Antimicrobial Resistant *Salmonella enterica* Typhimurium Colonizing Chickens: The Impact of Plasmids, Genotype, Bacterial Communities, and Antibiotic Administration on Resistance

Tameka N. Bythwood 1, Vivek Soni 1, Karen Lyons 1, Anne Hurley-Bacon 1, Margie D. Lee 1†, Charles Hofacre 1, Susan Sanchez 2 and John J. Maurer 1*

1 Department of Population Health, University of Georgia, Athens, GA, United States, 2 Department of Infectious Diseases, University of Georgia, Athens, GA, United States

The rise in *Salmonella* resistance to cephalosporins and fluoroquinolones has become a significant threat to public health. At issue, is whether agricultural use of antimicrobials is selecting antibiotic resistance in *Salmonella* and the degree to which large antimicrobial resistance gene reservoirs, present in animal manures, contribute to this resistance. Two in vivo studies were performed to address these questions. In the first study, chickens were administered *Salmonella* and commensals, including an *Escherichia coli* strain with a mobile, ceftiofur-resistance plasmid, in order to determine how antibiotic administration impacted resistance in *E. coli* and *Salmonella*. All antibiotics administered to chickens increased streptomycin resistance in *E. coli*. However, only ceftiofur administration increased resistance in *Salmonella* and specifically to extended-spectrum β-lactams (ESBL). There was no significant increase in ESBL-resistant *Salmonella* in chickens administered a ceftiofur-resistance plasmid donor. In the second study, chickens were administered two different isolates of *S. enterica* Typhimurium and a chicken resistome to serve as a gene donor. Antibiotic administration significantly altered aminoglycoside and tetracycline resistance in the *Enterobacteriaceae* population. However, there was no significant increase in antimicrobial resistant *Salmonella*. Administration of a chicken resistome had no significant impact on prevalence of resistance in *Enterobacteriaceae* populations, including *Salmonella*. Evident, from both studies, was that these treatments had minimal effect on increasing the prevalence of resistance in *Salmonella*, suggesting that other factors may be more important in dissemination of antimicrobial resistant *Salmonella* in chickens.

**Keywords:** *Salmonella*, antibiotic usage, antimicrobial resistance, poultry, plasmids
INTRODUCTION

*Salmonella enterica* is the 3rd leading cause of foodborne illnesses globally; accounting for 78 million illnesses and 59 thousand foodborne-related deaths annually (Havelaar et al., 2015). In the United States, non-typoidal *Salmonella* is responsible for 1 million illnesses, 19 thousand hospitalizations, and 378 deaths each year (Scallan et al., 2011). Children (<5 years old) and the elderly (>65 years old) are most susceptible to *Salmonella* infection, requiring some medical intervention (Scallan et al., 2013, 2015, 2018). Antibiotic therapeutics can lessen the severity and length of illness (Hu et al., 2014). However, the rise in antimicrobial-resistant *Salmonella* has become a significant public health concern. Especially problematic is its reduced susceptibility to antimicrobials commonly prescribed to treat *salmonellosis* (Wasyl et al., 2015; Iwamoto et al., 2017; Tyson et al., 2017; Duong et al., 2018) and documented cases of treatment failure (Collard et al., 2007; Tribble, 2017; Duong et al., 2018).

As most *Salmonella* infections are associated with consumption of meats, milk, and eggs, the veterinary use of antimicrobials, especially those important for treatment of humans, has long been a contentious issue (Cox and Ricci, 1969). While there is epidemiological data to support the link between antimicrobial use and resistance (Bortolaia et al., 2015; Noyes et al., 2016; Andersen et al., 2017; Caffrey et al., 2017; Shigemura et al., 2018), it is generally difficult to ascertain causal effect depending on the antimicrobial (Fairchild et al., 2005; Smith et al., 2007; Agga et al., 2016), food animal (Agga et al., 2016), level or scope of food animal production system (Iidris et al., 2006; Andersen et al., 2017; Liljebjelke et al., 2017), or method of quantifying resistance (Fairchild et al., 2005; Smith et al., 2007; Agga et al., 2016). In poultry production, the antimicrobials available for use in the US are limited to 15 drugs (Singer and Hofacre, 2006). The antimicrobials with Gram-negative spectrum are chlorotetracycline, oxytetracycline, streptomycin, gentamicin, and ceftiofur. The likelihood that any of these antimicrobials are used are mostly dependent on economics, weighing the cost of the drug against the level of disease (Singer and Hofacre, 2006). A commercial poultry farm may go a full year without any therapeutic antimicrobial use (Liljebjelke et al., 2017).

Antimicrobial resistance in *Salmonella* is dependent on the acquisition of resistance genes from its environment. As *Salmonella* is not naturally transformable (Lorenz and Wackernagel, 1994), resistance gene transfer is therefore reliant on cell-to-cell contact (Sieckmann et al., 1969) with another cell harboring a conjugative genetic element with resistance gene(s). Therefore, the population size of antibiotic resistance donors is an important factor, influencing antimicrobial resistance in *Salmonella*. If this is an especially small population, antibiotic selection pressure would be expected to play an important role in amplifying the donor population (Schjørring et al., 2008) or the newly acquired resistance in *Salmonella*.

The *Enterobacteriaceae*, which includes member species *Salmonella* and *Escherichia coli*, are a minor component of the chicken gastrointestinal microbiome (Lu et al., 2003a) and the poultry litter bacterial community (Lu et al., 2003b). However, *Salmonella* and *E. coli* isolated from the poultry environment tend to harbor class 1 integrons, which are genetic elements that can capture and integrate antimicrobial resistance genes within the bacterial host genome (Bass et al., 1999; Goldstein et al., 2001). The poultry litter bacterial community contains gram-positive species that have a very high prevalence of class 1 integrons and have the same integron-associated antimicrobial resistance genes as Gram-negatives cultured from the same environment (Nandi et al., 2004). This suggests that the poultry litter community is an important reservoir of resistance elements. There exists a diversity of integron-associated resistance genes in this environment, including resistances to β-lactams, phenicols, sulfonamides, aminoglycosides, and quaternary ammonium (Lu et al., 2003b; Nandi et al., 2004; Smith et al., 2007). In addition, the poultry environment also has a diverse array of tetracycline resistance genes with evidence of horizontal gene transfer among evolutionarily disparate genera, as evident in the presence of *tetO* in *Campylobacter* and *Enterococcus* (Fairchild et al., 2005). It is therefore not surprising that *Salmonella* isolated from the poultry environment can possess a diverse array of antimicrobial resistances, even on farms where there has been no therapeutic use of antimicrobials (Liljebjelke et al., 2017). However, it is not clear what factors have the highest impact on resistance in *Salmonella*. One would expect an increase in resistant *Salmonella* in birds raised in a deep litter system such as is common in the US, regardless of therapeutic antimicrobial treatment. The objective of this study was to determine the impact of resistance reservoirs and antimicrobial administration on prevalence of antimicrobial resistant *Salmonella* in chickens. The studies revealed that while there were significant changes in resistance in Gram-negative commensals, with the exception of ceftiofur, most antibiotics did not increase prevalence of antibiotic-resistant *Salmonella*.

MATERIALS AND METHODS

**Bacterial Strains, Plasmid Transfer Frequency, Segregation Rate, and Fitness Cost**

*Salmonella enterica* Typhimurium isolates, 934 and 3147, were used as recipients in antimicrobial resistance transfer experiments. The *Salmonella* isolates were obtained from a commercial poultry farm in northeast Georgia and represent the same strain as determined by PFGE (pulsed-field gel electrophoresis) (Liljebjelke et al., 2017). Typhimurium isolate 934 is resistant to streptomycin and sulfisoxazole and 3147 is only resistant to sulfisoxazole. Isolate 3147 contains a class 1 integron (*intI1*) with an empty integration site (Lévesque et al., 1995). Rifampicin and nalidixic acid resistant variants were selected by plating 100 μl of an overnight culture on Luria Bertani (LB)
(Murooka and Harada, 1994) agar with increasing concentrations of each antibiotic to obtain resistance to 64 µg/ml (Kruse and Sorum, 1994). Therefore, strains used in subsequent experiments will be referred to as R (rifampicin-resistant) or NR (nalidixic acid and rifampicin-resistant) if the phenotype was important for detection or selection of transconjugants.

