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Fluoroquinolone treatment of experimental *Salmonella enterica* serovar Typhimurium DT104 infections in chickens selects for both *gyrA* mutations and changes in efflux pump gene expression

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***Objectives*: To determine the efficacy of enrofloxacin (Baytril) in chickens in eradicating three different resistance phenotypes of *Salmonella enterica* and to examine the resistance mechanisms of resulting mutants.**

***Methods*: In two separate replicate experiments (I and II), three strains of *Salmonella enterica* serovar Typhimurium DT104 [strain A, fully antibiotic-sensitive strain; strain B, isogenic multiple antibiotic-resistant (MAR) derivative of A; strain C, veterinary penta-resistant phenotype strain containing GyrA Phe-83], were inoculated into day-old chicks at $\sim10^3$ cfu/bird. At day 10, groups of chicks (n =10) were given either enrofloxacin at 50 ppm in their drinking water for 5 days or water alone (control). Caecal contents were monitored for presence of *Salmonella* and colonies were replica plated to media containing antibiotics or overlaid with cyclohexane to determine the proportion of isolates with reduced susceptibility. The MICs of antibiotics and cyclohexane tolerance were determined for selected isolates from the chicks. Mutations in topoisomerase genes were examined by DHPLC and expression of *marA, soxS, acrB, acrD* and *acrF* by RT–PCR.**

***Results*: In experiment I, but not II, enrofloxacin significantly reduced the numbers of strain A compared with the untreated control group. In experiment II, but not I, enrofloxacin significantly reduced the numbers of strain B. Shedding of strain C was unaffected by enrofloxacin treatment. Birds infected with strains A and B gave rise to isolates with decreased fluoroquinolone susceptibility. Isolates derived from strain A or B requiring >128 mg/L nalidixic acid for inhibition contained GyrA Asn-82 or Phe-83. Isolates inhibited by 16 mg/L nalidixic acid were also less susceptible to antibiotics of other chemical classes and became cyclohexane-tolerant (e.g. MAR).**

***Conclusions*: These studies demonstrate that recommended enrofloxacin treatment of chicks rapidly selects for strains with reduced fluoroquinolone susceptibility from fully sensitive and MAR strains. It can also select for MAR isolates.**

Keywords: MAR, cyclohexane tolerance, quinolone resistance

Introduction

Salmonella enterica is still considered one of the major causes of gastro-enteritis in humans[1] with about 15 000 cases occurring in England and Wales in 2002.[2] As ciprofloxacin is the drug of choice for treating invasive human salmonellosis,[3–5] reduced susceptibility of *S. enterica* to fluoroquinolones is a matter of public concern, where patients infected with a quinolone-resistant serovar Typhimurium have been associated with a mortality rate 10.3 times higher than that for the general population.[6]

As *S. enterica* is primarily a zoonotic pathogen, strains from animals can infect and cause disease in humans. Thus, any concerns

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about reduced susceptibility to fluoroquinolones that might develop in animals is of obvious concern to humans. Fluoroquinolones have various uses in poultry and usage varies in different countries. For example, fluoroquinolones may be used to control colibacillosis,[7] or as part of mycoplasma eradication programmes in turkeys, or as an aid to control *S. enterica* and other bacterial infections in commercial poults.[8] As Salmonellosis is often associated with the consumption of contaminated raw or undercooked poultry products,[9,10] chickens are an ideal host for the development and dissemination to humans of *Salmonella* strains with reduced susceptibility to fluoroquinolones.[11] In a recent (~2000) survey in the UK, *S. enterica* were isolated from 29% of 300 chicken samples collected from supermarkets and butchers over a 7 month period.[12] In two processing plants in Turkey, the incidences of *S. enterica* in all broilers ($n = 90$ carcasses for each plant) were 36.6 and 31.1% at plants 1 and 2, respectively.[13]

Reduced susceptibility to fluoroquinolones (which may or may not be classified as clinical resistance depending on the degree of reduced susceptibility and the breakpoint concentration used) in *S. enterica* is usually mediated by one or more mutations in one or more topoisomerase genes.[14] However, in clinical human and veterinary isolates of *Salmonella* spp., mutations are usually only found in *gyrA*.[15–17] In addition to mutations in topoisomerase genes, some strains of *Salmonella enterica* serovar Typhimurium have been shown to be less susceptible to fluoroquinolones due to overexpression of the AcrAB efflux pump.[18,19] In *Escherichia coli*, the AcrAB efflux pump can be up-regulated by MarA and SoxS encoded by *marRAB* and *soxRS*, respectively.[20] As a result of considerable homology between *E. coli* and *S.* Typhimurium *acrA, acrB, marRAB,* and *soxRS*, it is thought that similar regulatory networks exist in *S.* Typhimurium.[21] Two other pumps, AcrD and AcrF, are homologous to AcrB, but the role of these pumps in antibiotic transport in *S.* Typhimurium is still unclear.[22,23]

With this background, it was hypothesized that mutants selected after exposure of *S.* Typhimurium to a fluoroquinolone *in vivo* would be dependent on the phenotype [e.g. fully sensitive, multiple antibiotic-resistant (MAR, ~two- to eightfold decrease in susceptibility to unrelated antibiotics such as β-lactams, chloramphenicol, quinolones and tetracycline)[24] or high level penta-resistant strain with reduced susceptibility to fluoroquinolones] of the infecting organism. Whilst it is possible to look at selection of reduced susceptibility or resistance in a laboratory setting, such studies have limited use when predicting what will happen in the much more complex environment of a living animal, where organisms will have to counter toxic substances such as bile, be exposed to changes in pH and compete with other organisms. Previous studies have shown that the AcrAB efflux system of *E. coli* appears to play a significant role in bile efflux.[25] Therefore, the aims of this study were to experimentally infect chicks with (i) a fully antibiotic-sensitive veterinary isolate of *S.* Typhimurium DT104 or (ii) a multiple antibiotic-resistant (MAR) isogenic mutant or (iii) a veterinary isolate with a typical pentavalent-resistance pattern associated with the presence of a type 1 integron which also had reduced susceptibility to fluoroquinolones (ciprofloxacin MIC 1 mg/L). The ability of the three strains to survive enrofloxacin treatment *in vivo* was monitored. Representative surviving isolates (mutants) of each resulting phenotype were examined for the level of reduced susceptibility to selected antibiotics, cyclohexane tolerance, mutations in *gyrA*, *gyrB*, *parC* and *parE* and for expression of genes involved in efflux such as *acrB*, *acrD*, *acrF*, *marA* and *soxS*.

