EFFECT OF CEFOVECIN ON THE FECAL FLORA OF HEALTHY DOGS

by

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A REPORT

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Abstract

Cefovecin is an extended-spectrum long-acting third generation cephalosporin used to treat canine infections. The study objective was to determine the effect of cefovecin on the absolute number and antimicrobial susceptibility of fecal enteric bacteria in healthy dogs. Fourteen Beagles were randomly assigned to a treated (n = 7, 8 mg/kg cefovecin subcutaneously on day 1) or untreated (n = 7) group. LC/MS was used to determine plasma cefovecin concentration on day 14. *E. coli*, enterococci, and *Salmonella* were isolated and enumerated from fecal samples collected on days 0, 3, 7, 14, and 28. Antimicrobial resistance was determined using disc diffusion, MIC, and detected using PCR for the $bla_{\text{CMY-2}}$ gene on select isolates.

Mean plasma concentration of cefovecin on day 14 was 9.59 µg/mL in treated dogs; untreated dogs had no measurable plasma cefovecin. The absolute number of *E. coli* was lower in treated dogs on day 3 ($P \leq 0.0001$), and the absolute number of cefovecin-resistant *E. coli* was higher in treated dogs on days 7 ($P = 0.002$), 14 ($P = 0.004$) and 28 ($P \leq 0.0001$), compared to untreated dogs. Enterococci increased and were higher in the treatment group on day 7 ($P = 0.0226$). Isolation of *Salmonella* was rare. After cefovecin treatment, beta-lactam resistance was more common in fecal *E. coli* from treated dogs than untreated dogs, while resistance of enterococci was not altered. On day 28, treated dogs were 3.25 times more likely to carry the $bla_{\text{CMY-2}}$ gene than untreated dogs (95% CI 1.27 – 8.35). The implications of these findings in clinically ill patients require further research.

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Chapter 1 - Introduction

Literature Review

Cefovecin is a semi-synthetic third-generation long-acting cephalosporin. It is authorized for use in the European Union, New Zealand, and in several countries in South America and Asia. In the United States, it is US Food and Drug Administration approved and labeled for the treatment of superficial pyodermas, abscesses, and wounds in dogs. In dogs, cefovecin has a half-life of 5.5 days, allowing a single subcutaneous (SC) injection to provide up to 14 days of treatment (Stegemann et al., 2006a). Cefovecin undergoes minimal hepatic metabolism and is primarily excreted unchanged in the urine, but excretion of unchanged drug also occurs in the bile resulting in exposure to gastrointestinal (GI) flora (Stegemann et al., 2006a).

Cephalosporins have a significant effect on the fecal flora of cattle. Calves administered intramuscular (IM) ceftiofur hydrochloride had a significantly increased number of ceftriaxone-resistant fecal bacteria following treatment (Jiang et al., 2006). Cattle administered ceftiofur crystalline-free acid had a 2-week increase in multidrug-resistant (ceftiofur, ampicillin, chloramphenicol, tetracycline, and sulfisoxazole) *Escherichia coli* (Lowrance et al., 2007). Ceftiofur crystalline-free acid-treated cattle also had increased levels of fecal $bla_{\text{CMY-2}}$ carriage (Alali et al., 2009). The $bla_{\text{CMY-2}}$ gene encodes cephalosporin resistance via AmpC β-lactamase and has been identified among commensal and pathogenic bacteria within the GI flora of humans and animals (Forward et al., 2004; Alali et al., 2009). In vitro conjugation studies have documented plasmid transfer of the $bla_{\text{CMY-2}}$ gene within and between fecal genera, specifically *E. coli* and *Salmonella* spp. (Jiang et al., 2006). Bacteria containing the $bla_{\text{CMY-2}}$ gene produce extended spectrum beta lactamases and therefore carry resistance to all beta lactams licensed for use in dogs (Damborg et al., 2011). This can pose an animal health problem when selecting an antimicrobial to be effective in treating canine infections.

Few data exist regarding the effect of antimicrobials on canine fecal flora. Oral enrofloxacin administered to dogs suppressed fecal coliforms throughout treatment and slowly normalized within 8 days after termination of treatment (Trott et al., 2004). A separate study evaluating fecal *E. coli* from dogs found an association between multidrug resistance and history

of receiving antimicrobials within 1 month of sampling, suggesting that selection pressure from antimicrobial therapy may influence the fecal flora (Stenske et al., 2009).

Study Objectives

The objectives of the present study were (1) to investigate the effect of cefovecin on the total number of coliforms and enterococci in the fecal flora of dogs, (2) to investigate the effect of cefovecin on the antimicrobial resistance of these bacteria, as determined by disc diffusion, minimum inhibitory concentration (MIC) determination, and presence of the antimicrobial resistance gene $bla_{\text{CMY-2}}$ on select isolates, and (3) to measure the plasma concentration of cefovecin on day 14 to ensure that untreated dogs did not inadvertently receive cefovecin.

Chapter 2 - Materials and Methods

Study Design and Enrolled Dogs

The Kansas State University (KSU) Institutional Animal Care and Use Committee approved the study (protocol number 2941).

Fourteen young adult Beagles living in a closed research facility were enrolled. A sample size of at least six dogs in each group was calculated based on an estimated 3-log difference in bacterial count between treatment groups, with a *P* value of 0.05, and power of 80 (Trott et al., 2004). No dogs had received antimicrobials at any time prior to the study, and all dogs were deemed healthy based on a physical examination, complete blood count, biochemical profile, and fecal flotation examination.

Dogs were randomly divided, with blocking by age, using a random numbers table into a treated group and untreated group. Seven treated dogs received 8 mg/kg cefovecin (Convenia, Zoetis) SC on day 1. Seven untreated dogs received no therapy. Dogs were housed in separate runs with no direct contact in the month prior to and throughout the study. They ate a dry maintenance dog food and received no medication other than the cefovecin (treated dogs) during the study. Authors performing bacterial isolation, identification, enumeration, and susceptibility testing were blinded to treatment group designation.

