

A norfloxacin dose finding study for selective decontamination of the digestive tract in pigs

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SUMMARY

Five pigs were treated orally with norfloxacin for 5 consecutive days in two well-separated periods. This was done to determine the lowest dose required to free the pigs of Enterobacteriaceae. In the first period of the study, the animals were treated with 400 mg per day while in the second treatment 800 mg norfloxacin was given. Daily faecal culturing indicated that the faeces became free of Enterobacteriaceae in 3–5 days when treated with 400 mg/day, while all animals were found negative on culturing after 3 days of treatment with 800 mg/day. Investigation of the concentration of norfloxacin in the faeces revealed that a substantial fraction of the dose was either absorbed or inactivated by faecal substances. An *in vitro* study in faeces confirmed that a substantial part, some 75% of the dose, may have been inactivated by intestinal contents. This finding helps to explain the much lower concentration of 120 mg norfloxacin per kg of faeces in the pig in comparison with the almost tenfold higher concentration reported to develop in man during treatment with an identical dose.

INTRODUCTION

The increased use of chemotherapy and irradiation for cancer treatment in patients makes it necessary to develop an animal model for investigating to what extent flora modulation can contribute to saving mucosal cells. For such studies large animals are an advantage because their clinical condition can be judged better than is possible in smaller animals. Careful clinical observation in addition to detailed histological investigations may contribute to better investigation of the pathogenesis and prevention of infectious complications, most of which originate from the digestive tract flora. In general dogs or monkeys are used for such studies. These species however, have the disadvantage of being respectively carnivorous (which implies that in microflora studies they may differ markedly from man) and very scarce. Therefore, the conventional pig – being omnivorous and readily available – was used for the present investigation.

Initially the feasibility of selective decontamination (SD) of the digestive tract of the pig was investigated. SD is the selective complete suppression of aerobic

Gram-negative bacilli in the digestive tract, while maintaining the protective indigenous anaerobic microflora intact (Sleijfer *et al.* 1980; Winston *et al.* 1987).

Norfloxacin, a new quinolone derivative, is in general well taken by patients and therefore was selected for our SD study. In addition, norfloxacin has recently been found very effective and safe for SD in man (Pecquet, Andreumont & Tancrede, 1985; Winston *et al.* 1987; Edlund *et al.* 1987). However, regardless of its apparent safety, there remains a question concerning the maintenance of the indigenous intestinal flora. Norfloxacin concentrations have been reported to be well in excess (more than 10 times) of the minimal inhibitory concentration (MIC) for bacteria which compose the indigenous flora. This is difficult to explain since *in vivo* much lower bio-active concentrations, which are compatible with the survival of the majority of the species of the anaerobic intestinal flora, exist apparently in the colonic contents. We have attempted to answer this question in our investigation.

To estimate the influence of norfloxacin on the indigenous intestinal microflora during oral treatment, direct microscopic clump counting and calculation of the number of colony forming units (c.f.u.) per gram of faeces was performed. Anaerobic culture in addition to aerobic culture, would have provided more detailed information. However, anaerobic culture is very laborious, expensive and may still be incomplete when constructing an inventory of the flora before, during and after treatment. It was therefore decided that detailed investigation of the composition of the indigenous microflora was better postponed to a subsequent study. In order to define whether the pig responded adequately to the oral treatment, it was decided that the number of days between the start of treatment and the first negative faecal sample should be identical to the interval found in man.

MATERIALS AND METHODS

Animals

Five outbred conventional Münchener Troll minipigs (two males and three females) of 30–36 kg bodyweight and aged 3 months were used. The animals were received from a commercial breeding unit: the Dr Brauer farm in Munich.

Housing

The pigs were housed one per cage under strict hygienic circumstances. The cages were cleaned and disinfected daily. The animals received (Enterobacteriaceae-free) pelleted pig food (Altromin, Haltungsdiet für Minischweinen; München) twice daily, while tap water was randomly available.

