

Correlation between Fecal Concentrations of Ciprofloxacin and Fecal Counts of Resistant Enterobacteriaceae in Piglets Treated with Ciprofloxacin: toward New Means To Control the Spread of Resistance?

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1 **Correlation between fecal concentrations of ciprofloxacin and fecal counts**
2 **of resistant *Enterobacteriaceae* in piglets treated with ciprofloxacin:**
3 **towards new means to control the spread of resistance?**

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12 **Running head**

13 **CIPROFLOXACIN LEVELS AND RESISTANT BACTERIA IN FECES**
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15 **Abstract**

16 We assessed in a piglet model the relationship between fecal ciprofloxacin concentrations and
17 ciprofloxacin resistant *Enterobacteriaceae* counts. Twenty-nine piglets were orally treated
18 with placebo, ciprofloxacin 1.5 or 15 mg/kg/day from day D1 to D5. Areas under the curve
19 (AUC) of concentrations increased sharply with dose and correlated positively with AUC of
20 resistant bacteria log counts between D1 and D9. Removing residual colonic quinolones could
21 help to control the emergence of resistance in fecal flora.

22

23 Increasing resistance to fluoroquinolones in Gram-negative bacilli is of major concern
24 because it compromises their therapeutic use (6, 7). Colonic flora is the reservoir of many of
25 the *Enterobacteriaceae* species with clinical significance (2) which are exposed to antibiotic
26 residues during fluoroquinolone treatments (9). It has been shown that administration of
27 enrofloxacin, a fluoroquinolone used in animals, could promote the emergence of quinolone
28 resistant enterobacteria in fecal flora of pigs (12). We also found that administration of oral
29 ciprofloxacin is associated with the emergence of quinolone resistant strains of
30 *Enterobacteriaceae* in fecal flora from human volunteers (3). However, rates of emergence of
31 resistance were not significantly different although the volunteers were exposed to different
32 antibiotic dosages. Of note, detection of resistance was only qualitative in that study, i.e.
33 absence versus presence of detectable resistant bacteria, and not quantitative, expressing the
34 densities of resistant bacteria in the feces. Indeed, several lines of evidence suggest that
35 quantitative aspects of emergence of resistance should also be analyzed. For instance, in
36 neutropenic patients, Gram-negative bacilli bacteremias are caused by the predominant fecal
37 clone of this type (11). Also, the density of a given *Escherichia coli* strain in a subject's fecal
38 flora can influence to some extent its ability to cause urinary tract infection (8). In order to
39 explore further the dynamics of fluoroquinolone resistance in the fecal flora during
40 treatments, we assessed here in a prospective randomized study in piglets the relationship
41 between fecal concentrations of ciprofloxacin and amounts of excreted ciprofloxacin resistant
42 *Enterobacteriaceae*.

43 Twenty-nine piglets from a single farm were included 4 weeks after birth. They were born
44 from sows that were treated at the time of parturition with 2 g oxytetracycline given
45 intramuscularly, but had not received directly any antimicrobials. Piglets were housed in
46 individual boxes for 21 days before the start of treatment (D1) and were randomly assigned to
47 oral treatment with placebo (9 piglets), ciprofloxacin 1.5 mg/kg/day (10 piglets) or 15

48 mg/kg/day (10 piglets) from D1 to D5. Ciprofloxacin suspension (Ciflox®, Bayer) or mineral
49 water (placebo group) was administered once a day at least 4 hours before food to animals
50 fasted for at least 12 hours. The protocol was approved by the local ethical committee.

51 Fecal samples were recovered from piglets after anal stimulation on mornings before
52 treatment (at D-1 and D1), during treatment (at D3 and D5, before ciprofloxacin
53 administration), and after treatment (at D7 and D9). Samples were stored frozen at -80°C until
54 microbiological analysis and ciprofloxacin assay were performed blinded of the treatment
55 group. Fecal concentrations of ciprofloxacin were measured by microbiological assay (5) after
56 ten-fold dilution in HCl 0.1N and then further dilutions in phosphate buffer pH 8.0. Counts of
57 total and resistant *Enterobacteriaceae* were obtained after plating serial dilutions of fecal
58 samples on Drigalski agar (BioRad laboratories, Marnes-la-Coquette, France) supplemented
59 or not with 20 mg/l of nalidixic acid or with 2 mg/l of ciprofloxacin. However, since there
60 was no significant differences in the counts obtained on the two types of media (data not
61 shown), only counts on ciprofloxacin containing agar were used for further statistical analysis.

