Administration of a *Salmonella* Enteritidis ΔhilAssrAfliG strain by coarse spray to newly hatched broilers reduces colonization and shedding of a *Salmonella* Enteritidis challenge strain

W. De Cort, F. Haesebrouck, R. Ducatelle, and F. van Immerseel

Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

**ABSTRACT** Consumption of contaminated poultry meat is still an important cause of *Salmonella* infections in humans. Colonization inhibition (CI) occurs when a live *Salmonella* strain is administered to chickens and subsequently protects against challenge with another *Salmonella* strain belonging to the same serotype. A *Salmonella* Enteritidis hilAssrAfliG deletion mutant has previously been proven to reduce colonization and shedding of a wild-type *Salmonella* Enteritidis strain in newly hatched broilers after experimental infection. In this study, we compared two administration routes for this strain. Administering the *Salmonella* Enteritidis ΔhilAssrAfliG strain through drinking water on the first day of life resulted in decreased fecal shedding and cecal colonization of a wild-type *Salmonella* Enteritidis challenge strain administered 24 h later using a seeder-bird model. When administering the CI strain by coarse spray on newly hatched broiler chicks, an even more pronounced reduction of cecal colonization was observed, and fecal shedding of the *Salmonella* Enteritidis challenge strain ceased during the course of the experiment. These data suggest that administering a *Salmonella* Enteritidis ΔhilAssrAfliG strain to newly hatched chicks using a coarse spray is a useful and effective method that reduces colonization and shedding of a wild-type *Salmonella* Enteritidis strain after early challenge.

**Key words:** broiler, *Salmonella*, colonization inhibition, administration route

**INTRODUCTION**

Despite the implementation of numerous monitoring and control measures in broiler production, poultry meat is still an important carrier of *Salmonella* that causes human infections (EFSA, 2013). Chickens are highly susceptible to *Salmonella* infection during their first days of life (Desmidt et al., 1997), and contact with *Salmonella*, even in very low numbers, can lead to persistent infections (Gast and Benson, 1995; Van Immerseel et al., 2004). These broilers often remain infected until slaughter age, which leads to introduction of *Salmonella* in the slaughterhouse and food chain (Heyndrickx et al., 2002). Consequently, prevention of infection during the early posthatch period is of utmost importance. Colonization inhibition (CI) occurs when a live *Salmonella* strain is orally administered to day-old chickens; it protects very rapidly against subsequent challenge with another *Salmonella* strain belonging to the same serotype (Barrow et al., 1987; Berchieri and Barrow, 1990; Methner et al., 1999; Bohez et al., 2008). CI can thus be used as a control method to prevent infection during the period in which the chick is highly susceptible to *Salmonella* infection (Bohez et al., 2008). Earlier research demonstrated that deletion of the *hilA*, *ssrA*, and *fliG* genes in a *Salmonella enterica* subspecies *enterica* serotype Enteritidis (*Salmonella* Enteritidis) strain resulted in a CI strain that was safe and effective in protecting broilers against challenge with a *Salmonella* Enteritidis wild-type strain (De Cort et al., 2013). This strain is considered safe because it is cleared by slaughter age, and it is considered effective because it lowers fecal shedding and cecal colonization of a wild-type *Salmonella* Enteritidis challenge strain. In this earlier study, the CI strain was administered by oral gavage in the crop, which is an unrealistic administration method in practice on a large scale. Because the level of protection offered by a CI strain depends on the administration route (Van Immerseel et al., 2005), the efficacy of the *Salmonella* Enteritidis ΔhilAssrAfliG strain might be different when this strain is administered by routes other than oral gavage. Therefore, two practically relevant administration methods were studied.
routes for the *Salmonella* Enteritidis *hilAssrAflG* deletion mutant, through drinking water and by coarse spray, were investigated and compared in this study.

**MATERIALS AND METHODS**

**Chickens**

One-day-old Ross 308 broiler chicks were obtained from a local hatchery and housed in isolation. Experimental groups were housed in separate rooms in containers on wood shavings. Commercial feed and drinking water were provided ad libitum. Cloacal swabs of all chicks were taken at the beginning of the experiment and cultured for *Salmonella* as described below to verify *Salmonella*-free status of the chickens prior to the experiment. Experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (experiment authorization number: EC2013/136).

