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Induction of the Carrier State in Pigeons Infected with *Salmonella enterica* Subspecies *enterica* Serovar Typhimurium PT99 by Treatment with Florfenicol: a Matter of Pharmacokinetics▽

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Paratyphoid caused by *Salmonella enterica* subsp. *enterica* serovar Typhimurium is the main bacterial disease in pigeons. The ability of *Salmonella* serovar Typhimurium to persist intracellularly inside pigeon macrophages results in the development of chronic carriers, which maintain the infection in the flock. In this study, the effect of drinking-water medication with florfenicol on *Salmonella* infection in pigeons was examined. The pharmacokinetics of florfenicol in pigeons revealed a relatively high volume of distribution of 2.02 liters/kg of body weight and maximum concentrations in plasma higher than the MICs for the *Salmonella* strain used (4 μg/ml) but quick clearance of florfenicol due to a short half-life of 1.73 h. Together with highly variable bioavailability and erratic drinking-water uptake, these parameters resulted in the inability to reach a steady-state concentration through the continuous administration of florfenicol in the drinking water. Florfenicol was capable of reducing only moderately the number of intracellular salmonellae in infected pigeon macrophages in vitro. Only at high extracellular concentrations (>16 μg/ml) was a more-than-10-fold reduction of the number of intracellular bacteria noticed. Florfenicol treatment of pigeons via the drinking water from 2 days after experimental inoculation with *Salmonella* serovar Typhimurium until euthanasia at 16 days postinoculation resulted in a reduction of *Salmonella* shedding and an improvement in the fecal consistency. However, internal organs in florfenicol-treated pigeons were significantly more heavily colonized than those in untreated pigeons. In conclusion, the oral application of florfenicol for the treatment of pigeon paratyphoid contributes to the development of carrier animals through sub-MIC concentrations in plasma that do not inhibit intracellular persistency.

Paratyphoid in pigeons is typically associated with *Salmonella enterica* subsp. *enterica* serovar Typhimurium variant Copenhagen PT2 or PT99 strains. These strains cause a typhoid condition and are the main cause of bacterial disease in pigeons, resulting in gastroenteritis, arthritis, oophoritis or orchitis, granulomatous inflammation in all possible organs, and high mortality (7). Homing pigeons, as dealt with in this study, are kept in groups in pigeon lofts, facilitating long-term maintenance of a *Salmonella* infection. Although vaccination is a valuable part of a control program, it does not eliminate the possibility of a clinical infection in a pigeon aviary but mainly reduces clinical symptoms and mortality (12, 15). Antimicrobial treatment is often used as an aid to control salmonellosis in the aviary. However, *Salmonella* bacteria may persist inside pigeon macrophages, a niche in which these microorganisms are well protected from most antimicrobial agents (11). It has been suggested previously, but not clearly proven, that antimicrobial treatment may promote the carrier state (16).

Florfenicol distributes easily throughout the body (1) and possibly reaches sufficient intracellular concentrations to assist the macrophage in killing intracellular salmonellae. Florfenicol is a fluorinated derivative of thiamphenicol, and neither of these two compounds contains the nitro group, which is the cause of aplastic anemia that is rarely seen after the use of chloramphenicol. Florfenicol blocks the peptidyltransferase at the 50S ribosome subunit and acts against a wide variety of both gram-positive and gram-negative bacteria (3, 9, 13). The pharmacokinetic parameters of florfenicol in pigeons are not available.

It was the aim of the present study to determine the ability of florfenicol to eliminate *Salmonella* serovar Typhimurium from experimentally infected pigeons and isolated pigeon macrophages and to correlate this ability with the drug's pharmacokinetics in pigeons by comparing naïve pooling with population analysis.

MATERIALS AND METHODS

Experimental animals. A total of 35 clinically healthy, 1- to 2-year-old homing pigeons (*Columba livia*) were used for the experimental infection and collection of macrophages. The animals were negative for the presence of *Salmonella* bacteria in the feces at sampling points at 2-week intervals and for the presence of agglutinating antibodies to *Salmonella* (14). For the pharmacokinetic experiment, 24 clinically healthy, 1- to 2-year-old homing pigeons were used. During the experiments, each animal was housed individually in a wire mesh cage. All

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experiments were carried out with the approval of the ethics committee of the Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.

Pharmacokinetics of florfenicol in pigeons after oral and intravenous administration. Both intravenous and oral administration of a single bolus of florfenicol, as well as continuous administration in drinking water, was performed to determine the pharmacokinetic parameters of florfenicol in pigeons. For the single-bolus study, a crossover design was used. Twenty-four pigeons received a single bolus of 30 mg of florfenicol per kg of body weight, either intravenously in the vena basilica (12 animals) as a 10-fold dilution of a 300-mg/ml florfenicol injectable solution (Nuflor; Schering-Plough Animal Health, NJ) in polyethylene glycol 400 (Sigma-Aldrich, Bornem, Belgium) or orally (12 animals) through a crop tube as an experimental drinking-water formulation containing 20% florfenicol. One week later, the pigeons that received florfenicol orally were injected intravenously with florfenicol at 30 mg/kg, and vice versa. After florfenicol administration, three blood samples from each pigeon were collected so that, per time point, six individual samples were obtained. The following time points for blood sampling were chosen, based on a preliminary experiment: 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, and 8 h after administration. For the continuous-administration study, florfenicol was administered in the drinking water at 0.5 mg/ml over 3 days to 30 pigeons housed in a group. This concentration in drinking water corresponds to an expected dose of 30 mg/kg. Blood samples were collected from six randomly chosen pigeons, with a maximum of three samples per pigeon, at 4, 8, 12, 24, 28, 32, 36, 48, 52, 56, and 60 h after the addition of florfenicol to the drinking water.

