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Modification of Enrofloxacin Treatment Regimens for Poultry Experimentally Infected with *Salmonella enterica* Serovar Typhimurium DT104 To Minimize Selection of Resistance▽

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We hypothesized that higher doses of fluoroquinolones for a shorter duration could maintain efficacy (as measured by reduction in bacterial count) while reducing selection in chickens of bacteria with reduced susceptibility. Chicks were infected with *Salmonella enterica* serovar Typhimurium DT104 and treated 1 week later with enrofloxacin at the recommended dose for 5 days (water dose adjusted to give 10 mg/kg of body weight of birds or equivalence, i.e., water at 50 ppm) or at 2.5 or 5 times the recommended dose for 2 days or 1 day, respectively. The dose was delivered continuously (ppm) or pulsed in the water (mg/kg) or by gavage (mg/kg). In vitro in sera, increasing concentrations of 0.5 to 8 μg/ml enrofloxacin correlated with increased activity. In vivo, the efficacy of the 1-day treatment was significantly less than that of the 2- and 5-day treatments. The 2-day treatments showed efficacy similar to that of the 5-day treatment in all but one repeat treatment group and significantly ($P < 0.01$) reduced the *Salmonella* counts. Dosing at 2.5× the recommended dose and pulsed dosing both increased the peak antibiotic concentrations in cecal contents, liver, lung, and sera as determined by high-pressure liquid chromatography. There was limited evidence that shorter treatment regimens (in particular the 1-day regimen) selected for fewer strains with reduced susceptibility. In conclusion, the 2-day treatment would overall require a shorter withholding time than the 5-day treatment and, in view of the increased peak antibiotic concentrations, may give rise to improved efficacy, in particular for treating respiratory and systemic infections. However, it would be necessary to validate the 2-day regimen in a field situation and in particular against respiratory and systemic infections to validate or refute this hypothesis.

Salmonella enterica serovars Enteritidis and Typhimurium are recognized as the leading *Salmonella* serovars that cause food poisoning in humans in the United States (33) and Europe (3, 4, 37). Illness associated with quinolone-resistant *S. enterica* serovar Typhimurium has been associated with a mortality rate 3.15 times higher than that observed with infection for pansusceptible strains (22). Ciprofloxacin is one of the drugs of choice for treating invasive human salmonellosis (17, 34, 39), and resistance of *S. enterica* to quinolones is a matter of public health concern.

Consumption of contaminated raw or undercooked poultry products (both eggs and meat) is the main vehicle of *Salmonella* infections of humans (11, 14, 33). Fluoroquinolones are used in the poultry industry in various ways in different countries. For example, fluoroquinolones may be used to control colibacillosis (12, 20) or mycoplasma infections (23, 35) or as an aid to control *S. enterica* and other bacterial infections in commercial poults (13). However, we have showed that the recommended fluoroquinolone treatment for chickens experimentally infected with *S. enterica* serovar Typhimurium DT104 rapidly selected for strains with reduced susceptibility to fluoroquinolones (30). Preventing the selective enrichment of mutant bacterial populations may help to restrict the development of antibiotic resistance, and the mutant prevention concentration (MPC) has been defined as the lowest concentration of antibiotic to inhibit the emergence of mutants from 10^{10} bacterial CFU (15).

Regimens that result in a C_{max}/MIC (C_{max}, maximum concentration of drug in serum) ratio of >10 have been considered optimal to prevent selection of resistance, and an AUC/MIC (AUC, area under the concentration-time curve) ratio of >125 in humans and animals is required for efficacy for gram-negative bacteria (26). However, many antibiotic dosage regimens were originally designed to maintain serum drug concentrations above the MIC for all or most of the dosing period, which assumes that the bactericidal action of the antibiotic is time, not concentration, dependent (26) and does not consider the concept of the mutant selection window (15). Several studies with humans and animals have indicated that fluoroquinolones should be administered at high doses over short periods to optimize their therapeutic effects (16, 18, 26, 32). For example, danofloxacin administered as a single intravenous bolus (0.738 mg/kg of body weight) was significantly better at reducing the counts of *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) in bronchial secretions of calves and resulted in a higher C_{max}/MIC ratio (14.5 compared to 2.3) than the same amount of danofloxacin given as a continuous intravenous infusion over 36 hours (25), and escalating the dose of

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enrofloxacin in pigs against experimental *S. enterica* serovar Typhimurium infection reduced the selection of resistant strains (40).

In view of the above, the aims of this study were to determine if modifying the dosage regimen of enrofloxacin in poultry could improve the pharmacokinetics and pharmacodynamics to maintain or improve efficacy but reduce selection of strains with reduced susceptibility/resistance.

MATERIALS AND METHODS

Bacterial strains. Strains A (fully antibiotic sensitive) and B (isogenic multiple-antibiotic-resistant [MAR] mutant of A) of *S. enterica* serovar Typhimurium DT104 described previously were used throughout (30). Two nonisogenic *gyrA* mutants (strain C, VLA 5120/98, cyclohexane sensitive, ciprofloxacin MIC of 0.25 μg/ml, and strain D, VLA 2590/99, cyclohexane tolerant, ciprofloxacin MIC of 1 μg/ml) were also used for in vitro studies only. For enrofloxacin (ciprofloxacin), the MICs/MPCs for strains A, B, and C were 0.03/0.5 (0.03/0.25) μg/ml, 0.06/4 (0.06/2) μg/ml, and 0.5/4 (0.5/1) μg/ml, respectively (29).

Strains NCTC 10418, AG100, and AG102 were used as controls for determination of MICs (2) and cyclohexane tolerance (5). Cultures were grown overnight at 37°C in Luria-Bertani (LB) broth prior to infection of chicks, MIC determinations, and time-kill studies.