**Salmonella Enteritidis Strains**

*Salmonella* Enteritidis 76Sa88 is a nalidixic acid–resistant, well-characterized strain originally isolated from a poultry farm (Desmidt et al., 1997; Van Immerseel et al., 2002). It was used for the production of isogenic mutants. Deletion of *hilA, ssrA,* and *fliG* genes was done using the one-step inactivation method described by Datsenko and Wanner, (2000). *Salmonella* Enteritidis 147, a streptomycin-resistant strain originally isolated from egg white, was used as a challenge strain. This strain is known to colonize the gut and internal organs of chickens to a high level (Methner et al., 1995a,b; Bochez et al., 2008).

**Experimental Design**

The total 180 day-old chicks were divided into 3 groups of 60 and each housed in a container of 2 m². The first group (Group C) was given sterile Hank’s Balanced Salt Solution (HBSS, 14175053, Invitrogen, Paisley, England) by oral gavage as a control on the first day of the experiment. Group D was given access to drinking water that initially contained 7.03×10⁸ CFU/ml of the *Salmonella* Enteritidis ΔhilAssrAflG strain for the first 24 h of the experiment. The chicks in Group S were transferred to their containers 10 min after treatment. On d 2 of the experiment, 12 chickens in each group (1 out of 5) were randomly selected and given 10⁹ CFU of the *Salmonella* Enteritidis challenge strain (seeder birds) by oral gavage and housed together again with the other animals of their group. To evaluate colonization by the *Salmonella* Enteritidis ΔhilAssrAflG strain and the wild-type challenge strain, their numbers in cecum and spleen were determined on 7, 21, and 42 d for 20 chickens. At each time point, 1 in 5 sampled animals were seeder birds. Shedding of both strains was evaluated during the experiment by bacteriological analysis of cloacal swabs taken on 2, 3, 9, 16, 23, 30, and 37 d.

**Bacteriological Analysis**

Cloacal swabs were directly inoculated on xylose lysine deoxycholate agar (XLD, CM0469, Oxoid, Basingstoke, England) plates supplemented with 20 µg/mL nalidixic acid (N8878, Sigma-Aldrich, St. Louis, MO) or 100 µg/mL streptomycin (S6501, Sigma-Aldrich, St. Louis, MO). Samples negative after direct inoculation were pre-enriched in buffered peptone water (BPW, CM0509, Oxoid, Basingstoke, England) and incubated overnight at 37°C. One mL of this suspension was further enriched by adding 9 mL tetrathionate-brilliant green broth (1.05178.0500, Merck, Darmstadt, Germany). After overnight incubation at 37°C, this suspension was plated on XLD plates with the appropriate antibiotic.

Samples of ceacum and spleen were homogenized in BPW and 10-fold dilutions were made in HBSS. Six droplets of 20 µl of each dilution were plated on XLD plates supplemented with the appropriate antibiotic. After overnight incubation at 37 °C, the number of CFU/g tissue was determined by counting the number of bacterial colonies. Negative samples were enriched as described above. Samples of the litter were taken after termination of the experiment and enriched as described above.

**Statistical Analysis**

GraphPad Prism software (Version 5.0, GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. A Fisher’s exact test (one-sided) was used to analyze differences in mortality between groups. A Kruskal-Wallis test (one-way ANOVA) was used to determine statistical differences of the number of *Salmonella*-positive cloaca swabs and (after enrichment) spleen and cecum samples between groups. Bacterial counts in cecum and spleen were converted into logarithmic form for statistical analysis. Samples of ceacum and spleen that were negative after direct plating were rated as log₁₀ = 0. Differences between groups were analyzed using a Kruskal-Wallis test (one-way ANOVA). Differences with *P*-values lower than 0.05 were considered to be significant.

**RESULTS**

In every group, 5 chickens died during the course of the experiment. Consequently, there is no statistical difference between groups in mortality. As shown in Table 1, shedding of the *Salmonella* Enteritidis ΔhilAssrAflG strain declined in both groups after...
Table 1. The number of cloacal swabs positive for a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain or a *Salmonella* Enteritidis challenge strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 9</th>
<th>Day 16</th>
<th>Day 23</th>
<th>Day 30</th>
<th>Day 37</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> Enteritidis</td>
<td>S</td>
<td>60/60</td>
<td>57/59</td>
<td>22/38</td>
<td>2/37</td>
<td>0/19</td>
<td>0/19</td>
<td>0/18</td>
</tr>
<tr>
<td>Δ<em>hilAssrAfliG</em></td>
<td></td>
<td>(54(^{\text{a}}))</td>
<td>(43(^{\text{a}}))</td>
<td>(4)</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis</td>
<td>D</td>
<td>55/60</td>
<td>57/59</td>
<td>17/38</td>
<td>12/38</td>
<td>0/18</td>
<td>0/18</td>
<td>0/17</td>
</tr>
<tr>
<td>challenge</td>
<td></td>
<td>(29(^{\text{a}}))</td>
<td>(22(^{\text{a}}))</td>
<td>(7)</td>
<td>(1)</td>
<td>(0)</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>NA</td>
<td>12/60</td>
<td>39/39</td>
<td>27/37(^{\text{a}})</td>
<td>3/18</td>
<td>4/16</td>
<td>6/16</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>NA</td>
<td>8/59</td>
<td>8/38(^{\text{a}})</td>
<td>22/38</td>
<td>4/18</td>
<td>7/18</td>
<td>5/17</td>
<td></td>
</tr>
</tbody>
</table>

