Colonization of Broiler Chicks by *Salmonella* Typhimurium

Definitive Phage Type 104

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ABSTRACT

The prevalence of an antibiotic-resistant strain of *Salmonella* Typhimurium definitive phage type 104 (DT104) has increased dramatically in recent years resulting in increased morbidity and mortality in both animals and humans. Colonization and shedding of *Salmonella* Typhimurium DT104 was studied in broiler chickens in two trials. In trial 1, 180 day-of-hatch chicks (n = 60 per group, n = 30 per replicate) were challenged with 10⁶ CFU DT104 (wild-type isolate from poultry) or were commingled with a seeder chick challenged with 10⁶ CFU DT104. In trial 2, 360 day-of-hatch chicks (n = 120 per treatment, n = 30 per rep) were divided into three groups. Chicks in the susceptible group were commingled with two seeder chicks that were orally challenged with 10⁷ CFU/bird of a pan-sensitive strain of *Salmonella* Typhimurium DT104. Chicks in the resistant group were commingled with two seeder chicks that were orally challenged with 10⁷ CFU/bird DT104 used in trial 1. For both trials, a control group was not exposed to DT104, composite fecal samples were evaluated twice weekly for levels of *Salmonella* shedding and 20 chicks per group were necropsied weekly and their cecal contents were cultured. At hatch all groups were colonized with naturally occurring *Salmonella* Senftenberg and *Salmonella* Mbandaka (trial 1) or *Salmonella* Senftenberg and *Salmonella* Ohio (trial 2) prior to exposure to DT104. Throughout the study, the level of *Salmonella* spp. shedding in feces (trial 1 means 3.1, 2.9, and 3.0 log_{10} CFU per g feces for challenged, seeder, and control groups, respectively) or ceca (trial 2 means 2.9, 2.9, and 2.5 log_{10} CFU per g ceca for resistant, susceptible, and control groups, respectively) did not differ among groups. In trial 1, colonization of DT104 remained constant at higher levels in the challenged group (mean 87%, P < 0.01), increased over time in the seeder group (10 to 50%, P < 0.02) and was not recovered from the control chicks. *Salmonella* Mbandaka colonization remained steady within each group with challenge and control groups maintaining higher levels of colonization than the control group. *Salmonella* Senftenberg colonization levels tended to decline (P = .058) over time in the challenged group (20 to 0%) and significantly decreased (P < 0.01) over time for both the seeder (80 to 0%) and control chicks (85 to 10%). In trial 2, the percentage of chicks colonized with susceptible DT104 declined (r = 0.90, P < 0.05) over the course of the trial from 45 to 0%, while recovery of the resistant DT104 persisted at a mean percentage of 27%. DT104 was not recovered from the control chicks. *Salmonella* Ohio colonization levels tended to decline (r = 0.79, P > 0.05) over time in the control group (75 to 20%) and significantly decreased (P < 0.05) over time in both susceptible and resistant groups (40 to 10%, r = 0.82 and 55 to 5%, r = 0.85, respectively). *Salmonella* Senftenberg was recovered from the control group at low frequency throughout the trial and was not recovered from the other groups. For either trial, no apparent affect on morbidity or mortality was observed. Introduction of DT104 by commingling may induce colonization resulting in persistent high levels of shedding in flocks simultaneously with other *Salmonella* species.

*Salmonella* Typhimurium definitive phage type (DT) 104 was first identified in humans in England and Wales in 1984 (26). This strain has a unique antimicrobial resistance pattern (R-type) with multiple resistance observed for ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), and tetracycline (T) (ACSSuT). The number of *Salmonella* Typhimurium DT104 isolations in the United Kingdom from humans rose slowly from 1984 to 1990, then more rapidly so that by 1993, DT104 with R-type ACSSuT accounted for over 80% of the isolations (30). In 1995, R-type ACSSuT DT104 accounted for over 87% of *Salmonella* Typhimurium isolates recovered from humans with 26.4 and 6.2% of these isolates having additional resistance to trimethoprim and ciprofloxacin, respectively (30, 31). Antimicrobial drug resistance in *Salmonella* species is most often plasmid mediated. This is advantageous as removal of the selective pressure typically results in a reversion to susceptibility. Although *Salmonella* Typhimurium DT104 harbors a 60-Mda plasmid, the resistance genes for R-type ACSSuT DT104 are chromosomally integrated (26). This is worrisome, as removal of the selective pressure is expected to have no effect on resistance. Additionally, DT104 appears to have the ability to acquire additional resistance as described above, rendering treatment with other drugs ineffective (11). Of importance in this observation is the fact that resistance to the newest class of antimicrobials, fluoroquinolones, has been compromised. Resistance to ciprofloxacin increased from 1% in 1994 to 6% resistance in 1995 (27). Resistance to ciprofloxacin is also chromosomally encoded.

