Fitness and dissemination of disinfectant-selected multiple-antibiotic-resistant (MAR) strains of *Salmonella enterica* serovar Typhimurium in chickens

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Objectives: The aims of this study were to determine whether strains of *Salmonella enterica* serovar Typhimurium which had acquired low-level multiple antibiotic resistance (MAR) through repeated exposure to farm disinfectants were able to colonize and transmit between chicks as easily as the parent strain and, if such strains were less susceptible to fluoroquinolones, would high-level resistance be selected after fluoroquinolone treatment.

Methods: Two mutants were compared with the isogenic parent. In the first experiment, day-old chicks were co-infected with both the parent and a mutant to determine their relative fitness. In the second experiment, parent and mutant strains (in separate groups of chicks) were assessed for their ability to transmit from infected (contact) to non-infected (naive) birds and with respect to their susceptibility to fluoroquinolone treatment. Birds were regularly monitored for the presence of *Salmonella* in caecal contents. Replica plating was used to monitor for the selection of antibiotic-resistant strains.

Results: The parent strain was shown to be significantly fitter than the two mutants and was more rapidly disseminated to naive birds. Antibiotic treatment did not preferentially select for the two mutants or for resistant strains.

Conclusions: The disinfectant-exposed strains, although MAR, were less fit, less able to disseminate than the parent strain and were not preferentially selected by therapeutic antibiotic treatment. As such, these strains are unlikely to present a greater problem than other salmonellae in chickens.

Keywords: fluoroquinolones, enrofloxacin, birds, microbial pathogenicity, multidrug exporter

Introduction

There are increasing concerns that disinfectants, which are used widely in farm environments, may select for antibiotic-resistant pathogens. If such strains are zoonotic pathogens, such as some *Escherichia coli* and *Salmonella*, then these could proceed to infect man with possible consequences for antibiotic therapy.

Certain disinfectants can select for bacteria with low-level multiple antibiotic resistance (MAR). In *E. coli* and *Salmonella*, MAR can be due to up-regulation of the AcrAB-TolC efflux pump, although down-regulation of porins may also be involved. MAR strains typically show an ~4–8-fold reduction in susceptibility to unrelated antibiotics such as β-lactams, chloramphenicol, fluoroquinolones and tetracyclines, organic solvent resistance and decreased susceptibility to disinfectants such as pine oil and triclosan. Such resistance is considered a possible stepping stone to higher-level antibiotic resistance.

In two recent studies, *Salmonella* exposed to farm disinfectants or triclosan were shown to have a small, but statistically significant, increased risk of selection of mutants with reduced susceptibility when exposed subsequently to ampicillin, ciprofloxacin, tetracycline or cyclohexane and some of the resulting mutants were MAR. RT–PCR analysis of MAR mutants derived from exposure to disinfectants showed up-regulation of acrB.
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*Salmonella enterica* serovars Enteritidis and Typhimurium are recognized as the leading *Salmonella* serovars that cause food poisoning in humans in the USA and Europe. As such, any practices that select for reduced antibiotic susceptibility in *Salmonella* should be avoided. Chicken is generally considered the main source of human infection with *Salmonella* and so a chick model is considered appropriate to investigate the colonization, dissemination, persistence and response to antibiotic treatment of *Salmonella*.

The aim of this study was to compare MAR mutants (derived by exposure to disinfectants) of *Salmonella Typhimurium* with their isogenic parent strain with respect to: (i) relative fitness; (ii) dissemination from infected contact chicks to non-infected naive chicks; (iii) response to fluoroquinolone treatment; and (iv) selection of higher-level resistance.

**Materials and methods**

**Bacterial strains**

*S. enterica* serovar Typhimurium SL 1344 and two isogenic MAR mutants were used to infect the chicks. The MAR mutants were derived from multiple passage with either an aldehyde-based farm disinfectant (mutant ABD1) or an oxidizing compound farm disinfectant (mutant OXC1).

