ORIGINAL ARTICLE

Effect of sterilized human fecal extract on the sensitivity of *Escherichia coli* ATCC 25922 to enrofloxacin

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The ingestion of antimicrobial residues in foods of animal origin has the potential risk of exposing colonic bacteria to small concentrations of antibiotics and inducing resistance in the colonic bacteria. To investigate whether human intestinal contents would influence resistance development in bacteria, *Escherichia coli* ATCC 25922 (MIC of enrofloxacin $< 0.03 \mu\text{g ml}^{-1}$) was exposed to 0.01 to $1 \mu\text{g ml}^{-1}$ of enrofloxacin in media supplemented with glucose, sucrose, sodium acetate or sterilized human fecal extract. In the first passage, only the medium containing sterilized fecal extract supported the growth of *E. coli* at an enrofloxacin concentration equal to the MIC. In the second and third passages following exposure to sub-inhibitory concentrations of the drug, the bacteria in media containing sterilized fecal extract grew at $0.1 \mu\text{g ml}^{-1}$ of enrofloxacin. The efflux pump inhibitors, reserpine and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), increased the sensitivity of bacteria to $0.1 \mu\text{g ml}^{-1}$ of enrofloxacin in the medium containing sucrose, but their effect was not observed in the medium supplemented with 2.5% sterilized fecal extract. The proportions of unsaturated and saturated fatty acids in *E. coli* grown in the medium with 2.5% sterilized fecal extract differed from those grown in the medium alone. Fecal extract may contain unknown factors that augment the ability of *E. coli* to grow in concentrations of enrofloxacin higher than MIC, both in the presence and absence of efflux pump inhibitors. This is the first study showing that fecal extract affects the level of sensitivity of *E. coli* to antimicrobial agents.

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INTRODUCTION

The use of antimicrobial agents in food-producing animals may result in the presence of low concentrations of drugs or their metabolites in food.^{1–5} The issue of whether residues of antimicrobial agents ingested in foods pose a danger to human health by increasing the population of bacteria that are insensitive to these substances has not been fully examined.^{6,7} Continuous exposure of the human colonic microbiota to antimicrobial agents from treated food animals may lead to elimination of highly sensitive commensal strains, allowing infection by opportunistic pathogens, or to exertion of selective pressure on the intestinal microbiota, favoring the growth of microorganisms less sensitive to the drugs and resistance development in bacteria that were previously sensitive.^{1,2,7–10}

Enrofloxacin is a fluoroquinolone antimicrobial agent effective against a broad spectrum of Gram-positive and Gram-negative bacteria.^{11,12} It is used in veterinary medicine for the treatment of a variety of infections, including those in food-producing animals. Fluoroquinolone-resistant strains of various colonic bacteria including *Escherichia coli*, may develop following exposure to these drugs.

Two main reasons for the fluoroquinolone-resistance are mutations in the drug targets gyrase A and topoisomerase IV, and extrusion of the drug via an efflux pump. Mutations at the quinolone resistance-determining region (QRDR) of target genes may alter the binding of quinolones to this region and lead to reduced sensitivity or resistance to quinolones.¹³ The *gyrA* mutations and over-expression of efflux pump gene appear to be prevalent in clinical isolates of various bacteria including *E. coli*.^{14–20}

Fecal slurries are a complex mixture of digested and undigested food, viable and non-viable bacteria, bacterial fermentation products and metabolites, and human cells and secretions.²¹ The inactivation of antibiotics by fecal materials has been shown *in vitro*.^{22–24} Drug inactivation can be due either to binding to insoluble components of fecal slurries,²⁵ or to enzymatic modification.²⁶ Unknown chemical properties of feces can affect binding or inactivation of antibiotic residues.²⁷ However, there are no studies investigating the effect of human fecal extracts on the sensitivity of *E. coli* to the veterinary antimicrobial enrofloxacin or on the ability of bacteria to develop resistance to this drug.