Materials and methods

Bacterial strains

Three strains of *Salmonella enterica* serovar Typhimurium DT104 were used to infect chicks in this study. VLA S3992/96, hereafter referred to as strain A (Table 1) is fully sensitive to a range of antibiotics including ciprofloxacin and has been studied previously in animal models.[26] Strain B (Table 1) is an isogenic multiple antibiotic-resistant (MAR, ~two- to eightfold decrease susceptibility to unrelated antibiotics such as β-lactams, chloramphenicol, quinolones and tetracycline) derivative from strain A by a single passage on solid media containing 2.5 mg/L tetracycline as described previously.[27,28] VLA S2950/99, hereafter referred to as strain C (Table 1) is a typical penta-resistant type (resistant to ampicillin, chloramphenicol, streptomycin, sulfadiazine and tetracycline) *S.* Typhimurium DT104s with a type 1 integron and the associated antibiotic resistance genes.[29] Additionally, strain C has reduced susceptibility to ciprofloxacin (MIC = 1 mg/L) and contains Phe-83 in GyrA. All strains were isolated from a farm environment in the UK and are therefore considered representative of the *Salmonella* that could infect chickens on a farm. Control strains included *E. coli* NCTC10418 as control for MICs; *E. coli* AG100 and AG102 as controls for determination of cyclohexane tolerance; *S.* Typhimurium NCTC74 as control for DHPLC and *S.* Typhimurium NCTC74 and SL1344[30] as controls for analysis of gene expression.

Antimicrobials and chemicals

Antibiotics and organic solvents used were obtained from Sigma–Aldrich (Poole, Dorset, UK) except ciprofloxacin and enrofloxacin which were kindly donated by Bayer Health care (Germany).

Infection of chicks and antibiotic treatment

All animal studies were conducted under the jurisdiction of the Animals Scientific Procedures Act (1986) and were reviewed by the local ethical review committee. Two separate experiments were performed to determine whether similar results were obtained. Inocula for infecting chickens were prepared from strains A, B and C (Table 1). Cultures were grown overnight at 37°C in Luria-Bertani (LB) broth without shaking. Sixty specific-pathogen-free (SPF; chicks specially reared and checked to be free of specific pathogens such as *Salmonella*) White Leghorn chicks (1-day-old) were randomly separated into three groups of 20 individuals and each group was then dosed orally by gastric intubations with strain A, strain B or strain C (~10^3 cfu in 0.1 mL of phosphate-buffered saline per chick).[31] Each group of 20 birds was then randomly sub-divided into two groups of 10 birds and each group was placed in six separate isolators where they received feed and water supplied *ad libitum* and were monitored for condition twice daily throughout the experiment.

At 7 days old, three groups of 10 birds infected with strain A, strain B or strain C (Table 1) received 50 ppm enrofloxacin in their drinking water for 5 days (Enrofloxacin Oral Doser) as per the manufacturer's instructions. The remaining three groups of 10 birds infected with strain A, strain B or strain C were not treated with antibiotic and served as controls.

The second experiment was identical to the first except that 56 birds were infected as above (20 with strain A, 20 with strain B and 16 with the strain C). Each group of birds was then divided into two further groups and each group was housed in a separate isolator (six isolators in total). For each two sets of birds infected with a specific strain, one group was treated with enrofloxacin and the other group was the untreated control as above.

Table 1. Phenotype and mutations in topoisomerase genes of colonizing strains and representative post-therapy isolates

			MIC (mg/L)								CIP SSC	
Strain	Baytril[a]	Day of isolation[b]	NAL	CIP	AMP	TET	CHL	TRI	CYC	GyrA	–CCCP	+CCCP
Control strains												
NCTC 74	–	–	2	0.015	1	0.5	1	0.06	S	wt	62.5 ± 2.6	105.7 ± 5.6
SL1344	–	–	4	0.06	1	1	2	0.06	S	wt	25.9 ± 5.7	27.7 ± 4.4
Experiment I												
Birds colonized with strain A (wild-type)												
A	–	pre	4	0.03	1	1	4	0.25	S	wt	39.86 ± 1.5	98.13 ± 1.2
D	–	7	4	0.03	1	1	4	0.25	S	wt	**15.92 ± 1.6**	69.67 ± 5.5
K	–	21	4	0.03	1	1	4	0.25	S	wt	**20.75 ± 1.5**	58.19 ± 2.8
E	+	7	4	0.03	2	2	4	0.5	S	wt	**13.11 ± 0.6**	42.28 ± 1.5
L	+	21	16	0.06	2	4	16	0.5	T	wt	**11.48 ± 2.4**	44.99 ± 2.0
M	+	21	16	0.06	2	2	4	0.5	T	wt	**30.76 ± 0.9**	53.62 ± 1.8
S	+	21	16	0.06	4	4	16	0.5	T	wt	**13.79 ± 0.9**	36.55 ± 2.4
T	+	35	16	0.12	4	4	16	0.5	T	wt	**18.23 ± 1.3**	44.84 ± 2.4
Birds colonized with strain B (multiple antibiotic-resistant, MAR)												
B	–	pre	16	0.06	4	4	16	0.5	T	wt	18.83 ± 1.5	100.26 ± 0.7
F	–	7	8	0.06	4	4	16	0.5	T	wt	22.36 ± 1.3	66.77 ± 2.1
N	–	21	8	0.06	4	4	16	0.5	T	wt	11.84 ± 1.7	71.13 ± 6.8
G	+	7	>128	0.25	4	4	16	0.5	T	Asn-82	38.39 ± 1.8	99.56 ± 0.9
H	+	7	>128	1	4	4	16	0.5	T	Phe-83	27.76 ± 2.3	101.81 ± 1.7
O	+	21	>128	0.25	4	4	16	0.5	T	Asn-82	**10.14 ± 0.2**	88.80 ± 0.5
P	+	21	>128	1	4	4	16	0.5	T	Phe-83	**9.73 ± 1.6**	56.87 ± 1.4
Birds colonized with strain C (penta-resistant, GyrA Phe-83)												
C	–	pre	>128	1	>128	128	128	0.5	T	Phe-83	19.9 ± 1.4	102.3 ± 1.7
I	–	7	>128	1	>128	128	>128	0.5	T	Phe-83	**13.6 ± 6.0**	70.3 ± 18.9
Q	–	21	>128	1	>128	128	>128	0.5	T	Phe-83	18.8 ± 0.7	89.8 ± 1.1
J	+	7	>128	1	>128	128	>128	0.5	T	Phe-83	**10.8 ± 1.9**	61.9 ± 3.3
R	+	21	>128	1	>128	128	>128	0.5	T	Phe-83	**12.5 ± 1.2**	96.78 ± 1.2
Experiment II												
Birds colonized with strain A (wild-type)												
A4	+	2	>128	0.5	2	1	4	0.25	S	Phe-83	ND	ND
A14	+	7	>128	0.25	2	1	4	0.25	S	Phe-83	ND	ND
A23	+	35	>128	0.12	2	1	4	0.25	S	Phe-83	ND	ND
Birds colonized with strain B (MAR)												
B8	+	2	32	0.12	4	4	16	0.25	T	wt	ND	ND
B18	+	7	32	0.12	4	4	16	0.5	T	wt	ND	ND
B24	+	35	32	0.25	4	4	16	0.5	T	wt	ND	ND