The blood samples were centrifuged at 3,000 × *g* for 10 min and stored at −20°C until further processing. Plasma samples (250 μl) were analyzed using a high-performance liquid chromatography-UV system (Thermo Separation Products, Fremont, CA) according to a procedure based on that of Varma et al. (17). The method was linear from 0.05 to 20 μg/ml, with correlation coefficients of >0.99. Trueness and precision were within acceptable limits as calculated with the Horwitz equation on three levels (limits of quantitation: 0.05, 1, and 10 μg/ml).

The protein binding was checked at three different concentrations (0.5, 2.5, and 10 μg/ml) in fresh pooled pigeon plasma by ultrafiltration with a Biomax 30K nominal molecular weight limit membrane filter (Millipore). The plasma with spiked florfenicol concentrations was allowed to equilibrate for 1 h at 40°C (the body temperature of a pigeon). After ultrafiltration, an aliquot of the ultrafiltrate containing free florfenicol was measured using the high-performance liquid chromatography method. The concentration of bound drug was calculated by subtracting the concentration of free drug from the total concentration introduced into the system.

Pharmacokinetic analyses. Pharmacokinetic analyses were conducted using both the naïve pooling approach and nonlinear mixed-effects modeling.

Naïve pooling approach. For intravenous administration, a biexponential equation describing a bicompartmental open model with first-order elimination was used to fit the pooled-concentration-versus-time profile:

$$Cp_{\text{i.v.}}(t) = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad (1)$$

where $Cp_{\text{i.v.}}(t)$ is the plasma florfenicol concentration at time t after intravenous administration, A and B are the concentrations in plasma extrapolated to time zero of the first and second phases of the florfenicol disposition curve, and α and β are the rate constants of the first and second phases.

For oral administration, a triexponential equation describing a bicompartmental open model with first-order elimination and first-order absorption was selected:

$$Cp_{\text{p.o.}}(t) = -(A + B) \cdot e^{-k_a t} + (A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}) \quad (2)$$

where $Cp_{\text{p.o.}}(t)$ is the plasma florfenicol concentration at time t after oral administration, A and B are the concentrations in plasma extrapolated to time zero of the first and second phases of the florfenicol concentration decrease, and k_a is the first-order absorption rate constant.

The curve fitting for both intravenous and oral data was performed using a weighted least-squares nonlinear regression with WinNonlin (version 5.0.1; Pharsight). The data points were weighted by the inverse of the squared fitted concentration.

The pharmacokinetic parameters were calculated using classical equations (8).

Nonlinear mixed-effects modeling. Individual concentration-time data were simultaneously analyzed by applying a nonlinear mixed-effects regression model as implemented by using a software package (WinNonMix, version 2.0.1; Pharsight). A general least-squares method with a simplex algorithm was used to estimate fixed-effect and random-effect parameters.

The pharmacokinetic models used to predict plasma florfenicol concentrations after intravenous and oral administration are described by equations 1 and 2, respectively.

The two models were parameterized in terms of systemic clearance (CL), intercompartmental-distribution clearance (CL_D), the volume of distribution in the central compartment (V_1), and the volume of distribution in the peripheral compartment (V_2). For oral administration, V_1/F, V_2/F, CL/F, and CL_D/F ratios, in which F is the absolute bioavailability for the oral route, were estimated.

The pharmacokinetic parameters were assumed to be log-normally distributed, as indicated in the following equation:

$$\ln \text{Par}_i = \theta_{\text{Par}} + \eta_{\text{Par}_i} \quad (3)$$

in which Par_i is the parameter (CL_i, $CL_D i$, $V_1 i$, $V_2 i$, or $k_a i$) for the i-th pigeon and θ_{Par} is the population mean of the logarithm of the parameter. Also, η_{Par_i} is a centered independent random variable assumed to be normally distributed, with a variance of $\omega^2\text{Par}$. These variances were estimated by the software, accounting for interindividual errors.

The concentration-time profile was described by the following equation:

$$Cp_{ij} = E\{Cp_{ij}\} \cdot (1 + \varepsilon_{ij}) \quad (4)$$

where Cp_{ij} is the observed concentration in plasma of the i-th pigeon at time j, $E\{Cp_{ij}\}$ is the expected concentration in plasma of the i-th pigeon at time j, and ε_{ij} is a normally distributed random variable with a mean of zero and a variance of σ^2 estimated by the software. ε_{ij} accounts for the residual variability of the data resulting from intraindividual variability, assay errors, model misspecification, and any other sources of variability.

Comparison of concurrent models. The bicompartmental models described by equations 1 and 2 were compared to the corresponding monocompartmental models (for intravenous and oral administration routes). The comparison was based on Akaike's information criterion, conducing to the selection of the two bicompartmental models presented above (see Discussion).

***Salmonella* serovar Typhimurium strains and growth conditions.** In all experiments, *Salmonella* serovar Typhimurium PT99 strain DAB66 was used. This strain was isolated from pigeons and has been proven to be highly pathogenic to pigeons (11). In all experiments, the strain was grown in Luria-Bertani broth at 37°C for 16 h without shaking. The MIC of florfenicol for this strain, as determined using both agar and broth dilution according to CLSI guideline M31-A2 (5), was 4 μg/ml. The minimal bactericidal concentration for the strain, determined accordingly, was >256 μg/ml. In vitro killing curves were prepared by plating 10-fold dilutions of Mueller-Hinton broth, inoculated with 5×10^5 CFU of the *Salmonella* strain and exposed to 0, 2, 4, 32, or 128 μg of florfenicol/ml, onto Mueller-Hinton agar at different time points postinoculation (see Fig. 5).

