Additional research on heterophil and PBMC function is needed in order to determine further variations in the innate immune response of turkeys originating from different genetic lines (e.g., intercrosses) as well as the degree of such responses in commercial lines.

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