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Ingestion of residues of antimicrobial agents in foods from treated animals may have the potential to influence the antimicrobial sensitivity of the members of the colonic microbial community. In this study, we have measured the effect of a low concentration of enrofloxacin on the sensitivity of an *E. coli* strain grown in media with and without sterilized fecal extract.

MATERIALS AND METHODS

Bacterial strain and growth media

E. coli ATCC 25922, for which the MIC of enrofloxacin is reported to be $0.03 \mu\text{g ml}^{-1}$,²⁸ was grown in 10 ml of Mueller–Hinton broth (MHB, LabM, Farmingdale, NY, USA), at 37 °C with shaking at 200 r.p.m. Autoclaved MHB was supplemented with filter-sterilized solutions of glucose, sucrose or sodium acetate to a final concentration of 5 mM or with 1 and 2.5% of human fecal extract.

Preparation of sterilized fecal extract

Fresh feces collected from three healthy individual volunteers, who had not been treated with any antibiotic for 6 months, were added to equal amounts (w/v) of MHB. The use of human fecal samples was approved by the FDA Research Involving Human Subjects Committee (approval number 09-033T). The samples were mixed extensively to produce homogeneous suspensions. The fecal suspensions were further diluted with MHB to produce 10 and 25% (w/v) dilutions and sterilized by autoclaving at 121 °C for 30 min. The sterile fecal suspensions were centrifuged at $10\,000\times g$ for 10 min and filtered (0.2 μm , 25 mm, Millipore, Billerica, MA, USA). The extracts were transferred to new tubes for use in experiments. As the concentrated fecal extract is opaque and its OD is higher than the limit of detection by spectrophotometer, it was diluted in MBH medium to final concentration of 1.0 or 2.5%, suitable for monitoring its effect on the growth of bacteria.

Verification of the MIC of enrofloxacin

The MIC of enrofloxacin for *E. coli* ATCC 25922 was verified in a 96-well microtiter plate. Serial dilutions of enrofloxacin, in concentrations ranging from 0.01 to $1 \mu\text{g ml}^{-1}$, were prepared in 200 μl of MHB medium. The wells were inoculated with 2×10^5 CFU ml^{-1} and the plates were incubated for 24 h at 37 °C. The MIC of enrofloxacin, in which no bacterial growth was observed, was then determined.

Kinetics of bacterial growth with enrofloxacin in supplemented MHB

The effects of enrofloxacin on the kinetics of growth of *E. coli* ATCC 25922 in MHB, in the presence and absence of 5 mM glucose, sucrose or acetate, or of 1.0 or 2.5% sterilized fecal extract, were measured as follows: 200 μl of each of the media containing different additives was added to duplicate wells of 96-well plates. Dilutions of 0.01, 0.02, 0.03, 0.06, 0.1 and $1 \mu\text{g ml}^{-1}$ of enrofloxacin were made in each of the media containing different additives. *E. coli* cells (2×10^5 CFU ml^{-1}) were added to each of the wells. Duplicate control wells, containing media without antibiotics and with and without additives, were also inoculated with the same number of cells. The microtiter plates were directly incubated in a Synergy MX spectrophotometer (BioTek Instruments, Winooski, VT, USA).

The kinetics absorbance assay protocol of the Gen5 microplate data collection and analysis software (BioTek Instruments) were used to measure and record the OD₆₀₀ of the cells every 30 min for 15 h with shaking for various intervals. The kinetics of growth of *E. coli* grown with each of the concentrations of enrofloxacin in the medium with each of the additives was determined, subtracting the OD₆₀₀ value of the non-inoculated medium with each of the additives. These experiments were repeated for three passages. *E. coli* grown in MHB was used for inoculation of plates in the first passage. Cells that had survived in MHB containing $0.01 \mu\text{g ml}^{-1}$ of enrofloxacin with different supplements were used for inoculation of each of the wells in the second passage. For the third passage, bacteria grown with $0.01 \mu\text{g ml}^{-1}$ of enrofloxacin in the second passage were used. Statistical analyses were performed using

a one-way analysis of variance, with a *P* value of <0.05 being considered significant.