Minimal concentration of florfenicol inhibiting proliferation of *Salmonella* serovar Typhimurium inside pigeon macrophages. The minimal concentration of florfenicol in the extracellular environment needed to inhibit intracellular proliferation inside pigeon macrophages was determined as follows. Pigeon respiratory macrophages were collected according to a previously described method (10). Briefly, a pigeon was humanely killed, the trachea was aseptically exposed and cut in the midgular region, and a tracheotube was inserted. The respiratory tract was flushed through this tube with 150 ml of Hanks' balanced salt solution without Ca^{2+}-Mg^{2+} at room temperature. The aspirate was centrifuged at 350 × *g* at 4°C, and the pellet was resuspended in RPMI medium with 1% glutamine and 1% pyruvate at 0°C. The cells were counted and seeded into a 96-well plate at 10^3 macrophages per well. After 2 h of incubation at 41°C and 5% CO_2, the wells were rinsed to remove nonadherent cells. Cell sample purity was determined using nonspecific esterase (Sigma Diagnostics, St. Louis, MO) and Haemacolor (Merck, Darmstadt, Germany) staining. Then the cells were exposed to *Salmonella* bacteria at a concentration of 5×10^4 CFU/ml (a multiplicity of infection of 10), centrifuged at 350 × *g* for 10 min at 41°C, and incubated for 1 h at 41°C in 5% CO_2. Cell culture medium containing gentamicin (final concentration of 50 μg/ml; Gibco) and different concentrations of florfenicol (0, 1, 2, 4, 8, 16, and 32 μg/ml) were added, and the wells were incubated for 16 h at 41°C in 5% CO_2. Finally, the wells were rinsed to remove the gentamicin and extracellular florfenicol, after which the macrophages were lysed by the addition of 1% Triton X-100 (Acros, NJ). Bacteria were enumerated by plating six drops of 20 μl of 10-fold dilutions onto brilliant green agar (BGA; Oxoid Ltd., Hampshire, United Kingdom). The experiment was repeated with macrophages from five different pigeons.

Oral florfenicol treatment of pigeons inoculated with *Salmonella* serovar Typhimurium. Thirty pigeons were divided into the following three groups of 10 pigeons each: (i) pigeons inoculated and not treated, (ii) pigeons inoculated and treated with florfenicol, and (iii) pigeons not inoculated and not treated. Each pigeon was housed individually. The animals of the first two groups were inoculated in the crop with 10^8 CFU of the *Salmonella* serovar Typhimurium strain DAB69 in 1 ml of phosphate-buffered saline. Treatment consisted of the addition

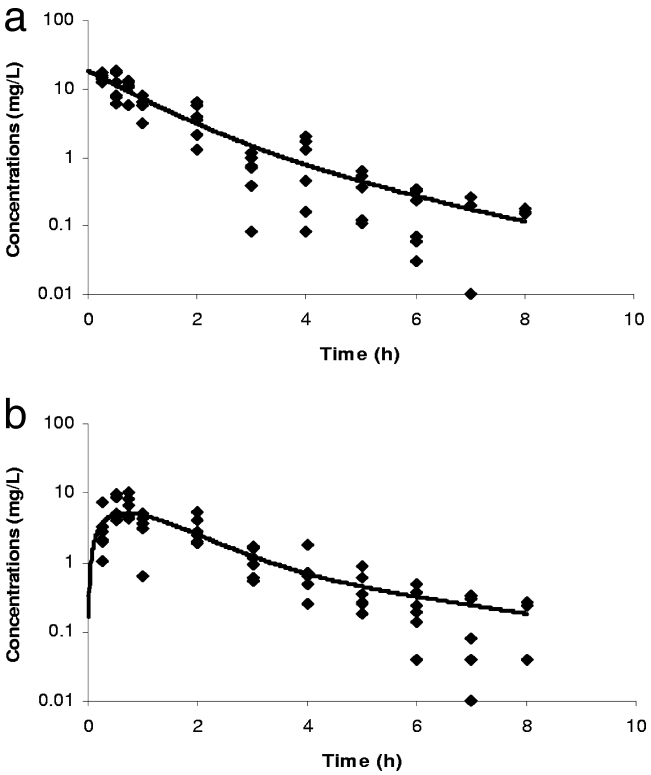

FIG. 1. Plasma florfenicol concentrations after administration of an intravenous (a) or oral (b) bolus of 30 mg of florfenicol/kg to pigeons as determined by the naïve pooling approach. The equations of the curves are given in the text.

of florfenicol to the sterilized drinking water at a concentration of 0.5 mg/ml (the solubility of florfenicol in water is 0.68 mg/ml maximum) and was started 48 h postinoculation and continued until euthanasia at 16 days postinoculation. Counts of *Salmonella* bacteria in the excreta were determined daily as numbers of CFU per gram by plating 10-fold dilutions of excreta onto BGA. If negative after direct plating, the samples were preenriched in buffered peptone water (Oxoid), enriched in tetrathionate brilliant green broth (Oxoid), and plated onto BGA. The fecal consistency was scored daily as a measure of the severity of gastrointestinal symptoms, as follows: 0, normal feces; 1, feces not well formed; 2, watery feces; 3, severe diarrhea; 4, presence of blood in the stools; 5, absence of fecal production combined with anorexia. Daily water intake was measured to the nearest milliliter. Polydypsia was defined as a level of water consumption higher than the sum of the average consumption by the negative control pigeons plus two times the standard deviation for these control animals, combined with the presence of polyuria. The pigeons were kept until 16 days postinoculation, after which they were humanely killed, weighed, and necropsied. The numbers of CFU per gram of tissue from the lungs, livers, spleens, kidneys, gonads, and intestines were determined as described above. Significant weight loss was defined as weight loss equal to the sum of the average weight loss of the negative controls plus two times the standard deviation for these controls.