NAL, nalidixic acid; CIP, ciprofloxacin; AMP, ampicillin; CHL, chloramphenicol; TET, tetracycline; TRI, triclosan; CYC, cyclohexane; S, sensitive; T, tolerant; CIP SSC, steady-state concentration after 5 min of exposure to ciprofloxacin (ng ciprofloxacin/mg dry weight cells); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ND, not determined; wt, wild-type. Bold, indicates those isolates for which the concentration of ciprofloxacin accumulated is significantly (Student's *t*-test) lower than the originating inoculum strain.

[a]Birds with (+) or without (–) enrofloxacin (Baytril) treatment.

[b]Days post the end of antibiotic treatment.

Cloacal swabbing and shedding of Salmonella

A semi-quantitative system was used to monitor the colonization of chicks by each strain. For both experiments, cloacal swabs were taken when the birds were 6 days old (pre-antibiotic challenge) and then 1 or 2, 7, 14, 21, 28 and 35 days after the end of antibiotic dosing as described previously[31] with 10 birds swabbed at each time point. Swabs were emulsified in 1 mL of sterile saline, decimal dilutions were then prepared from the original saline suspension to a maximum dilution of 1/10 000 and 100 µL of relevant dilutions was spread onto Brilliant Green Agar (BGA, Oxoid CM 329). If plating of cloacal swab dilutions failed to yield suitable numbers of colonies for replica plating, an enrichment procedure was used; 10 mL of selenite broth (Oxoid R39) was inoculated with 0.5 mL of the neat cloacal swab suspension and subsequently incubated at 37°C for up to 7 days.[31]

At 35 days post-antibiotic treatment, all birds were sacrificed following swabbing. In order to relate the numbers of colonies recovered from swabs to cfu/g of faeces, viable counts of *S. enterica* in caecal contents from representative birds were determined using the method described previously.[31,32]

In experiment II, swabs were taken from birds dosed with strain A or strain B (but not for birds dosed with strain C, as in the previous

experiment this strain did not show increased susceptibility to fluoroquinolones) during each day of antibiotic treatment in addition to the swabbing before and after treatment as in experiment I for all groups.

At each stage of sampling, five isolates were selected at random from each group of both treated and untreated birds from either the original isolation plates or from replica plates containing antibiotic. MIC values were determined for representative isolates from experiment I (n = 101) and from experiment II (n = 91). These isolates represented all the different phenotypes observed by replica plating.

Replica plating

To determine the emergence of strains with reduced susceptibility during and after antibiotic treatment, original swab isolation plates were replica plated onto BGA containing four times the MIC of ciprofloxacin or nalidixic acid as determined in BGA (e.g. to detect strains with reduced susceptibility). To determine the emergence of tolerance to cyclohexane, an indicator of MAR, original swab isolation plates were replica plated onto LB agar in glass Petri dishes and tolerance determined as described previously.[33]

Determination of MIC values

The susceptibility of all strains to ampicillin, ciprofloxacin, nalidixic acid, tetracycline and triclosan was determined by the method of the British Society for Antimicrobial Chemotherapy (BSAC).[34] MIC values were also determined on BGA to ensure the correct concentration of each agent was used for replica plating.

Strain identification post-infection

Isolates were confirmed as presumptive *Salmonella* on the basis of distinctive red colour colonies on BGA. Some isolates, including strains named D to T in Table 1 were confirmed as *S.* Typhimurium DT104 using methods described previously.[35,36] Plasmid profiles were determined for strains A to T (Table 1) as previously described.[37] This was to ensure strains had not acquired plasmids from the resident chick gut flora. In addition, PFGE profiles were determined for strains A to T (Table 1) using methods previously described.[38]

Determination of mutations within the quinolone resistance determining regions (QRDRs) of gyrA, gyrB, parC and parE

Isolates were cultured overnight in LB at 37°C and harvested for subsequent isolation of DNA for use in the PCR using DNAace spin cell culture kits (Bioline). The QRDRs of *gyrA*, *gyrB*, *parC* and *parE* were amplified as described previously.[15,16] Mutations within the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* were determined by DHPLC analyses using the WAVE DNA fragment analysis system (Transgenomic Inc., USA) as described previously.[15,16]