Selective decontamination treatment

Two dosages of norfloxacin (Merck Sharpe and Dohme, Darmstadt, F.R.G.) have been investigated: 400 mg or 800 mg per day. Both dosages were given orally in two daily portions for 5 successive days. Treatment periods with these two dosages were separated by 2 weeks during which no antimicrobial treatment was given. For treatment, norfloxacin tablets were ground and mixed in appropriate amounts with a few food pellets for each pig. After the pigs had taken the medicated pellets they received the remaining part of their food ration.

Faecal sampling

Fresh faeces were sampled daily to determine the weight of output. In addition, a sample of the faeces was used for aerobic culture for investigation of the concentration and inactivation of norfloxacin by faecal substances, as well as for direct microscopic clump counting (DMCC) (Holdeman, Cato & More 1977).

Bacteriological culturing

Qualitative counts: faeces was directly inoculated on to McConkey agar No 3 (Merck). After overnight incubation all morphologically different looking colonies were subcultured to purity and then typed with the API 20E system (Analytab, Lyon).

Quantitative counts: one gram of faeces was suspended in 9 ml of distilled water by short vortex mixing. After 30 min of standing on the bench to permit sedimentation, one drop of the supernatant (10^{-3} ml) was inoculated on to McConkey agar and another on to Aesculin azide agar (Merck). After overnight incubation, the concentration of Gram-negative bacilli and of enterococci was estimated by comparing the number of colonies with those on culture plates that had been inoculated with suspensions with known numbers of *Escherichia coli*.

Direct microscopic clump counting

To estimate the number of anaerobic bacteria per gram of faeces, the method described by Holdeman, Cato & More (1977) was used. Briefly a faecal suspension of 1 g of faeces in 9 ml of saline was gently centrifuged to eliminate particles greater than $5\ \mu\text{m}$. Then 0.01 ml of the supernatant was smeared over a surface of exactly $1\ \text{cm}^2$ on a microscopic slide. After drying, fixation and staining the number of microscopic clumps (bacteria) was counted. In this way the approximate number of bacteria per gram of faeces could be calculated.

Norfloxacin concentration

Reference graphs were constructed showing the relation between the concentration of norfloxacin and the diameter of inhibition zones on agar. Norfloxacin was dissolved in an acetate buffer solution (pH 4.5). A reference graph was then constructed by a saturation of standard-sized neutral discs (diameter 9 mm) in buffered (pH 7) norfloxacin solutions of seven different concentrations (1000, 5000, 2500, 1000, 300, 100, 30 mg/l). After saturation, the disks were placed on DST agar (Merck, Darmstadt) previously seeded with a 10^7 c.f.u./ml suspension of an *E. coli* strain which had an MIC to norfloxacin of 0.1 mg/l. After overnight incubation, the inhibition zones around the disks were measured. The reference graph was made by plotting the diameter of the inhibition zones against the \log_{10} of the corresponding norfloxacin concentrations in the standard solutions. For each determination of the norfloxacin concentration in faeces, a new reference graph was determined.

The degree of inactivation of norfloxacin by faecal substance

This was investigated before treatment by suspending 1 gram of faeces from each pig in 3 ml of each of the norfloxacin solutions mentioned in the previous

paragraph. After standing on the bench for 30 min, the suspensions were centrifuged at 2000g for 30 min. The norfloxacin concentration in the supernatants was then determined with the disk method outlined in the previous paragraph. In addition a new reference graph norfloxacin was determined. The norfloxacin concentration in the faecal suspensions was then determined by the horizontal distance between the graphs formed by the reference solutions and the bio-active norfloxacin concentrations in the *in vitro* mixed faeces. The difference between the concentration in the control solutions and those in the faeces mixed with a corresponding concentration of norfloxacin was taken as a measure of the degree of inactivation.