The multi-drug resistance plasmid (floR, blaCMY2, strA, tetA) harbored by S. Newport strain 14407 was moved into nalidixic-resistant E. coli strain 1932 (Wooley et al., 1992) through filter mating (Taylor et al., 1981). Escherichia coli strain 1932 harboring the resistance plasmid was used as a plasmid donor in both in vitro and in vivo studies. Ceftiofur and florfenicol-susceptible Salmonella enterica serovars Enteritidis, Heidelberg, Infantis, Kentucky, Montevideo, and Typhimurium isolates were made rifampicin (64 µg/ml) resistant as previously described, and used in in vitro conjugation experiments in order to assess the plasmid transfer rate in multiple strain backgrounds. The florfenicol resistance gene, floR was used as a marker in PCR screening of transconjugants (Keyes et al., 2000).

Plasmid donor and rifampicin-resistant Salmonella recipient strains were grown in LB at 37°C overnight in standing culture. Strains were diluted in 5 ml of fresh LB broth, 1/500, at a donor to recipient ratio of 1:1. Cultures were subsequently incubated at 37°C standing overnight then diluted 10-fold in PBS and plated on LB agar with rifampicin alone (recipients), and LB agar with rifampicin and florfenicol (transconjugants), for enumeration. Plates were incubated overnight at 37°C. Plasmid transfer frequency was calculated by taking the total number of transconjugants divided by the total number of recipients.

Plasmid segregation rate was determined for a Typhimurium 934R transconjugant passed for 5 serial cultures in LB broth with no antibiotic selection using the following protocol. Transconjugant was streaked onto LB with florfenicol and incubated overnight at 37°C, as a standing culture then a single colony was used to inoculate LB broth with florfenicol and incubated overnight at 37°C. One hundred microliters was used to inoculate 100 ml of LB with no antibiotics, and incubated overnight at 37°C. Following overnight incubation, the broth culture was diluted 10-fold and plated onto LB agar with no antibiotics and LB agar with florfenicol. The overnight culture was used to inoculate fresh LB and repeated for four more passages. Plate counts were recorded daily and plasmid segregation rate was calculated as described by Modi and Adams (Modi and Adams, 1991).

To measure the fitness cost of the multidrug-resistance plasmid on isolate 934R, transconjugant and recipient were grown alone or together in LB broth with no antibiotics. Seed cultures in LB broth with and without antibiotics were inoculated overnight at 37°C. The next day, 1 ml of the overnight broth culture was transferred to microfuge tubes and centrifuged at 4,500 × g for 10 min to pellet bacterial cells. The supernatant was decanted and the cells were suspended in 1 ml of deionized H2O. Fifty microliters of each cell suspension was used to inoculate 10 ml of LB with no antibiotics, alone or together, of which 10-fold serial dilutions (10−4 – 10−7) were plated onto LB agar with and without florfenicol (0 h). After incubation at 37°C for 24 h serial 10-fold dilutions were plated onto LB agar with and without florfenicol and plates were incubated at 37°C for 24 h. Fitness cost was calculated as described by Melnyk et al. (2015) and Lenski et al. (1994).

### Table 1: Animal Study 1: Does antibiotic administration enhance acquisition of the Newport multidrug-resistance plasmid by Typhimurium 934R in chickens?

| Antibiotic administration | #Birds | Oral administration |
|---------------------------|--------|---------------------|
| None                      | 30     | S. Typhimurium 934R alone |
| Ceftriофor<sup>a</sup>    | 30     | S. Typhimurium 934R alone |
| Oxytetracycline<sup>b</sup> | 30      | S. Typhimurium 934R alone |
| Streptomycin<sup>c</sup>  | 30     | S. Typhimurium 934R alone |
| None                      | 30     | S. Typhimurium 934R + E. coli with MDR plasmid<sup>d</sup> |
| Ceftriофor<sup>a</sup>    | 30     | S. Typhimurium 934R + E. coli with MDR plasmid<sup>d</sup> |
| Oxytetracycline<sup>b</sup> | 30      | S. Typhimurium 934R + E. coli with MDR plasmid<sup>d</sup> |
| Streptomycin<sup>c</sup>  | 30     | S. Typhimurium 934R + E. coli with MDR plasmid<sup>d</sup> |

<sup>a</sup>1 day old, chicks received a subcutaneous injection of ceftriофor.

<sup>b</sup>At 14 days of age, some groups were given antibiotics in their drinking water for three consecutive days.

<sup>c</sup>Escherichia coli strain 1932 with Newport MDR plasmid.

<sup>d</sup>Plasmid segregates with MDR.

### Animal Study 1: Does Antibiotic Administration Enhance Acquisition of the Newport Multidrug-Resistance Plasmid by Typhimurium 934R in Chickens?

Three hundred and sixty, 1 day-old SPF chicks were separated into 12 groups and placed into individual pens (30 birds per pen) in a research flock house, as described in Table 1. The pen floors had been sanitized with Vircon S (Lanxess Corp.; Pittsburgh, PA) and covered with fresh pine shavings. For ceftriофor administration group, 1 day old, chicks received a subcutaneous injection of ceftriофor (0.1 mg/chick). At 1 day of age, chicks were orally inoculated with Typhimurium 934R (10<sup>6</sup> CFUs/chick) or Typhimurium 934R with Escherichia coli strain 1932 harboring Newport multidrug-resistance plasmid (10<sup>6</sup> CFUs/chick). At 14 days of age, some groups were given antibiotics in their drinking water continuously for 3 consecutive days (0.60 g/gallon streptomycin or 1.45 g/gallon oxytetracycline).

Fresh cecal droppings were collected from the pens on days 13, 15, and 20 by placing 0.75 m × 1 m sheets of paper in each pen. Three pools of 10 cecal samples were recovered from each pen and placed into 50 ml centrifuge tube, weighed and diluted 1/10 ml in freezer stock solution (1% peptone and 15% glycerol in water), and aliquots were placed into three 1 ml Eppendorf tubes for freezer storage at −80°C.

Resistant bacteria were isolated by plating onto selective media as follows. Salmonella were isolated onto XYlose Lysine Tergitol 4 (XLT4) agar (Sigma; St. Louis, MO) plates containing ampicillin (25 µg/ml), florfenicol (32 µg/ml), streptomycin (32 µg/ml), or tetracycline (10 µg/ml). Total Salmonella Typhimurium 934R were enumerated by plating onto XLT containing rifampicin
(64 µg/ml). Resistant coliforms were enumerated by plating onto MacConkey agar containing ampicillin (25 µg/ml), florfenicol (32 µg/ml), streptomycin (32 µg/ml), or tetracycline (10 µg/ml). Plates were incubated at 37°C for 18–22 h. Isolates were stocked in freezer stock medium and stored at −80°C.

Animal Study 2: Does Antibiotic Administration Enhance Acquisition of Antimicrobial Resistance in Salmonella in vivo From the Chicken Resistome?