Bacterial Isolation

One gram of fresh feces was collected from each of the 14 dogs, via rectal palpation with a sterile glove, on days 0, 3, 7, 14, and 28. Feces were diluted 10-fold in 0.1% peptone water and spread-plated on EC containing 4-methylumbelliferyl-beta-D-glucuronide agar (EC), Hektoen enteric agar (HEA), and mEnterococcus agar (mENT). Additionally, diluted fecal samples were spread-plated onto these three media each containing 1 µg/mL cefovecin (EC-CEF, HEA-CEF, and mENT-CEF). EC plates were incubated at 44 °C for 24 hours, HEA plates at 37 °C for 24 hours, and mENT plates at 37 °C for 48 hours.

Bacterial Identification

Isolates on EC plates were considered *E. coli* if they had the desired morphology, fluoresced under 366 nm ultraviolet light, were indole positive, and oxidase negative.

Salmonella enrichment was performed by adding 1 mL of the 10^{-1} dilution to 9 mL Rappaport broth, incubating for 24 hours at 44 °C, plating on HEA and incubating for an additional 24 hours. Isolates on HEA were considered *Salmonella* if they were blue-green colonies that were indole negative and oxidase negative. Esculin hydrolysis was used to confirm isolates from mENT on the genus level as enterococci. A multiplex PCR assay was performed on three random enterococcal isolates grown on mENT plates per fecal sample, to determine the species as *Enterococcus faecium* or *Enterococcus faecalis*, as previously described (Kariyama et al., 2000; Poyart et al., 2000). Isolates of *E. coli*, *Salmonella*, and enterococci were enumerated as colony forming units (CFU) per gram of feces from each fecal sample. Isolates were stored in brain heart infusion broth with glycerol at -80 °C.

Plasma Cefovecin Concentration Analysis

Plasma samples were obtained on day 14 from each dog for the determination of cefovecin concentration using liquid chromatography (LC) (Shimadzu Prominence, Shimadzu Scientific Instruments) with mass spectrometry (MS) (API 2000, Applied Biosystems). Plasma standards and samples were processed according to Table 1; the LC settings are described in Table 2, and the MS settings in Table 3.

Antimicrobial Susceptibility Testing

To determine the absolute number of cefovecin-resistant fecal bacteria after administration of cefovecin, *E. coli*, *Salmonella*, and enterococci were enumerated from EC-CEF, HEA-CEF, and mENT-CEF plates, respectively, at each sampling time. Antimicrobial susceptibility testing using the disc diffusion method with Mueller-Hinton agar was performed on up to five randomly selected *E. coli* isolates from each media not containing cefovecin per fecal sample on each sampling day. *E. coli* were tested for susceptibility to 12 antimicrobials, enterococci were tested for susceptibility to 10 antimicrobials, and *Salmonella* were not tested for susceptibility.

Determination of the MIC was performed on five *E. coli* isolates from each EC plate on days 0, 7, and 28 for cefovecin, ceftriaxone, enrofloxacin, gentamicin, and imipenem. Testing was performed and interpreted in accordance with the recommendations of the Clinical Laboratory Standards Institute (CLSI), using zone diameters and interpretive breakpoints for *Enterobacteriaceae* and *Enterococcus* spp. for dogs when available, otherwise guidelines for

humans were used (CLSI M31-A3 2008; CLSI M100-S20 2010). The CLSI has not approved zone diameters or MIC breakpoint criteria for cefovecin, thus breakpoints used were based on recommendations from Zoetis (disc diffusion: S ≥ 24, I 21-23, R ≤ 20 mm; MIC S ≤ 2, I 4, R ≥ 8 µg/mL).

Three randomly selected *E. coli* isolates from EC plates and three randomly selected *E. coli* isolates from EC-CEF plates from each dog on each day were tested using PCR for the presence of the bla_{CMY-2} gene (not necessarily the same isolates as those selected for susceptibility testing). *E. coli* DNA was isolated by boil-prep, and spectrophotometry was used to ensure nucleic acid concentration and quality from each *E. coli* isolate. Real-time PCR was performed to determine the presence of the bla_{CMY-2} gene compared to the *eub* housekeeping gene in the overall fecal population of each dog on each sampling day. Total DNA from 200 µg of feces was extracted using QIAamp DNA stool mini kit (Qiagen), following the manufacturer's protocol.

The bla_{CMY-2} gene was amplified using forward primer 5^1GACAGCCTCTTTCTCCACA (reference number 59972060), and reverse primer 5^1GAATAGCCTGCTCCTGCATC (reference number 59972061) (Zhao et al, 2001). PCR amplification (denaturation for 30 seconds at 95 °C, primer annealing for 30 seconds at 55 °C, and extension for 30 seconds at 72 °C) consistently yields a product size of 101 bp and has been successfully used in DNA from pure cultures or total fecal DNA (Zhao et al, 2001).

Statistical Method

Mean age and body weight of enrolled dogs with one standard deviation were calculated, and a Student's *t* test was used to compared these values between treated and untreated groups. A random effects-mixed model was used to analyze bacterial counts in a repeated measures format, and data were presented as mean CFU/gram feces ± standard error of the mean. Distribution of enterococcal species, antimicrobial resistance testing by disc diffusion, and presence of the bla_{CMY-2} gene in *E. coli* isolates were analyzed using the Fisher's exact test and relative risk. MIC results were reported as range and MIC_{90}. The presence of the bla_{CMY-2} gene from the total fecal population was achieved by comparing the ratio of the bla_{CMY-2} gene to the universal eubacterial *eub* gene (*bla*:*eub*) in collected fecal samples on each day using a random effects-mixed model in a repeated measures format. A *P*-value of <0.05 was considered significant for

all analyses. Statistical analyses were evaluated using the commercial software program JMP 9 (SAS Institute).

Chapter 3 - Results

Enrolled Dogs

Six male intact and eight female intact Beagles were enrolled. Mean age was 11.57±0.94 months and body weight was 9.69±1.26 kg; there was no difference in age ($P = 0.589$) or body weight ($P = 0.921$) between groups. No adverse clinical signs were noted in any dog.

Plasma Cefovecin Concentration

The average plasma cefovecin concentration on day 14 in treated dogs was 9.59 µg/mL (range 3.20-12.90 µg/mL). Cefovecin was not detected in any untreated dogs.