62 Individual area under the curve (AUC) from D1 to D9 of ciprofloxacin fecal concentrations
63 (AUC_CIP), of log counts (AUC_RES) and of percentages (AUC_%RES) of ciprofloxacin
64 resistant *Enterobacteriaceae* above pre-treatment baseline values were computed by
65 trapezoidal approach. These criteria were compared across groups by nonparametric ANOVA
66 and pairwise Wilcoxon tests if global tests were statistically significant (with significance
67 level at 0.05). All statistical analyses were performed using SAS 9.1 (SAS, Cary, NC).

68 Concentrations of ciprofloxacin increased with the dose administered and peaked at 84.8 ± 57.9
69 *versus* 11.6 ± 12.6 µg/g of feces at D5 in piglets treated with 15 and 1.5 mg/kg/day of
70 ciprofloxacin respectively ($p < 0.001$) (Fig. 1A). Fecal antibiotic activity was detectable to
71 some extent in all animals 2 days after cessation of treatment (D7) but no activity was
72 detected in any animal at D9. AUC_CIP was significantly higher for the group treated with 15

73 mg/kg/day of ciprofloxacin than that for the group treated with 1.5 mg/kg/day ($p < 0.0005$)
74 (Table 1).

75 Most of the piglets (25/29, 86%) had ciprofloxacin resistant *Enterobacteriaceae* detected in
76 fecal samples before treatment. Administration of ciprofloxacin promoted the increase of both
77 absolute counts (Fig. 1B) and percentages of ciprofloxacin resistant enterobacteria (Fig. 1C)
78 in the 2 treatment groups when compared with the placebo group. AUC_RES and
79 AUC_%RES were significantly different between the groups treated with 1.5 mg/kg/day and
80 15 mg/kg/day *versus* the placebo group (both $p < 0.01$ for AUC_RES and both $p < 0.0005$ for
81 AUC_%RES). AUC_RES and AUC_%RES were also significantly higher for the group
82 treated with 15 mg/kg/day than that for the group treated with 1.5 mg/kg/day ($p < 0.05$ and
83 $p < 0.001$ respectively) (Table 1).

84 There were significant correlations between AUC_CIP and AUC_RES as well as between
85 AUC_CIP and AUC_%RES (both $p < 0.0001$, Spearman tests). These relationships were
86 adequately described using an Emax

87 model: $AUC_RES = AUC_RES_0 + \frac{AUC_RES_{max} \times AUC_CIP}{AUC_CIP_{50} + AUC_CIP}$ where AUC_RES_0 ,

88 AUC_RES_{max} and AUC_CIP_{50} are respectively the baseline, the maximal effect and the value
89 of AUC_CIP to obtain 50% of the maximal effect (Fig. 2).

90 We showed a significant positive correlation between the AUC of fecal ciprofloxacin
91 concentrations and the AUC of log ciprofloxacin resistant *Enterobacteriaceae* counts from
92 D1 of treatment to D9 post treatment. Rapid increase of ciprofloxacin resistant enterobacteria
93 from the beginning of ciprofloxacin administration probably resulted from the selection of
94 resistant bacteria which were already present in low counts before treatment in the fecal flora
95 of most (86%) of our piglets. Indeed, in a previous study in human volunteers (3), only 6 of
96 48 (12.5%) had quinolone resistant enterobacteria detected before treatment and the increased