In the animal population in the United Kingdom,
DT104 was first recovered in 1989 from cattle. Only one herd was identified. Since that time isolations have continued to rise and now account for the majority of Salmonella Typhimurium isolates. Recovery has also been documented from sheep, pigs, poultry, goats, rabbits, dogs, seabirds, rodents, porpoises, cats, horses, and animal feed (29, 30). Contact with ill farm animals, particularly cattle, is implicated as a primary factor for transmission (29, 30). Long-term carriage has also been observed in all species, particularly in cats and cattle (19, 28).

Interestingly, invasiveness of DT104 in humans does not appear to differ from other salmonellae, however, an increase in severe illness is noted with 36% of 105 patients in one case control study requiring hospitalization (5, 6). A rise in the human DT104 isolations in the Pacific Northwest was also observed with only 2 of 46 isolates identified from 1989 and 80 of 188 (42.5%) identified in 1994 (3). Additionally, it has been suggested that person-to-person transmission may also play a significant role in transmission.

Prior to 1986, DT104 was not recognized in the United States. In the Pacific Northwest, from 1986 to 1991, 13% of Salmonella Typhimurium isolates of bovine origin were R-type ACSSuT compared to 64% for the period 1992 to 1995 (5, 6). A rise in the human DT104 isolations in the Pacific Northwest was also observed with only 2 of 46 isolates identified from 1989 and 80 of 188 (42.5%) identified in 1994 (5, 6). Interestingly, no tendency for an increase in the annual number of reported animal or human Salmonella Typhimurium cases has been evident in the Pacific Northwest (7).

Broiler chickens can harbor Salmonella spp. from hatch through grow-out although numbers of birds shedding Salmonella appear to decline over time (8, 17, 18). Additionally, salmonellae in chickens appear to have a commensal relationship as the bird's health is not impaired and birds do little to exclude the organism once Salmonella is established (3). In this study, we conducted two trials. In trial 1, we evaluated the colonization of DT104 in addition to assessing its virulence (as defined by morbidity and mortality) in broiler chickens from hatch through 3 weeks of age using two different challenge routes. In trial 2, we compared the colonization patterns and virulence of pan-susceptible and penta-resistant strains of DT104 following commingling of chicks that had been challenged by oral gavage.

**MATERIALS AND METHODS**

**Bacterial strains and challenge.** Two strains of Salmonella Typhimurium DT104 were used in these experiments. The resistant strain was obtained from a water cup sample from a broiler house that was experiencing morbidity and mortality in the chicks and was subsequently identified as Salmonella Typhimurium DT104 at the National Veterinary Services Laboratories, Ames, Iowa. This strain was resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline and is considered the wild-type strain. A sensitive DT104 isolate was obtained from Denmark (kindly provided by D. Emborg) and was pansensitive to the above antimicrobials.

Both strains were grown stationary overnight in Luria Bertani broth (Difco, Detroit, Mich.) at 37°C. The following morning a 1% inoculum was transferred into fresh Luria Bertani broth and agitated at 200 rpm and 37°C for approximately 3 h. The culture was centrifuged and the pellet was resuspended in 0.5 volume of phosphate-buffered saline and adjusted to a final concentration of 10^5 CFU/ml (optical density at 600 nm = 0.850) with phosphate-buffered saline. The 10^5 and 10^6 CFU/ml challenge cultures were obtained by diluting the 10^6 CFU/ml culture 1:10 or 1:100, respectively, in phosphate-buffered saline. Approximately 0.1 ml of the challenge culture was administered by oral gavage to the chicks.

**Experimental design: trial 1.** One hundred eighty day-old hatch chicks were allotted to one of three groups and each group was replicated. Chicks were placed in isolation units and were provided unmedicated feed and water ad libitum. The challenge group was comprised of 30 chicks, each of which was challenged by oral gavage with 10^6 CFU/ml wild-type DT104. The seeder group chicks (n = 30) were commingled with one chick that had been challenged by oral gavage with 10^6 CFU/ml wild-type DT104. The control group was not exposed to DT104.