Fecal Bacterial Counts

On day 0, a mean of 6.7±1.8 x 10^6 CFU of *E. coli* per gram of feces was isolated, with no difference identified between treated and untreated dogs (Figure 1). Thereafter, a significant time * treatment group interaction was identified ($P < 0.0001$), and on day 3 the absolute number of fecal *E. coli* dropped significantly in the treated group (Figure 1). Based on the absolute number of *E. coli* grown on media containing cefovecin, resistance to cefovecin among *E. coli* increased throughout the study and was significantly greater in the treated vs. untreated dogs on days 7, 14, and 28 (Figure 2).

Salmonella isolation was rare, even with enrichment. *Salmonella* spp. were isolated from the feces of six dogs (3 treated, 3 untreated), but no effect of treatment ($P = 1.000$) or interaction between treatment and time ($P = 0.676$) was identified. Cefovecin-resistant *Salmonella* were isolated from one treated and one untreated dog on day 28 but not on other days.

On day 0, a mean of 1.3±0.7 x 10^7 CFU of enterococci per gram of feces was found, with no difference between treated and untreated dogs ($P = 1.000$). However, a significant time * treatment group interaction was identified ($P = 0.002$), with more enterococci isolated from feces of treated dogs compared with untreated dogs on day 7 ($P = 0.023$). No differences were seen in enumeration of cefovecin-resistant enterococci between groups at any time point. All enterococcal isolates were identified with PCR as either *E. faecalis* or *E. faecium*. *E. faecalis* were more common than *E. faecium* in both groups on day 0, but the percentages of *E. faecalis* isolates decreased and *E. faecium* isolates increased until day 14 (Figure 3). By day 3 isolates

from treated dogs (12/21, 66.7%) were 14 times as likely as from untreated dogs (1/21, 4.8%) to be *E. faecium* ($P < 0.0001$, 95% CI 2.0-97.5), but by day 7 there was no difference in species distribution between groups ($P = 0.520$), with feces from both treated and untreated dogs having *E. faecium* percentage peaking at 14 days.

Antimicrobial Resistance

Based on disc diffusion testing, there were no differences in susceptibility between *E. coli* (EC plates) from treated and untreated dogs on day 0, but resistance to cefovecin was high in both groups (Figure 4). On days 3, 7, 14, and 28, significant differences were observed, with fecal *E. coli* from treated dogs being more likely to be resistant to numerous tested antimicrobials than feces from untreated dogs (Figures 5-8).

Based on disc diffusion testing, from the population of *E. coli* that grew on media containing cefovecin (EC-CEF), all isolates from all treated and untreated dogs on all sampling days were resistant to cefovecin, as well as to ampicillin, cefazolin, and cefpodoxime. Of tested isolates from treated and untreated dogs, >97% were resistant to amoxicillin-clavulanic acid at each sampling time, and >33% were resistant to ceftiofur. In these isolates, ceftriaxone resistance increased in both groups from day 0 (20% treated, 28% untreated) to day 28 (82.9% treated, 77.1% untreated), with no group effect. All *E. coli* isolates from EC-CEF were susceptible (via disc diffusion) to gentamicin and imipenem, while resistance to doxycycline, enrofloxacin, and TMS increased throughout the study, with no group effect.

MIC was performed on *E. coli* isolated from EC plates from treated and untreated dogs on days 0, 7, and 28 for cefovecin, ceftriaxone, enrofloxacin, gentamicin, and imipenem, and compared based on previous categorization (via disc diffusion) of *E. coli* as being cefovecin-susceptible or cefovecin-resistant (Table 4).

At baseline, presence of the $bla_{\text{CMY-2}}$ gene was isolated from the fecal *E. coli* of 14.3% (3/21) of treated and 4.8% (1/21) of untreated dogs ($P = 0.606$). No treatment group differences were noted until day 28 when fecal *E. coli* (from EC plates) from treated dogs (62%, 13/21) were 3.25 times more likely (95% CI 1.27-8.35) than fecal *E. coli* from untreated dogs (19%, 4/21) to carry the $bla_{\text{CMY-2}}$ gene ($P = 0.010$). While the percent of fecal *E. coli* containing the $bla_{\text{CMY-2}}$ gene increased over the study period (14-76%) among those isolates tested from EC-CEF plates, no significant treatment group differences were documented. The ratio of the $bla_{\text{CMY-2}}$ gene to

the universal eubacterial gene, *bla*:*eub*, was then assessed in the total fecal bacterial population of enrolled dogs. Contrary to what was found when assessing presence of $bla_{\text{CMY-2}}$ in *E. coli* alone, the feces of untreated dogs had a higher mean *bla*:*eub* (2.714±0.84) compared with the feces of treated dogs (1.667±0.28) on day 28 ($P < 0.0001$); no differences were found on other days, and there was no significant change over time ($P = 0.145$).

Treatment group had no effect on antibiotic susceptibility within *E. faecalis* or *E. faecium* isolates over the study time period, with the exception of day 28 *E. faecium* isolates from treated dogs (94.4%) which were more frequently resistant to tigecycline than isolates from untreated dogs (54.6%) ($P = 0.019$). Percent resistance to each antimicrobial of all *E. faecalis* and *E. faecium* isolates together are presented in Figure 9.

Chapter 4 - Discussion

Plasma cefovecin concentrations confirmed that no untreated dog inadvertently received cefovecin. Cefovecin concentrations in treated dogs on day 14 (3.20–12.90 µg/mL) were similar to a previous study which reported 5.6 ± 1.8 µg/mL and 15.2 ± 3.2 µg/mL in two groups of dogs 14 days after administration (Stegemann et al., 2006a). A second study reported the in vitro MIC_{90} of cefovecin for *E. coli* isolates from the United States was 1 µg/mL; however the correlation between in vitro MIC_{90} and in vivo plasma concentration, especially for highly protein bound drugs such as cefovecin, has not been determined (Stegemann et al., 2006b).

Due to the bacterial isolation and identification techniques used, only typical *Salmonella*, *E. coli*, and enterococci were selected for analysis. Any atypical isolates that might have been present were not analyzed.