97 prevalence of resistance was only observed after the end of the treatment without significant
98 dose relationship. The piglet study described here was much more fitted to evidence
99 pharmacokinetic/pharmacodynamic relationship since the doses varied by a factor of 10,
100 starting from a very low dose to a clinical dose (from 1.5 to 15 mg/kg/day) whereas they only
101 varied by a factor of 3 (250 mg twice/day to 750 mg twice/day) in the volunteers. Median
102 ciprofloxacin fecal concentrations at steady state were also much lower in piglet than in
103 human volunteers groups of treatment (7 and 73 versus 845 and 1938 $\mu\text{g/g}$ of feces
104 respectively). An increase in the percentage of ciprofloxacin resistant *Bacteroides fragilis*
105 strains in feces of human-flora-associated mice treated with various doses of ciprofloxacin has
106 been also reported as dose-related (10). However, our results differ from the absence of
107 correlation between antibiotic dosage and the percentages of fecal quinolone resistant
108 enterobacteria during treatment that was observed by others in pigs receiving several doses of
109 enrofloxacin varying in a range of 1 to 6 (12). Dynamic of emergence of resistant bacteria is
110 dependant of several parameters among which the concentrations of free and active antibiotic
111 reaching the colonic flora, MICs of bacterial populations and barrier effects exerted in the
112 colonic ecosystem. This may account for the nonlinear correlation between fecal antibiotic
113 concentrations and level of resistant bacteria we observed and, also, for differences in results
114 from similar studies.

115 We used AUCs to characterize the fecal pharmacokinetic/pharmacodynamic relationship for
116 ciprofloxacin, taking into consideration the whole time-course of concentrations and bacteria
117 counts. We believe that AUCs of fecal counts are the most relevant end-points because they
118 describe, better than single time point values, the total amount of resistant bacteria excreted,
119 which is actually what one wants to decrease to control the spread of resistance.

120 Indeed, the link we showed between intestinal concentration of ciprofloxacin and the amount
121 of ciprofloxacin-resistant enterobacteria excreted by the animals may be of utmost

122 importance in the current context of the spread of bacterial resistance. It has been shown in a
123 rat model that the administration of a compound consisting of activated charcoal entrapped
124 within zinc-pectinate beads could remove residual colonic ciprofloxacin (4). Thus, if such a
125 removal could be obtained in animals or humans receiving therapeutic doses of quinolones
126 without impairing the pharmacokinetics of the drug in plasma, this would open new avenues
127 within the scope of the recently signaled eco-evo drugs (1) to help to control of the emergence
128 and spread of quinolone resistance in gut-originating Gram-negative bacteria.