Antimicrobials, chemicals, and sera. Enrofloxacin (Baytril 10% oral solution) for dosing poultry was obtained from the National Veterinary Services, United Kingdom. Ciprofloxacin and enrofloxacin for MIC determinations and high-pressure liquid chromatography (HPLC) were kindly donated by Bayer HealthCare AG (Germany). Other antibiotics, organic solvents, and chicken sera for in vitro studies were obtained from Sigma-Aldrich (Poole, Dorset, United Kingdom).

Activity of enrofloxacin in vitro. The effect of increasing concentrations of enrofloxacin was determined in vitro against *Salmonella* in cecal contents, sera, and physiological (0.85%) saline. Cecal contents were collected from healthy untreated birds at necropsy. Serum (2 ml) was inoculated with $\sim 10^5$ CFU/ml (to correspond to the level of *Salmonella* in a severe systemic infection) of strain A, B, C, or D; cecal contents (2 g) and saline (2 ml) were inoculated with $\sim 10^8$ CFU/gram/ml (to correspond to the upper level of *Salmonella* in cecal contents of infected birds) of strain A, B, or D. For serum, enrofloxacin was added at 0 (control), 0.5, 1, 2, 4, and 8 μg/ml (to correspond to the determined concentration range in sera following recommended and modified dosage regimens). For cecal contents and saline sample, enrofloxacin was added at 0, 12.5, 25, 50, 100, and 200 μg/ml (to correspond to the determined concentration range in cecal contents following recommended and modified dosage regimens). The effect of enrofloxacin against strain A or B was determined only for cecal contents. Samples were incubated at 41.5°C (the normal body temperature of chickens), and viable counts of *Salmonella* were performed on selective agar (Rambach agar; Merck, Darmstadt, Germany) by the method of Miles et al. (27) at 0, 3, 6, and 24 h after addition of enrofloxacin.

Binding of enrofloxacin to cecal contents and sera. Sera and saline (as control) were spiked with 2 and 20 μg/ml and cecal contents with 20 and 200 μg/ml enrofloxacin and allowed to stand for 1 hour at 37°C (for binding equilibrium to occur). Complexes were then centrifuged for ~30 min (until enough filtrate was collected) in a Vivaspin 15 (Vivascience AG, Hannover, Germany) column with a molecular mass cutoff of 10,000 Da. Original samples and filtrates were frozen at −80°C prior to quantification of enrofloxacin (and ciprofloxacin if present) by HPLC.

Dose calculations and routes of administration. Current data sheets for Baytril 10% oral solution recommend adding enrofloxacin to the drinking water, based on the calculated amount of water drunk in the house, to give 10 mg/kg of body weight of birds per day for 5 to 10 days for treatment of salmonellosis in poultry. However, previous data sheets (2003) recommended a dose of 50 ppm enrofloxacin added to the drinking water each day for 5 to 10 days; the two approaches are considered equivalent. Routes of administration were compared and included the data sheet-recommended method (continuously medicated water) compared to pulsed water treatment ("breakfast regimen"; dose given to birds before lights come on and before they start eating or drinking) and dosing by oral gavage.

Antibiotic dose was calculated as mg/kg per individual bird (gavage dosing) or mg/kg per group of birds given in one-third of the previously calculated amount of water drunk daily (pulsed dosing) or water at 50, 125, or 250 ppm enrofloxacin (continuous dosing). For the pulsed dose, once the water was drunk, it was replaced with fresh water. Birds received antibiotic at the beginning of each day before the lights in their accommodation came on.

Chick experiments—efficacy. All animal studies were conducted under the jurisdiction of the Animals Scientific Procedures Act (1986) and were reviewed by the local ethical review committee. For experiments I, II, III, and IV, 95, 150, 103. and 100 specific-pathogen-free (SPAFAS chicks specially reared and checked to be free of specific pathogens such as *Salmonella*) White Leghorn chicks (1 day old) were randomly separated into the appropriate number of groups for the experiment (Table 1). Each group of birds was housed together, but separately from other groups, with appropriate biosecurity between groups. Chicks received feed and water ad libitum and were monitored for condition twice daily throughout the experiments.

At 3 to 4 weeks of age birds (Table 1 shows bird numbers per group) were infected by gastric gavage (10) with a standardized inoculum of $\sim 10^6$ CFU per bird of *S. enterica* serovar Typhimurium strains A and B in a 1:1 mix in phosphate-buffered saline (0.1 ml of phosphate-buffered saline containing $\sim 10^7$ CFU/ml *Salmonella*), although in experiment III the birds received a lower ($\sim 10^4$ CFU per bird) dose in order to investigate response to antibiotic treatment in birds with a lower colonization level. Strains A and B were given together to try to establish if antibiotic treatment selected strain B (the isogenic MAR mutant) in preference to strain A. In order to neutralize the crop acid, chicks were dosed orally by gavage with 0.2 ml of 10% sodium bicarbonate approximately 30 min prior to dosing by oral gavage with 0.1 ml of the *Salmonella* solution (7).

One week postinfection birds were treated with the different treatment regimens of enrofloxacin (Baytril 10% oral solution). In each experiment the 5-day data sheet-recommended treatment for salmonellosis (10 mg/kg or 50 ppm) was compared with 2 days at 125 ppm or 25 mg/kg. In experiments I, II, and III, comparisons were also made to a 1-day treatment at 250 ppm or 50 mg/kg. The shorter courses of antibiotic overall delivered the same amount of antibiotic as the 5-day treatment regimen. In experiment II half of the birds for each treated group were moved to clean accommodation 6 hours after the last antibiotic dose to determine if this affected recolonization of the birds and the extent of antibiotic resistance seen post-antibiotic treatment. The *Salmonella* organisms present before, during, and after antibiotic treatment were enumerated by both semiquantitative (cloacal swabs) and quantitative (enumeration of *Salmonella* organisms from 1 g of cecal contents) methods as described in our previous study (30).