The chicken resistome was prepared by administering used litter to chicks and collecting the subsequent resistant cecal microbiome. Eighteen days old, embryonating chicken eggs from specific pathogen free (SPF), white leghorn chickens (Charles River Laboratories; Willington, MA) were cleaned and disinfected by immersing in pre-warmed (100°F) 10% commercial bleach solution (James Austin Co.; Mars, PA) for approximately 3 min as previously described (Wang and Slavik, 1998). Fifty-eight day of hatch, SPF chickens were separated into four groups containing 14 chickens per Horsfall unit. Prior to placement of chicks, HEPA-filtered, Horsfall isolator units were disinfected by washing with bleach solution followed by fumigation with formaldehyde for approximately 24 h (Williams, 1970). Each bird was orally inoculated with 0.5 ml of pooled, poultry litter samples harvested from commercial broiler farms in the Northeast Georgia area; this litter microbiota had been shown previously to harbor a variety of antimicrobial resistance genes (Lu et al., 2003b). At 3 weeks, post-inoculation, chicks were sacrificed by cervical dislocation. Theecal contents were pooled, homogenized for 5 min at maximum speed using a stomacher (Tekmar Company; Cincinnati, OH) with 1 ml of phosphate buffered saline (PBS, pH = 8). Homogenates were filtered through gauze and confirmed to be Salmonella-free by culture as described below. The resistome homogenate was administered to broiler chicks in subsequent experiments.

Experiments were conducted with day of hatch, broiler chicks housed in research flock houses at stocking densities simulating commercial conditions. Broiler eggs, hatching incubators, and floor pen houses at the Poultry Diagnostic and Research Center were disinfected and fumigated with formaldehyde. Five days prior to placing broiler chicks, the floor pens, walls and floors were randomly sampled with drag swabs (Liljebjelke et al., 2005) and confirmed as Salmonella-negative according to procedures described below. Cobb/Cobb broiler chicks (Siloam Springs, AR) were collected from incubators at day of hatch and transferred to an adjacent floor pen house containing 3 × 3 m pens lined with fresh softwood shavings. Chicks were divided into 16 groups of ~50 birds per pen, as described in Table 2.

Salmonella inoculum was prepared by streaking onto XLT4NR (containing nalidixic acid and rifampicin), incubating at 37°C overnight which was used to inoculate 500 ml Brain Heart Infusion broth (Sigma) which was incubated at 37°C standing for 4 h. Approximately 10⁷ CFU were administered to the broiler chicks on day of hatch. One day following oral inoculation, a 1 ml suspension of litter resistome was added to the chicks’ drinking water. All birds were given clean drinking water daily ad libitum and fed commercial feed supplemented with monensin (Elanco Animal Health; Indianapolis, IN) (55 g/ton). At 2 weeks of age, the birds were administered chlorotetracycline (55 mg/kg, body weight) or streptomycin (33 mg/kg, body weight) via their water drinkers for 1 week. Control birds received no chlorotetracycline or streptomycin. The colony house environment was sampled weekly for Salmonella by drag swabs (Sigma) as previously described (Liljebjelke et al., 2005). To assay for resistant Salmonella, 10 µl of tetraethionate brilliant green broth (TTBG) drag swab enrichment were streaked onto XLT4 agar with no antibiotics or XLT4NR in order to detect 934NR and 3147NR. Additionally, TTBG enrichments were streaked onto XLT4NR and one of the following antibiotics: ampicillin (10 µg/ml), chloramphenicol (25 µg/ml), gentamicin (16 µg/ml), kanamycin (25 µg/ml), streptomycin (25 µg/ml), or tetracycline (10 µg/ml). Plates were incubated for 24 h at 37°C; presumptive positive isolates were stocked in freezer stock medium (1% peptone, 15% glycerol) and stored at −80°C.

Poultry litter, water and feed from each pen (n = 16) were collected and processed on a weekly basis, as follows. Chicken litter samples were randomly collected from five general areas within a pen then pooled (n = 16 pens) in order to enumerate Gram-negative enterics. Five grams of litter

**Table 2** | Animal Study 2: Does antibiotic administration enhance acquisition of antimicrobial resistance in Salmonella in vivo from the chicken resistome?

| Antibiotic administered | #Birds/PEN | Oral microbial administration |
|-------------------------|------------|------------------------------|
| None                   | 50         | None                         |
| Chlorotetracycline      | 50         | Chicken resistome            |
| Streptomycin            | 50         | Chicken resistome            |
| None                   | 50         | S. Typhimurium 934NR         |
| Chlorotetracycline      | 50         | S. Typhimurium 934NR         |
| Streptomycin            | 50         | S. Typhimurium 934NR         |
| None                   | 50         | S. Typhimurium 3147NR        |
| Chlorotetracycline      | 50         | S. Typhimurium 3147NR        |
| Streptomycin            | 50         | S. Typhimurium 3147NR        |
| None                   | 50         | S. Typhimurium 3147NR + chicken resistome² |
| Chlorotetracycline      | 50         | S. Typhimurium 3147NR + chicken resistome² |
| Streptomycin            | 50         | S. Typhimurium 3147NR + chicken resistome² |
| None                   | 50         | S. Typhimurium 3147NR + chicken resistome² |

a At 2 weeks of age, chickens were administered chlorotetracycline or streptomycin in their drinking water.

b Untreated experimental groups received sterile phosphate buffered saline (PBS) as a placebo.

c 1 day-old chicks were orally administered chicken resistome alone, Typhimurium strain alone, or combination chicken resistome and Salmonella.

d Cecal intestinal microbiome from chickens seeded with poultry litter resistome.
was suspended in approximately 30 ml of PBS and vigorously shaken for 10 min with a “wrist-action” shaker set at maximum speed (Burrell Scientific; Pittsburgh, PA). Samples were then filtered through sterile gauze and centrifuged at low speed (50 × g for 15 min at 4°C) to remove litter debris, the bacteria were pelleted by high-speed centrifugation (3,650 × g for 15 min at 4°C); supernatant was discarded and the bacterial pellet suspended in 1 ml PBS. A 0.5 ml aliquot was collected by high-speed centrifugation, and the bacterial pellet was suspended in Superbroth (Murooka and Harada, 1994) with 20% sterile glycerol and stored at −80°C. Enumeration of resistant, Gram-negative enterics was done by serially diluting frozen-glycerol stocks of litter suspensions in PBS (dilution range: 10<sup>−1</sup> – 10<sup>−5</sup>), spread-plating onto MacConkey agar, MacConkey agar supplemented with nalidixic acid and rifampicin to enumerate <em>Salmonella</em>, and MacConkey agar with other antibiotics (ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin or tetracycline), at same concentrations used to enumerate antimicrobial resistant <em>Salmonella</em>. Plates were incubated for 18–24 h at 37°C.

Water and feed samples were screened for <em>Salmonella</em> by culture as follows. One hundred ml of water was added to 2X TTGB, 25 grams of feed was added to 225 ml TTGB, and both were incubated at 41.5°C overnight. TTGB enrichments were then plated onto XLT4 agar and incubated at 37°C for 18–24 h. Black colonies were confirmed as <em>S. enterica</em> Typhimurium by whole-cell agglutination test using polyvalent <em>Salmonella</em> “O” and Group B antisera (Sigma).

**Plasmid and Strain Typing of Bacterial Isolates**

<em>Salmonella</em> isolates cultured from the chickens were strain typed using PGFE with 2.5 U of restriction endonuclease, <em>Xba I</em> (Roche; Indianapolis, IN) as previously described. DNA fragments were separated with a CHEF DR-II electrophoresis apparatus (Bio-Rad; Hercules, CA) at 200 V for 25 h and pulse time of 2–40 s. (Liljebljelke et al., 2005) To increase the resolution of DNA band patterns for some isolates, 50 μM thiourea (Sigma Aldrich) was added to the running buffer (Koort et al., 2002). Yeast strain <em>Saccharomyces cerevisiae</em> YPH80 served as the molecular weight marker (220-1100 kb) (BioWhittaker Molecular Applications; Rockland, ME).