*E. coli* strains NCTC 10418, AG100 and AG102 were used as controls for determination of MICs and cyclohexane tolerance. Cultures were grown overnight at 37°C in Luria–Bertani (LB) broth prior to infection of chicks and determination of MICs.

**Antimicrobials and chemicals**

Enrofloxacin [Baytril 10% (w/v) oral solution] for dosing poultry was obtained from the National Veterinary Services, UK. Ciprofloxacin and enrofloxacin for MICs were kindly donated by Bayer HealthCare AG (Germany). Triclosan was kindly donated by Ciba specialty chemicals, PLC, UK. Other antibiotics and organic solvents were obtained from Sigma-Aldrich (Poole, Dorset, UK).

**Chick experiments**

All animal studies were conducted under the jurisdiction of the animals scientific procedures act (1986) and were reviewed by the local Ethics Review Committee. In all experiments, chicks received all animal studies were conducted under the jurisdiction of the animals scientific procedures act (1986) and were reviewed by the local Ethics Review Committee. In all experiments, chicks received feed and water *ad libitum* and were monitored for their condition twice daily.

**Experiment (i). Fitness of the mutants when in competition with the parent strain.** Sixty specific-pathogen-free (SPF; SPAFAS chicks specially reared and checked to be free of specific pathogens such as *Salmonella* species) White Leghorn chicks (1-day-old) were randomly separated into two groups of 30 birds and housed in separate bio-secure isolators. One-day-old birds were infected by gastric gavage with a standardized inoculum of ~10³ cfu per bird of *Salmonella Typhimurium* SL1344 and one mutant in a 0.1 mL volume of PBS in a 1:1 ratio.

At 2, 9, 16, 23 and 30 days of age, 9–10 birds per group were swabbed to check for the presence of salmonellae (Table 2). Swabs were plated onto Rambach (Merk) agar without ciprofloxacin to determine the number of salmonellae enumerated as above. At 7, 14, 21 and 35 days, 5 birds (9–10 birds at 35 days) from each group were killed by cervical dislocation and the number of *Salmonella* in caecal contents enumerated as above.

**Experiment (ii). Ability of mutants to be transmitted from contact (e.g. infected) to naive (e.g. un-infected) chicks and the effectiveness of antibiotic treatment.** One hundred and thirty-two specific-pathogen-free White Leghorn chicks (1-day-old) were randomly separated into six groups (A, B, C, D, E and F) of 22 birds each, with each group of birds housed in a separate bio-secure room. At 1-day-old, five chicks per room were infected by gastric gavage with a standardized inoculum of ~10³ cfu per bird of *Salmonella Typhimurium*. Birds that were infected by gastric gavage (rather than infected via transmission) are hereafter referred to as contact birds whereas other birds are referred to as naive birds. Birds in groups A and D received strain SL1344, birds in groups B and E received the mutant ABD1 and birds in groups C and F received the mutant OXC1 (Table 3).

Groups D, E and F were replicates for groups A, B and C, respectively, except that birds in these groups had antibiotic treatment (Baytril 10% oral solution in the drinking water for 5 days as described below) at ~3 weeks old. The number of *Salmonella* present before and after antibiotic treatment was enumerated on Rambach agar without antibiotics from cloacal swabs or from caecal contents following post-mortem on the days shown in Table 3.

For all experiments, the limit of detection was ~100 cfu/g of faeces.

**Antibiotic dose calculations and methodology**

For treatment of salmonellosis in poultry, current data sheets for Baytril 10% oral solution recommend to add enrofloxacin to the drinking water to give 10 mg/kg bodyweight of birds per day for 5–10 days. These data sheets also recommend that medicated water should be made up immediately prior to provision, medication of the water supply should be continuous during the treatment period with no other source of water available and the product should be given over a minimum period of 6 h per day to allow all birds to drink. The dosing regimen for groups D, E and F was based on these guidelines with the exception that the antibiotic dose was given as a pulsed dose to conform with previous studies. Pulsed dosing was achieved by making up the dose in one-third of the previously calculated amount of water drunk daily. Once the medicated water was consumed, it was replaced with fresh water. Birds received antibiotic at the start of each day before the lights in their accommodation came on and the dose was consumed over ~6–7 h. Giving provision of the dose in this way ensured that the birds received the full antibiotic dose, which may not have been achieved if the birds were given the dose in larger volumes of water that may not have been consumed.