The significant decline in total number of *E. coli* within 72 hours of cefovecin administration was similar to that seen with oral enrofloxacin administration to dogs (Trott et al., 2004). However, in that study, total coliform numbers remained below detectable limits throughout antimicrobial administration (21 days) followed by a return to untreated levels within 8 days after ceasing administration, while in the current study total *E. coli* levels returned to levels consistent with untreated dogs by day 7 despite treated dogs having measurable cefovecin in their plasma on day 14 (Trott et al., 2004). One potential explanation would be differences in gastrointestinal exposure of cefovecin and enrofloxacin.

The absolute number of cefovecin-resistant *E. coli* increased over the study period in untreated dogs as well as treated dogs. The authors hypothesized that this was caused by fecal oral cross-contamination which may have occurred between the two groups of dogs despite deliberate efforts to prevent this, including prohibiting direct contact among dogs, housing in runs with solid walls to prevent fecal spread between kennels, and keeping kennels clean. Suspected cross-contamination is a limitation of the present study, and more aggressive attempts to prevent any contact between the dogs or their feces should be made for future research studies (i.e. separate wards, foot baths, etc.). However, the effects of suspected cross-contamination may be relevant to a clinical hospital setting, where dogs are in kennels next to each other and similar precautions (no direct contact, keeping kennels clean, wearing gloves) are taken to minimize

disease transmission. Thus, it is reasonable to believe that resistant bacteria could be shed from a patient receiving antimicrobials and transferred to a dog in a nearby kennel, which could be deleterious if the recipient is highly susceptible to infection, such as an immunosuppressed patient. A public health risk could also be inferred, as resistant fecal bacteria could be spread to the veterinary staff or dog's owner.

Disc diffusion and MIC testing of *E. coli* from EC plates identified a high percentage of cefovecin-resistant *E. coli* at baseline and throughout the study in treated and untreated dogs. The most reliable measure of cefovecin resistance in this study was from enumeration of absolute numbers of cefovecin-resistant *E. coli* grown on EC–CEF media at each sampling day, and this technique found a lower rate of cefovecin-resistance at baseline in both groups. One explanation for this discrepancy could be that without CLSI approved zone diameters and breakpoint criteria, the manufacturer-recommended breakpoint criteria may need to be re-evaluated. It was also possible that the enrolled dogs may have had cefovecin resistance prior to the study from maternal or environmental exposure to resistant bacteria.

Significant differences occurred between treated and untreated dogs in the percentage of resistant *E. coli* isolates via disc diffusion to other beta-lactams after administration of cefovecin. The difference in $bla_{\text{CMY-2}}$ carriage identified on day 28 from fecal *E. coli* of treated and untreated dogs may indicate that cefovecin treatment selects for *E. coli* producing a plasmid-borne CMY-2 beta-lactamase. A study of dogs with pyoderma also found that treatment with cephalexin resulted in fecal *E. coli* from 8/13 dogs carrying plasmid-mediated $bla_{\text{CMY-2}}$ (Damborg et al., 2011). Acquisition of this gene may explain the cross resistance seen among β-lactams in this study. Furthermore, co-residence of multiple resistance genes has been documented in *E. coli* carrying the $bla_{\text{CMY-2}}$ gene, which may help to explain the resistance seen in the fecal *E. coli* of the present study to doxycycline, enrofloxacin, gentamicin, and imipenem (Winokur et al., 2001).

The significant increase in the number of enterococci isolated from treated dogs compared with untreated dogs on day 7 was not surprising, as enterococci are not susceptible to cefovecin and would have a competitive advantage when other enteric flora (*E. coli*) are eliminated by cefovecin. At day 0 of this study, *E. faecalis* was the predominant enterococcal species (88%), which is consistent with a previous report of canine rectal swabs (n = 86) that had a species distribution of *E. faecalis* (60%), *E. hirae* (15%), and *E. faecium* (8%) (Jackson et al.,

2009). It is possible that cefovecin contributed to the change in species distribution towards a higher percentage of *E. faecium*, but no mechanism for this effect is known; sampling bias from random selection of colonies may have also played a role in this change.

Chapter 5 - Conclusion

This study documented that cefovecin administration impacted both the number and antimicrobial resistance of fecal *E. coli* in healthy dogs. However, the clinical implications of the results are unknown as the work was performed using young healthy dogs in a research setting. Future studies are recommended to assess the effect of other cephalosporins, route of administration, and other classes of antimicrobials on the canine gastrointestinal flora, as well as the effect of these drugs on the flora of clinically ill patients. The results from this study suggest that antimicrobials should only be prescribed to dogs when benefits to the patient clearly out-weigh the risks.

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Figures and Tables

Figure 1. Absolute number of fecal *E. coli* presented as log CFU/g feces from treated and untreated dogs. Error bars represent standard error. *$P \leq 0.0001$

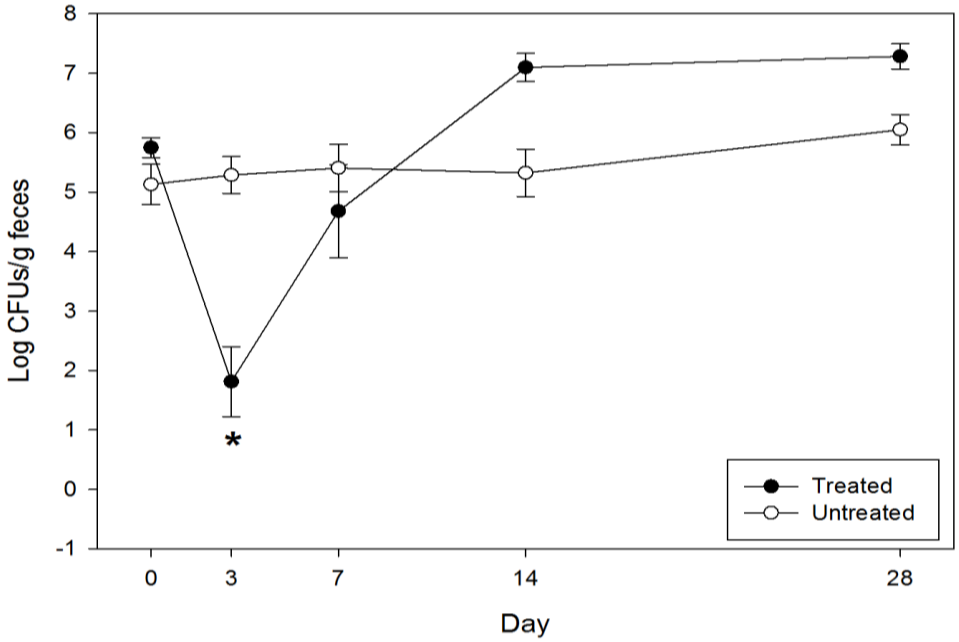

Figure 2. Absolute number of fecal cefovecin-resistant *E. coli* isolated from EC-CEF plates presented as log CFU/g feces from treated and untreated dogs. Error bars represent standard error. *$P = 0.002$, ^$P = 0.004$, $^{\#}P < 0.001$.