Chick experiments—pharmacokinetics. To determine the antibiotic concentrations in cecal contents, liver, and sera following the different antibiotic gavage treatments in experiment II (Table 1), birds were killed at 3, 6, and 24 h (four birds per group) after antibiotic treatment.

To determine the pharmacokinetics of continuous versus pulsed water treatments, a separate experiment was performed. Eighty-day-old specific-pathogen-free White Leghorn chicks were randomly divided into four groups of 20 birds, and at 4 weeks of age the groups were treated with the continuous or pulsed water treatments at the recommended dose and 2.5× the recommended dose. Three birds were killed from each group at 2, 4, 6, 8, 10, and 24 h after the start of antibiotic treatment.

Samples of cecal contents, liver, lung, and sera taken were frozen at −80°C for subsequent analysis by HPLC. The concentrations of enrofloxacin and its metabolite ciprofloxacin in these samples were measured by HPLC (9) based on a method described previously (40).

Basic pharmacokinetic parameters such as AUC, half-life, and mean residence time were calculated using PK-Solutions software (Summit Research Services, Montrose, CO).

Replica plating, determination of MICs, cyclohexane tolerance, and *gyrA* mutations. To determine the emergence of strains with reduced susceptibility during and after antibiotic treatment, replica plating was performed as previously described (30) but only onto medium with 4× MICs (for strains A and B) of nalidixic acid or ciprofloxacin or medium overlaid with cyclohexane to detect the presence of MAR *Salmonella* (5, 28). Isolates obtained from postmortem counts, swabbing controls, and all treatment groups of birds in all experiments at 1, 7, 14, 21, and 28 days post-antibiotic treatment were replica plated. Additionally, some isolates from before treatment and during treatment (experiments I and II only) were replica plated.

MICs of ciprofloxacin, nalidixic acid, and tetracycline were determined by the method of the British Society for Antimicrobial Chemotherapy (2) against post-treatment isolates ($n \sim 40$ per experiment) selected from all treatment groups and time periods. Isolates were deliberately selected to include fully sensitive strains and strains with reduced susceptibility.

Fifty strains from experiment II were tested for mutations in the quinolone resistance-determining region of *gyrA* as previously described (31).

TABLE 1. Efficacy of recommended and modified enrofloxacin treatments against experimental *Salmonella* serovar Typhimurium infections in chickens and selection of reduced fluoroquinolone susceptibility[a]

Dose method	Expt no.	Dose level	No. of chicks/group	No. of days of dose	Mean log CFU/g cecal contents[b] (± SEM)	*P* value, treatment vs control[c]	No. of colonies replica plated during/after treatment[d]	% Colonies with reduced susceptibility[e] during/after treatment
Water, continuous	I	Control	10	NA	6.8 (0.76)	NA	400/187	0/0
		50 ppm[f]	30	5	**0.2 (1.14)**	**<0.001**	1,328/752	0.4/43
		125 ppm	30	2	**0.4 (0.86)**	**<0.001**	1,824/3,859	0/96
		250 ppm	25	1	4.3 (0.77)	0.213	969/3,089	0/14
	IV	Control	20	NA	6.0 (0.67)	NA	ND/672	ND/0
		50 ppm[f]	20	5	**<1.0 (0)**	**<0.001**	ND/825	ND/0
		125 ppm	20	2	3.5 (0.94)	0.006	ND/632	ND/0
Water, pulsed	III[g]	Control	13	NA	5.1 (0.25)	NA	ND/206	ND/0
		10 mg/kg	15	5	**0.6 (0.87)**	**<0.001**	ND/33	ND/0
		25 mg/kg	15	2	**<1.0 (0)**	**<0.001**	ND/293	ND/0
		50 mg/kg	15	1	1.9 (0.25)	<0.001	ND/335	ND/0
	IV	Control	20	NA	6.0 (0.67)	NA	ND/672	ND/0
		50 ppm	20	5	4.9 (2.84)	0.493	ND/974	ND/42
		125 ppm	20	2	3.9 (0.24)	0.005	ND/1,255	ND/23
Gavage	II	Control	24	NA	8.2 (0.69)	NA	394/1,242	0/0
		10 mg/kg	51	5	**2.7 (1.25)**	**<0.001**	2,920/2,474	42/100
		25 mg/kg	41	2	**2.0 (1.40)**	**<0.001**	1,206/2,010	0/0
		50 mg/kg	34	1	5.2 (0.69)	0.051	1,455/1,999	0/14
	III[g]	Control	13	NA	5.1 (0.25)	NA	ND/206	ND/0
		10 mg/kg	15	5	**<1.0 (0)**	**<0.001**	ND/62	ND/0
		25 mg/kg	15	2	**0.4 (1.47)**	**<0.001**	ND/206	ND/0
		50 mg/kg	15	1	2.4 (0.25)	<0.001	ND/128	ND/0

[a] NA, not applicable; ND, not determined. Values in boldface indicate counts reduced by at least 4 logs.
[b] Counts were made 1 day after the last antibiotic dose to reduce the effect of any recolonization following the end of therapy.
[c] *P* value denotes significance of antibiotic therapy in reducing *Salmonella* counts compared to control.
[d] Colonies were replica plated from many representative plates (and hence different birds) from all time points for up to 4 weeks post-antibiotic treatment.
[e] Reduced susceptibility, e.g., able to grow in the presence of 4× the ciprofloxacin MIC of the original strains.
[f] The current and recent data sheet-recommended treatment is water adjusted to give 10 mg/kg of bird or water at 50 ppm for 5 days supplied continuously in the water.
[g] In experiment III birds received a lower challenge of *Salmonella*, which resulted in a lower level of colonization.