Large molecular weight plasmids were isolated and analyzed as follows. Cultures were grown to an OD<sub>600</sub>nm of 0.8–1.0 in 3 ml LB broth at 37°C with aeration (shaking: 220 rpm). Plasmid isolation, was carried out as described by Williams et al. (2006) using the CosMC Prep Tube Protocol for High and Low Copy Plasmid Purification (Agencourt Bioscience; Beverly, MA). Plasmids were separated on a 0.5% Seakem Gold agarose (Lonza; Rockland, ME) gel by electrophoresis at 5.14V/cm for 18 h at room temperature. Ten microliter of BAC-Tracker Supercoiled DNA ladder (Epicenter Biotech; Madison, WI) was used as molecular weight markers. Gels were stained with SYBR green I nucleic acid gel stain (Lonza) for 60 min. The DNA band, containing the plasmid of interest, was excised from the agarose gel and rinsed in cold 1:10 strength TE buffer pH 8.0 (10 mM Tris HCl, 1 mM EDTA) at 4°C. The agarose gel slice was subsequently washed with 100 μl SuRE/Cut Buffer B (10 mM Tris HCl, 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 M 2-mercaptoethanol), replaced with fresh enzyme buffer with <em>Hind III</em> (1U) (Roche), and incubated at 37°C for 2 h. The cut DNA was embedded in 0.75% Seakem Gold agarose gel and fragments were separated by gel electrophoresis at 45V, for 18 h at room temperature. One Kilobase DNA Ladder Plus (Fermentas; Glen Burnie, MD), and <em>Hind III</em> digest of λ DNA were used as the molecular weight markers. Gels were stained with Sybr Green for 60 min. DNA fragment sizes were then determined using Gene Profiler 4.05 by Scanalytics, Inc. (Fairfax, VA).

**Antibiotic Susceptibility Profile**

<em>Salmonella</em> isolates were initially screened for resistance using the Kirby-Bauer disk diffusion method against a panel of eight antimicrobials (CLSI, 2017). This panel included ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), rifampicin (5 μg), streptomycin (10 μg), and tetracycline (30 μg) (Sigma). The <em>Salmonella</em> parent strain was run as the negative control in order to infer acquired resistances. The Sensititre<sup>®</sup> susceptibility system (Trek Diagnostics Systems, Ltd.; Oakwood Village, OH) was used to determine the minimum inhibitory concentration (MIC) for select <em>Salmonella</em> isolates (CLSI, 2017). The antimicrobials contained in each Sensititre<sup>®</sup> plate were: enrofloxacin, gentamicin, cefotiofur, neomycin, oxytetracycline, tetracycline, amoxicillin, spectinomycin, sulphadimethoxine, trimethoprim/sulphamethoxazole, sarafloxacin, sulphathiazole, sulphadimidine, streptomycin, tetracycline, ampicillin, chloramphenicol, kanamycin, and vancomycin.
TABLE 3 | PCR primers.

| Target | Primer sequence | PCR conditions | References |
|--------|-----------------|----------------|------------|
| intI   | F:CTCTCCGCGACAGTGATC R:TCCAGCGATGCTGAAGCC | 55<sup>a</sup> | 280 | Bass et al., 1999 |
| suIT   | F:GGGTTTCCGGAAGGTTGATTGC R:TTGCGGCTTGCTTATGCTC | 60<sup>a</sup> | 187 | Lévesque et al., 1995 |
| Tn21   | F:GATAGCCTCCACCGCCGACA R:AGGATGCTGCGCCCATTC | 55<sup>a</sup> | 595 | Lévesque et al., 1995 |
| blaTEM | F:ATAAAATCTTGAGAAGAAA R:GAAGTACCAATGCTTAATCA | 55<sup>a</sup> | 1,079 | Mabilat et al., 1990 |
| tetA   | F:GCAGACGACTGCTGCTTC R:ATACATGCGTCGTAATGGA | 55<sup>b</sup> | 210 | Ng et al., 2001 |
| aadA1  | F:GCCAGACGACTCCATTCGC | 60<sup>a</sup> | 307 | Shahi and Kumar, 2015 |
| aadB   | F:AGGATGTGACGAGGTACAG R:CGGCATAGTAAGAGTAATCC | 60<sup>a</sup> | 400 | Shahi and Kumar, 2015 |

<sup>a</sup>2 mM MgCl<sub>2</sub>.  
<sup>b</sup>3 mM MgCl<sub>2</sub>.

TABLE 4 | In vitro Salmonella serovar transfer frequency of Newport multi-drug resistance plasmid.

| Salmonella serovar<sup>a</sup> | Plasmid transfer frequency<sup>b</sup> |
|-------------------------------|-----------------------------------|
| S. Typhimurium 934            | 8.0 × 10<sup>−6</sup>             |
| S. Heidelberg                 | 1.8 × 10<sup>−7</sup>             |
| S. Infantis                   | 3.8 × 10<sup>−7</sup>             |
| S. Kentucky                   | 3.3 × 10<sup>−7</sup>             |
| S. Enteritidis                | 1.4 × 10<sup>−7</sup>             |
| S. Montevideo                 | 1.7 × 10<sup>−7</sup>             |

<sup>a</sup>Salmonella isolates were chosen based on their susceptibility to florfenicol at 32 µg/ml. 
<sup>b</sup>Rifampicin-resistant Salmonella derivatives were selected by plating 10<sup>8</sup> cells on Luria- 
Bertani (LB) with rifampicin 64 µg/ml.

TABLE 5 | In vitro maintenance of Newport MDR plasmid in S. Typhimurium 934R: stability in the absence of antibiotic selection pressure.

| Time   | Total population<sup>a</sup> (CFU/ml) | Florfenicol-Resistant population<sup>b</sup> (CFU/ml) | Ratio of resistant cells to total population |
|--------|--------------------------------------|-------------------------------------------------|------------------------------------------|
| Day 1  | 1.1 × 10<sup>9</sup>                 | 8.9 × 10<sup>8</sup>                            | 0.81                                     |
| Day 2  | 1.0 × 10<sup>9</sup>                 | 7.7 × 10<sup>8</sup>                            | 0.77                                     |
| Day 3  | 2.0 × 10<sup>9</sup>                 | 1.2 × 10<sup>8</sup>                            | 0.60                                     |
| Day 4  | 1.6 × 10<sup>9</sup>                 | 1.5 × 10<sup>8</sup>                            | 0.94                                     |
| Day 5  | 2.6 × 10<sup>9</sup>                 | 2.7 × 10<sup>8</sup>                            | 1.04                                     |

<sup>a</sup>Enumeration on Luria-Bertani (LB) agar with no antibiotics.  
<sup>b</sup>Enumeration on LB agar with florfenicol (32 µg/ml).

and streptomycin. Antimicrobial susceptibilities for *Salmonella* isolated in Animal Study 2 were assessed using a Sensititre® plate that contained 17 antimicrobials including: amikacin, amoxicillin, ampicillin, cefazolin, cefotaxin, cefpodoxime, ceftiofur, cephalothin, chloramphenicol, enrofloxacin, gentamicin, imipenem, marbofloxacin, orfloxacin, rifampicin, tetracycline, ticarcillin, clavulanic acid, and trimethoprim. Susceptibilities were controlled and interpreted according to guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2017).

**Statistical Analysis**

The mean and standard deviations were computed for each experimental group and therapeutic treatment.

Paired and unpaired student *t*-test analyses were used to evaluate the effects of therapeutic treatments over time on the degree of resistance to each antibiotic tested. *X<sup>2</sup>* test was used to determine if there was a random association between administration and antimicrobial resistance.