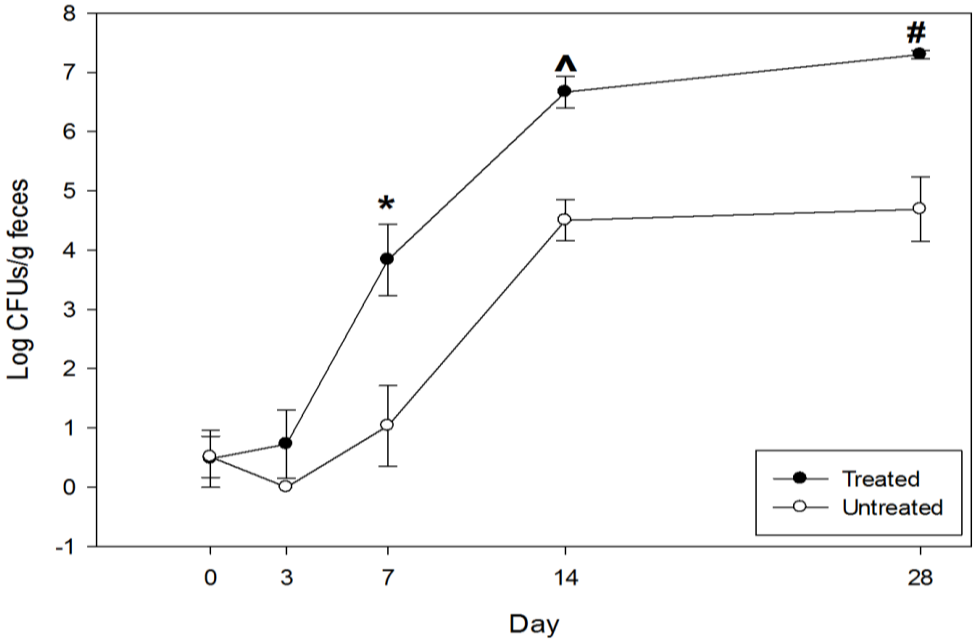

Figure 3. Percent of each enterococcal species by PCR on each sampling day from both groups combined.

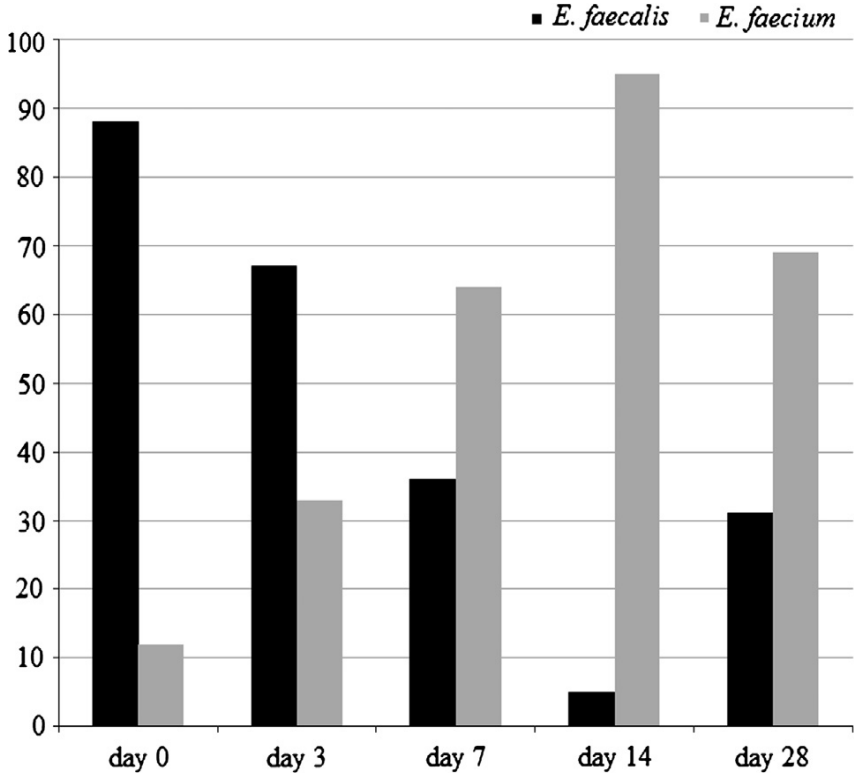

Figure 4. Percent of fecal *E. coli* that were resistant to tested antimicrobials via the disc diffusion method on day 0. No differences were documented between treated and untreated dogs.