Birds in groups D, E and F were weighed the day before treatment, and on days 2 and 4 of treatment. The collective weight was used to calculate the antibiotic dose for the following day for each group of birds.

**Replica plating, determination of MIC values and cyclohexane tolerance**

To determine the emergence of strains with reduced susceptibility after antibiotic treatment only, replica plating was performed as previously described, but only onto media with 4× the MIC amount of nalidixic acid or ciprofloxacin for respective strains or media. The viable count of SL1344 was derived by subtracting the count of the mutants on agar with ciprofloxacin from the total count on agar with no antibiotic.
overlaid with cyclohexane. Bacteria from representative plates (mainly post-antibiotic treatment) representing all groups of birds with and without antibiotic treatment were replica plated.

MICs of ampicillin, chloramphenicol, ciprofloxacin, nalidixic acid, triclosan and tetracycline were determined by the method of the BSAC\(^{23}\) against isolates \((n = 45)\) selected from all groups and time periods from birds that received antibiotic treatment.

Statistical analyses

For animal experiments, there is an on-going requirement to use as few animals as possible. The number of chickens that were used in each experiment was based on numbers used in previous studies where statistically significant differences were shown between \(Salmonella\) counts in enrofloxacin-treated and -untreated birds.\(^{1,22}\)

In the first experiment, the non-parametric Wilcoxon matched pairs test was used to compare the \(Salmonella\) parent and mutant counts for each time point.

In the second experiment, because many of the \(Salmonella\) counts were below the lower limit of detection, non-parametric methods were also used to compare \(Salmonella\) counts between different groups of birds. The given counts were transformed to \(\log_{10} (\text{count} + 1)\) and the non-parametric Mann–Whitney test was used to compare the means of the \(Salmonella\) counts in the contact and naive birds for each group and day. The Kruskal–Wallis test was used for overall comparisons of \(Salmonella\) counts in groups A/B/C and D/E/F. These were followed by the specific comparisons of the mutants to the parent strain using Dunn’s method as implemented in the Unistat software package.

Results

Phenotype and fitness of the mutant strains

The two mutants ABD1 and OXC1 were MAR (Table 1). The mutants were both cyclohexane tolerant and showed an \(\sim 4\)-fold reduction in susceptibility to the antibiotics tested and to triclosan compared with the parent strain.

When the mutants were in competition with the parent strain SL1344 in the same birds \(\{(\text{experiment i})\}\), they were isolated in lower numbers than SL1344 on most occasions and in most cases these differences were statistically significant (Table 2).

Table 1. MICs of antibiotics and disinfectants for \(Salmonella\) Typhimurium SL1344 parent strain and disinfectant-derived MAR mutants

<table>
<thead>
<tr>
<th>Strain details</th>
<th>MICs of antibiotics (mg/L) or disinfectants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344</td>
<td>AMP 4 CHL 0.03 CIP 4 NAL 0.13 TET 0.03 TRIC 0.25 ABD OXC CYX 8</td>
</tr>
<tr>
<td>ABD1</td>
<td>4 16 0.13 16 8 0.5 0.03 0.25 R</td>
</tr>
<tr>
<td>OXC1</td>
<td>4 16 0.13 16 8 0.5 0.03 0.25 R</td>
</tr>
</tbody>
</table>

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; TRIC, triclosan; ABD, aldehyde-based disinfectant; OXC, oxidizing compound disinfectant; CYX, cyclohexane; S, susceptible, R, resistant.