Statistical analysis. A one-way analysis of variance of the cumulative daily fecal consistency scores, the cumulative measurements of daily fecal shedding of *Salmonella*, and the bacteriological counts per pigeon and per each separate organ was performed. Pairwise comparisons between the treatment groups were done using the least-significant-difference test with a confidence interval of 95%. A statistical software package (SPSS, version 12) was used for these calculations.

RESULTS

Pharmacokinetics of florfenicol in pigeons after oral and intravenous administration. Intravenous administration of the florfenicol solution induced a brief period of vomiting in 70% of the pigeons. No side effects after oral administration were noticed. Figure 1 presents the individual plasma florfenicol concentrations measured after intravenous and oral administration and the corresponding predicted concentration-versus-time curves obtained after the fitting of the pooled data. The pharmacokinetic parameters derived from this analysis are summarized in Table 1. Both absorption and elimination occurred rapidly. The CL for florfenicol was estimated to be 1.41 liters kg^{-1} h^{-1}, and F for the oral route based on the average areas under the curve from time zero to infinity (AUCs) was about 59%.

Figure 2 presents the individual plasma florfenicol concentrations determined after intravenous and oral administration and the predicted population concentration-versus-time curve obtained after nonlinear mixed-effects modeling. The pharmacokinetic parameters derived from this analysis are summarized in Table 2. The median population estimates were a florfenicol CL of 1.32 liters kg^{-1} h^{-1} and F of 60% for the oral route. Interindividual variability of CL and F, expressed as percentages corresponding to coefficients of variation, were 29 and 49%, respectively. The residual errors of the models, expressed as percentages corresponding to coefficients of variation, were 55% for intravenous administration and 56% for oral administration by naïve pooling and 9.6% for intravenous administration and 8.8% for oral administration by nonlinear mixed-effects modeling.

The average concentrations of florfenicol in plasma after continuous administration in the drinking water are depicted in Fig. 3. Continuous administration did not result in a steady state. In 13 of 66 samples, no florfenicol was detected. In eight samples, a concentration in plasma higher than 1 mg/liter was detected. The highest concentration detected in plasma was 3.31 mg/liter.

The levels of protein binding of florfenicol in pigeon plasma were 88.8% at 0.5 μg/ml, 79.8% at 2.5 μg/ml, and 71.2% at 10 μg/ml.

Minimal concentration of florfenicol inhibiting proliferation of *Salmonella* serovar Typhimurium strain DAB69 inside pigeon macrophages. The addition of florfenicol to the extracellular medium reduced the number of viable intracellular bacteria compared to the numbers inside the untreated mac-

TABLE 1. Pharamacokinetic parameters of florfenicol obtained by the naïve pooling approach after intravenous or oral administration of a single bolus of 30 mg/kg[a]

Parameter	Median value after:	
	Intravenous administration	Oral administration
CL (liters kg^{-1} h^{-1})	1.41	
V_{ss} (liters kg^{-1})	2.02	
Mean residence time (h)	1.43	
$t_{1/2\beta}$ (h)	1.73	2.69
AUC (mg · h $liter^{-1}$)	21.30	12.47
F (%)		58.54
k_a		1.25
$t_{1/2ka}$ (h)		0.56
T_{max} (h)		0.69
C_{max} (mg $liter^{-1}$)		5.10

[a] Abbreviations: V_{ss}, volume of distribution at steady state; $t_{1/2\beta}$, half-life at β phase; $t_{1/2ka}$, absorption half-life; T_{max}, time to reach the maximum concentration; C_{max}, maximum concentration in plasma.

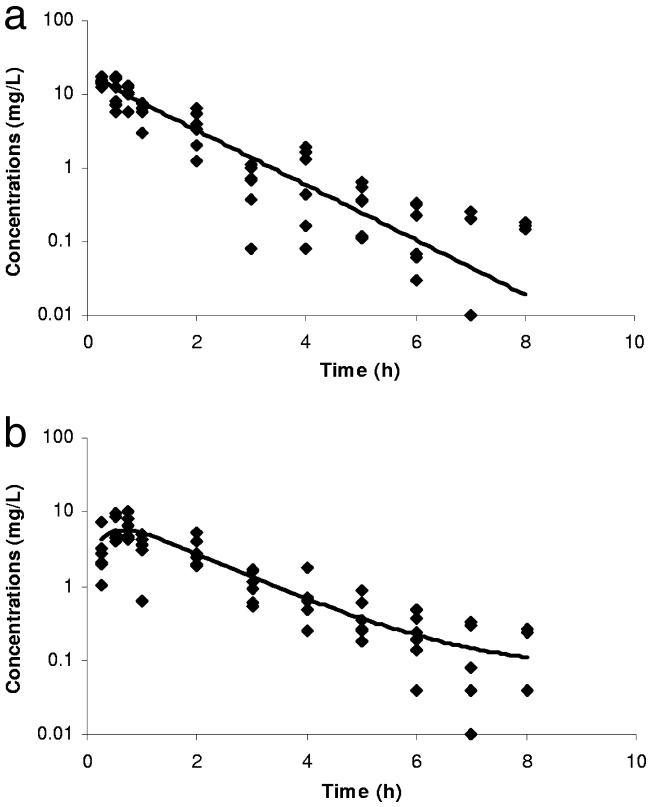

FIG. 2. Plasma florfenicol concentrations after administration of an intravenous (a) or oral (b) bolus of 30 mg of florfenicol/kg to pigeons as determined using nonlinear mixed-effects modeling. The equations of the curves are given in the text.