PCR amplification and sequencing analysis of *gyrA*

PCR amplification of the QRDR of *gyrA* was carried out by using the primers, GYRAF (5'-CGTTGGTGACGTAATCGGTA-3') and GYRAR (5'-CCGTGCCGTCATAGTTATCA-3').²⁹ Amplification was performed in a reaction volume of 25 μl by using FastStart Taq DNA Polymerase (Roche Applied Science, Indianapolis, IN, USA). Each reaction tube contained 5 μl of cell suspension, 2 μl of a 10 μM primer mixture, 2.5 μl of 10 $\times$ PCR reaction buffer with 20 mM MgCl₂, 0.5 μl of 10 mM dNTP mix and 0.2 μl of 2 U FastStart Taq DNA polymerase. Each 35 cycles consisted of 95 °C denaturation for 30 s, 51 °C annealing for 30 s and 72 °C extension for 1 min. The first denaturation and the last extension steps were extended for 4 and 5 min, respectively. PCR products were purified by a Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and directly sequenced with PCR forward and reverse primers. MEGA version 4 software (Tempe, AZ, USA) was employed for mutation analysis of *gyrA* sequences.³⁰

Effects of efflux pump inhibitors on enrofloxacin sensitivity

To investigate the effects of efflux pump inhibitors on the sensitivity of *E. coli* to enrofloxacin, 4 μl reserpine (Aldrich, Milwaukee, WI, USA) or carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP, Sigma, St Louis, MO, USA), dissolved in dimethyl sulfoxide, was added to one set of duplicate wells in the microtiter plates. The final concentrations of reserpine and CCCP were 20 $\mu\text{g ml}^{-1}$ and 10 μM , respectively.^{20,31–32} The kinetics of growth were monitored in a Synergy MX spectrophotometer.

Microscopic observation

The effect of various concentrations of enrofloxacin on the morphology of *E. coli* ATCC 25922 were examined using a phase-contrast microscope (Nikon Eclipse 80i, Nikon, Melville, NY, USA) with an oil-immersion objective (UPlanF1 100/1.3) after overnight growth of cells in the wells of 96-well microtiter plate without and with 0.01, 0.02, 0.03, 0.06 and $0.1 \mu\text{g ml}^{-1}$ of enrofloxacin.

Fatty acid analysis

Cellular lipids from *E. coli* grown in MHB with and without 2.5% fecal extract and enrofloxacin were saponified, methylated and extracted using hexane/methyl tertiary butyl ether as described by Song *et al.*³³ Fatty acid methyl esters were analyzed via GC (Agilent series 6890, Agilent, Santa Clara, CA, USA) by using the SHERLOCK Microbial Identification System (MIDI, Newark, DE, USA) and identification was verified by GC-MS with a Thermo Scientific DSQ II (Thermo Scientific, West Palm Beach, FL, USA), equipped with a DB5-MS capillary column (30 m $\times$ 0.25 mm inner diameter, film thickness 0.25 mm) with helium as the carrier gas.

RESULTS

Effect of enrofloxacin on bacterial growth

The kinetics of growth of *E. coli* ATCC 25922 were compared in various substrates with and without enrofloxacin (Figure 1). The MIC of enrofloxacin for wild-type *E. coli* ATCC 25922 was verified to be $0.03 \mu\text{g ml}^{-1}$. In the first passage, in the absence of enrofloxacin, the maximum growth of *E. coli* was similar in all media after 15 h of incubation (Figure 1a), but its growth rates in different media varied in the first 5 h (Figure 1b). In the presence of sub-MIC ($0.01 \mu\text{g ml}^{-1}$) of enrofloxacin, growth of *E. coli* was substantially higher in the medium supplemented with 1 or 2.5% sterilized fecal extract than in other media (Figures 1a and c). The medium with sterilized fecal extract (1 and 2.5%) also allowed the growth of *E. coli* in the presence of 0.03 and $0.06 \mu\text{g ml}^{-1}$ enrofloxacin, which are equal to 1 and 2 times the MIC of the drug, respectively, for this strain (Figure 1a).