Concentration of norfloxacin in stools of treated animals

To determine the amount of biologically active norfloxacin per gram of faeces from treated animals, neutral disks were saturated in the supernatant of a 1:3 (w/v) suspension of faeces of pigs before, during and shortly after treatment. These disks were then placed on to DST agar preseeded with the same *E. coli* strain as used for the reference line. After measuring the inhibition zone, the norfloxacin concentration could be determined with the help of the reference line.

Sensitivity of Enterobacteriaceae for norfloxacin

Determination of the minimal inhibitory concentration (MIC) of norfloxacin of each Enterobacteriaceae strain isolated, was performed by agar diffusion. This was done with three of the seven norfloxacin solutions (2500, 1000, 100 mg/l). The isolates were seeded in the same way as the reference *E. coli* strain on DST agar, before the disks were placed on to it. The slope of the line, which represented the relation between the inhibition zones and the three standard solutions, was determined as described for the reference line. Its intersection with the intercept was used to calculate the MIC of the strains.

Investigation of the pH of the faeces

Because the activity of norfloxacin is influenced by the pH of the milieu in which it is suspended, the pH of fresh faeces of each individual pig was investigated six times during the study.

Reconventionalization

One week after stopping oral norfloxacin treatment, the animals were orally inoculated with 10^9 bacteria of each of the strains of Enterobacteriaceae species which had been isolated before the onset of norfloxacin treatment. This was done to bring the Gram-negative flora back to the composition it had before the first treatment course.

RESULTS

The quantitative results of faecal culturing regarding the Enterobacteriaceae species before, during and after norfloxacin treatment, are depicted in Fig. 1 and 2. In all five animals, the stool cultures were negative on day 5 of treatment with 400 mg/day and on day 3 during daily treatment with 800 mg. Enterococci were

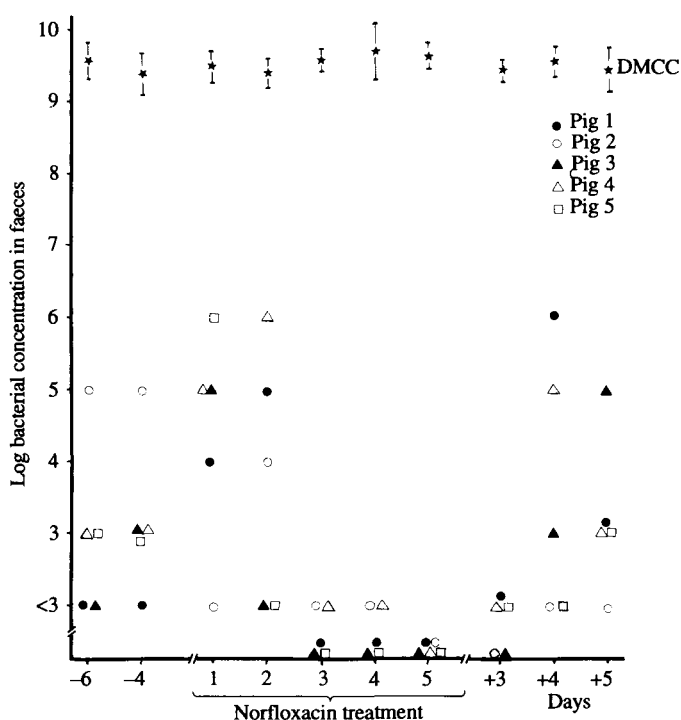


Fig. 1. Log concentrations of total numbers of intestinal bacteria (DMCC) as well as of Enterobacteriaceae species per gram of faeces before, during and after 5 days of oral treatment of five pigs with 400 mg norfloxacin per day. Mean DMCC's and the standard deviation of the means are presented in the top of the figure.

only slightly suppressed by the norfloxacin concentrations established by treatment in all five animals (data not shown).

The direct microscopic clump counting (DMCC) of the daily collected faecal samples, revealed no detectable suppression of the faecal microflora (Figs 1 and 2).