Accumulation of ciprofloxacin

The accumulation of ciprofloxacin (10 mg/L) with and without the presence of 100 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was determined as described previously.[39]

Expression of acrB, acrD, acrF, marA and soxS

Bacteria were cultured with shaking until the cell density reached an absorbance at 600 nm of 0.4–0.5. Cultures (3 mL) were chilled on ice and harvested by centrifugation at 4°C. Total RNA was isolated on at least three separate occasions, using RNAce Spin Cell Mini Kits (Bioline) as described previously.[23] Expression of each gene was determined by comparative RT–PCR (C-RT–PCR) as follows. Complementary DNA (cDNA) was synthesized from RNA template using Superscript II H^- Reverse transcriptase (GibcoBRL) and random primer oligonucleotides (mostly hexamers) (Invitrogen) according to the manufacturer's instructions. PCR amplification of cDNA was carried out using primer sets for 16S rRNA, *soxS*, *marA*, *acrB*, *acrF* and *acrD*. The PCR products were quantified by DHPLC using the WAVE DNA fragment analysis system (Transgenomic Inc.), as described previously.[23] Data are presented as mean ± standard deviation (SD) from at least three independent PCR amplifications from each RNA preparation.

Statistics

For the chicken studies, a two-way analysis of variance (ANOVA) was used. The counts were transformed to their logarithm to base 10 (x+1) because of the dependence of the variance on the mean. For each time point, an analysis of variance was performed to test for the effects of antibiotic (–/+), strain (A/B/C) and their interaction. This measured whether the effect of the antibiotic varied between strains. For data where the ANOVA analyses indicated that $P \leq 0.05$, Student's t-tests were performed to test the effect of antibiotic exposure for each strain separately.

For the expression studies (C-RT–PCR) and accumulation experiments, all values were compared to those obtained for the respective colonizing strain (A, B or C) in a two-tailed Student's t-test. P values <0.05 were taken as significant.

Results

Shedding of Salmonella from infected chicks

In experiment I, shedding of strains A, B and C was constant and in similar numbers from all untreated birds over the course of the experiment (Figure 1a). Treatment of birds colonized with strain A with enrofloxacin reduced significantly ($P < 0.001$) the numbers of *Salmonella* shed from a mean of $\sim 10^5$ cfu/swab before antibiotic treatment to means of ~40 and 2 cfu/swab at days 1 and 7 post-treatment, respectively. During this period, only 1/9 birds infected with strain A and treated with enrofloxacin was colonized with *Salmonella*, but the numbers of birds in this group colonized increased after day 7 with 5/9, 8/8, 8/8 and 8/8 birds colonized at days 14, 21, 28 and 35, respectively (Figure 1a). Shedding of strains B and C were largely unaffected by enrofloxacin treatment in experiment I (Figure 1a). Of interest, the numbers of strains B and C were higher after enrofloxacin treatment than in the antibiotic-free control ($P < 0.001$).

For experiment I, the mean *Salmonella* counts from the caecal contents on day 35 when the experiment was terminated were $\sim 10^7$ cfu/g faeces and these were $2–3 \times \log_{10}$ greater than the counts from the cloacal swabs taken just before the birds were killed. If one applies a correction factor to the swab counts on the basis of this difference between the counts from cloacal swabs and the cfu/g caecal contents, then the chickens were shedding $\sim 10^9$ cfu/g of faeces 6 days post-infection and this was reduced to $\sim 10^6–10^7$ cfu/g of faeces by 21 and 35 days post-treatment.

In experiment II, the mean counts of *Salmonella* were ~10-fold less than in experiment I (Figure 1b). For this experiment, birds were monitored for *Salmonella* during the 5 days of antibiotic treatment as well as before and after antibiotic treatment, as in experiment I. During the 5 days of antibiotic treatment, with the exception of one bird from which *Salmonella* were recovered

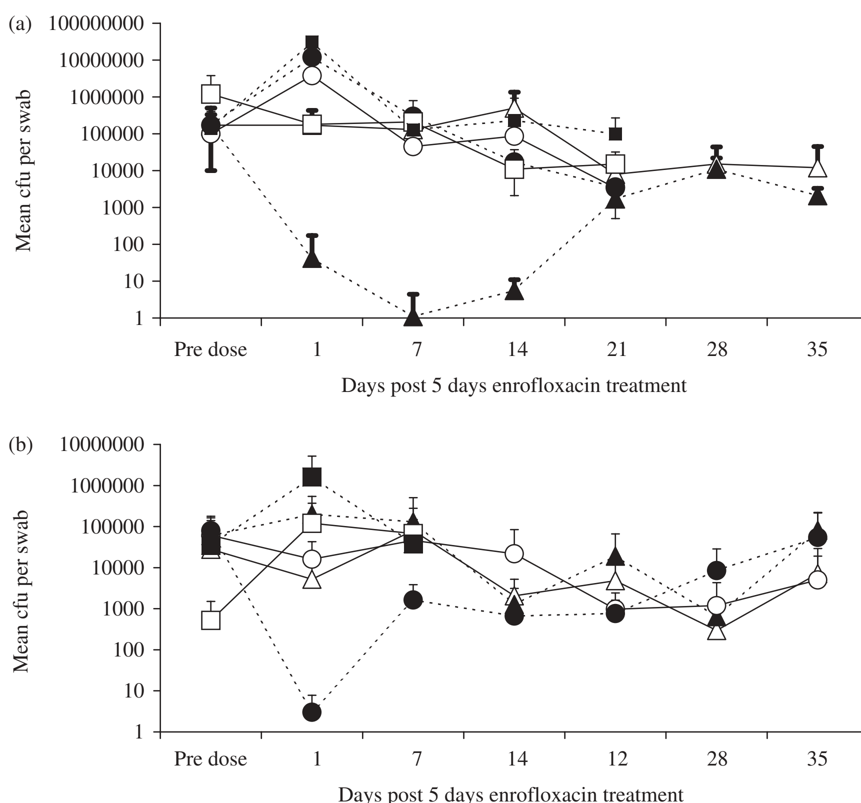

Figure 1. Mean semi-quantitative counts (cloacal swabs) of *Salmonella* Typhimurium DT104 from chickens before and after enrofloxacin treatment. (a) Experiment I and (b) Experiment II. Open triangles, strain A; filled triangles, strain A plus enrofloxacin; open circles, strain B; filled circles, strain B plus enrofloxacin; open squares, strain C; filled squares, strain C plus enrofloxacin.