Composite fecal samples per group were collected from the pen floor and cultured (quantitative) twice weekly for 3 weeks to determine levels of Salmonella being shed. Twenty chicks per group (10 per replicate) were necropsied weekly at 1, 2, and 3 weeks postchallenge, and their cecal contents were individually cultured (qualitative) as described below. For the seeder group, the seeder chick was permanently marked with color to prevent inclusion in necropsy.

**Trial 2.** Three hundred sixty day-old hatch chicks were allotted to one of three groups and each group was replicated four times. Facilities and conditions were the same as the previous trial. The resistant group chicks (n = 30) were commingled with two chicks that had been challenged by oral gavage with 10^5 CFU/ml wild-type DT104 used in trial 1. The susceptible group chicks (n = 30) were commingled with two chicks that had been challenged by oral gavage with 10^5 CFU/ml pan-sensitive DT104. The control group was not exposed to DT104.

Twenty chicks per group (five per replicate) were necropsied weekly for 6 weeks postchallenge, and their cecal contents were both pooled per replicate and cultured (quantitative) and cultured individually (qualitative) as described below. The seeder chicks were permanently marked to prevent inclusion in necropsy.

Paper pads used to line the transport boxes from all chicks were cultured (qualitative) for both trials as described below to determine the Salmonella serotypes that the birds were exposed to and/or shedding upon arrival from the hatchery.

**Bacterial culture.** Qualitative bacteriology was conducted with modification as previously described (9). Briefly, all samples were pre-enriched in buffered peptone water (Difco) overnight at 37°C. One-tenth (0.1) ml was subsequently transferred into 10 ml of tetrathionate broth (Difco) for enrichment at 37°C for 48 h. Then an aliquot was transferred to Rappaport R1-10 medium (Difco). After incubation at 37°C overnight, samples were streaked onto xylose-lysine-tergitol 4 agar (Difco) plates and incubated for 24 h at 37°C. Quantitative bacteriology was conducted as previously described (33). Briefly, a five-tube, most probable number
calculation was performed, utilizing tetraionate broth enrichment and a secondary enrichment in Rappaport R-10 medium. Incubation conditions and plating were similar to qualitative methods. For both qualitative and quantitative bacteriology, a minimum of five colonies per plate were serogrouped by agglutination using *Salmonella* O antiserum (Difco). Presumptive positive colonies were inoculated into triple sugar iron agar (Difco) and lysine iron agar (Difco) for biochemical confirmation. A representative number of isolates with different serogroups were sent to National Veterinary Services Laboratories for serotyping.

**Statistical analysis.** The relationship between colonization and sampling time was evaluated using Pearson correlation. Differences in colonization were determined using the chi-square test for independence, and differences in shedding levels were determined using the analysis of variance and Tukey’s honest significant difference test.

**RESULTS**

**Trial 1.** *Salmonella* Mbandaka and *Salmonella* Senftenberg were cultured from the transport paper pads, indicating exposure from the hatchery prior to challenge with *Salmonella* Typhimurium DT104. Levels of total *Salmonella* spp. shed in feces did not differ between groups. *Salmonella* levels per group were 3.1, 2.9, and 2.4 mean CFU log₁₀ per g of feces over the course of the trial for the challenge, seeder, and control groups, respectively. Group CFU/g by day are shown in Table 1. The percentage of chicks that were colonized following oral challenge remained at approximately 87% throughout the study (Fig. 1), and colonization was significantly higher (*P* = 0.01) when compared to the other groups. The percentage of chicks that became colonized with DT104 over time following commingling with the seeder bird increased (*P* < 0.02) from 10 to 50% over the 3-week period (Fig. 1). DT104 was never recovered from the control chicks. *Salmonella* Mbandaka colonization remained relatively stable from weeks 1 to 3 in the seeder and control groups (mean percentages equal to 35 and 33%, respectively) (Fig. 2A). Fewer birds in the challenge group (mean percentage 3%) were colonized with *Salmonella* Mbandaka compared to the other groups. *Salmonella* Senftenberg colonization decreased (*P* < 0.01) over time in both seeder (80 to 0%) and control chicks (85 to 10%). Colonization levels tended to decline (*P* = 0.58) over time for the challenge group (20 to 0%). As was observed for *Salmonella* Mbandaka, fewer birds in the challenge group (20%) as compared to the other groups (80 and 85% for the seeder and control groups, respectively) were colonized by week 1 (Fig. 2B). No *Salmonella* Senftenberg was recovered from the challenge and seeder group at 3 weeks.