Group C was given sterile HBSS as a control on d 1. Group S was sprayed with 125 mL of a suspension containing ± 10⁶ CFU/mL *Salmonella* Enteritidis Δ*hilAssrAfliG* strain on d 1. Group D was given access to drinking water containing ± 10⁶ CFU/mL of a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain for the first 24 h of the experiment. Twelve chickens in each group (1 out of 5) were challenged with 10⁵ CFU of a *Salmonella* Enteritidis challenge strain on d 2 of the experiment (seeder birds). Samples were taken at d 2, 3, 9, 16, 23, 30, and 37 of the experiment.

\(^{\text{a}}\)Number of positive samples after enrichment/total number of samples

\(^{\text{b}}\)Number of positive samples after direct plating

\(^{*}\)Significant difference between groups (P-value < 0.05)

NA = Not available

Figure 1. Cecal (A, B, C) and spleen (D, E, F) colonization by a *Salmonella* Enteritidis challenge strain. Group C was given sterile HBSS as a control on d 1. Group S was sprayed with a suspension containing a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain on d 1. Group D was given access to drinking water containing a *Salmonella* Enteritidis Δ*hilAssrAfliG* strain for the first 24 h of the experiment. Twelve chickens in each group (1 out of 5) were challenged with a *Salmonella* Enteritidis challenge strain on d 2 of the experiment (seeder birds). Subfigures A and D show colonization on d 7, B and E on d 21, and C and F on d 42. Values shown are log₁₀ of CFU/g sample. The horizontal lines represent the mean, and the error bars represent the standard error of mean (SEM). Significant differences between groups are indicated with \(^{*}\) (P-value < 0.05).
inoculation, and the strain was no longer shed from 23 d of age onward. Shedding of the *Salmonella* Enteritidis challenge strain was lower in Group S than in Group C for the entire duration of the experiment, while there was only initially a difference between Groups C and D. Colonization by the *Salmonella* Enteritidis ΔhilAssrAfliG strain was initially high in the cecum, where the bacterial load amounted to 6.67 ± 0.029 log CFU/g and 6.82 ± 0.115 log CFU/g after direct plating, respectively, in Groups S and D on d 7 of the experiment. On d 21, the colonization inhibition (CI) strain could not be detected after direct plating in any sample belonging to Group S, and only one sample was positive in Group D; the bacterial load amounted to 4.28 log CFU/g. The CI strain could not be detected after direct plating in any of the cecum samples on d 42 or in spleen samples for the entire duration of the experiment. Colonization by the *Salmonella* Enteritidis challenge strain is shown in Figure 1. Colonization of the cecum was significantly lower in Group S than in Group C for the entire duration of the experiment. Colonization of the cecum in Group D was significantly lower than the control group on d 7 and 42. There was no difference between the mean log CFU/g spleen of the different groups at any time point. Enrichment of cecum and spleen samples showed that the *Salmonella* Enteritidis ΔhilAssrAfliG strain was present in only a few samples at d 21 of the experiment and could no longer be detected at d 42 (Table 2). The number of cecum and spleen samples positive for the *Salmonella* Enteritidis challenge strain was lower in both Groups S and D than in Group C. However, this difference was statistically significant only on d 42 for the cecum when comparing Group S to the control group. The *Salmonella* Enteritidis ΔhilAssrAfliG strain could not be detected after enrichment of the litter samples. The *Salmonella* Enteritidis challenge strain could be detected in the litter of Group C but was not present in the litter of the treated groups.