on the third day of antibiotic treatment, *Salmonella* were only recovered after the first day of treatment from birds infected with strain A (mean $\sim 10^3$ cfu/swab) and birds infected with strain B (mean $\sim 10^1$ cfu/swab). Although by day 4 of antibiotic treatment *Salmonella* were not recovered from any of the birds, they were recovered after the end of antibiotic treatment. In contrast to experiment I the numbers of strain A shed after the end of antibiotic treatment were similar to those for untreated birds (Figure 1b). In addition, and again in contrast to experiment I, the numbers of strain B shed immediately (1 day) after the end of antibiotic treatment were reduced (Figure 1b). Subsequently, by day 7 and onwards, strain B in treated birds was shed in similar numbers to birds not treated with enrofloxacin (Figure 1b).

Swabs were only taken from birds infected with strain C up to 21 days (experiment I, Figure 1a) and 7 days (experiment II, Figure 1b) after the end of antibiotic treatment as there was no evidence that the phenotype of this organism was affected by antibiotic treatment. Additionally, for experiment I (Figure 1a), swabs were not taken beyond 21 days after the end of antibiotic treatment of strain B, as in this time period, enough isolates were collected for further examination and counts were similar to those seen in birds from the control group.

Susceptibility of surviving strains

In experiment I, a mean of 1269 (±SD 878) colonies were replica plated (from a total of ~10 chickens/plates) at each time point for each strain type (e.g. A or B or C) and condition (e.g. with or without antibiotic treatment). In total, for experiments I and II, up to ~35 000 and 20 000 colonies, respectively, from many non-selective media plates, were replica plated onto media containing ciprofloxacin or nalidixic acid. For isolates obtained from birds inoculated with strain A, strains were also replica plated to plates overlaid with cyclohexane. The MICs of ciprofloxacin and nalidixic acid for isolates collected from birds colonized with strains A, B or C before antibiotic treatment were within one doubling dilution of the inoculum strain. This was also true of isolates collected from untreated birds (Table 1).

In experiment I, isolates recovered from birds colonized with strain A had the same susceptibility to antibiotics as the original strain A during the first 14 days post-antibiotic treatment (Table 1). By 21, 28 and 35 days post-treatment 0.3, 8 and 2%, respectively, of the replica plated colonies from strain A showed reduced susceptibility to nalidixic acid, but all were susceptible to ciprofloxacin, as determined by replica plating to BGA + 4× MIC concentrations of nalidixic acid or ciprofloxacin (representative isolates shown in Table 1). Between 1 and 21 days post-enrofloxacin treatment, all isolates recovered from strain B showed reduced susceptibility to nalidixic acid (representative isolates shown in Table 1). At day 1, 50% of isolates showed reduced susceptibility to ciprofloxacin rising to 91% of isolates 21 days after enrofloxacin treatment as determined by replica plating to BGA + 4× MIC concentrations of nalidixic acid or ciprofloxacin (representative isolates shown in Table 1). All strain C isolates had the same susceptibility to antibiotics as the original strain C.

In experiment II, for birds inoculated with strain C, similar data were obtained as in experiment I. However, in this experiment, after treatment with enrofloxacin, 100% of colonies from birds

colonized with strain A showed reduced susceptibility to nalidixic acid and ciprofloxacin as determined by replica plating to BGA + 4× MIC concentrations of nalidixic acid or ciprofloxacin (representative isolates shown in Table 1). The isolates from birds inoculated with strain B had the same susceptibility as the inoculating strain B.

MICs and cyclohexane tolerance

Strains B and C were originally cyclohexane-tolerant and all isolates recovered from birds treated with strain B and C were tolerant to cyclohexane.

In experiment I, at 21, 28 and 35 days post-treatment 47, 64 and 79%, respectively, of the replica-plated colonies from strain A were cyclohexane-tolerant (Table 1). From experiment I, most post-treatment isolates of strain A from treated birds that were tested had become cyclohexane-tolerant (e.g. strains L, M, S and T). These were less susceptible to nalidixic acid and ciprofloxacin, than isolates for strain A from untreated birds. The MICs of ampicillin, chloramphenicol, tetracycline and triclosan were unchanged for most isolates, with the exception of some isolates of strain A which had become cyclohexane-tolerant, and these in some instances showed a two- to fourfold decrease in susceptibility. Post-treatment isolates collected from birds colonized with strain B (e.g. strains G and H) were less susceptible to nalidixic acid and ciprofloxacin than those isolated from birds colonized with strain A (Table 1). The MICs of ampicillin, tetracycline and triclosan were essentially the same for strain B pre- and post-treatment. Strain C was resistant to nalidixic acid, ampicillin, tetracycline and cyclohexane and showed reduced susceptibility to ciprofloxacin and triclosan, and all isolates collected from birds colonized with this strain showed a similar phenotype.

Typing of strains

As birds were obtained as specific-pathogen-free, housed in isolators which had been sterilized, and were infected at 1 day old, it was highly unlikely that any *Salmonella* obtained from the birds were other than the strains used to infect the birds. However, to test that *Salmonella* obtained from birds were identical to parent strains, representative isolates (strains D to T of Table 1) from experiment I obtained at different sampling times were analysed by serotyping, phage typing, plasmid profiling and PFGE.