The faecal norfloxacin concentration found during and after treatment with 400 mg and 800 mg per day are depicted separately for each pig and varied between 10 and 25 mg/l suspension (40 and 100 mg/kg faeces) as depicted in Figs 3 and 4. The bioactive norfloxacin concentration in the faeces appeared to increase to relatively high concentrations. On the third day of treatment, concentrations averaging 10 mg/l faecal suspension (40 mg/kg faeces) were found. After stopping treatment, it took 4 days before the faeces were found free of detectable norfloxacin. During treatment with 800 mg the concentration was found to be of the same magnitude as during treatment with 400 mg/day, on the third day of treatment.

The degree of bio-inactivation of norfloxacin by experimental mixing of seven different concentrations of norfloxacin with faecal suspensions prepared from all five pigs, is shown in Fig. 5. The data which determined these graphs had a high correlation with the reference line ($r = 0.98$). The horizontal distance between the mean concentrations found in the faecal suspensions after mixing *in vitro* and the concentrations found in corresponding solutions made in buffered saline, indicate

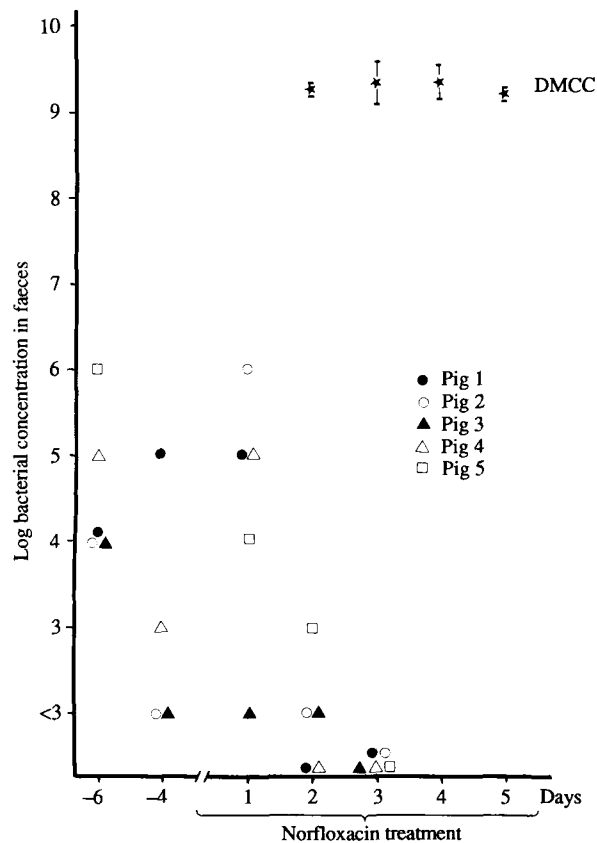


Fig. 2. Log concentrations of total numbers of intestinal bacteria (DMCC) as well as of *Enterobacteriaceae* species per gram of faeces before and during oral treatment of five pigs with 800 mg norfloxacin per day. Mean DMCC's and the standard deviation of the means are presented in the top of the figure.

considerable bio-inactivation of norfloxacin by faecal substances of all pigs. At the concentration levels investigated, the degree of inactivation appeared independent of the original norfloxacin concentration. In all faecal suspensions after incubation approximately 75% of the premixed norfloxacin could not be recovered (Fig. 5).

The sensitivities for norfloxacin of the biotypes of *Enterobacteriaceae* species isolated before treatment are presented in Table 1. All isolates were sensitive to 10 mg norfloxacin/l or less (accuracy of reference graphs determined for these investigations: $r > 0.96$).

The pH of the stools was close to 7 in all five animals although slightly lower in pigs 2 and 3 than in pigs 1, 4 and 5 (Table 2). The amount of stools produced per day varied between 380 and 550 g/pig (mean, 490 g).