Bold font indicates mutants at least \(4\times\) less susceptible to compound than parent strain.

The results of experiment (ii) confirmed the findings of experiment (i) and indicated that the mutants were less fit even when they were not directly in competition with SL1344 in the same birds (Table 3, Figures 1 and 2). In experiment (ii), on most occasions up to 17 days post-infection, the mean \(Salmonella\) counts for SL1344 \(\{(\text{e.g. birds in groups A and D}\}\) were significantly higher than the mean \(Salmonella\) counts for the mutants \(\{(\text{e.g. birds in groups B, C, E and F}\}\) (Table 3, Figures 1 and 2).

After antibiotic treatment, the counts of \(Salmonella\) derived for mutants in groups E and F were all \(< 100\). At days 25, 29, 36 and 43, however, there were counts for SL1344 for birds in group D (Table 3).

Transmission of strains from contact to naive birds

In experiment (ii), the mutants were less able to transmit from infected contact birds to naive chicks compared with SL1344 (Table 3, Figures 1 and 2). On the first occasion of swabbing \(\{(3\text{ days after infection of the contact birds}\}\), SL1344 had been transmitted to most naive birds in groups A and D (Table 3 and Figures 1 and 2), and by 7 days post-infection, all naive birds tested in groups A and D were infected (Table 3). Conversely, at 3 days, none of the naive birds was infected with either of the mutant strains and by 7 days, out of a possible total of 20 naive birds tested that could have been infected with the two mutants \(\{(\text{combined tested birds for groups B, C, E and F}\}\), only 7 were infected (Table 3). However, differences in the mean \(Salmonella\) counts from contact and naive birds were only significant \(\{(P \leq 0.05)\}\) for the two mutants in groups C, E and F at 3 days post-infection.

Efficacy of enrofloxacin treatment for the different strains

Infected the birds with \(Salmonella\) did not make them clinically ill, so antibiotic efficacy was related to the ability to reduce \(Salmonella\) counts in caecal contents.

In groups A, B and C \(\{(\text{no antibiotic treatment}\}\) of experiment (ii), the numbers of \(Salmonella\) isolated from birds decreased over time. However, at 36 days post-infection, \(Salmonella\) was still isolated from birds in all groups, and by the end of the experiment \(\{(44\text{ days}\}\), \(Salmonella\) was still isolated from birds infected with SL1344 and birds infected with one mutant ABD1 (Table 3). Conversely, after antibiotic treatment, no mutant strains were recovered from any birds and antibiotic treatment reduced the number of birds infected with SL1344.

Selection of \(Salmonella\) with reduced susceptibility

There was no evidence in experiment (ii) from the replica plating or the MICs that the enrofloxacin treatment of birds in groups D, E and F selected for SL1344 with reduced susceptibility, or further reduced susceptibility of the two mutants.

Discussion

Previously, we examined populations of \(Salmonella\) from animals and found that MAR strains were relatively common.\(^{25}\) It is well known that disinfectants such as pine oil and triclosan can select for MAR strains of \(E.\) coli.\(^{9,10}\) We have also
Disinfectant mutants of *Salmonella* in chickens