Statistical analyses. For animal experiments, there is an ongoing requirement to use as few animals as possible. The numbers of chickens that were used in each experiment were based on numbers used in previous studies where it was possible to show statistically significant differences between *Salmonella* counts in enrofloxacin-treated and untreated birds (30) or on the numbers used in previous pharmacokinetic studies with enrofloxacin in animals (40).

The *Salmonella* counts were transformed to their logarithm to base 10. One-way or two-way analyses of variance according to the design were performed to test for treatment effects. In some of the experiments small counts were recorded as an upper limit; these were regarded as left-censored observations, and the appropriate censored regression model was fitted instead. Following a significant F test ($P < 0.05$) for the treatment effects or interactions in the analyses of variance, treatment contrasts were tested using *t* tests. Likelihood ratio tests were used for the corresponding tests in the censored regression models. Where the majority of pairwise treatment differences were of interest, Bonferroni adjustments were made to the *P* values. In one experiment where all of the observations for a treatment were censored, the Kruskal-Wallis nonparametric analysis of variance was followed by Dunnett's test for comparisons with a control treatment.

RESULTS

In vitro activity of enrofloxacin. In sera at all concentrations tested, enrofloxacin rapidly killed the fully sensitive strain A (reduction of $>10^4$ CFU/ml in 3 h), although at 24 h there was some regrowth in the presence of 0.5 and 1 μg/ml (Fig. 1A). The activity of enrofloxacin was progressively less for strains B, C, and D (Fig. 1B, 1C, and 1D, respectively).

In saline, enrofloxacin at 50 μg/ml at 6/24 h reduced the counts for strains A, B, and D to 0.025%/0.001%, 0.23%/0.023%, and 6.55%/1.04%, respectively, of the control counts at that time (results not shown).

Enrofloxacin at 50 μg/ml in cecal contents at 6 h did not reduce the counts for strains B and D (compared to controls) and reduced the counts for strain A only to 80% of control (results not shown). At 24 h there were no viable *Salmonella* organisms in control cecal contents in vitro. The pH of the cecal contents was in the range of 4.6 to 5.2.

When enrofloxacin was tested at 0, 12.5, 25, 50, 100, and 200 μg/ml against strain A or B only in cecal contents, there was no increased enrofloxacin activity with increasing concentrations of enrofloxacin as seen with sera (results not shown).

Binding of enrofloxacin to cecal contents and sera. Enrofloxacin (mean ± standard deviation) was bound 45.3% (±9.7%) and 54.8% (not determined) to cecal contents fortified with 20 and 200 μg/ml enrofloxacin, respectively, and 56.4% (±31.8%) and 50.1% (±1.6%) to sera fortified with 2 and 20 μg/ml enrofloxacin, respectively.

Calculated dose and dose uptake time. For birds that received the dose continuously in the drinking water at 50 and 125 ppm, the mean dose (± standard deviation) that they actually received when calculated for two separate days of dosing was 14.8 (±0.71) mg/kg and 32.3 (±0.42) mg/kg, respectively. On the basis of these results, the birds that received