**RESULTS**

**Does Antibiotic Administration Enhance Acquisition of the Newport Multidrug-Resistance Plasmid by Typhimurium 934R in Chickens?**

*In vitro*, the Newport multidrug-resistance plasmid was readily transmitted to multiple *Salmonella* serovars at frequencies ranging from 3.8 × 10<sup>−7</sup> to 8.0 × 10<sup>−6</sup> (Table 4). This plasmid was stably maintained in Typhimurium 934R even after 50 generations in the absence of antibiotic selective pressure (day
5: Table 6) and the plasmid segregation rate was low ($\nu = 8.6 \times 10^{-2}$). This indicates that plasmid acquisition would not be a barrier to Salmonella acquiring antibiotic resistance. However, the plasmid did impair Typhimurium 934R fitness ($S = -0.10, S_0 = 3.86; S_1 = -0.01$) (Lenski et al., 1994; Melnyk et al., 2015), indicating that plasmid maintenance may be a challenge in vivo. Since there was a fitness cost, acquisition, and maintenance of the plasmid in vivo may be enhanced with antimicrobial selection pressure. Therefore, administration of streptomycin, tetracyclines, or the cephalosporins would be expected to increase plasmid-encoded resistance in the microbiome of chickens.

In vivo, none of the antibiotics increased abundance of resistant Salmonella (Table 6). In addition, no florfenicol-resistant Salmonella were detected suggesting that the Newport multidrug-resistance plasmid was not transferred and/or maintained in Salmonella in vivo. There was no increase in Salmonella extended-spectrum β-lactam (ESBL) resistance in birds co-infected with E. coli harboring the Newmport MDR plasmid compared to group colonized with Salmonella alone (61.4% vs. 67.5%). This was confirmed in that the plasmid phenotype of Typhimurium expressing ESBL resistance, gentamicin resistance or antimicrobial susceptible phenotypes were identical to Salmonella Typhimurium LT2 sspB-virulence plasmid control using Hind III restriction enzyme mapping (Supplemental Figure 1). There were only three antimicrobial resistance phenotypes detected: ESBL resistance to amoxicillin, ampicillin, cefazolin, cephalothin, cefoxitin, cefpodoxime, and ceftiofur (59.8%); gentamicin resistance (0.5%); or pan-susceptible (39.7%). Cefotiofur administration was the only antibiotic that significantly increased ESBL resistance abundance in Typhimurium 934R compared to the untreated group (37.5% vs. 88.5%; $p = 1.1 \times 10^{-7}$) (Table 7).

### TABLE 6 | Impact of antibiotic administration on antimicrobial resistance in Gram-negative bacteria colonizing chickens.

| Resistance | Organism | 13 Days | 15 Days | 20 Days | 13 vs. 15 Days | 15 vs. 20 Days | 13 vs. 20 Days |
|------------|----------|---------|---------|---------|--------------|--------------|--------------|
| **NO ADMINISTRATION** | | | | | | | |
| Ampicillin | *E. coli* | 7.11 ± 0.21 | 7.58 ± 0.12 | 7.28 ± 0.16 | $p = 0.11$ | $p = 0.10$ | $p = 0.46$ |
| | *Salmonella* | 2.40 ± 1.37 | 2.55 ± 1.28 | 3.15 ± 0.84 | $p = 0.47$ | $p = 0.36$ | $p = 0.33$ |
| Streptomycin | *E. coli* | 5.68 ± 0.13 | 5.49 ± 0.30 | 6.29 ± 0.36 | $p = 0.32$ | $p = 0.46$ | $p = 0.11$ |
| | *Salmonella* | 0.49 | 0.00 | 0.00 | | | |
| Tetracycline | *E. coli* | 7.73 ± 0.20 | 7.67 ± 0.06 | 7.28 ± 0.05 | $p = 0.12$ | $p = 0.27$ | $p = 0.22$ |
| | *Salmonella* | 3.80 ± 0.62 | 2.32 ± 1.16 | 3.10 ± 1.56 | $p = 0.17$ | $p = 0.36$ | $p = 0.35$ |
| **CEFTIOFUR ADMINISTRATION** | | | | | | | |
| Ampicillin | *E. coli* | 7.26 ± 0.21 | 7.55 ± 0.12 | 7.23 ± 0.16 | $p = 0.16$ | $p = 0.10$ | $p = 0.46$ |
| | *Salmonella* | 4.24 ± 0.52 | 4.74 ± 0.34 | 4.00 ± 0.41 | $p = 0.24$ | $p = 0.12$ | $p = 0.36$ |
| Streptomycin | *E. coli* | 5.05 ± 0.17 | 5.83 ± 0.11 | 5.79 ± 0.20 | $p = 0.05$ | $p = 0.46$ | $p = 1.3 \times 10^{-2}$ |
| | *Salmonella* | 0.00 | 0.00 | 0.00 | | | |
| Tetracycline | *E. coli* | 6.47 ± 0.26 | 6.92 ± 0.20 | 6.74 ± 0.18 | $p = 0.12$ | $p = 0.27$ | $p = 0.22$ |
| | *Salmonella* | 4.17 ± 0.38 | 3.25 ± 1.66 | 4.48 ± 0.39 | $p = 0.32$ | $p = 0.27$ | $p = 0.30$ |
| **STREPTOMYCIN ADMINISTRATION** | | | | | | | |
| Ampicillin | *E. coli* | 7.61 ± 0.26 | 4.70 ± 0.18 | 7.96 ± 0.24 | $p = 7.0 \times 10^{-4}$ | $p = 3.1 \times 10^{-4}$ | $p = 0.19$ |
| | *Salmonella* | 4.77 ± 0.29 | 0.51 ± 0.51 | 4.52 ± 0.37 | $p = 2.1 \times 10^{-3}$ | $p = 2.0 \times 10^{-3}$ | $p = 0.32$ |
| Streptomycin | *E. coli* | 6.10 ± 0.55 | 5.34 ± 0.03 | 7.99 ± 0.29 | $p = 0.15$ | $p = 5.6 \times 10^{-3}$ | $p = 2.7 \times 10^{-2}$ |
| | *Salmonella* | 0.00 | 0.00 | 0.00 | | | |
| Tetracycline | *E. coli* | 7.18 ± 0.77 | 5.18 ± 0.19 | 7.97 ± 0.21 | $p = 0.08$ | $p = 2.9 \times 10^{-4}$ | $p = 0.21$ |
| | *Salmonella* | 4.50 ± 0.36 | 0.00 | 5.03 ± 0.05 | $p = 3.1 \times 10^{-3}$ | $p = 4.7 \times 10^{-5}$ | $p = 0.14$ |
| **OXYTETRACYCLINE ADMINISTRATION** | | | | | | | |
| Ampicillin | *E. coli* | 7.53 ± 0.54 | 7.12 ± 0.39 | 7.48 ± 0.53 | $p = 0.29$ | $p = 0.31$ | $p = 0.48$ |
| | *Salmonella* | 4.57 ± 0.25 | 4.26 ± 0.17 | 4.18 ± 0.40 | $p = 0.19$ | $p = 0.39$ | $p = 0.20$ |
| Streptomycin | *E. coli* | 5.67 ± 0.31 | 6.50 ± 0.51 | 6.89 ± 0.26 | $p = 0.13$ | $p = 0.27$ | $p = 2.0 \times 10^{-2}$ |
| | *Salmonella* | 0.00 | 0.00 | 0.00 | | | |
| Tetracycline | *E. coli* | 7.26 ± 0.49 | 4.60 ± 2.43 | 7.85 ± 0.49 | $p = 0.19$ | $p = 0.16$ | $p = 0.22$ |
| | *Salmonella* | 2.92 ± 1.48 | 2.95 ± 1.51 | 4.12 ± 0.72 | $p = 0.49$ | $p = 0.26$ | $p = 0.25$ |

*Resistant Gram-negative population was calculated from colony counts on MacConkey (lactose-positive; *E. coli*) or XLT4 (black colonies; *Salmonella*) agar plus antibiotic.

[1] Log_{10} transformed data.

[2] Day chickens were administered antibiotic, except for ceftiofur, which was administered to chickens at day of hatch.

[3] Unpaired, Student T-test.

[4] No colonies were detected on XLT4 with florfenicol at any dilution.