All strains were confirmed as *S.* Typhimurium DT104. The plasmid profiles of the representative isolates collected from each group of birds were identical with those of the original colonizing strain A, B or C, suggesting no plasmids were acquired during passage through the birds and the PGFE profiles of strains derived from parents strains A or B or C were identical with the parent strains (Figure 2, PFGE profiles for strain B and derivatives). This indicates that all birds were colonized with the parent strains or mutants derived from them and not with any contaminating strains. It also indicates that the cyclohexane-tolerant strain that re-colonized birds 14 days and onwards after enrofloxacin treatment (Figure 1a) was a mutant of the parent strain and not due to contamination from outside the environment of the isolators.

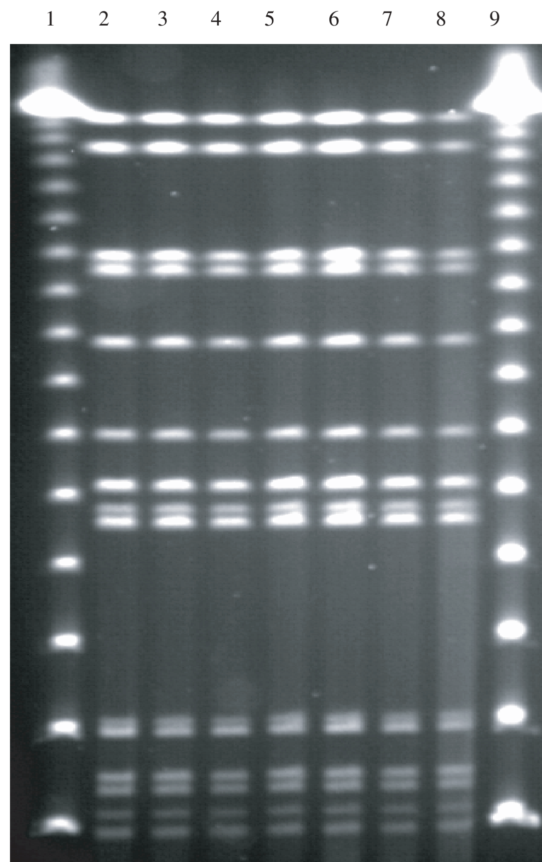

Figure 2. PFGE profile of strain B and derivatives from chicks. Lanes 1 and 9, Lambda DNA PFG ladder; lane 2, strain B; lane 3, strain F; lane 4, strain N; lane 5, strain G; lane 6, strain H; lane 7, strain O; lane 8, strain P.

Determination of mutations within the QRDRs of gyrA, gyrB, parC and parE

Strains A, B and C plus 17 and 21 isolates (from experiments I and II, respectively), representative of those isolated from birds colonized with each strain were tested for mutations within the QRDRs of *gyrA*, *gyrB*, parC and *parE* by DHPLC. Parent strains A and B had wild-type QRDR regions of *gyrA*. Parent strain C contained a single mutation encoding a Phe-83 substitution. None of the parent strains had a mutation within the QRDR regions of *gyrB*, *parC* or *parE*. In experiment I, post enrofloxacin exposure, all isolates collected from birds colonized with strain A had wild-type QRDRs in *gyrA*. Isolates F and N were collected from birds colonized with strain B and had not been treated with enrofloxacin; these contained wild-type *gyrA* QRDRs. However, the post-treatment isolates from these birds (isolates G and O) contained mutations that encoded an Asn-82 substitution within the QRDR of GyrA. The post-treatment isolates H and P, also from birds colonized with strain B, contained mutations that encoded a Phe-83 substitution within the QRDR. All isolates collected from experiment I birds colonized with strain C contained the same substitution in GyrA as the parent strain C. In experiment II post enrofloxacin exposure, all isolates collected from birds colonized with strain A contained a mutation that encoded a Phe-83 substitution in GyrA. All isolates colonized with strain B had wild-type GyrA QRDR. Isolates from strain C were not examined.

Accumulation of ciprofloxacin

Only isolates from experiment I were examined (Figure 3). Strain A accumulated twofold more ciprofloxacin than strains B and C