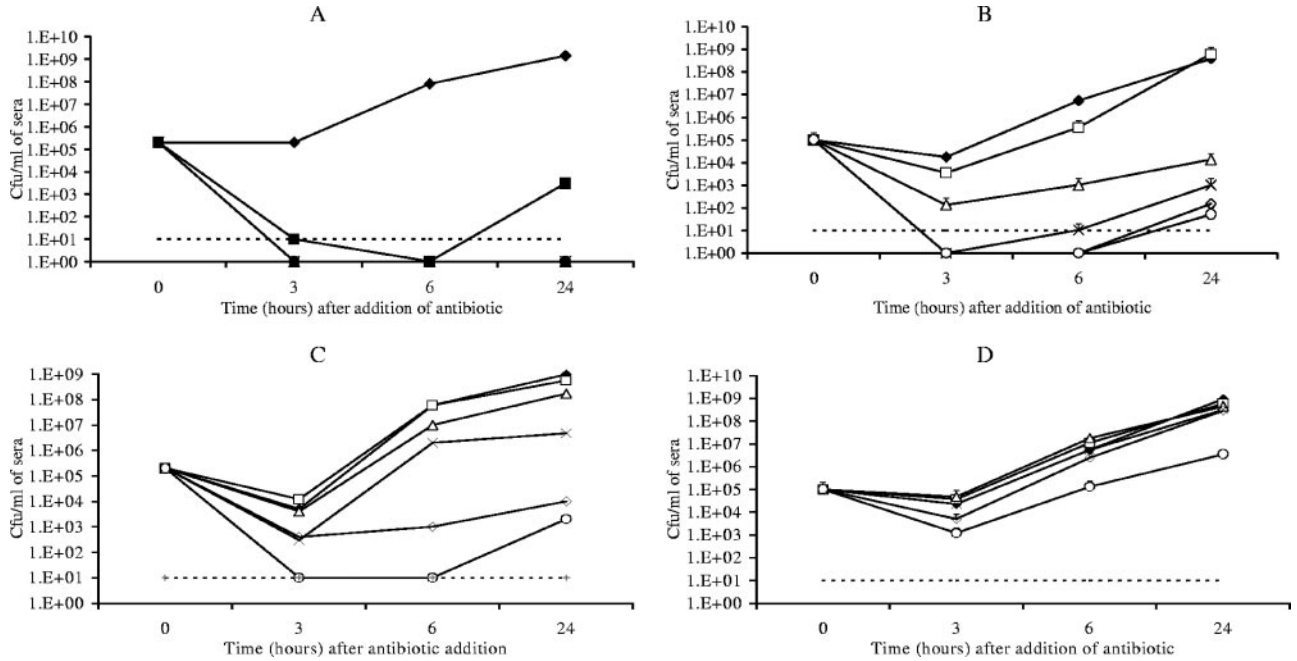

FIG. 1. Effects of various concentrations of enrofloxacin on survival of *Salmonella* serovar Typhimurium strains in chicken sera at 41°C. Counts are means of two experiments, and error bars are standard errors of the means. The dotted line is the limit of detection. A. Parent strain A (fully sensitive; ciprofloxacin MIC, 0.03 μg/ml). B. MAR strain B (isogenic mutant of strain A; ciprofloxacin MIC, 0.6 μg/ml). C. VLA 512/98 (strain C, nonisogenic *gyrA* mutant, cyclohexane sensitive; ciprofloxacin MIC, 0.25 μg/ml). D. VLA 2590/99 (strain D, nonisogenic *gyrA* mutant, cyclohexane tolerant; ciprofloxacin MIC, 1 μg/ml). Symbols: filled diamonds, control; open squares, 0.5 μg/ml enrofloxacin; open triangles, 1.0 μg/ml enrofloxacin; crosses, 2.0 μg/ml enrofloxacin; open diamonds, 4.0 μg/ml enrofloxacin; open circles, 8.0 μg/ml enrofloxacin.

the 50-ppm dose received 1.48× the dose of those that received the 10-mg/kg dose and the birds that received the 125-ppm dose received 1.29× the 25-mg/kg dose. For the birds that received the dose pulsed in the water, the dose was drunk in ca. 4½ h.

Efficacy of the different enrofloxacin treatment regimens. Infecting the birds with *Salmonella* did not make them clinically ill, and so efficacy was related to the ability to reduce *Salmonella* counts in cecal contents. The different 1-day treatments at 5× the recommended treatment concentration did not reduce the *Salmonella* counts to the same extent as the 2- or 5-day treatments did but in some instances still significantly reduced the *Salmonella* counts compared to bacterial counts in control birds (Table 1). The 2-day treatments significantly reduced the *Salmonella* counts compared to counts in control birds ($P < 0.01$), and in all but one treatment group, the 2-day treatment showed reductions similar to those of the 5-day treatment.

Pharmacokinetics. The higher doses of antibiotic resulted in a proportionate increase in antibiotic concentration in sera and tissues (Tables 2 and 3; Fig. 2). The mean peak antibiotic concentrations obtained following different dosage routes were gavage > pulsed water > continuous water treatment, and this corresponded to a shorter time to reach the peak antibiotic concentrations and less time that antibiotic was present in sera or tissues for gavage or pulsed treatments compared to antibiotic given continuously in the water (Table 2; Fig. 2).

Some basic pharmacokinetic parameters are shown in Table 3 for pulsed and continuous water treatments at recommended and 2.5× the recommended treatment dose only. As would be expected, the pulsed treatment led to higher C_{max}/MIC ratios but to slightly lower AUC/MIC ratios.

Recolonization of chicken gut post-antibiotic treatment. Overall, there were no statistical differences in the extent of *Salmonella* recolonization after the end of antibiotic treatment for the different treatment groups within an experiment, including for birds that were moved to clean accommodation 6 h after their last antibiotic dose in experiment II (results not shown). Moving the birds to clean accommodation also did not affect whether the birds were shedding reduced-susceptibility *Salmonella* organisms or not (results not shown). However, there was considerable variation in the extent of recolonization between different experiments; in particular in experiments I and III, at 4 weeks after the end of antibiotic treatment, mean counts for different treatment groups were all ≤log 1.5, whereas in experiments II and IV mean counts for different treatment groups ranged from log 3.6 to 6.8 (results not shown).

Selection of *Salmonella* isolates with reduced susceptibility. The number of colonies replica plated and the percentages of strains with reduced susceptibility to ciprofloxacin for each treatment group and experiment are shown in Table 1. None of the control (Table 1) or pretreatment (results not shown) birds gave rise to strains with reduced susceptibility. The cyclohexane-tolerant MAR strain B was present in high numbers during the first few days of antibiotic therapy, but then the numbers rapidly decreased (results not shown).

In experiment III, where the lower dose of *Salmonella* was given to birds, there were no strains with reduced susceptibility isolated (Table 1).

TABLE 2. Mean fluoroquinolone (ciprofloxacin and enrofloxacin) concentrations in sera and tissues of chickens following different treatment regimens of Bayril 10% oral solution