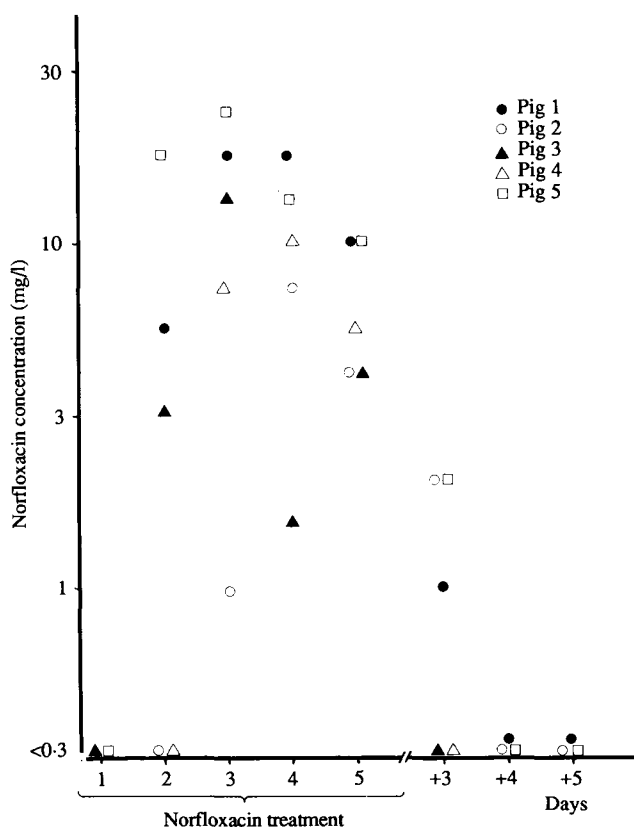


Fig. 3. Norfloxacin concentrations found in suspensions of faecal samples of five individual pigs, during and shortly after five days of oral treatment with 400 mg/day.

DISCUSSION

The results of the present study show good correlation in dose-effectiveness between human subjects and the pigs. De Vries-Hospers, Welling & van der Waaij (1985) found that, in ten human volunteers, daily oral treatment with 200 mg, 400 mg or 800 mg norfloxacin resulted in Enterobacteriaceae-free stool cultures after 3–5 days. A similar period of norfloxacin treatment was required for negative stool cultures in another study by Pecquet and co-authors (1985) as well as by Edlund *et al.* (1987). In our pigs, it also took 5 or 3 days of treatment with the dosages used (400 mg/day and 800 mg/day respectively) before their stools were negative. However, the DMCC and the concentration of enterococci were not changed. The fact that the DMCC was not changed during norfloxacin treatment, regardless the fact that (perhaps inactive) concentrations existed in the faeces far in excess of the MIC's of most if not all anaerobic intestinal bacteria, could be explained if, as described by Edlund *et al.* (1987), faecal substance complexes strongly with norfloxacin. After suspending faeces with (bio-inactive) norfloxacin, part of the complexed norfloxacin may be dissociated and return to activity. This supposition is to some extent supported by our *in vitro* norfloxacin inactivation

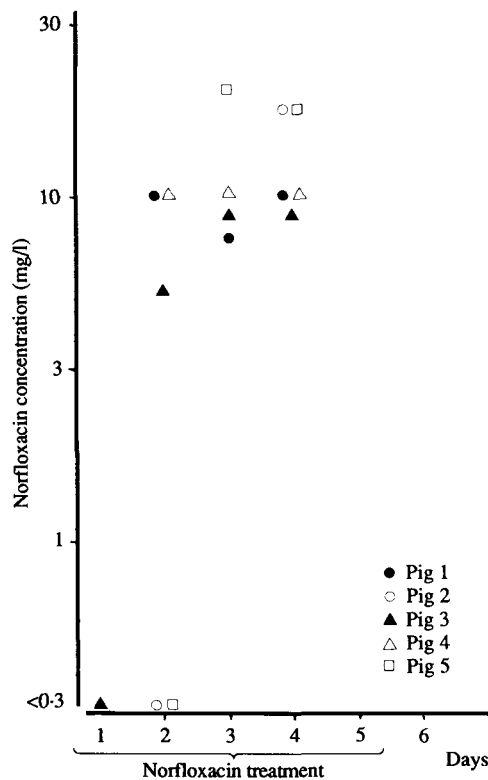


Fig. 4. Norfloxacin concentrations found in suspensions of faecal samples of five individual pigs, during oral treatment with 800 mg/day.