[5] No statistically significant difference comparing no antibiotic treatment groups vs. ceftiofur treatment groups ($p > 0.05$).
### TABLE 7 | Impact of antibiotic administration on S. Typhimurium 934R antimicrobial resistance phenotype.

| Administration                  | ESBLa,b | Gentamicina | Sensitivea,c |
|----------------------------------|---------|-------------|-------------|
|                                  |         |             |             |
| None (n = 48)                    |         |             |             |
| 13 (n = 20)                      | 30.0%   | 0.0%        | 70.0%       |
| 15 (n = 12)                      | 41.7%   | 0.0%        | 58.3%       |
| 20 (n = 16)                      | 43.8%   | 0.0%        | 56.3%       |
| 13 (n = 16)                      | 93.8%   | 0.0%        | 6.3%        |
| Cefiofur (n = 52)                |         |             |             |
| 15 (n = 16)                      | 81.3%   | 0.0%        | 18.8%       |
| 20 (n = 20)                      | 90.0%   | 0.0%        | 10.0%       |
| 13 (n = 14)                      | 64.3%   | 0.0%        | 35.7%       |
| Streptomycin (n = 40)            |         |             |             |
| 15 (n = 5)                       | 80.0%   | 0.0%        | 20.0%       |
| 20 (n = 21)                      | 47.0%   | 3.1%        | 32.3%       |
| 13 (n = 15)                      | 60.0%   | 0.0%        | 40.0%       |
| Oxytetracycline (n = 54)         |         |             |             |
| 15 (n = 18)                      | 50.0%   | 0.0%        | 50.0%       |
| 20 (n = 21)                      | 54.2%   | 0.0%        | 47.6%       |
| No Treatment vs. Cefiofurd       |         |             |             |
| ESBL                             | p = 1.1 × 10⁻⁷ |
| No Treatment vs. Streptomycind   |         |             |             |
| ESBL                             | p = 0.06 |
| No Treatment vs. Oxytetracyclined |     |             |             |
| ESBL                             | p = 0.10 |
| Salmonella alone vs. E. coli with S. Newport MDRd plasmid | ESBL | p = 0.46 |

a All isolates were resistant to rifampicin.
b Extended-spectrum β-lactam (ESBL) resistance. Resistant to β-lactams: ampicillin and amoxicillin; and 1st, 2nd, 3rd generation cephalosporins: cefazolin, cephalothin, cefotaxin, ceftriaxone, and cefotiofur.
c Isolates were susceptible to the panel of 19 antimicrobials excluding rifampicin.
d Chi-squared test.
e Cefiofur, florfenicol, multi-drug resistance (MDR) plasmid.

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### Does Antibiotic Administration Enhance Acquisition of Antimicrobial Resistance in Salmonella in vivo From the Chicken Resistome?

In this experiment, two S. Typhimurium isolates (934NR and 3147NRint11) of the same strain (see Supplemental Figure 2) were used in order to determine if antimicrobial resistance genotype influenced the acquisition of new resistances. Isolate 3147NR contained a type 1 integron with an empty integration site enabling easy PCR screening of integron-associated resistance. Because inflammation has been shown to affect the antimicrobial resistance in Salmonella (Stecher et al., 2012), birds were administered an infectious dose high enough to cause clinical symptoms. A difference in mortality (Table 8) and poultry environment abundance (Figure 1) was shown between the isolates. Mortality was reduced in chickens fed litter resistome then administered isolate 3147NRint11 (31.8% vs. 45.4%; p = 0.035) and the isolate was less abundant in the poultry environment compared to isolate 934NR (Figure 1). Isolate 934NR was also more likely to be ampicillin resistant (91.7% vs. 67.4%; p = 3.7 × 10⁻³) (Table 9).

Antibiotic administration had a minor effect on Salmonella resistance while it increased resistance in other Gram-negative enterics. The total Gram-negative enteric population peaked at 10⁹ CFU/g in the feces of 2 weeks old birds, and remained high (~10⁸ CFU/g) up to 6 weeks of age (see Figure 2) however for the most part the resistant Gram-negatives did not exceed the abundance of susceptible population. There were also no significant differences in abundance of resistant Gram-negative enteric in the feces of birds colonized with the litter resistome. Nor did administering litter resistome as a gene donor affect resistance in Salmonella.
TABLE 8 | Mortality in chickens orally inoculated with nalidixic acid, rifampicin-resistant Salmonella Typhimurium strains.

| Oral administration       | % Mortality |
|---------------------------|-------------|
| None                      | 2.0% (176)  |
| Chicken resistome only    | 2.7% (221)  |
| S. Typhimurium 934        | 46.2% (132) |
| S. Typhimurium 934 + chicken resistome | 41.7% (132) |
| S. Typhimurium 3,147      | 45.4% (130) |
| S. Typhimurium 3,147 + chicken resistome | 31.8% (129) |

*Cecal microbiome collected from chickens administered poultry litter resistome at day of hatch.*

**Chi-squared test:** $p = 0.035$. There were no statistically significant differences in mortality associated with S. Typhimurium isolate (934 vs. 3,147; $p = 0.83$) or administration of litter resistome (Control vs. Litter resistome alone; $p = 0.65$).

However, antimicrobial administration had a significant impact on resistance to certain antibiotics (Table 10). Streptomycin administration increased aminoglycoside and tetracycline resistance in Gram-negative enterics cultured from the poultry environment. Chlortetracycline usage also increased tetracycline resistance ($p = 1.9 \times 10^{-3}$) but had no effect on resistance to the other antimicrobials. There was no increase in resistance to chloramphenicol or ampicillin in birds administered antibiotics, nor was there a significant change in Salmonella abundance. The prevalence of resistance in Salmonella was impacted by the source (bird or environment) and the age of the birds (Tables 9, 11). A significantly greater proportion of Salmonella isolated from the poultry environment were resistant to ampicillin, streptomycin, and tetracycline, compared to Salmonella isolated from birds (80.6% vs. 52.6%; $p = 2.2 \times 10^{-3}$). In addition, ampicillin and tetracycline resistance was more likely in isolates cultured from the environment of 4–6 weeks old birds compared to 1–3 weeks old. Resistance to two or more antibiotics was also significantly more likely in isolates from the environment of older birds (97.1% vs. 51.4%; $p = 1.5 \times 10^{-5}$).

Antimicrobial resistance phenotype and genotype was characterized in Salmonella isolated from the poultry environment in order to verify gene transfer to the Typhimurium strain. Isolates were screened for class 1 integrons and the integron-associated resistance genes sul1, adaA1, and adaB; bllTEM1; and tetA. Isolate 934NR, naturally resistant to streptomycin and sulfoxazole, acquired resistances to ampicillin, amoxicillin, kanamycin, or tetracycline (Table 11). However, half of the 934NR isolated in this experiment, were sensitive to streptomycin. Seven resistance phenotypes were identified with sulfoxazole, ampicillin, and amoxicillin...
resistance as the most common. Eighty-seven percent of isolates were resistant to three or more antimicrobials. All β-lactam resistant 934NR isolates were positive for the blaTEM β-lactamase gene allele. The only other antimicrobial resistance gene detected was aadA1 in one isolate that was resistant to streptomycin and sulfisoxazole, however none of the 934NR isolates were positive for class 1 integrons. Of the three tetracycline-resistant isolates examined, none were positive for tetA.