Dose method	Tissue	Dose	Mean (SEM) antibiotic concn[a] (μg/ml) at h after start of antibiotic treatment (n = 4 for gavage; n = 3 for other treatments)					
			2	4	6	8	10	24
Gavage[b]	Cecal[c]	1×	20.10 (5.95)	ND	78.01 (9.44)	ND	ND	10.79 (4.07)
		2.5×	46.46 (6.59)	ND	115.86 (35.33)	ND	ND	50.53 (5.34)
	Liver	1×	10.61 (0.50)	ND	5.46 (0.76)	ND	ND	1.86 (0.57)
		2.5×	20.74 (0.86)	ND	15.79 (1.24)	ND	ND	2.6 (0.15)
	Sera	1×	1.81 (0.14)	ND	0.72 (0.14)	ND	ND	0.08 (0.01)
		2.5×	3.83 (0.35)	ND	2.16 (0.19)	ND	ND	0.18 (0.01)
Water, continuous	Cecal	1×	3.79 (0.42)	9.88 (2.25)	20.64 (1.63)	24.73 (1.82)	20.28 (0.86)	23.59 (4.01)
		2.5×	6.61 (1.00)	30.51 (2.40)	41.54 (0.75)	52.69 (8.05)	63.23 (5.75)	68.02 (0.92)
	Liver	1×	2.85 (0.74)	3.81 (1.60)	4.97 (0.15)	5.15 (0.21)	6.23 (0.81)	4.94 (0.63)
		2.5×	5.86 (3.05)	6.53 (3.32)	9.17 (1.13)	12.67 (1.88)	10.54 (0.81)	11.46 (1.40)
	Lung	1x	0.85 (0.20)	1.14 (0.52)	1.38 (0.06)	1.80 (0.44)	1.38 (0.08)	1.00 (0.14)
		2.5×	1.53 (0.73)	3.06 (0.73)	2.97 (0.27)	3.93 (0.06)	3.39 (0.25)	2.35 (0.39)
	Sera	1×	0.46 (0.14)	0.53 (0.09)	0.59 (0.006)	0.50 (0.03)	0.73 (0.03)	0.51 (0.15)
		2.5×	0.58 (0.29)	0.91 (0.19)	1.42 (0.07)	1.54 (0.19)	1.54 (0.11)	1.11 (0.12)
Water, pulsed	Cecal	1×	9.48 (0.60)	32.28 (2.47)	43.13 (7.20)	53.19 (8.86)	35.15 (5.77)	12.00 (0.33)
		2.5×	17.35 (0.73)	55.52 (10.82)	58.72 (14.27)	115.63 (17.31)	85.70 (38.25)	18.26 (6.65)
	Liver	1×	3.98 (0.31)	9.13 (0.56)	11.94 (0.96)	9.49 (1.32)	4.93 (0.60)	1.29 (0.25)
		2.5×	9.82 (0.73)	11.06 (5.57)	17.87 (2.61)	13.86 (0.35)	8.77 (1.80)	2.61 (0.14)
	Lung	1×	1.28 (0.23)	2.36 (0.16)	2.79 (0.31)	2.23 (0.35)	1.20 (0.19)	0.20 (0.02)
		2.5×	4.91 (8.15)	8.15 (0.92)	6.81 (1.93)	4.41 (0.64)	2.07 (0.25)	0.45 (0.06)
	Sera	1×	0.45 (0.05)	0.77 (0.007)	1.17 (0.11)	0.97 (0.12)	0.30 (0.13)	0.07 (0.02)
		2.5×	1.17 (0.05)	2.74 (0.17)	2.57 (0.50)	1.76 (0.23)	1.84 (0.89)	0.01 (0.001)

[a] As enrofloxacin can be metabolized to ciprofloxacin in vivo, various percentages (the highest percentage being seen in the liver) of the antibiotic detected were ciprofloxacin, but values are for total antibiotic. ND, not determined.
[b] Antibiotic concentrations are recorded on day 1 of treatment with the exception of the 24-h results for birds dosed by gavage, which are 24 h after the last dose of the 5-day (recommended dose) or 2-day (2.5× recommended dose) treatment regimens.
[c] Cecal refers to cecal luminal contents.

Excluding isolates from experiment III, the numbers of colonies replica plated from all experiments and treatment routes after the end of antibiotic treatment were 5,025 for the 5-day treatments, 7,756 for the 2-day treatments, and 5,088 for the 1-day treatments. The percentages of these colonies/isolates that showed reduced susceptibility (i.e., ability to grow on agar with 4× MICs for the respective strains) to ciprofloxacin were 64% for the 5-day treatment, 52% for the 2-day treatment, and 14% for the 1-day treatment (results not shown).

MICs of ciprofloxacin against *Salmonella* isolates recovered from different treatment groups. Based on the MICs against selected isolates, the 5-day treatments tended to select for strains with a higher level of reduced susceptibility than the 2- or 1-day treatment regimens. The ciprofloxacin MIC_{100} was 0.03, 0.5, 0.5, and 2 μg/ml for isolates (n = 12, 35, 57, and 66) from control groups and from all of the 1-, 2-, and 5-day treatment regimens, respectively (results not shown). Compared with the pulsed or continuous regimens, the gavage regimen appeared to select for strains with a lower level of reduced susceptibility with MIC_{100}s of 0.25, 0.06, and 0.5 μg/ml for isolates (n = 16, 13, and 24) from the gavage 1-, 2-, and 5-day treatment regimens, respectively (results not shown).

Determination of mutations within the quinolone resistance-determining region of *gyrA*. Fifty isolates from experiment II were examined for mutations in *gyrA* by denaturing HPLC. Isolates were taken from all treatment groups and the control group and from birds during (n = 21 isolates) and after (n = 29 isolates) antibiotic treatment. Isolates were wild type

TABLE 3. Pharmacokinetic parameters (based on mean values) for enrofloxacin in sera of poultry[a]