rophages at 16 h after inoculation at all concentrations of florfenicol tested (Fig. 4). Only at 32 and 64 μg/ml was a more-than-10-fold reduction of the numbers of bacteria noticed. The inhibition of *Salmonella* growth in Mueller-Hinton medium after exposure to florfenicol was much more pronounced than the inhibition inside macrophages, with reductions of nearly 1 and 2 $\log_{10}$ CFU at 2 and 4 μg/ml, respectively, at 16 h postinoculation (Fig. 5).

Oral florfenicol treatment of pigeons inoculated with *Salmonella* serovar Typhimurium strain DAB69. The average daily level of florfenicol exposure for all birds ± the standard deviation, calculated from the drinking-water uptake, was 35 ± 5 mg/kg, and daily levels of exposure ranged from 26 to 45 mg/kg. The average minimum and maximum daily levels of florfenicol exposure were 17 ± 8 and 59 ± 19 mg/kg, respectively.

TABLE 2. Pharamacokinetic parameters of florfenicol obtained from nonlinear mixed-effects modeling after intravenous or oral administration of a single bolus of 30 mg/kg

Parameter	Median value (% variation) after:	
	Intravenous administration	Oral administration
CL (liters kg^{-1} h^{-1})	1.32 (29)	
CL_D (liters kg^{-1} h^{-1})	2.97 (29)	
V_1 (liters kg^{-1})	0.88	
V_2 (liters kg^{-1})	0.56 (88)	
AUC (mg · h $liter^{-1}$)	22.72 (29)	13.09 (49)
F (%)		60.0 (46)
k_a (h^{-1})		0.775 (33)

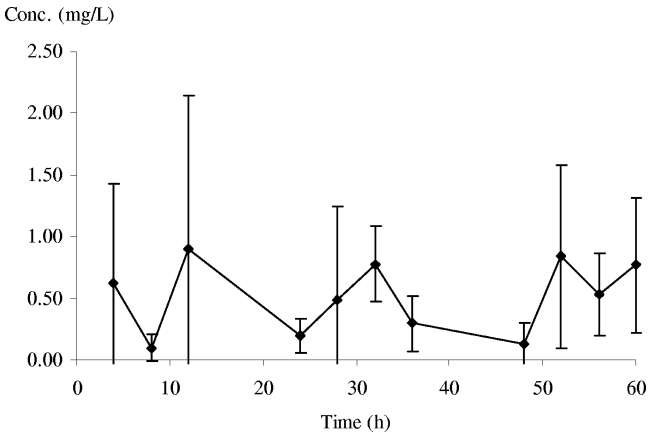

FIG. 3. Average concentrations (conc.) ± standard deviations of florfenicol in pigeon plasma after continuous administration in drinking water at 0.5 mg/ml.

Significant weight loss was determined to be a loss of ≥17% of the initial body weight. One inoculated and florfenicol-treated pigeon and two inoculated untreated pigeons showed significant weight loss (23, 17, and 37%, respectively). The average fecal consistency score for the negative control pigeons remained 0 throughout the experiment (i.e., normally formed stools). Average scores for the inoculated pigeons declined after inoculation with the *Salmonella* strain but were restored to earlier levels after 10 days of treatment with florfenicol (Fig. 6). Over the whole experimental period, the florfenicol-treated pigeons showed significantly better fecal consistency than the untreated pigeons ($P = 0.019$). Polydypsia was determined to correspond to a water uptake of ≥63 ml per day and was noticed in only one inoculated and untreated pigeon (with an average daily water uptake of 89 ml). *Salmonella* was isolated from all inoculated pigeons and from none of the negative controls. Florfenicol-treated pigeons shed significantly fewer *Salmonella* bacteria than the untreated ones ($P < 0.01$) (Fig. 7). On average, levels of fecal shedding by the untreated animals between days 5 and 13 postinoculation were more than 100 times higher than those by the florfenicol-treated pigeons during this time period. At 16 days postinoculation, 9 of 10 untreated pigeons were still shedding *Salmonella* in the feces, as opposed to 4 of 10 treated animals. The sum of the bacteriological counts for all *Salmonella*-infected organs in the treated pigeons was higher than that for the untreated pigeons, but the difference was not significant ($P = 0.166$) (Fig. 8). The bacteriological counts of *Salmonella* CFU in the spleens and kidneys of treated pigeons were significantly higher ($P < 0.05$) than those in the spleens and kidneys of the untreated animals.