Similarly, isolate 3147NRintI1, naturally sulfisoxazole resistant, acquired resistances including ampicillin, amoxicillin, gentamicin, kanamycin, streptomycin and tetracycline. However, one third of 3147NRintI1 isolates were sensitive to sulfisoxazole. Eight antimicrobial resistance phenotypes were identified in 3147NRintI1, with resistances to streptomycin and sulfisoxazole the most common. Twenty-percent of these isolates were resistant to three or more antibiotics. However, three 3147NRintI1 isolates were cultured that were resistant to six or more antimicrobials. All β-lactam resistant 3147NRintI1 isolates were positive for blatem. Four of five gentamycin-resistant isolates were positive for the aadB; and all tetracycline resistant 3147NRintI1 isolates were positive for tetA. The most common antimicrobial resistance genotype identified in 3147NRintI1 isolates was aadA1, aadB, blatem, sul1, tetA. The other antimicrobial resistance genotypes identified included blatem; and aadA1, blatem, sul1, tetA. All 3147NRintI1 isolates harboring aadA1, aadB, blatem, sul1, tetA or aadA1, blatem, sul1, tetA were positive for the class 1 integron. While the original Salmonella 3147NRintI1 isolate administered to the birds contained the class 1 integron, only 28% of antimicrobial resistant 3147 isolates cultured from the birds contained an integron. And Tn21, a transposon responsible for dissemination of class 1 integrons in avian E. coli (Bass et al., 1999), was not detected in any antimicrobial-resistant Salmonella isolates indicating that the integron was encoded on a different element.

**DISCUSSION**

While acquisition of antimicrobial resistance in Salmonella occurs in nature, how quickly does this occur in vivo and what is the impact of antibiotic usage on the acquisition of resistance? We performed two trials in birds using multiple genotypes of Salmonella and two sources of resistant genes: a defined MDR plasmid and an environmental resistome. This study documented an abundant resistant Enterobacteriaceae population in the birds and the poultry litter in the absence of treatments. Others have reported similar findings; abundant antimicrobial resistance in the absence of antimicrobial usage (Fairchild et al., 2005; Smith et al., 2007; Agga et al., 2016; Liljebljekke et al., 2017). Emergence of antimicrobial-resistant Salmonella in chickens occurred readily despite its existence as a minor population in bird and litter bacterial community outnumbered by gram-positives 100-1,000 to 1 (Lu et al., 2003a,b). Salmonella colonizing chickens easily acquire
Antimicrobial resistance from the resident bacteria present in chickens, even in the absence of antimicrobial use. For Salmonella to acquire resistance genes there needs to be a sufficient antibiotic resistance reservoir. Poultry litter has a rich resistome with a high abundance of class 1 integrons at 1 copy per 1-100 bacterial genomes (Nandi et al., 2004). The most abundant integron associated antimicrobial resistance genes in poultry Salmonella tend to be the same as those present in litter (Nandi et al., 2004; Liljebjelke et al., 2017). Emergence of extended-spectrum β-lactam/cephalosporin resistance in Salmonella has been attributed to acquisition of conjugative plasmids (Winkurk et al., 2000). These plasmids often contain other resistances including chloramphenicol, florfenicol, streptomycin, and tetracycline (Winkurk et al., 2000; Doublet et al., 2004; Fernández-Alarcón et al., 2011). In nature, Salmonella acquires MDR plasmids from commensals that inhabit the same environment (Winkurk et al., 2001; Fricke et al., 2009; McCollister et al., 2016) and its acquisition of antimicrobial resistance is dependent on an abundant, antimicrobial-resistant donor population (Smith, 1975; Schjørring et al., 2008; Faure et al., 2009; Card et al., 2017). How quickly Salmonella acquires plasmids and associated antimicrobial resistances are dependent on the presence and abundance of the plasmid in the intestinal microbiome, rate of plasmid transmission, plasmid stability and fitness cost (Stewart and Levin, 1977; Ponciano et al., 2007). Antimicrobial usage may also play an important role in the acquisition and maintenance of plasmid-borne resistance in Salmonella by ameliorating the fitness cost.

With the exception of ceftiofur, antibiotic administration did not significantly increase antimicrobial resistance in Salmonella. ESBL-resistance in Salmonella was not due to horizontal transmission of the Newport MDR-plasmid. Subbiah et al. reported an ESBL incA/C plasmid imposed a fitness cost in vitro and in vivo (Subbiah et al., 2011) therefore it is possible that ESBL-resistant Salmonella acquired the resistance gene on a transposon. Given that the ESBL-resistance gene bla<sub>CMY</sub> is adjacent to a transposon in the S. Newport MDR plasmid (Giles et al., 2004) and a recent report of bla<sub>C</sub>MX in a transposon (Huang et al., 2017), one possible explanation for plasmid-less ESBL resistance is bla<sub>C</sub>MX transposition on and off a conjugative plasmid that ferried this element between cells. Whole genome sequencing will determine the nature of ESBL resistance in these Salmonella isolates; where the resistance gene resides; and the genetic element that bears it.

In other animal models, the intestinal microbiome can be a formidable barrier to the transfer of antimicrobial resistance (Schjørring et al., 2008; Faure et al., 2009; Stecher et al., 2012). However, this study showed that antimicrobial-resistant Salmonella occurred in the absence of administration of a resistant microbiome. This finding shows that ample reservoirs exist among the normal microbiome of baby chicks, even in a sanitized environment.

**CONCLUSIONS**

Salmonella can easily acquire antimicrobial resistance from the chicken resistome. With the exception of ceftiofur use, antimicrobial resistance in Salmonella occurred even in the absence of antibiotic administration. The animal resistome is one important contributor to antimicrobial resistance in Salmonella. A detailed analysis of the animal

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**TABLE 10 | Antimicrobial resistance in Gram-negative bacteria isolated from chickens administered antimicrobials and chicken resistome.**

| Resistance<sup>a</sup> | Administration | No antibiotics vs. chlorotetracycline<sup>b</sup> | No antibiotics vs. streptomycin<sup>b</sup> |
|---------------------|----------------|---------------------------------------------|---------------------------------------------|
|                     | No antibiotics | Chlorotetracycline | Streptomycin | p               | p               |
| Streptomycin        | Average: 21.1%  | 34.3%            | 46.2%        | 0.14            | 2.4 x 10<sup>-3</sup> |
|                     | Range: 0.0–97.7%| 0.0–96.6%       | 4.0–99.3%    | 0.30            | 2.8 x 10<sup>-2</sup> |
| Gentamicin          | Average: 2.7%  | 3.3%             | 7.4%         | 0.07            | 1.7 x 10<sup>-2</sup> |
|                     | Range: 0.1–10.6%| 0.0–15.0%       | 0.5–36.5%    | 0.07            | 1.7 x 10<sup>-2</sup> |
| Kanamycin           | Average: 12.3% | 23.4%            | 32.0%        | 1.9 x 10<sup>-3</sup> | 3.2 x 10<sup>-2</sup> |
|                     | Range: 1.9–64.8%| 0.8–100.0%     | 1.6–100%     | 0.08            | 0.39            |
| Tetracycline        | Average: 6.1%  | 24.4%            | 16.7%        | 0.28            | 0.35            |
|                     | Range: 1.2–16.0%| 4.8–100.0%     | 1.7–97.4%    | 0.28            | 0.35            |
| Chloramphenicol     | Average: 6.6%  | 8.8%             | 7.9%         | 0.08            | 0.39            |
|                     | Range: 0.0–28.5%| 0.0–61.6%      | 0.0–45.4%    | 0.08            | 0.39            |
| Ampicillin          | Average: 31.8% | 40.9%            | 33.7%        | 0.30            | 0.21            |
|                     | Range: 7.4–62.1%| 10.6–97.7%     | 3.6–84.9%    | 0.30            | 0.21            |
| NAL/RIF<sup>c</sup> | Average: 2.1%  | 1.3%             | 6.4%         | 0.07            | 1.7 x 10<sup>-2</sup> |
|                     | Range: 0.0–10.0%| 0.0–6.5%       | 0.0–41.2%    | 0.07            | 1.7 x 10<sup>-2</sup> |

<sup>a</sup>Antimicrobial resistance was calculated from paired enumeration of resistant population (MacConkey agar + antibiotic) divided by the total Gram-negatives (MacConkey agar with no antibiotics) for a given sample set, and averaged for weeks 3–5. Chickens were administered chlorotetracycline or streptomycin for 1 week, at 2 weeks of age. For the no antibiotic-treatment control group, birds were orally given PBS as placebo.