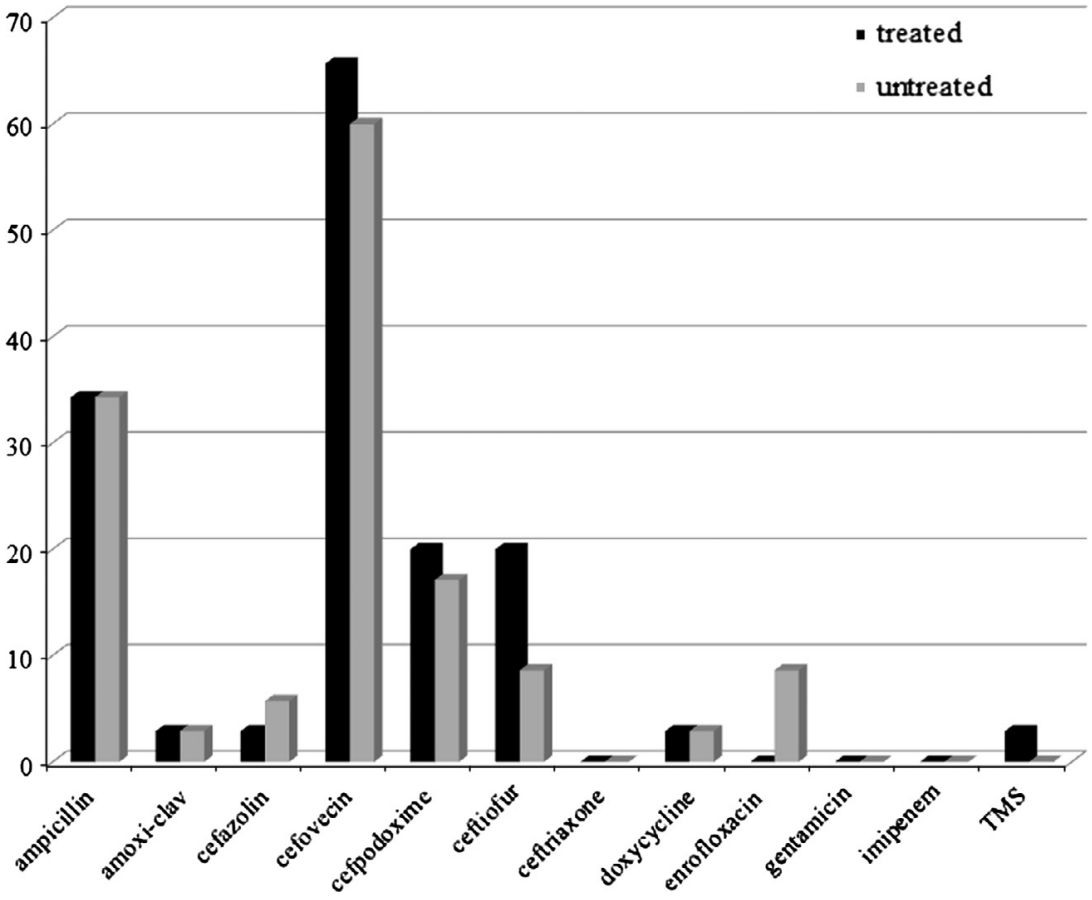

Figure 5. Percent of fecal *E. coli* that were resistant to tested antimicrobials via the disc diffusion method on day 3. *Signifies $P < 0.05$.

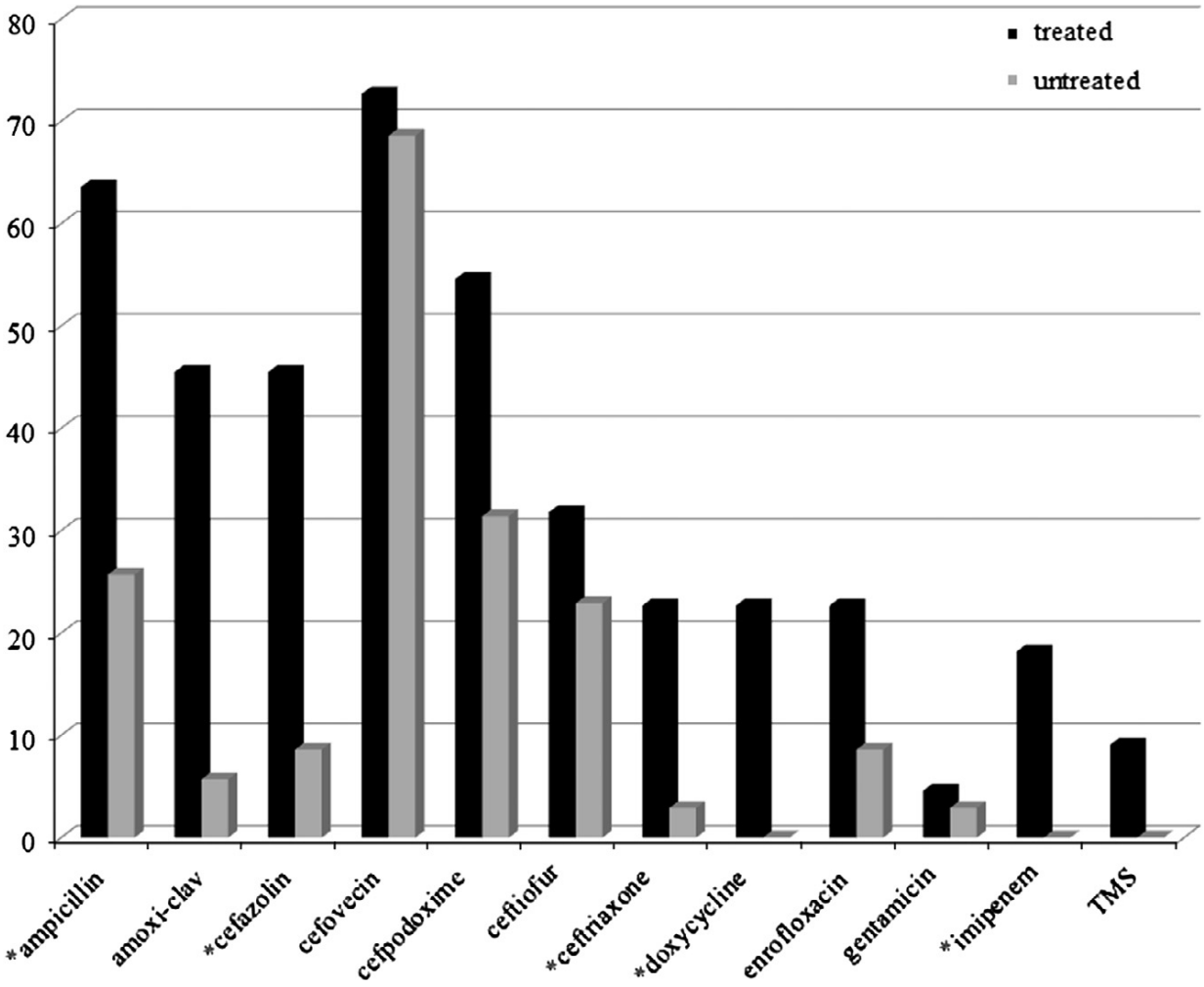

Figure 6. Percent of fecal *E. coli* that were resistant to tested antimicrobials via the disc diffusion method on day 7. *Signifies $P < 0.05$.