DISCUSSION

The naïve pooling approach is classically used to deal with sparse data in pharmacokinetic experiments. The main drawbacks of this approach are the risk of pharmacokinetic-model

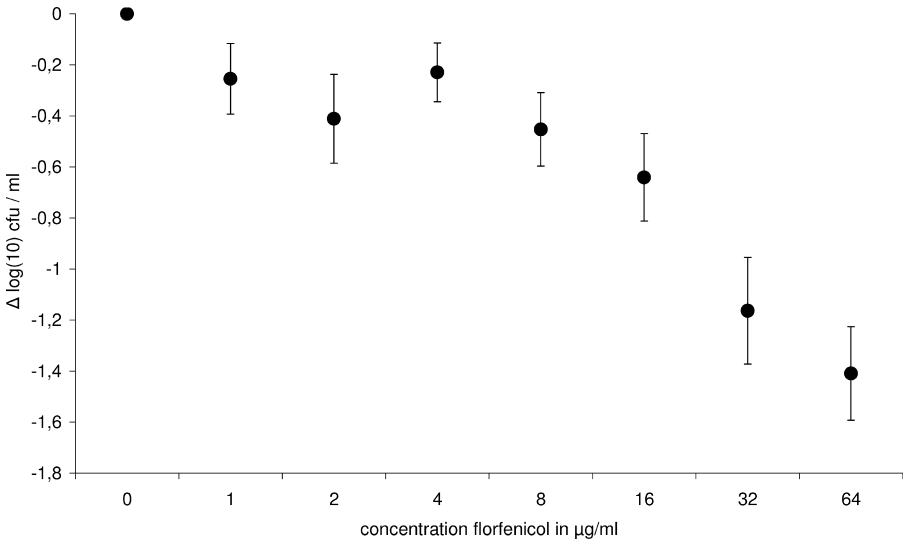

FIG. 4. Numbers of viable intracellular *Salmonella* bacteria recovered 16 h after the inoculation of pigeon macrophages with *Salmonella* at a multiplicity of infection of 10 and the subsequent addition of different concentrations of florfenicol to the cell culture medium. Data are presented as the mean differences (Δ) between the $\log_{10}$ numbers of CFU of intracellular bacteria per milliliter in cultures with the given concentrations of florfenicol and the $\log_{10}$ numbers of CFU of intracellular bacteria per milliliter in cultures not exposed to florfenicol. Bars indicate standard errors of the means.

misspecification and the absence of estimation of the interindividual variability in drug disposition. The application of nonlinear mixed-effects modeling is appropriate for the unbiased estimation of population means and the interindividual variability of pharmacokinetic parameters. In the present study, the naïve pooling approach resulted in similar fitting performances with monocompartmental and bicompartmental models (based on Akaike's information criterion) whereas nonlinear mixed-effects modeling indicated that the best fit was obtained with a bicompartmental model. This model was finally used with both the naïve pooling approach and nonlinear mixed-effects modeling. The inability of naïve pooling to distinguish both inter- and intraindividual variabilities was highlighted by the comparison of residual errors obtained with naïve pooling and nonlinear mixed-effects modeling: these errors were close to the analytical error for florfenicol determination (about 10%) with nonlinear mixed-effects modeling, whereas they were much higher (more than 55%) with naïve pooling.

In contrast, the comparison of pharmacokinetic parameter estimates indicated reasonable agreement between results from the naïve pooling approach and nonlinear mixed-effect modeling. Only the second approach was able to estimate the interindividual variabilities of estimated parameters, expressed as percentages corresponding to coefficients of variation. For intravenous administration, the interindividual variabilities of

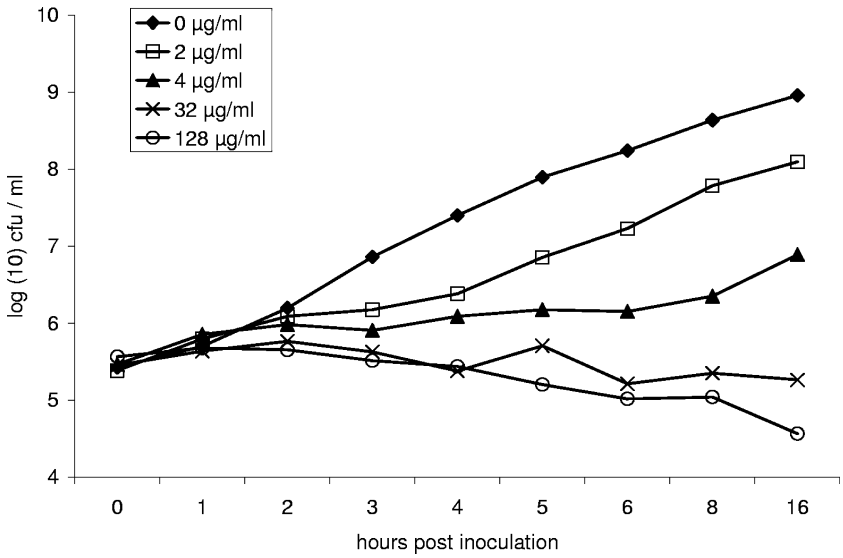

FIG. 5. In vitro killing curves for *Salmonella* serovar Typhimurium strain DAB69.