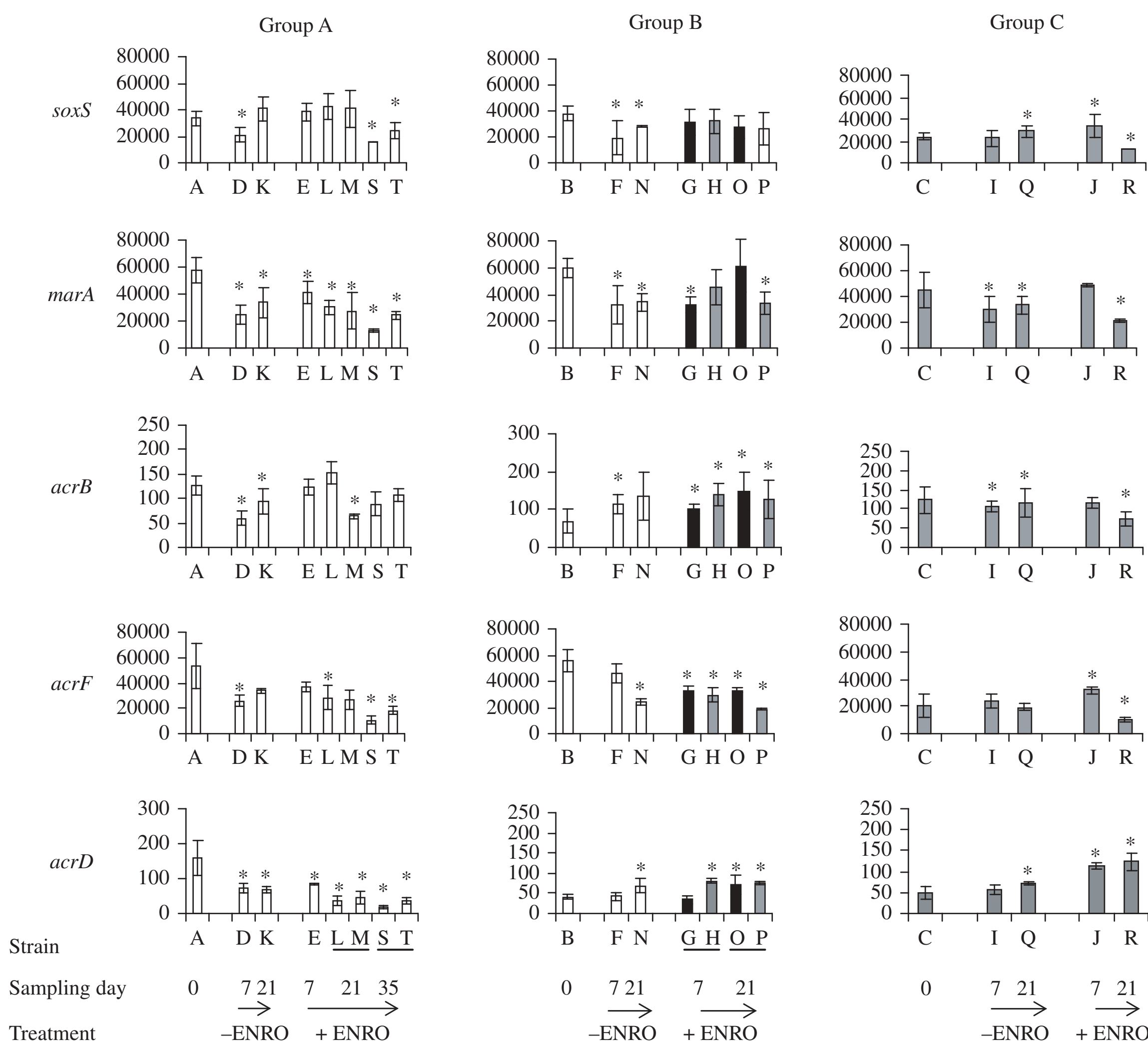

Figure 3. Expression of *soxS, marA, acrB, acrD* and *acrF* in representative isolates from experiment I. Arbitrary units ($\times 10^3$). Open bars, no mutation in *gyrA*; grey bars, GyrA Phe-83; filled bars, GyrA Asn-82. Capital letters refer to individual strains as shown in Table 1. *Mean values significantly different from those for respective parent strains.

(Table 1). The uncoupler CCCP increased the accumulation of ciprofloxacin to equivalent levels in strains A, B and C. All isolates collected from birds colonized with strain A accumulated less ciprofloxacin (11.48 to 30.76 ng ciprofloxacin/mg dry weight cells) than did the parent strain A (39.86 ng ciprofloxacin/mg dry weight cells). After the addition of CCCP, the amount of ciprofloxacin accumulated did not resume to that found in the parent strain A (Table 1). The concentration of ciprofloxacin accumulated after the addition of CCCP was less for the isolates from enrofloxacin-treated birds (isolates E, L, M, S, T) than those from the untreated ones (isolates D, K). Three isolates (F, G and H) collected from birds colonized with strain B accumulated more ciprofloxacin (22.36 to 38.39 ng ciprofloxacin/mg dry weight cells) than did the parent strain B (18.83 ng ciprofloxacin/mg dry weight cells). Isolates N, O and P accumulated less ciprofloxacin (9.73 to 11.84 ng ciprofloxacin/mg dry weight cells) than the parent strain B. After the addition of CCCP, accumulation of ciprofloxacin resumed to levels equivalent to parent strain B in isolates G and H (7 days post-enrofloxacin treatment) but not in isolates F, N, O and P (Table 1). Three (I, J, R) out of four isolates collected from birds colonized with strain C accumulated less ciprofloxacin (10.80 to 13.60 ng ciprofloxacin/mg dry weight cells) than strain C (19.90 ng ciprofloxacin/mg dry weight cells). After the addition of CCCP, the concentration accumulated was increased.

Expression of soxS, marA, acrB, acrF and acrD

Only isolates from experiment I were analysed (Figure 3). The levels of expression of *soxS, marA* and *acrF* in strain B were similar to those in strain A. The expression of *acrB* and *acrD* in strain B was significantly lower than in strain A (P = 0.008 and 0.0487, respectively).

Expression of *marA* was decreased 0.2- to 0.8-fold in all strains isolated from all three groups of birds irrespective of antibiotic treatment. Expression of *soxS* varied ±0.5-fold, with no association with treatment or phenotype of the isolate. Expression of *acrB* was increased 0.45- to 1.16-fold in all post-treatment isolates, but not those from untreated birds, colonized with strain B. These were statistically significant differences ($P < 0.05$). Isolates H, O and P also expressed more *acrD* than did strain B (0.77- to 0.99-fold

more; $P < 0.05$). Four of seven isolates from birds colonized with strain A and 5/6 isolates from birds colonized with strain B expressed 0.4- to 0.8-fold less *acrF* than strains A and B, respectively. These were statistically significant differences ($P < 0.05$). Post-treatment isolates J and R expressed more *acrD* (1.40- to 1.58-fold) than did strain C.

Discussion

Barrow *et al.*[40] showed that in chickens, whilst enrofloxacin treatment largely eliminated an experimental infection with *S.* Enteritidis, it caused the resident *E. coli* population to become fluoroquinolone-resistant. Subsequently, Wilkinson *et al.*[41] showed that enrofloxacin treatment failed to eradicate an *S. enterica* infection in a colony of laboratory short-tail grey opossums. More recently, Wiuff *et al.*[42] showed that enrofloxacin treatment of pigs selected for fluoroquinolone resistance in the resident coliform population and selects for an experimentally introduced strain of *Salmonella* with reduced susceptibility to ciprofloxacin.

In our study, we examined strains with different resistance phenotypes. Our data indicate that strain C, *S. enterica* serovar Typhimurium DT104 (ciprofloxacin MIC 1 mg/L), which also had a pentavalent resistance pattern and a type 1 integron, survived enrofloxacin treatment without attaining a higher level of resistance. It therefore seems likely that the reduced susceptibility to fluoroquinolones of strain C was sufficient to allow it to survive antibiotic challenge in chickens. However, both the quinolone-susceptible strain (strain A, which lacked a type 1 integron) and the isogenic MAR derivative (strain B) only survived enrofloxacin treatment in high numbers by becoming less susceptible.