**Trial 2.** *Salmonella* Ohio and *Salmonella* Senftenberg were cultured from the transport paper pads, indicating exposure from the hatchery, prior to exposure to either resis-

**FIGURE 1.** Percentage of birds colonized by *Salmonella* Typhimurium DT104. These data represent the percentage of birds colonized with DT104 at necropsy on weeks 1 through 3 following exposure to DT104 via oral challenge (10⁶ CFU/bird) (■) or exposure by commingling with a seeder chick that received 10⁶ CFU DT104 (●). The control group was not exposed to DT104 and no colonization is observed (▲). Number of birds necropsied per week per group equals 20 (n = 10 per replicate; two replicates/group).
Colonization of broiler chicks by DT104

**Figure 2.** (A) These data represent the percentage of birds colonized at hatch with Salmonella Mbandaka on weeks 1 through 3 following exposure to DT104 via oral challenge (10^6 CFU/bird) (■) or exposure by commingling with a seeder chick that received 10^6 CFU DT104 (▲). Colonization for the control group is represented by ●. Number of birds necropsied per week per group equals 20 (√10 per replicate; two replicates/group). (B) These data represent the percentage of birds colonized at hatch with Salmonella Senftenberg on weeks 1 through 3 following exposure to DT104 via oral challenge (10^6 CFU/bird) (▲) or exposure by commingling with a seeder chick that received 10^6 CFU DT104 (■). Colonization for the control group is represented by ●. Number of birds necropsied per week per group equals 20 (√10 per replicate; two replicates/group).

The mean percentage of chicks that were colonized with the resistant strain of DT104 was 27% throughout the 6-week period. At week 1, 45% of birds were colonized that decreased to 5% by week 4 followed by an increase to 30% by week 6 (Fig. 3). For birds exposed to the susceptible strain, 45% were colonized at week 1, which decreased (r = 0.90, P < 0.05) to 0% by week 6. No DT104 was recovered from the control group.

*Salmonella* Ohio colonization (Fig. 4A) tended to decline (r = 0.79, P > 0.05) over time in the control group.

**Table 2.** Salmonella levels in broiler chicks ceca, trial 2

<table>
<thead>
<tr>
<th>Weeks postchallenge</th>
<th>Mean log_{10} CFU/g (a)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>1.73 ± 0.85</td>
</tr>
<tr>
<td>Susceptible</td>
<td>3.70 ± 0.43</td>
</tr>
<tr>
<td>Resistant</td>
<td>3.15 ± 0.67</td>
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(a) Ceca were pooled by repetition, means of four repetitions ± standard error.
FIGURE 3. Percentage of birds colonized by Salmonella Typhimurium DT104. These data represent the percentage of birds colonized with DT104 at necropsy on weeks 1 through 6 following challenge with 10⁷ CFU/bird with a resistant strain of DT104 (▲) or a susceptible strain of DT104 (■). The control group did not receive DT104 and no colonization is observed (◆). Number of birds necropsied per week per group equals 20 (n = 5 per replicate; four replicates/group).

(75 to 20%) and significantly decreased (P < 0.05) over time in both susceptible and resistant groups (40 to 10%, r = 0.82 and 55% to 5%, r = 0.85, respectively). All groups were still colonized at week 6 postchallenge.

Salmonella Senftenberg was observed at a low frequency (20% at week 1 and 5% at week 5) in the control group (Fig. 4B) and was never recovered from birds in either the resistant or susceptible groups.

DISCUSSION

From an animal disease standpoint, there is a paucity of information regarding the pathogenesis of disease induced by DT104 in all animal species. These data indicate that following exposure of day-of-hatch chicks to Salmonella Typhimurium DT104, either by direct (oral) or indirect (commingling with an infected chick) challenge, chicks will remain persistently colonized with DT104 throughout the grow-out period, particularly with a multiresistant strain of DT104 (Fig. 3). Intensive rearing practices have been implicated in the emergence and dissemination of DT104 (34). Of particular concern with this strain is the tendency for a possible competitive advantage to occur over other strains as observed in this paper and elsewhere (7). Although dissemination has been implicated through foods and direct contact with ill animals (1), no transmission to humans via poultry has been reported. Recovery of DT104 from chickens has been reported (24). However, because no increase in virulence has ever been observed in poultry, this strain may persist undetected within a poultry environment and may persist through processing and be transmitted to humans. The absence of overt virulence (morbidity and mortality) in resistant strains does not necessarily indicate a complete absence of virulence, as the increased tendency for persistence could also be considered a virulence attribute as resistant strains outcompete other strains and predominate within a population. These strains may then be transmitted to more susceptible hosts, such as humans, a phenomenon that has been observed (5–7).