In the second and third passages, the bacteria that had survived in $0.01 \mu\text{g ml}^{-1}$ of enrofloxacin (sub-MIC) were used for inoculation. They grew well in all media containing up to the MIC ($0.03 \mu\text{g ml}^{-1}$) of enrofloxacin. Better growth was observed in media supplemented

Figure 1 Effects of different concentrations of enrofloxacin on growth of *E. coli* ATCC 25922 in media containing 5 mM glucose, sucrose or sodium acetate, or 1 or 2.5% sterilized extract from a human fecal sample, in the first passage. Symbols represent averages of triplicates from three samples and error bars represent the standard deviations. (a) Maximum growth of *E. coli* (OD₆₀₀) observed in various media with each enrofloxacin concentration. (b) The kinetics of growth of *E. coli* in media with different supplements in the absence (b) and presence (c) of 0.01 µg ml⁻¹ enrofloxacin. *Indicates statistically significant differences from control ($P < 0.05$).

with sugars and sterilized fecal extracts (Figure 2). Media supplemented with sterilized fecal extracts and sugars also better supported the growth of *E. coli* with 0.06 and 0.1 µg ml⁻¹ of enrofloxacin in the second (Figure 2a) and third (Figure 2b) passages. Strains showed a slower rate of growth in the first 5 h of incubation in the media supplemented with sterilized fecal extracts than in others, but then the rate of growth increased substantially in comparison with the rates of growth in other media (Figures 2c and d). This increase in the rate of growth was more pronounced with concentrations of enrofloxacin higher than the MIC (0.03 µg ml⁻¹).

Comparison of the sequences of the QRDR of *E. coli* strains

To find out if the bacteria that survived in higher concentrations of enrofloxacin than the MIC had mutations in the QRDR of *gyrA*, the QRDR primers were used to amplify 251 bp fragments from the wild-type and the cell mixtures that grew with different concentrations of enrofloxacin in the second and third passages. The PCR amplicons were sequenced and analyzed. The sequences of the QRDR from all of the *E. coli* strains were identical, regardless of the level of sensitivity to enrofloxacin.

Effects of efflux pump inhibitors on sensitivity of *E. coli* to enrofloxacin

The effect of efflux pump inhibitors on sensitivity of *E. coli* to 0.1 µg ml⁻¹ enrofloxacin was also examined in MHB supplemented with 5 mM sucrose or 2.5% sterilized fecal extract in the second and third passages. Reserpine and CCCP increased the sensitivity of bacteria to 0.1 µg ml⁻¹ of enrofloxacin in the medium containing sucrose, but their effect was not observed in the medium supplemented with sterilized fecal extract (Figure 3).

Effect of enrofloxacin on cell structure

Enrofloxacin, even at low concentrations, affected the morphology of *E. coli* cells. The bacteria exposed to 0.01 ~ 0.1 µg ml⁻¹ of enrofloxacin were elongated. The increase in the number of elongated cells varied with the concentration of enrofloxacin (Figure 4).

Effects of sterilized fecal extract on lipid composition

The major saturated and unsaturated fatty acids were measured in *E. coli* grown in the medium with and without 2.5% sterilized fecal extract. In the bacteria grown with 2.5% sterilized fecal extract, the proportions of the saturated fatty acids, heptadecanoic acid (C_{17:0}) and octadecanoic acid (C_{18:0}) and of the cyclopropane-substituted methylene-heptadecanoic acid (C_{17:0 cyclo}) and cyclopropane-substituted methylene-nonadecanoic acid (C_{19:0 cyclo}) had increased, but the main saturated fatty acid hexadecanoic acid (C_{16:0}) had decreased. The decrease was also observed in the proportion of the two unsaturated fatty acids, (*Z*)-9-hexadecenoic acid (C_{16:1 ω7c}) and (*E*)-11-octadecenoic acid (C_{18:1 ω7c}) (Figure 5a).