study. Observations reported by Cofsky, DuBouchet & Landesman (1984) however, may provide more evidence. These authors washed faeces from treated volunteers several times; each time more norfloxacin was recovered in high concentrations in the supernatant.

In the studies of human subjects by De Vries-Hospers, Welling & van der Waaij (1985), the average faecal norfloxacin concentration during treatment with 200 mg, 400 mg and 800 mg per day was higher than we found in the pig. Also Gilfillan, Pelak & Bland (1984) as well as Edlund *et al.* (1987) also reported relatively high average concentrations of 700–1000 mg/kg of faeces at the third day of treatment with 400 mg per day. Pecquet and co-authors (1985), also studying human volunteers, reported even higher concentrations between 1000 and 5000 mg/kg of faeces with a mean of 2500 mg/kg during treatment with 200 mg norfloxacin per day and 4000 mg/kg faeces during daily administration of 400 mg.

We found mean bio-active norfloxacin concentrations of between 10 and 25 mg/l faecal suspension (this equals 40 and 100 mg/kg faeces) in our pig-study during daily treatment with 400 and 800 mg. Assuming that the percentage of the drug that is absorbed following oral administration is about equal in man and in the pig, the difference in bio-active norfloxacin concentration in man and in pigs could be explained as follows. The amount of faeces produced by a human individual is

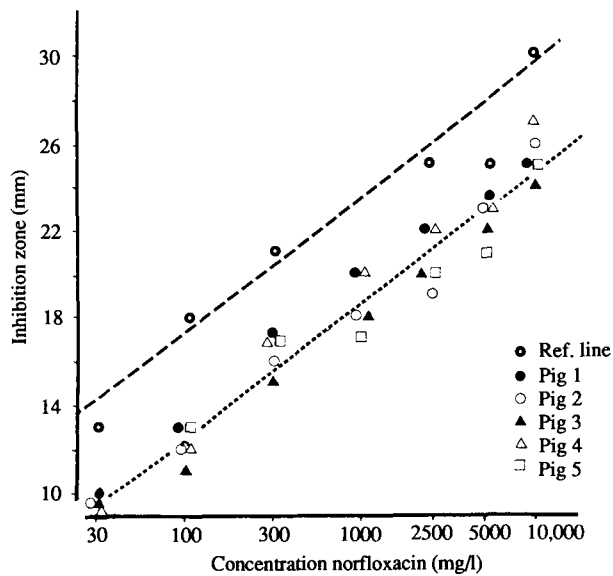


Fig. 5. Relation between the concentration of norfloxacin after *in vitro* mixing with faeces and the corresponding inhibition zone on agar preseeded with a sensitive strain of *E. coli*. The horizontal distance between the reference graph of norfloxacin in buffered saline and the values in faeces reflects the degree of norfloxacin inactivation by intestinal contents (faeces).

about 100 g/day (Drasar, Jenkins & Cummings, 1976). This is two to five times less than the mean amount of faeces excreted by our minipigs. At a daily dose of 400 mg norfloxacin and an average absorption of 40% (Stein, 1987) in our pigs, 240 mg may have mixed daily with 500 g colonic contents. Our *in vitro* bio-inactivation study has shown that, after suspending of faeces in a norfloxacin solution, only 25% remains bio-active. This implies that 25% of the 240 mg of unabsorbed norfloxacin (e.g. 60 mg/500 g faeces) may have become bio-active upon suspending a faecal sample in buffered saline, as the suspensions were prepared in the same way as was used in the *in vitro* study. With this pharmacodynamic explanation the bio-active norfloxacin concentration of between 40 and 100 mg/kg faeces found in our pig study is in fact only a little lower than the concentration reported in man, whose daily production of faeces is five times less than that of the pig.