Table 2. Presence of *Salmonella* Typhimurium parent strains and isogenic disinfectant-passaged strain when in competition with each other in chicks – Experiment (i)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Days post-infection</th>
<th>Swab or post-mortem (PM)</th>
<th>No. of birds tested</th>
<th>Log cfu/g count parent (SD)</th>
<th>Log cfu/g count mutant (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD1 mutant versus parent SL1344</td>
<td>0</td>
<td>swab</td>
<td>10</td>
<td>5.22 (2.88)</td>
<td>6.39 (1.75)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>PM</td>
<td>5</td>
<td>8.63 (0.24)</td>
<td><strong>6.77</strong> (0.57)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>swab</td>
<td>10</td>
<td>5.50 (2.00)</td>
<td><strong>3.99</strong> (1.87)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>PM</td>
<td>5</td>
<td>7.16 (0.25)</td>
<td><strong>4.40</strong> (0.36)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>swab</td>
<td>10</td>
<td>5.12 (2.90)</td>
<td>5.38 (0.79)</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>PM</td>
<td>5</td>
<td>7.35 (0.14)</td>
<td><strong>3.39</strong> (1.19)</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>swab</td>
<td>10</td>
<td>5.52 (0.93)</td>
<td>&lt;100 (NA)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>swab</td>
<td>5</td>
<td>5.39 (0.50)</td>
<td>&lt;100 (0)</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>PM</td>
<td>10</td>
<td>6.25 (0.58)</td>
<td><strong>2.13</strong> (0.87)</td>
</tr>
<tr>
<td>OXC1 mutant versus parent SL1344</td>
<td>0</td>
<td>swab</td>
<td>10</td>
<td>7.11 (1.12)</td>
<td><strong>4.10</strong> (2.30)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>PM</td>
<td>5</td>
<td>8.79 (0.36)</td>
<td><strong>3.70</strong> (2.58)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>swab</td>
<td>10</td>
<td>3.85 (3.34)</td>
<td>5.36 (2.03)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>PM</td>
<td>5</td>
<td>6.76 (0.84)</td>
<td><strong>2.47</strong> (1.42)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>swab</td>
<td>10</td>
<td>6.59 (0.86)</td>
<td><strong>4.10</strong> (2.26)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>swab</td>
<td>5</td>
<td>5.51 (0.71)</td>
<td><strong>0.78</strong> (1.11)</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>PM</td>
<td>5</td>
<td>4.37 (2.16)</td>
<td><strong>0.60</strong> (1.19)</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>swab</td>
<td>9</td>
<td>5.51 (0.35)</td>
<td>&lt;100 (NA)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>swab</td>
<td>5</td>
<td>6.27 (0.69)</td>
<td>&lt;100 (NA)</td>
</tr>
</tbody>
</table>

NA, not applicable.

Bold font indicates that mutant counts were significantly (*P < 0.05*) lower than counts for parent strain.

Table 3. Shedding of *Salmonella* Typhimurium SL1344 parent strain and disinfectant-derived MAR mutants before and after antibiotic treatment – Experiment (ii)

<table>
<thead>
<tr>
<th>Pre- or post-antibiotic</th>
<th>Day post-infection</th>
<th>Mean <em>Salmonella</em> in caecal contents as log cfu/g (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swabs or post-mortem (PM)</td>
<td>No. of chickens positive <em>Salmonella</em>—contact/naive*</td>
</tr>
<tr>
<td></td>
<td>group A SL1344</td>
<td>group B ABD1</td>
</tr>
<tr>
<td>Pre (contact) birds</td>
<td>swabs (n = 10)</td>
<td>5.17 (2.04)</td>
</tr>
<tr>
<td>infected at 0 days old</td>
<td>swabs (n = 10)</td>
<td>6.33 (0.67)</td>
</tr>
<tr>
<td>Post (birds treated)</td>
<td>PM (n = 4)</td>
<td>5.53 (0.91)</td>
</tr>
<tr>
<td>with antibiotic</td>
<td>swabs (n = 10)</td>
<td>5.14 (1.39)</td>
</tr>
<tr>
<td>at 20–24 days old</td>
<td>PM (n = 4)</td>
<td>5.70 (3.80)</td>
</tr>
</tbody>
</table>

*, birds not treated with antibiotic; +, birds treated with antibiotic; ND, none detected; NA, not applicable.

*For all pre-antibiotic treatment samples and the first post-antibiotic post-mortem samples, equal numbers of contact and naive birds were sampled. Therefore results are for all birds as there was only one contact bird left per group.