Variable and unit[d]	Value for treatment regimen[b]			
	WC		WP	
	R	2.5×	R	2.5×
C_{max} (μg/ml)	0.73	1.54	1.17	2.74
T_{max} (h)	10	8	6	4
$AUC_{0\text{-}24}$ (μg · h/ml)	13.6	29	9.6	31.3
MRT (h)	42.4	46.2	9.2	7.5
$t_{1/2}$ (h)	27.05	29.63	6.47	1.99
C_{max}/MIC (ratio of C_{max}/MIC for strains[c])				
A	24.3	51.3	39.0	91.3
B	12.1	25.7	19.5	45.7
C	1.46	3.08	2.3	5.5
D	0.73	1.5	1.17	2.7
AUIC (ratio of AUC/MIC for strains[c])				
A	453.3	966.7	320	1,043.3
B	226.7	483.3	160	512.7
C	27.2	58	19.2	62.6
D	13.6	29	9.6	31.3

[a] Birds were killed to obtain sera and tissues, and so values are calculated from mean serum concentrations from three different birds at each time point.
[b] WC, continuous water treatment; WP, pulsed water treatment; R, recommended treatment of water at 50 ppm (continuous) or water adjusted to give 10 mg/kg of bird (pulsed); 2.5×, 2.5× recommended treatment.
[c] Strain A, B, C, or D.
[d] C_{max}, maximum concentration in serum after administration; T_{max}, time needed to reach C_{max}; $AUC_{0\text{-}24}$, area under the concentration-time curve for 0 to 24 h; MRT, mean residence time (time for 63.2% of administered dose to be eliminated); $t_{1/2}$, time for concentration to diminish by one-half.

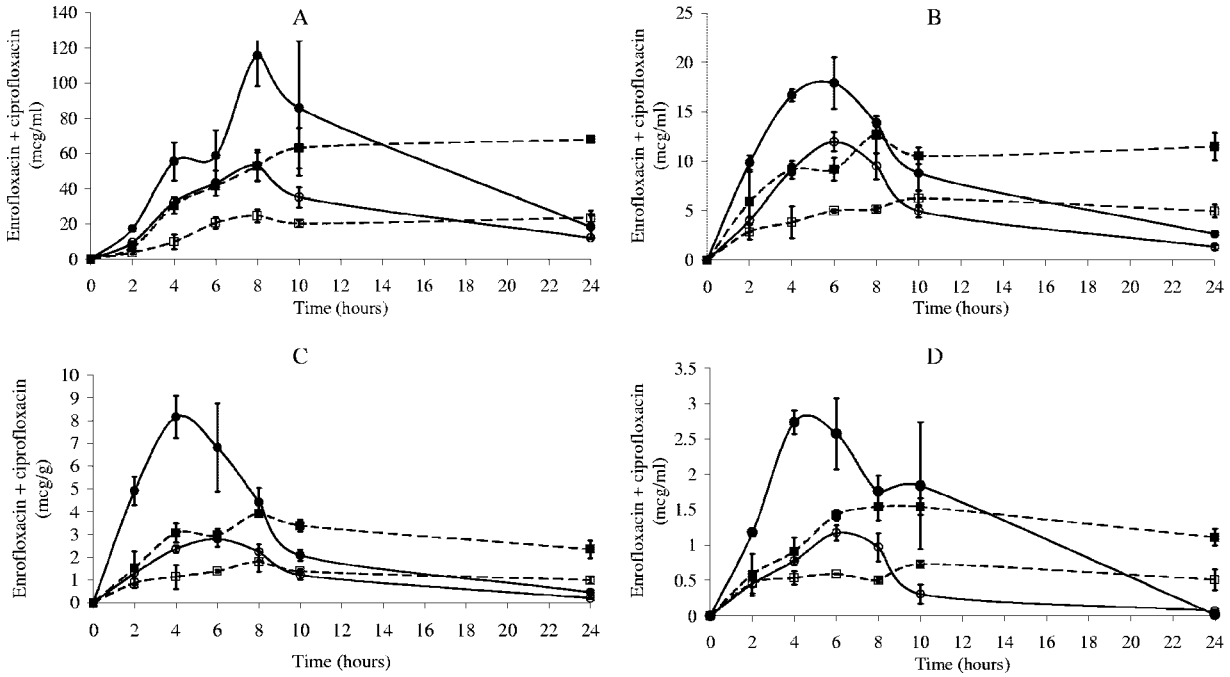

FIG. 2. Graphs showing mean (n = 3) fluoroquinolone (enrofloxacin and ciprofloxacin) concentrations in chicken sera and tissues at 0, 2, 4, 6, 8, 10, and 24 h after dosing with Baytril 10% oral solution. (As enrofloxacin can be metabolized to ciprofloxacin in vivo, a percentage of the antibiotic detected was ciprofloxacin.) (A) Cecal contents; (B) liver; (C) lung; (D) sera. Values are for total antibiotic. Symbols: dashed lines and squares, continuous dosing; solid lines and circles, pulsed dosing; open squares or circles, recommended dose; filled squares or circles, 2.5× standard dose.

(n = 24) or had the following mutations: Asp87-Asn (n = 7), Asp87-Tyr (n = 8), and Ser83-Phe (n = 11). There was no evidence that specific mutations were associated with specific treatment regimens. Most of the strains which had mutations in *gyrA* and were also cyclohexane resistant had an Asp87-Asn mutation (seven/eight strains). The presence of a mutation in *gyrA* was associated with reduced susceptibility to ciprofloxacin and resistance to nalidixic acid. No isolates with mutations in *gyrA* were obtained from control birds.

DISCUSSION

Chickens represent a tremendous challenge for effective antibiotic treatment of enteric pathogens without selection of antibiotic resistance, because they live in the same environment pre- and post-antibiotic treatment of a particular bacterial infection and thus may be reexposed to the disease organism post-antibiotic treatment by accidental ingestion of contaminated fecal material. Indeed, several workers have shown that enrofloxacin treatment of poultry can rapidly select for *Campylobacter jejuni* (24), *Escherichia coli* (6), and *Salmonella* (30) with reduced fluoroquinolone susceptibility. Concerns over selection of antibiotic-resistant *Campylobacter* have led the FDA to ban the use of enrofloxacin in poultry in the United States in July 2005 (36). However, fluoroquinolones are still used to treat disease in poultry in most other parts of the world, and so it is timely to determine if dosing strategies of fluoroquinolones for poultry can be optimized in such a way as to maintain efficacy but reduce selection of resistance in zoonotic human pathogens.