**DISCUSSION**

Recent research demonstrated that a *Salmonella* Enteritidis hilAssrAfliG deletion mutant is a colonization inhibition (CI) strain that lowers colonization of a *Salmonella* Enteritidis wild-type strain after experimental infection. In addition, this deletion mutant could no longer be detected at slaughter age (De Cort et al., 2013). Because the level of protection offered by live vaccine strains depends on the administration route (Van Immerseel et al., 2005), two practically relevant administration methods for the *Salmonella* Enteritidis ΔhilAssrAfliG strain were investigated in this study. Only one *Salmonella* Enteritidis challenge strain was used in this study, as protection offered by CI is often similar for heterologous strains within the same serotype (Methner et al., 2011). Neither administration method investigated in this study offered protection against mortality caused by the *Salmonella* Enteritidis challenge strain, as there was no significant difference in mortality between the untreated group and treated groups. However, by adding the *Salmonella* Enteritidis ΔhilAssrAfliG strain to the chicks’ drinking water, a significant reduction in colonization of the cecum could be obtained by slaughter age, and shedding of the wild-type strain was reduced during the course of the experiment. An even more distinct reduction of cecal colonization was obtained when the strain was administered by coarse spray, and this also resulted in a significantly higher number of ceca negative for the wild-type strain. Additionally, shedding of the challenge strain ceased during the course of the experiment in the spray-treated group. This may be because spraying allows a more uniform and simultaneous distribution of the CI strain among the chickens, as spraying results in the formation of droplets on the birds that are taken up orally quickly after administration during preening (Caldwell et al., 2001a,b). Recent research has, however, suggested the respiratory route as a viable route

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**Table 2.** The number of ceca and spleen samples positive for a *Salmonella* Enteritidis ΔhilAssrAfliG strain or a *Salmonella* Enteritidis challenge strain after enrichment.

<table>
<thead>
<tr>
<th>Day</th>
<th>CECUM</th>
<th>Spleen</th>
<th>CECUM</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Group S</strong></td>
<td><strong>Group D</strong></td>
<td><strong>Group S</strong></td>
<td><strong>Group D</strong></td>
</tr>
<tr>
<td>7</td>
<td>16/19*</td>
<td>16/20</td>
<td>16/18</td>
<td>9/20</td>
</tr>
<tr>
<td>21</td>
<td>3/18</td>
<td>1/18</td>
<td>0/18</td>
<td>0/18</td>
</tr>
<tr>
<td>42</td>
<td>0/18</td>
<td>0/17</td>
<td>0/18</td>
<td>0/17</td>
</tr>
<tr>
<td>2020</td>
<td>14/19</td>
<td>14/20</td>
<td>11/20</td>
<td>5/18</td>
</tr>
<tr>
<td>19/19*</td>
<td>12/18</td>
<td>16/18</td>
<td>19/19*</td>
<td>5/18</td>
</tr>
<tr>
<td>16/16*</td>
<td>1/18*</td>
<td>15/17</td>
<td>7/16</td>
<td>3/18</td>
</tr>
</tbody>
</table>

Group C was given sterile HBSS as a control on d 1. Group S was sprayed with 125 mL of a suspension containing ± 100 CFU/mL *Salmonella* Enteritidis ΔhilAssrAfliG strain on d 1. Group D was given access to drinking water containing ± 100 CFU/mL of a *Salmonella* Enteritidis ΔhilAssrAfliG strain for the first 24 h of the experiment. Twelve chickens in each group (1 out of 5) were challenged with 10⁵ CFU of a *Salmonella* Enteritidis challenge strain on d 2 of the experiment (seeder birds). Samples were taken at d 7, 21, and 42 of the experiment.

*Number of positive samples after enrichment

*Total number of samples

*Significant difference between groups (P-value < 0.05)
of entry for *Salmonella* in poultry (Kallapura et al., 2013). Consequently, it is possible that the CI strain is also taken up through the respiratory route when it is sprayed. Additionally, spraying newly hatched chicks ensures better uptake of the CI strain quickly after hatching. In contrast, drinking water consumption may ensure better uptake of the CI strain quickly after spraying. Additionally, spraying newly hatched chicks also taken up through the respiratory route when it is sprayed. Consequently, it is possible that the CI strain is required to inhibit growth of the wild-type strain (Berchieri and Barrow, 1991; Barrow et al., 1996).

We conclude that the *Salmonella* Enteritidis ΔhilA Δassr ΔfltG strain should ideally be administered via a coarse spray to newly hatched chicks for the purpose of colonization inhibition, as this resulted in the most profound reduction in cecal colonization and fecal shedding of a wild-type *Salmonella* Enteritidis strain in an early challenge model.

**REFERENCES**