<sup>b</sup>Unpaired Student T-test.

<sup>c</sup>Nalidixic acid (NAL) and rifampicin (RIF).
### TABLE 11 | Antimicrobial resistance genotype and phenotypes of *S*. Typhimurium recovered from poultry environment of chickens experimentally colonized with *Salmonella*.

| Isolate<sup>a-f</sup> | Time of isolation (week) | Antimicrobial resistance profile<sup>g</sup> | integr<sup>h</sup> | Antimicrobial resistance genes<sup>i</sup> |
|----------------------|-------------------------|------------------------------------------|----------------|---------------------------------|
| 934                  |                         | STR SMX                                  |                |                                 |
| 8756<sup>a,c,d</sup> | 2                      | STR SMX TET                              |                |                                 |
| 8757<sup>a,c,d</sup> | 2                      | STR SMX                                  |                |                                 |
| 8758<sup>a,d</sup>   | 2                      | STR SMX TET KAN                          |                |                                 |
| 8759<sup>a,f</sup>   | 2                      | STR SMX AMP AMX                          |                |                                 |
| 8763<sup>a</sup>     | 2                      | STR SMX TET                              |                |                                 |
| 8765<sup>a,d</sup>   | 3                      | STR SMX AMP                              |                |                                 |
| 8766<sup>a,d</sup>   | 3                      | STR SMX AMP AMX                          |                |                                 |
| 8767<sup>a,d</sup>   | 3                      | SMX AMP AMX                              |                |                                 |
| 8768<sup>a</sup>     | 3                      | STR SMX AMP                              |                |                                 |
| 8769<sup>a,d</sup>   | 5                      | STR SMX AMP                              |                |                                 |
| 3147                 | 5                      | STR SMX AMP                              |                |                                 |
| 8755<sup>b,e</sup>   | 1                      | STR SMX                                  |                |                                 |
| 8760<sup>a,d</sup>   | 2                      | STR SMX TET KAN GEN AMP                  |                |                                 |
| 8761<sup>b</sup>     | 2                      | STR SMX                                  |                |                                 |
| 8762<sup>b</sup>     | 2                      | STR                                      |                |                                 |
| 8763<sup>e</sup>     | 2                      | STR SMX                                  |                |                                 |
| 8764<sup>e</sup>     | 2                      | STR SMX                                  |                |                                 |
| 8766<sup>f</sup>     | 2                      | STR                                      |                |                                 |
| 8776<sup>f</sup>     | 2                      | STR                                      |                |                                 |
| 8777<sup>f</sup>     | 2                      | STR                                      |                |                                 |
| 8778<sup>c,f</sup>   | 2                      | STR SMX                                  |                |                                 |
| 8779<sup>c</sup>     | 2                      | STR SMX                                  |                |                                 |
| 8780<sup>c,f</sup>   | 2                      | STR SMX KAN GEN                          |                |                                 |
| 8781<sup>b,d</sup>   | 2                      | STR SMX                                  |                |                                 |
| 8782<sup>b,c</sup>   | 2                      | STR SMX                                  |                |                                 |
| 8786<sup>f</sup>     | 3                      | STR SMX                                  |                |                                 |
| 8788<sup>f</sup>     | 4                      | STR SMX                                  |                |                                 |
| 8789<sup>f</sup>     | 4                      | STR SMX                                  |                |                                 |
| 8790<sup>e</sup>     | 4                      | STR SMX                                  |                |                                 |
| 8791<sup>b</sup>     | 4                      | STR                                      |                |                                 |
| 8797<sup>e</sup>     | 4                      | STR                                      |                |                                 |
| 8770<sup>b</sup>     | 5                      | STR SMX TET KAN GEN AMP                  |                |                                 |
| 8771<sup>b</sup>     | 5                      | STR SMX TET KAN GEN AMP AMX              |                |                                 |
| 8772<sup>b</sup>     | 5                      | STR SMX TET KAN GEN AMP AMX              |                |                                 |
| 8773<sup>c,f</sup>   | 5                      | STR AMP                                  |                |                                 |
| 8774<sup>b,e</sup>   | 5                      | STR AMP                                  |                |                                 |
| 8775<sup>f</sup>     | 5                      | STR AMP                                  |                |                                 |

*Salmonella* recovered from poultry environment of chickens infected with nalidixic acid/ rifampicin-resistant *S*. Typhimurium strains 934<sup>a</sup> or 3147<sup>b</sup> alone or chickens also seeded with a chicken resistome<sup>c</sup>. Isolates produced PFGE pattern identical to *Salmonella* strains administered to birds. Birds were administered, at 2 weeks of age, chlortetracycline or streptomycin.<sup>d</sup>

<sup>a</sup>No antibiotic administered, experimental group.

<sup>b</sup>No antibiotic administered, control group.

<sup>c</sup>Antimicrobial resistance abbreviations: AMP, ampicillin; AMX, amoxicillin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; SMX, sulfisoxazole; TET, tetracycline.

<sup>d</sup>Or for 1 week.

<sup>e</sup>Antimicrobial resistance genes detected in a PCR screen for aadA1, aadB, bl</sup>TEM<sup>, sul1</sup>, tetA.
resistome will provide insights into important players in the dissemination of antimicrobial resistance within these production systems.

ETHICS STATEMENT

The University of Georgia’s, Institutional Animal Care and Use Committee approved all animal care protocols described in this work.

AUTHOR CONTRIBUTIONS

JM, CH, and ML contributed to the conception and design of this study. JM, TB, AH-B, VS, and KL were responsible for the acquisition of data analyzed in this study. JM was involved in the analysis and interpretation associated with this work. SS directed antimicrobial susceptibility testing and interpretation of susceptibility results. All authors were involved in manuscript writing, revisions, and final approval of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2019.00020/full#supplementary-material

Supplemental Figure 1 | Detection of high molecular weight plasmids in antimicrobial-resistant Salmonella cultured from the environment of chickens administered S. Typhimurium 934R. (A) Uncut plasmid DNA. Lane 1: BAC-Tracker Supercoiled DNA ladder; Lane 2: RP4 plasmid control; lane 3: S. Typhimurium 934R; lanes 4–12: antimicrobial-resistant isolates: 1543Rc (lane 4), 1546Ra (lane 5), 1546Rb (lane 6), 1546Rc (lane 7), 1549Rb (lane 8), 1549Rac (lane 9), 1552Raa (lane 10), 1552Rab (lane 11), and 1552Rbc (lane 12). (B) Hind III restriction digestion of plasmid DNA. Lane 1: λ DNA ladder; lane 2: λ DNA; lane 3: Hind III-digested λ DNA; lane 4: S. Typhimurium LT2 (virulence plasmid control); lane 5: S. Typhimurium 934R; lanes 6–11: antimicrobial-resistant isolates: 1543Rc (lane 6), 1546Rfa (lane 7), 1546Rbc (lane 8), 1546Rc (lane 9), 1549Rb (lane 10), and 1549Rab (lane 11). DNA was separated on 0.5% agarose gel at 5.15V/cm for 18 h at room temperature and stained with Sybr-Green.

Supplemental Figure 2 | Pulsed-field gel electrophoresis (PFGE XbaI) confirms antibiotic-resistant Salmonella recovered from the poultry environment as the S. Typhimurium strain administered to the birds as isolates 934R and 3147. Lanes 1 and 16: MW DNA standard Saccharomyces cerevisiae chromosomes; Lane 2: laboratory strain S. Typhimurium SR11; Lane 3: S. Typhimurium 934R; lane 4: S. Typhimurium 3147R; lanes 5–15: antibiotic resistant Salmonella isolated from poultry environment of chickens administered S. Typhimurium strains 934R or 3147R.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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