Interestingly, the percentage of chicks colonized with resistant DT104 between trials within the challenge group in trial 1 and the resistant group in trial 2 varied at week 1 by almost 40%. Challenge dose could not be implicated in this difference as the dose in trial 1 was 1 log less than trial 2. Challenge dose may also account for an observed lack of virulence as disease may become more evident with the use of a higher challenge dose. However, comparison of routes of challenge may also account for this difference in colonization (13, 14). Direct oral challenge may result in higher numbers of Salmonella being introduced into each bird (trial 1). Therefore, colonization levels may increase as numbers of Salmonella within the host increases. Although we only sampled birds through to week 3 in trial 1, it is likely that persistence through slaughter in the challenge group would have occurred as 85% of the birds were colonized at week 3. Further, we would speculate that the percentage of birds that would have remained colonized would be higher than observed for chicks exposed to seeder birds.

Comparison of seeder groups between trials 1 and 2 provides an interesting contrast. Use of only one bird in trial 1 in the seeder group compared to two birds used in trial 2 may account for the difference in the percentage of birds colonized at week 1 (10% in trial 1 versus 45% in trial 2). However, by week 3 in trial 1, colonization levels were the same between trials, suggesting that exposure dose over time does not affect colonization within the flock. Bailey et al. (4) reported that only one contaminated egg in a hatching cabinet will lead to substantial spread. This leads to production of seeder birds at hatch that then lead to colonization of other birds through grow-out. Although aerosol transmission of Salmonella has been reported in other animal species (10, 15), it is difficult to determine the exact mode of transmission in the poultry environment. Contamination by litter, insects, and other means is also likely (17).

Although DT104 may expand within the environment, these data indicate that not all serotypes may be crowded out. It may be more likely that DT104 may outcompete other serotypes in animals that have longer production
schedules such as cattle (6) than those with shorter production schedules such as chickens. Additionally, there may be differences between serogroups that may account for persistence/colonization within a host. *Salmonella* Mbandaka and *Salmonella* Ohio belong to serogroup C1. *Salmonella* Choleraesuis is defined as the host-adapted serotype in swine (32), and an increase of C1 *Salmonella* serotypes has been observed in cattle (9). These data provide additional evidence for an increased likelihood of colonization with C1 salmonellae that may persist through to slaughter in the poultry environment. In contrast, it appears to be less likely that *Salmonella* Senftenberg (serogroup E4) will persist in many birds within the production environment.

Because all chicks in both trials were colonized at hatch, percent colonization with these serotypes may be underestimated. It is possible that as colonization with DT104 spreads throughout the flock and crowding of other serotypes occurs, low levels of colonization may, and do, occur as demonstrated in this study. However, because only five colonies were picked for analysis, it is likely that we would have missed selection of different serotypes. This may be circumvented with the development of multiplex polymerase chain reaction or other tests that would have been able to determine if multiple serotypes occurred within a single sample. Additionally, CFU/g over time indicated little difference between groups regardless of whether feces or ceca were cultured. However, it is noted that variability occurred throughout both trial periods. This may be attributed to a calculation of total salmonellae, as individual serotypes were not differentiated for quantitative bacteriology.

Use of antimicrobials within poultry production (16, 20) may provide the selective pressure to ensure some level of persistence of resistant bacteria in the production environment that may be transmitted to subsequent flocks. No information is available on the effect of antimicrobial use and persistence of DT104 or acquisition of additional re-

![Figure 4](image-url)
istance in DT104. Multiple drug resistance in the United States in *Salmonella* species is observed in both humans (12, 21, 23) and animals (22, 24). Additional characterization of the epidemiology and transmission of DT104 is required, particularly in poultry in which clinical disease can be inapparent. These data provide a baseline of information for future studies.

**REFERENCES**