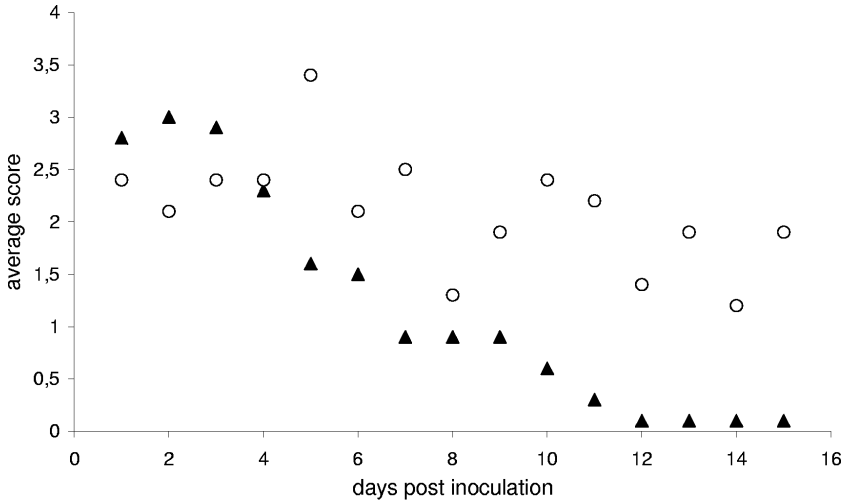

FIG. 6. Daily scores of fecal consistency after experimental infection of pigeons with *Salmonella* serovar Typhimurium. Circles and triangles represent the average daily scores for untreated and florfenicol-treated pigeons, respectively.

the AUC and CL for florfenicol ranged between 24 and 29%, whereas AUC variability after oral administration was about 50%. The resulting *F* of orally administered florfenicol was rather high (60%), but it could be deduced from the degree of variation (46%) that 95% of the pigeons exhibited *F* values in the range from 25 to 100%. In other words, the same dose given orally can result in fourfold variations in levels of systemic exposure to florfenicol among pigeons. Such variability should be taken into account in evaluations of the ability of a given dose to provide appropriate exposure levels for a given percentage of animals in a group.

Florfenicol is a time-dependent antimicrobial agent that shows strong bactericidal activity at MICs for *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Mannheimia haemolytica*, and *Histophilus somni* and bacteriostatic activity at MICs for *Staphylococcus aureus* (2, 6). Therefore, it seems advisable to maintain concentrations in plasma above the MIC to successfully control a *Salmonella* infection in pigeons. The average peak concentration of florfenicol in the serum after oral bolus administration was slightly higher than the MIC for the *Salmonella* strain tested. Combined with the rather high volume of distribution, this pharmacokinetic parameter suggests that the oral florfenicol medication of pigeons with salmonellosis results in a bacteriostatic effect on *Salmonella* in vivo. However, the short half-life of the drug, together with the erratic drinking-water uptake patterns of the pigeons, resulted in the absence of a steady state and the absence of blood samples containing concentrations higher than 4 μg/ml in the plasma when florfenicol was given at a dose of 0.5 mg/ml in the drinking water. Oral resorption of florfenicol by pigeons occurred rapidly, but the oral *F* was moderate (60%) and variable. This result may be due to incomplete intestinal resorption and/or a first-pass effect.

The volume of distribution of florfenicol in pigeons is rather

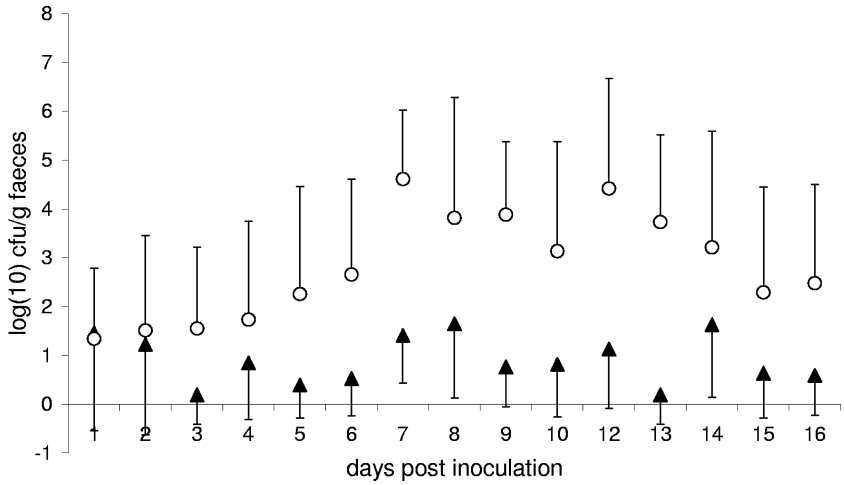

FIG. 7. Fecal shedding of *Salmonella* serovar Typhimurium after experimental infection of pigeons. Circles and triangles represent the average daily shedding by untreated and florfenicol-treated pigeons, respectively, expressed as the arithmetic means of the $\log_{10}$ numbers of CFU ± the standard deviations.