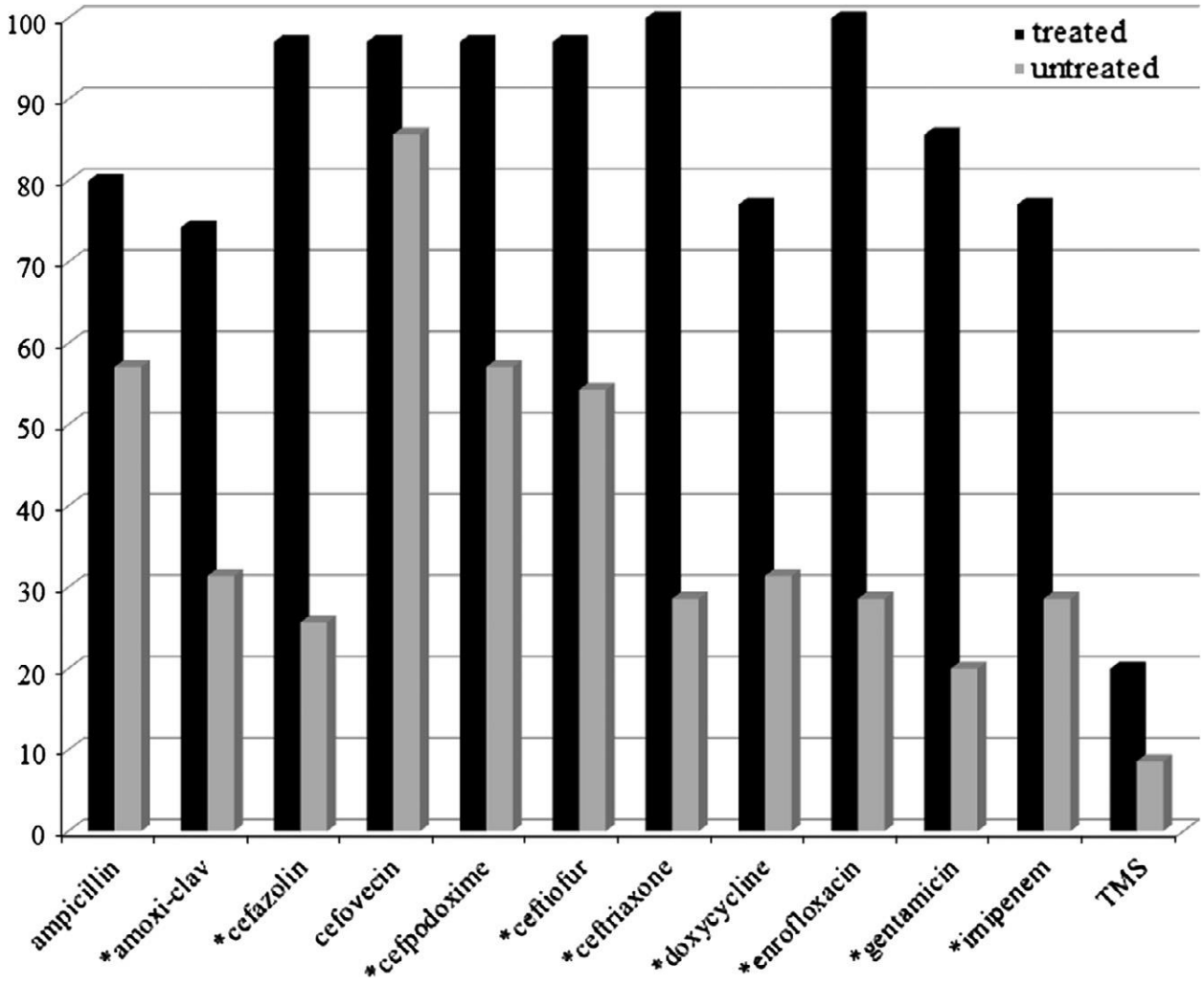

Figure 7. Percent of fecal *E. coli* that were resistant to tested antimicrobials via the disc diffusion method on day 14. *Signifies $P < 0.05$.