129

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133

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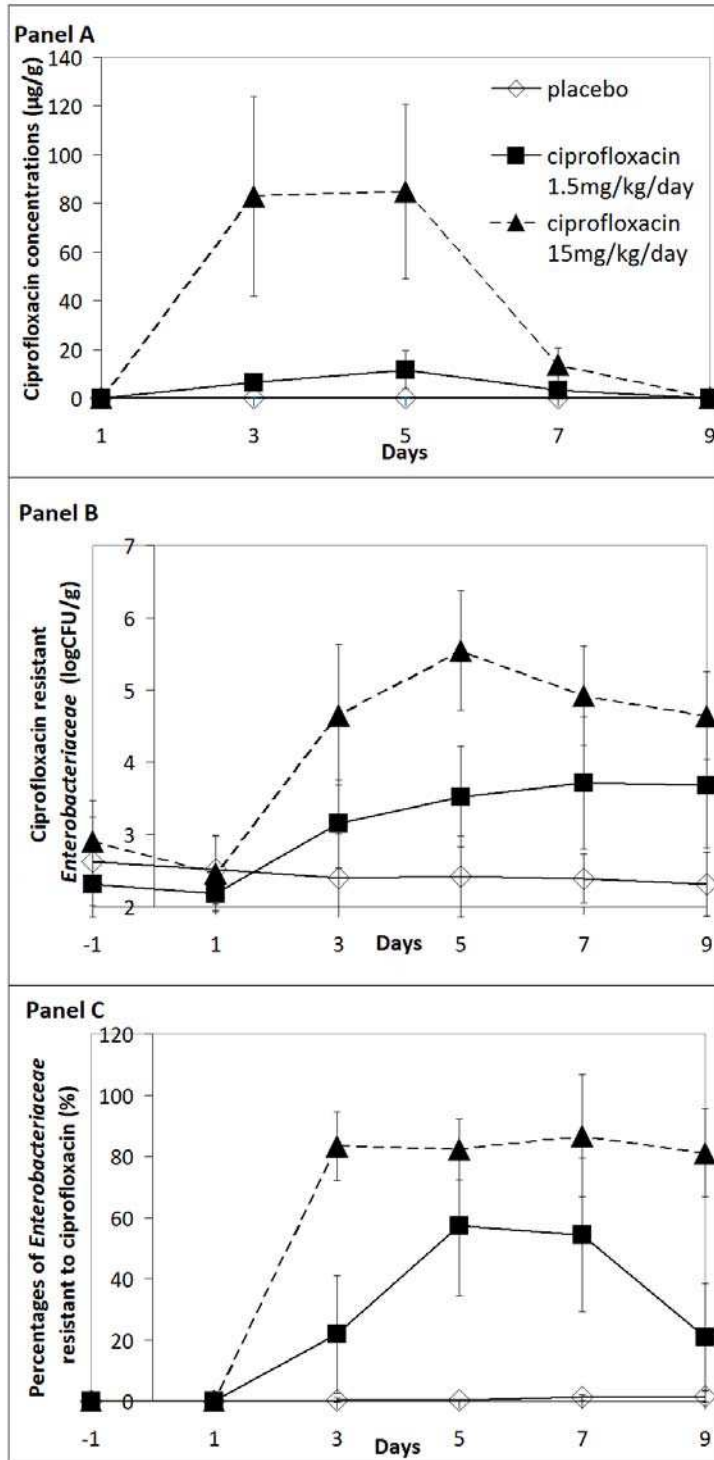
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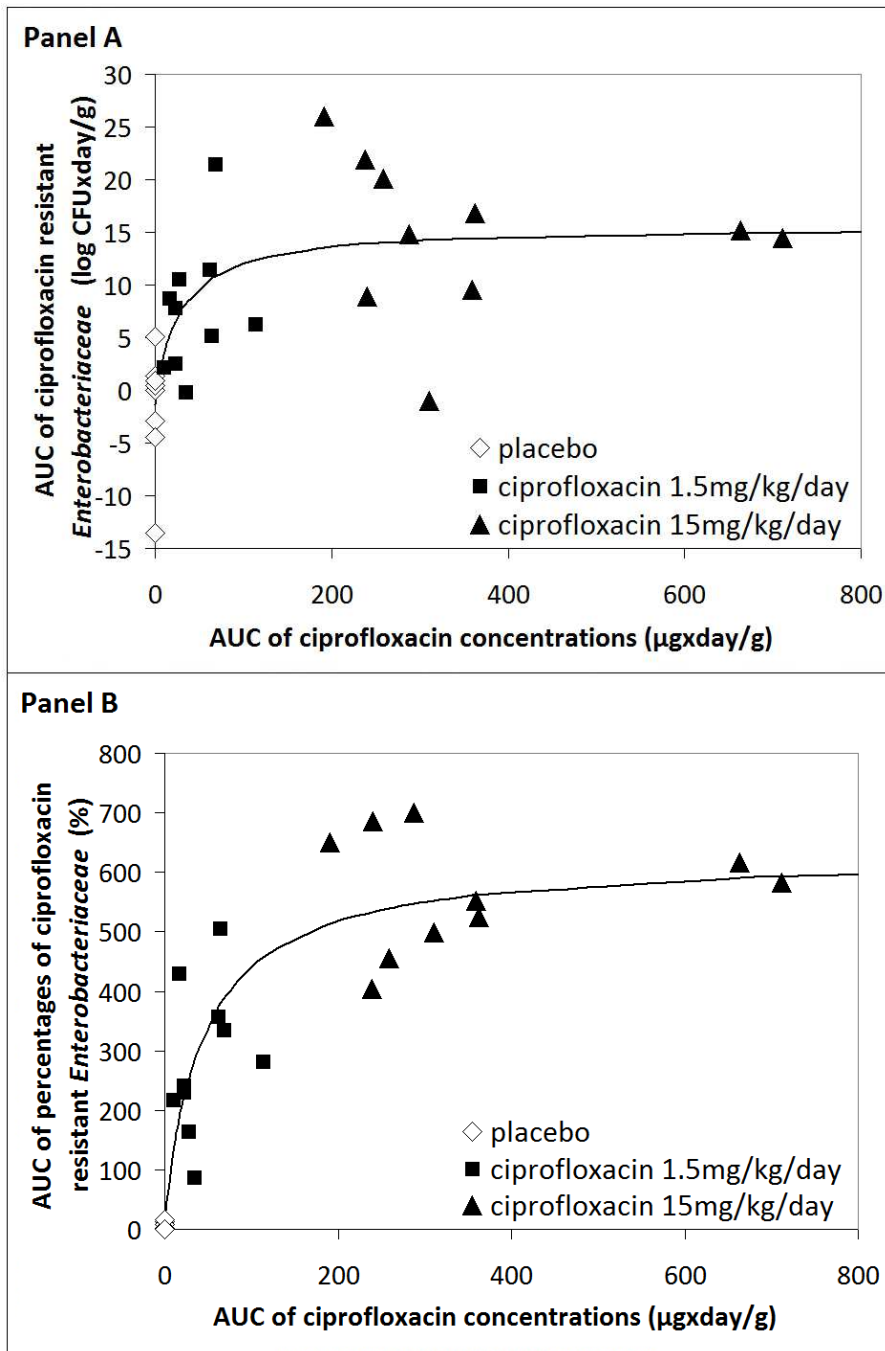
169 TABLE 1. Descriptive statistics by treatment group on AUC of ciprofloxacin concentrations
 170 (AUC_CIP), AUC of log counts of ciprofloxacin resistant *Enterobacteriaceae* above baseline
 171 (AUC_RES)^a and AUC of percentages of *Enterobacteriaceae* resistant to ciprofloxacin
 172 (AUC_%RES).