In vitro, the enrofloxacin range of 0.5 to 8 μg/ml for time-kill studies in serum corresponded to different serum and lung C_{max} values obtained following current recommended and modified treatment regimens and increased concentrations of enrofloxacin in serum over the above-described range led to an increased rate of killing and an increased ability to kill strains which were MAR (strain B) or had a mutation in *gyrA* (strain C). This would suggest an advantage for any dosing strategies that could increase the enrofloxacin C_{max} in sera or tissues.

Previous studies in which enrofloxacin has been given as a 10-mg/kg bolus to chickens have resulted in serum C_{max}/T_{max} values of 2.44 μg/ml/1.68 h (1) and 2.29 μg/ml/3 h (21), whereas in our study the mean concentration in sera 2 h post-treatment by gavage was 1.81 μg/ml. The mean serum C_{max} following pulsed water treatment was lower than that observed for gavage and lower again for the continuous water treatment, and this presumably reflects the longer time of uptake for the antibiotic dose by pulsed and continuous water treatments. The effect of increasing the dose by 2.5× in the 2-day treatment increased the serum AUC by 2.1× and 3.3× and the serum C_{max} by 2.1× and 2.3× for the continuous and pulsed water treatments, respectively. These relative differences were also observed in the antibiotic concentrations in cecal contents, liver, and lung. Comparing the 2-day pulsed dosing to the 5-day continuous water treatment, an increase in mean C_{max} values of 4.7×, 2.9×, 4.5×, and 3.8× was observed for cecal contents, liver, lung, and sera, respectively. However, could these increases in C_{max} be related to improved efficacy, as measured by the reduction of *Salmonella* counts?

Based on a C_{max}/MIC ratio of >10 being required to not

select for reduced susceptibility (26), coupled with the gut concentrations of fluoroquinolones being above the previously determined in vitro MPCs for strains A and B (29), in theory all treatment regimens should not have selected for reduced susceptibility. However, strains with reduced susceptibility were selected, and based on both in vitro results and in vivo counts after 1 day, this may be because the presence of cecal contents seemed to reduce the activity of enrofloxacin compared to its activity in sera.

In vivo, the 1-day treatment in chicks reduced *Salmonella* counts, but to a significantly lesser extent than did the 2-day treatments. As such, a 1-day treatment would not be viable for treating enteric infections, although it is possible that it would be efficacious for treating systemic or respiratory infections, but further work would be needed to ascertain this.

In all instances, with one exception in experiment IV, the 2-day enrofloxacin treatment regimen showed an ability similar to that of the 5-day regimen to reduce *Salmonella* counts in cecal contents in chickens. However, in experiment IV, the 2-day regimen still significantly reduced *Salmonella* counts compared to counts in control birds.

The pulsed dosing clearly showed an advantage compared to the continuous dosing with respect to C_{max}, but this advantage was not clearly linked to improved efficacy of pulsed treatment against *Salmonella* infections in chickens. In view of the vastly reduced activity of enrofloxacin in vitro in cecal contents, it is possible that improved efficacy of the pulsed versus continuous water treatments may be seen only when treating systemic or lung infections. However, when pulsed and continuous enrofloxacin water treatment regimens were compared against colisepticemia in chickens, there was a lower mortality associated with birds which received the continuous water regimen (8).

Reinfection after antibiotic treatment occurred with all treatment routes and doses, although there were limited instances when reinfection was significantly less following some treatments. In experiment III, where the birds received a lower dose of *Salmonella* and shed lower numbers of *Salmonella* at the time of antibiotic treatment, there were low numbers of *Salmonella* shed at 4 weeks after the end of antibiotic treatment for all of the treatment regimens and no resistant organisms were selected. There is some evidence to suggest that the level of *Salmonella* colonization in commercial layer flocks would usually be less than 10^4 CFU/gram feces (38). Other studies have shown that for chicks infected experimentally with *S. enterica* serovar Enteritidis at 1 day of age, the mean counts from cecal samples dropped from ca. log 8 at 1 week old to ca. $\leq$log 2 at 8 to 16 weeks of age (19). Thus, if commercial birds do have a lower level of *Salmonella* colonization than that seen in experiments I, II, and IV of this study, then based on the results of experiment III, it is likely that not only will recolonization after the end of treatment be less likely but also efficacy will be better than for birds with higher numbers of *Salmonella* organisms, and selection of resistance should be less.

In conclusion, modifications of the current approved dosing regimen of enrofloxacin for chickens that still used the same overall amount of enrofloxacin gave rise to a considerably increased C_{max} of fluoroquinolones in the sera and tissues. The 2-day 2.5× dosing regimen showed an ability to reduce *Salmonella* counts in cecal contents similar to that of the 5-day regimen, and there was some evidence that the shorter treatments selected for lower percentages of strains with reduced susceptibility and with lower levels of reduced susceptibility. Additionally, the 2-day regimen would be easier for farmers to administer and would overall result in a shorter withholding time. The increased concentrations of antibiotic following the 2-day treatment at 2.5× the recommended dose may be particularly advantageous for treating respiratory or systemic infections, where the enrofloxacin activity will not be compromised by the presence of fecal material. However, further studies are required to validate this hypothesis.

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