It is unclear why the results from the two experiments were different, with *gyrA* mutants being selected only from strain B in experiment I and only from strain A in experiment II. Mutant selection is itself a random and variable process and previous *in vitro* data showed that fluoroquinolones can select either MAR or GyrA mutants.[43] Nonetheless, this study indicates that fluoroquinolones can select for reduced susceptibility from fully sensitive or MAR strains. The re-colonization of birds in experiment I after the end of antibiotic treatment with the fully sensitive strain A isolates probably represents re-colonization from the environment in the isolators when there is no longer any antibiotic selective pressure. Since these experiments were performed, a further two experiments have been performed as part of a separate study. Enrofloxacin treatment of chicks again selected for *Salmonella* strains with reduced susceptibility to fluoroquinolones.

The serotyping, phage typing, plasmid and PFGE profiling of the isolates derived from strains A and B showed that they were identical (except in some instances in antibiotic and cyclohexane sensitivity) to those used to dose the chicks. Coupled with the fact that the birds were specific-pathogen-free and were housed in isolators, this confirms that the isolates with differing reduced susceptibility phenotypes selected by antibiotic treatment represented mutants of the parent strains rather than any sort of contamination. Experiment II confirmed the observations of experiment I. However, in experiment II, strain A, not strain B, showed reduced susceptibility via mutation in *gyrA*. These data confirm that enrofloxacin treatment of chicks can select for reduced susceptibility from fully quinolone-susceptible strains of *S.* Typhimurium DT104 or MAR isolates of *S.* Typhimurium DT104.

Essentially, isolates from birds colonized with strain A, B or C fell into two phenotypes: those that were MAR and those that showed reduced susceptibility to quinolone only. All isolates with high MICs (>128 mg/L) of nalidixic acid contained a mutation in *gyrA*. In experiment I, all isolates from birds colonized with strain A had no mutation in *gyrA*, or increased expression of any gene examined. However, low concentrations of ciprofloxacin were accumulated (compared with strain A) by these isolates irrespective of antibiotic treatment. In experiment I, most of the isolates from birds colonized with strain B treated with enrofloxacin accumulated lower concentrations of ciprofloxacin, and this was associated with increased expression of *acrB* and *acrD*. Isolates from birds colonized with strain C treated with enrofloxacin also accumulated lower concentrations of ciprofloxacin but only expressed more *acrD*.

Despite focusing upon determining the expression of genes previously implicated in MAR, there was no clear relationship between increased gene expression and phenotype. It is hypothesized that the variation in expression of *soxS* reflected the natural variation in expression of this gene. The observation that expression of *marA* was consistently decreased suggests that expression of this gene was influenced by passage through the chicken gut, although this would require further work to confirm. Expression of the efflux pump genes *acrB*, *acrD* and *acrF* has previously been shown to influence expression of each other and *marA*,[23] and so the varying levels of expression observed in this study may reflect this feedback mechanism. Although it is tempting to speculate that increased expression of *acrB* in the isolates from birds colonized with strain B confers MAR, or that increased expression of *acrD* in the isolates from birds colonized with strain C confers MAR, further experimentation is warranted. It is possible that some bacterial genes were up-regulated within chickens, but that this was transient and therefore not possible to measure once the bacteria were outside the environment of the chicken. It is interesting to note that previous workers have shown that the AcrAB system of *E. coli* appears to play a significant role in bile efflux.[25]

In experiment II, strains with reduced susceptibility to quinolones and mutations in *gyrA* were selected from strain A and not strain B. Strain B colonized chickens to a similar extent as strain A (the isogenic parent strain), suggesting no attenuation, at least of colonization potential, of the avian gastrointestinal tract, with development of MAR, although to fully confirm this, the two strains would need to be in competition with each other in the same birds and such experiments are planned. It was interesting to note that in experiment I, the MICs of ciprofloxacin for *gyrA* mutants selected following antibiotic treatment of strain B (MAR) were higher (MIC 0.25–1 mg/L) than *gyrA* mutants selected following antibiotic treatment of strain A in experiment II (MICs 0.12–0.5 mg/L). This correlates with previous findings that *Salmonella* which are both cyclohexane-tolerant (e.g. have a MAR phenotype) and have a mutation in *gyrA* tend to show higher MICs to ciprofloxacin than cyclohexane-sensitive strains with a mutation in *gyrA*.[24] These data suggest that MAR strains, although initially sensitive to ciprofloxacin, may give cause for extra concern as they can attain a higher level of reduced susceptibility upon acquisition of a mutation in *gyrA*. Recent *in vitro* studies have also demonstrated that exposure of wild-type susceptible *Salmonella* to ciprofloxacin gave rise to strains inhibited by ciprofloxacin MICs of 0.12 mg/L, whereas MAR strains gave rise to strains inhibited by 2 mg/L ciprofloxacin.[43]

Giraud *et al.*[44] recently suggested that *S.* Typhimurium with mutation in *gyrA* were less fit and less able to colonize chicks than the wild-type isolate from which they were derived. In the

study of Giraud *et al.*,[44] the stains colonizing the chickens carried a different mutation in *gyrA* from those seen in this study and so are not directly comparable with the results of this study. However, data from this study demonstrated that *gyrA* mutants were able to colonize chicks, and once established in the chicks, in both experiments, they persisted until the end of the experiment. This was demonstrated for *gyrA* mutants derived from a fully sensitive strain, from a MAR derivative of a fully sensitive strain and for a *gyrA* mutant in the background of a pentavalent-resistant strain with a class 1 integron. Therefore, this study demonstrated that not only can enrofloxacin treatment of *Salmonella* in chickens rapidly select from strains with reduced susceptibility to fluoroquinolones, but also that such strains can persist and thus could be a threat to human health through contamination along the food chain.

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