In the cells grown with and without enrofloxacin in the absence of fecal extract, the ratios of saturated to unsaturated fatty acids were 1.7 and 2.2, respectively, but in the presence of 2.5% sterilized fecal extract, the ratios increased to 2.4 and 3.9, respectively (Figure 5b). This increase in the ratio in the presence of fecal extract was the result of increases in the amounts of heptadecanoic acid, octadecanoic acid and cyclopropane fatty acids (C_{17:0 cyclo} and C_{19:0 cyclo}), even though the amount of hexadecanoic acid had decreased.

DISCUSSION

Colonic bacteria are exposed to antibiotics and may develop resistance to them. We used enrofloxacin as a model compound to examine the

Figure 2 Comparison of the growth (OD_{600}) of *E. coli* ATCC 25922 that had survived a sub-MIC concentration of enrofloxacin ($0.01 \mu\text{g ml}^{-1}$), with different concentrations of enrofloxacin ($0.01 \sim 0.1 \mu\text{g ml}^{-1}$) in the second and third passages. The bacteria were incubated for 15 h in Mueller–Hinton broth containing 5 mM glucose, sucrose or sodium acetate, or 1 or 2.5% extract from a human fecal sample. Symbols represent averages of triplicates from three human fecal samples and error bars represent the standard deviations. Maximum cell growth was measured in the second (a) and third (b) passages in media with different supplements. Kinetics of growth of *E. coli* in $0.1 \mu\text{g ml}^{-1}$ of enrofloxacin were measured in the second (c) and third (d) passages in media with different supplements. *Indicates statistically significant differences from control ($P < 0.05$).

Figure 3 Effects of reserpine and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) on the growth of *E. coli* ATCC 25922 with $0.1 \mu\text{g ml}^{-1}$ enrofloxacin in Mueller–Hinton broth containing sucrose and 2.5% sterilized human fecal extract in the third passage. Symbols represent averages of triplicates from three samples and error bars represent the standard deviations.

effect of low concentrations of a fluoroquinolone on the resistance development in *E. coli* in the presence of fecal extract. We found that at low concentration (2.5%) of sterilized fecal extract, the growth of *E. coli* was enhanced in MIC and sub-MIC levels of enrofloxacin in the first passage. The strain grew in 2–3 times the MIC of enrofloxacin in the second and third passages without developing mutations in the QRDR region of *gyrA*. Efflux pump inhibitors increased the sensitivity of the resistant strain to enrofloxacin, but had less effect on increasing sensitivity of the cells in the presence of sterilized fecal extract. The fecal extract had affected the lipid composition of the bacterial membrane in the resistant strain.

Decreased sensitivity of *E. coli* in sterilized fecal extract in the first passage may indicate either decreased potency of antibiotics or reduced cell penetration. Kamberi *et al.*³⁴ reported that at lower pH (for example, in urine) the piperazine-containing fluoroquinolones, such as enrofloxacin, are positively charged, which may decrease their penetration into bacteria and thus decrease their activity. Fecal pH generally is 6.4, but ranges from 5.4 to 7.8 among healthy adults³⁵

Figure 4 *E. coli* cells grown with different concentrations of enrofloxacin. (a) Without enrofloxacin, (b) 0.01 $\mu\text{g ml}^{-1}$, (c) 0.02 $\mu\text{g ml}^{-1}$, (d) 0.03 $\mu\text{g ml}^{-1}$, (e) 0.06 $\mu\text{g ml}^{-1}$ and (f) 0.1 $\mu\text{g ml}^{-1}$. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Figure 5 Fatty acid composition of lipids of *E. coli* grown with and without 0.06 $\mu\text{g ml}^{-1}$ enrofloxacin in Mueller–Hinton broth with and without 2.5% fecal extract (a). Symbols represent averages of triplicates from one human fecal sample and error bars represent the standard deviations. Ratio of saturated to unsaturated fatty acids (b). *Indicates statistically significant differences from control ($P < 0.05$).