The difference in response between Enterobacteriaceae species and the other enteric bacteria requires further investigation. There could be a parallel with the mode of action of trimethoprim in selective decontamination as described by Toorop-Bouma & Van der Waaij (1987). They found that trimethoprim acts mainly after absorption in the gut and subsequent secretion in the mucus. Trimethoprim is therefore supposed predominantly to suppress growth of Enterobacteriaceae in the mucus layer and to a much lower extent in the intestinal contents. Like norfloxacin, trimethoprim is also found to be considerably inactivated by faecal substance (Veringa & Van der Waaij, 1984). To suppress growth of colonizing Enterobacteriaceae, norfloxacin may have to be absorbed and excreted by intestinal mucus. In our pigs, Gram-negative enterobacilli may

Table 1. *Sensitivity of Enterobacteriaceae species isolated from pigs before norfloxacin treatment*

Pig	Enterobacteriaceae species	API-profile number	MIC*
1	<i>E. coli</i>	5044552	0.38
	<i>E. coli</i>	5044550	0.54
	<i>E. coli</i>	5144552	3.90
	<i>E. coli</i>	5144552	0.36
	<i>E. coli</i>	5144572	0.54
2	<i>E. coli</i>	5044552	0.46
	<i>Ent. hafniae</i>	5104113	6.30
	<i>E. coli</i>	5144113	0.53
	<i>E. coli</i>	5144553	0.29
	<i>E. coli</i>	5144572	0.54
	<i>Ent. aerogenes</i>	5304773	0.46
3	<i>Ent. hafniae</i>	4144113	4.80
	<i>E. coli</i>	4144372	3.50
	<i>E. coli</i>	5144572	1.00
	<i>E. coli</i>	5144572	2.20
4	<i>E. coli</i>	5144172	2.60
	<i>E. coli</i>	5144572	2.60
5	<i>E. coli</i>	4144572	3.20
	<i>E. coli</i>	5144142	2.50
	<i>E. coli</i>	5144552	2.30
	<i>E. coli</i>	5144552	5.20
	<i>E. coli</i>	5144572	2.40

* MIC, minimal inhibitory concentration of norfloxacin (mg/l).

Table 2. *Mean and standard deviation (s.d.) of the pH of five different faecal samples per pig*

pH	Pig				
	1	2	3	4	5
Mean	7.7	7.1	6.5	7.4	7.6
s.d.	0.45	0.56	0.79	0.25	0.15

have been affected by norfloxacin in the mucus layer in a direct way. In the colon contents, the concentration of bio-active norfloxacin may be below the MIC of these Gram-negatives as well as of that of the great majority of the bacteria in the colon. We assume therefore, that like trimethoprim, norfloxacin may dissociate and become active upon suspending of faeces from an inactive complexed form.

Another possible explanation for the discrepancy between the MIC's of intestinal bacteria and the norfloxacin concentrations found in faeces of treated subjects has recently been put forward by Goldstein, Citron and Corrado (1987). These investigators have found a marked inoculum effect at 10^9 c.f.u./ml for most anaerobic isolates, but not for *E. coli*. At 256 mg/l all *E. coli* were killed while the anaerobic bacteria maintained colony counts greater than 10^9 c.f.u./ml. The anaerobes, however, had MIC's which were only two to fourfold greater than those of the three *E. coli* strains tested.

In conclusion, the present study indicates that the results of selective decontamination of the intestinal tract by oral norfloxacin treatment is comparable between man and pigs in respect of the daily dose of norfloxacin required to obtain after several days of treatment stool cultures free of Enterobacteriaceae species the number of treatment days required for Enterobacteriaceae negative faecal cultures and the average 'faecal norfloxacin (steady state) concentration' accomplished during a 5-day treatment period.

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