Demonstrated that farm disinfectants can select for MAR salmonella.\textsuperscript{13,14} Here, we compared MAR salmonella selected by exposure to farm disinfectants with their isogenic parent, SL1344, for fitness and ability to survive and be transmitted in a farm environment. We also investigated whether enrofloxacin could eradicate such strains or if resistant mutants were more readily selected from the disinfectant-selected mutants.

Although the two mutants ABD1 and OXC1 showed a classical MAR phenotype as evidenced by ~4-fold reduced susceptibility to antibiotics such as ampicillin, chloramphenicol, nalidixic acid and tetracycline, they did not show reduced susceptibility to the selecting disinfectants. Related studies have shown that *Salmonella* Typhimurium SL1344 following repeated exposure to disinfectants have reduced levels of outer membrane proteins.
(OMPs) and in some instances high levels of AcrAB-TolC.26 These changes must represent a survival mechanism of the bacteria in a toxic environment. It would seem likely that reduced levels of OMPs and possible high levels of AcrAB-TolC are responsible for the reduced susceptibility to some antibiotics, but presumably these changes are not sufficient to alter the disinfectant MICs by amounts measurable by doubling dilution MICs. However, other recent related studies have shown that disinfectant mutants of *Salmonella* Typhimurium SL1344 can have subtle levels of increased disinfectant tolerance that are picked up by methods other than MICs.27

Experiment (i) showed that the mutant strains were attenuated when in competition with the parent strain. However, such an experiment is limited in that it does not assess the fitness of the strains when they are not in competition with the parent strain, nor does it assess the transmissibility of the mutants compared with the parent strain. As such, in experiment (ii), the fitness of the mutants when they were not in competition with the parent

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**Figure 1.** Shedding (swab results only) of *Salmonella* strains from birds without antibiotic treatment in experiment (ii). Filled black squares, contact birds; open white squares, naive birds.

**Figure 2.** Shedding (swab results only) of *Salmonella* strains from birds with antibiotic treatment in experiment (ii). Filled black squares, contact birds; open white squares, naive birds. *Five days enrofloxacin treatment.*
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Strains are maintained at a high level and in some batches of chicks infected at 1-day-old, the higher-level antibiotic-resistant strains. It is also possible that important to assess the ability of antibiotic treatment to strain was assessed, as was their transmissibility. Additionally, it is observed that the presence of normal flora both in the environment and in animals offers a greater possibility of non-MAR resistance mechanisms involving horizontal gene transfer between the same or different bacterial species.

Over the years we have performed many chick studies, and in some batches of chicks infected at 1-day-old, the *Salmonella* strains are maintained at high levels (10⁸ cfu/g caecal contents) for many weeks, whereas in other batches of chicks, the chicks gradually clear the *Salmonella*. In experiment (ii) where the birds received antibiotic, the numbers of SL1344 in chicks fell to ~1000 cfu/g of caecal contents by the time of antibiotic-treatment. It is possible that this low number of bacteria led to the observation that no antibiotic-resistant mutants were selected. In our previous studies with other batches of chicks, the *Salmonella* numbers were higher, antibiotic treatment selected for *gyrA* mutants in both the parent strain and the MAR mutants. However, the lower level of *Salmonella* colonization is more likely to equate to the general level of colonization in a real farm situation, but this is not to say that higher levels of colonization do not occur in a farm situation.

In conclusion, these data show that strains of *Salmonella* that have had multiple exposure to disinfectants, although MAR, were less fit and less able to disperse into a chick model than the isogenic parent strain. Additionally, the mutants were not selected over the background flora by fluoroquinolone treatment, nor were fluoroquinolone-resistant mutants isolated. These results suggest that such isolates do not pose a threat to human health above that of other *Salmonella*. However, it is still possible that the combination of inadequate disinfection regimens coupled with antibiotic usage may select for higher-level resistance in bacteria. As such, care should be taken in a farm environment to ensure that both disinfection and antibiotic treatment are appropriate and delivered correctly.

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Transparency declarations

None to declare.

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