and is important in determining binding of ionizable drugs. In our study, the average fecal pH was 6.3 and the pH of media supplemented with the sterilized fecal extract was the same as the pH of other media, indicating that decrease in sensitivity to enrofloxacin in the presence of sterilized fecal extract was not related to change in pH. HPLC analysis showed that fecal extract did not change the structure of enrofloxacin (data not shown). The recovery rate was more than 90%, indicating that it was not eliminated by binding to the fecal extract. However, we can not preclude unknown conformational changes that may have occurred, either in the cells or in the drug that may have affected interaction of the drug with the cells or the drug penetration into the cell. Interaction of fluoroquinolones with organic matter has been shown.^{36,37}

The effect of sterilized fecal extract was more pronounced in the second and third passages, in which the *E. coli* cells were able to grow in the medium with $\geq 3 \times \text{MIC}$. Also, the cells grown with enrofloxacin were elongated (Figure 4), which was similar to previously reported observations.³⁸ The cells were not elongated in the medium without enrofloxacin. Bacteria growing in higher concentrations of enrofloxacin (0.1 $\mu\text{g ml}^{-1}$) had more elongated cells and did not have mutations in *gyrA*, which has been shown to be the reason for decreased sensitivity of *E. coli*, including those exposed to low concentrations of fluoroquinolones.³⁹ Both inhibitors of the efflux pump, reserpine and CCCP, inhibited the growth of *E. coli* in the presence of enrofloxacin (data not shown), as has been shown previously for other fluoroquinolones.³² However, in the media containing sterilized fecal extracts, no increase in sensitivity to enrofloxacin was observed in the presence of these pump inhibitors. It could be hypothesized that fecal extract may have decreased the permeability of the cells to enrofloxacin, either by affecting the membrane or by binding to the drug, similar to those reported for other organic matter.^{36,37} We observed that 2.5% sterilized fecal extract affected the ratio of saturated to unsaturated fatty acids in *E. coli*. Cells grown with 2.5% sterilized fecal extract had a higher ratio of saturated to unsaturated fatty acids than that in the

control cells grown with and without enrofloxacin. Changes in relative proportions of saturated and unsaturated fatty acids affect the fluidity and permeability of the cell membrane, its physical state and functions under various conditions.^{40–42} The alteration in the fatty acid composition caused by sterilized fecal extract may have affected not only bacterial membrane functions but also the sensitivity of bacteria to antimicrobial agents, possibly by influencing its entry into the cell.

The CLSI breakpoint for enrofloxacin is $1 \mu\text{g ml}^{-1}$ for *E. coli*.³⁴ The rapid threefold increase in the MIC of enrofloxacin for *E. coli*, after only one passage at a sub-MIC concentration in sterilized fecal extract, may indicate potential for rapid growth of this bacterium in the presence of enrofloxacin under colonic conditions. Catry *et al.*,⁴³ evaluating resistance of *E. coli* through the digestive tract of calves (duodenum, jejunum, cecum, colon and rectum), found the highest percentage of enrofloxacin-resistant *E. coli* in the colon.

In conclusion, exposure to sub-inhibitory concentrations of enrofloxacin in media supplemented with sterilized human fecal extract reduced sensitivity of a strain of *E. coli* to a concentration of enrofloxacin three times higher than the MIC. The sterilized fecal extract altered the proportion of unsaturated and saturated fatty acids in *E. coli*, which may have affected the transport of enrofloxacin into the cells. This is the first study in which the influence of fecal extract on bacterial sensitivity to a drug has been investigated. The exact component(s) of the fecal extract that has affected the *E. coli* sensitivity to enrofloxacin is not known and merits investigation.

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