| Treatment group | Mean±SD | | |
|--------------------------------------|-----------------------|----------------------------|---------------------|
| | Median (Range) | | |
| | AUC_CIP (µg×day/g) | AUC_RES (log CFU×day/g) | AUC_%RES (%×day) |
| Placebo (n=9) | 0 | -1.3±5.3 | 4.5±6.1 |
| | 0 | 0.5 (-13.5-5.1) | 0.9 (0.1-15.6) |
| Ciprofloxacin 1.5mg/kg/day (n=10) | 44.0±32.2 | 7.6±6.2 | 284.9±124.8 |
| | 30.6 (10.1-113.5) | 7.1 (-0.2-21.5) | 261.8 (86.5-504.8) |
| Ciprofloxacin 15mg/kg/day (n=10) | 362.5±179.8 | 14.7±7.6 | 567.1±98.2 |
| | 299.3 (191.2-711.5) | 15.0 (-1.0-26.0) | 568.0 (403.9-700.0) |

173 ^a Some values of AUC_RES are negative because of the normalisation by the baseline.



174
 175 FIG. 1. Mean (and 95% confidence intervals) of ciprofloxacin concentrations (Panel A) and of log
 176 counts of ciprofloxacin resistant *Enterobacteriaceae* (Panel B) as well as of percentages of
 177 *Enterobacteriaceae* resistant to ciprofloxacin (Panel C) in fecal samples from piglets treated with
 178 placebo (n = 9), 1.5 mg/kg/day (n = 10) and 15 mg/kg/day (n = 10) of oral ciprofloxacin from day 1 to
 179 day 9.



180

181 FIG. 2. Relationship between individual AUC of fecal concentrations of ciprofloxacin (AUC_CIP)
 182 versus AUC of log counts of fecal ciprofloxacin resistant *Enterobacteriaceae* (AUC_RES) (Panel A)
 183 and versus AUC of percentages of ciprofloxacin resistant *Enterobacteriaceae* (AUC_%RES) (Panel B)
 184 for 29 piglets in 3 treatment groups. Each symbol represents the AUC_CIP and AUC_RES or
 185 AUC_%RES values for one piglet. The black curve among the symbols is the mean curve predicted by
 186 the Emax model.