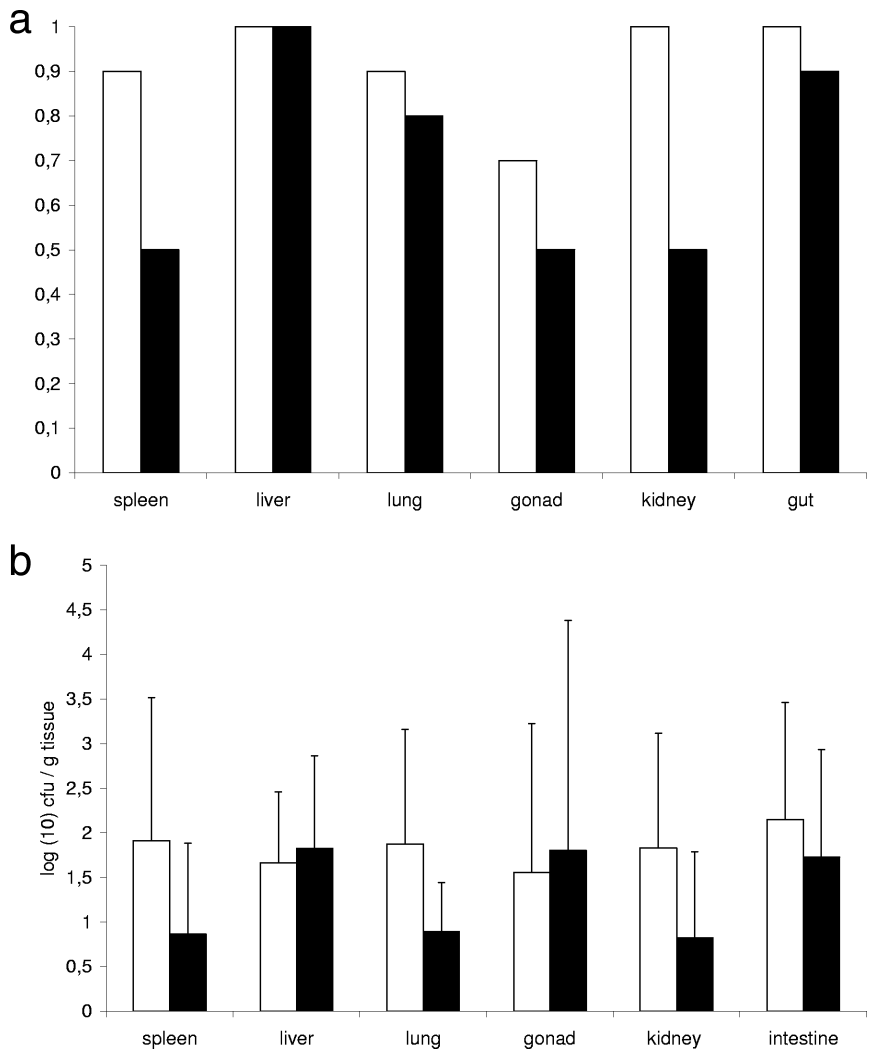

FIG. 8. Results of the bacteriological examination of tissues from pigeons inoculated with *Salmonella* serovar Typhimurium. Panel *a* shows the fraction of *Salmonella*-positive tissues from untreated (black bars) and florfenicol-treated (white bars) pigeons. In panel *b*, the average *Salmonella* loads ± the standard deviations in the tissues of treated (white bars) and untreated (black bars) pigeons are presented.

high, indicating elevated tissue concentrations and possibly high intracellular concentrations. In the in vitro experiment with the pigeon macrophages, in which the macrophages were exposed to different florfenicol concentrations for 16 h, a marked reduction of the number of intracellular salmonellae was obtained only at high (>16 μg/ml) concentrations of florfenicol in the extracellular environment. The pronounced growth inhibition observed in broth combined with the limited inhibition in the pigeon macrophages at similar concentrations of florfenicol would even suggest that the *Salmonella* bacteria are protected from the florfenicol in *Salmonella*-containing vacuoles. The dose dependency of florfenicol for the reduction of intracellular salmonellae was not observed previously (4) for chloramphenicol by using murine macrophages. Chiu et al. noticed a significant reduction of intracellular viable bacteria at extracellular chloramphenicol concentrations equal to the MICs and 10 times the MICs, whereas in the present study, no significant decrease of bacterial numbers was noticed at an extracellular florfenicol concentration equal to the MIC. The reasons for these differences are not clear but may include the use of chloramphenicol instead of florfenicol, the use of murine instead of pigeon cells, and/or the use of a non-gentamicin-based intracellular proliferation assay. If the data concerning the reduction of intracellular bacteria are combined with the pharmacokinetic data and the erratic drinking behavior of the animals, these results indicate that it is highly improbable that the intracellular persistency of *Salmonella* inside pigeon macrophages could be effectively inhibited by the administration of florfenicol via the drinking water. Drinking-water medication of pigeons with florfenicol for the treatment of *Salmonella* infections would thus promote therapeutic failure.

The predicted therapeutic failure of the oral florfenicol treatment of pigeons after inoculation with *Salmonella* serovar Typhimurium was reflected in the creation of *Salmonella* carriers (6 of the 10 animals) that did not shed the bacteria in detectable numbers in the feces but in which high numbers of *Salmonella* bacteria persisted in the tissues. Actually, the internal organs of the florfenicol-treated pigeons exhibited

higher *Salmonella* burdens than those of the untreated ones. This result is in agreement with the finding that the concentrations of florfenicol in plasma were not high enough to inhibit intracellular persistency inside macrophages. Such pigeons thus would pose a serious health threat to previously unexposed animals. This finding supports the hypothesis that the antimicrobial treatment of animals for *Salmonella* infection may promote the *Salmonella* carrier state, which would be of special importance for food-producing animals such as poultry and pigs.

Despite the persistency of *Salmonella* in high numbers in the pigeon tissues, oral florfenicol treatment of pigeons experimentally inoculated with *Salmonella* serovar Typhimurium markedly reduced fecal shedding and improved the fecal consistency. This finding suggests that the clinical recovery of pigeons from paratyphoid due to antimicrobial treatment is not necessarily correlated with decreases of the *Salmonella* loads in the internal organs.

In conclusion, the oral application of florfenicol for the treatment of paratyphoid in pigeons results in lower fecal shedding and less severe clinical symptoms than those in untreated animals but contributes to the development of carrier animals through sub-MIC concentrations in plasma that do not inhibit intracellular persistency.

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