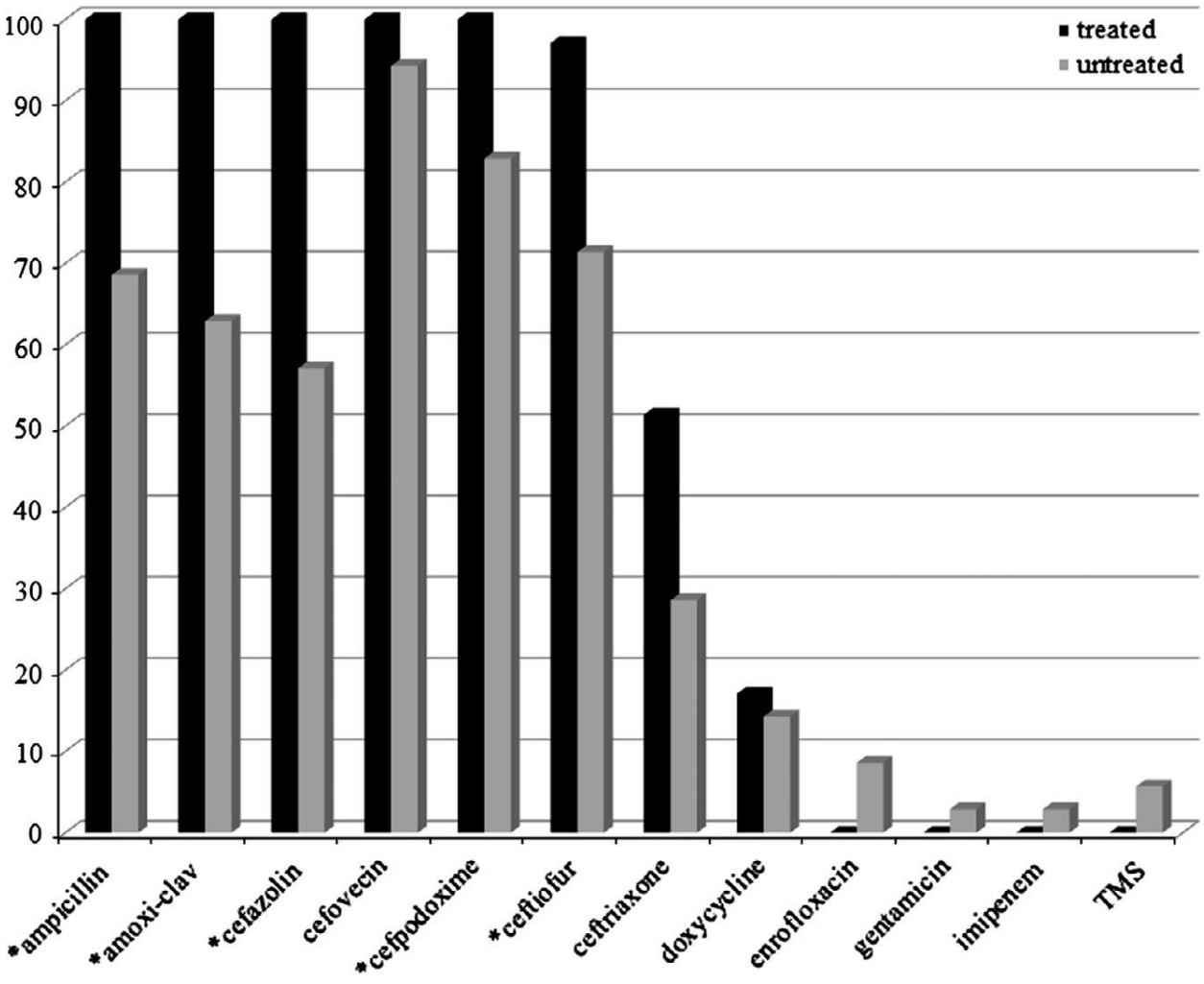

Figure 8. Percent of fecal *E. coli* that were resistant to tested antimicrobials via the disc diffusion method on day 28. *Signifies $P < 0.05$.

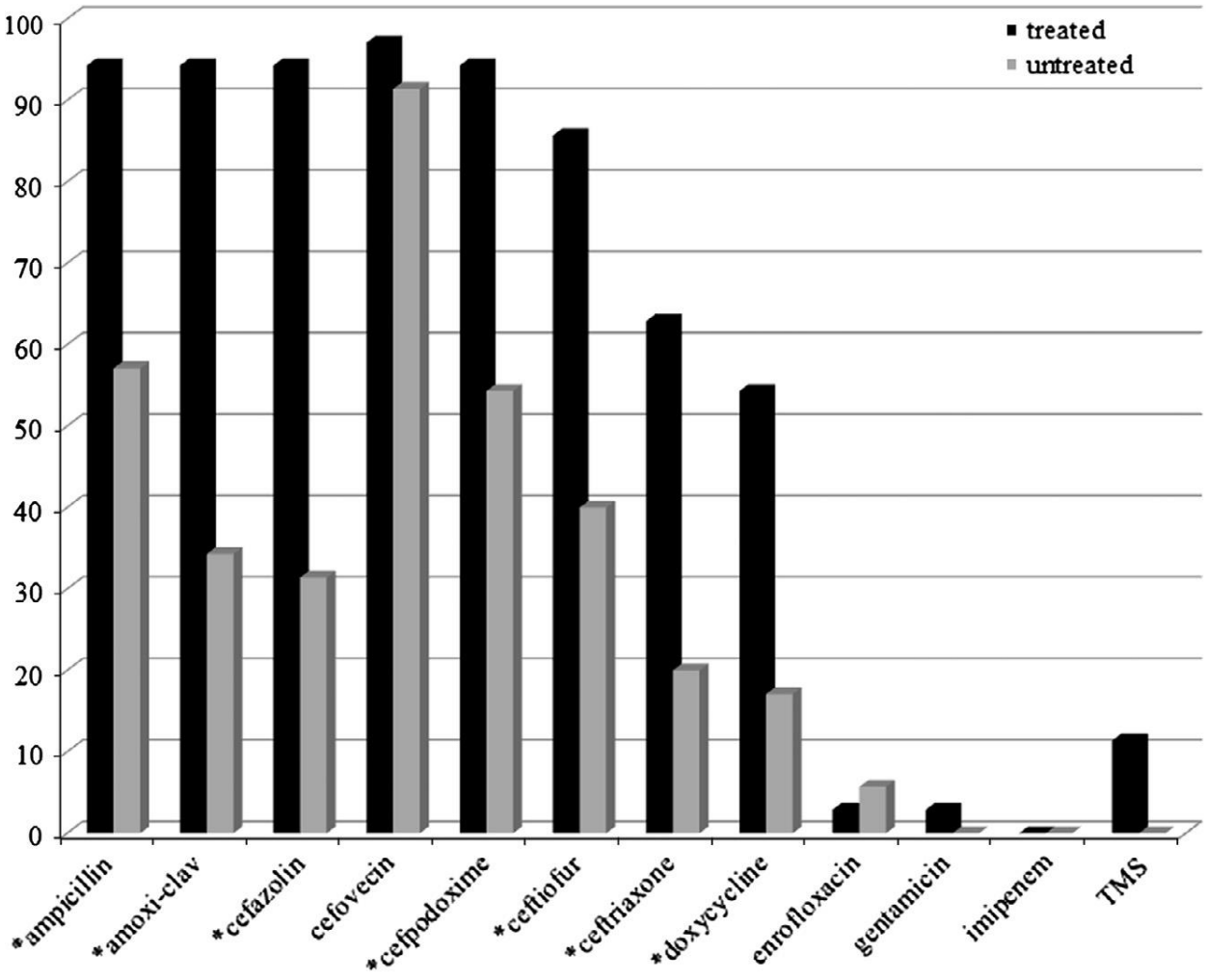

Figure 9. Percent of fecal *E. faecalis* and *E. faecium* resistant to each antimicrobial, based on disc diffusion testing. q/d, quinupristin-dalfopristin. There was no significant difference between the treatment groups for each antimicrobial.

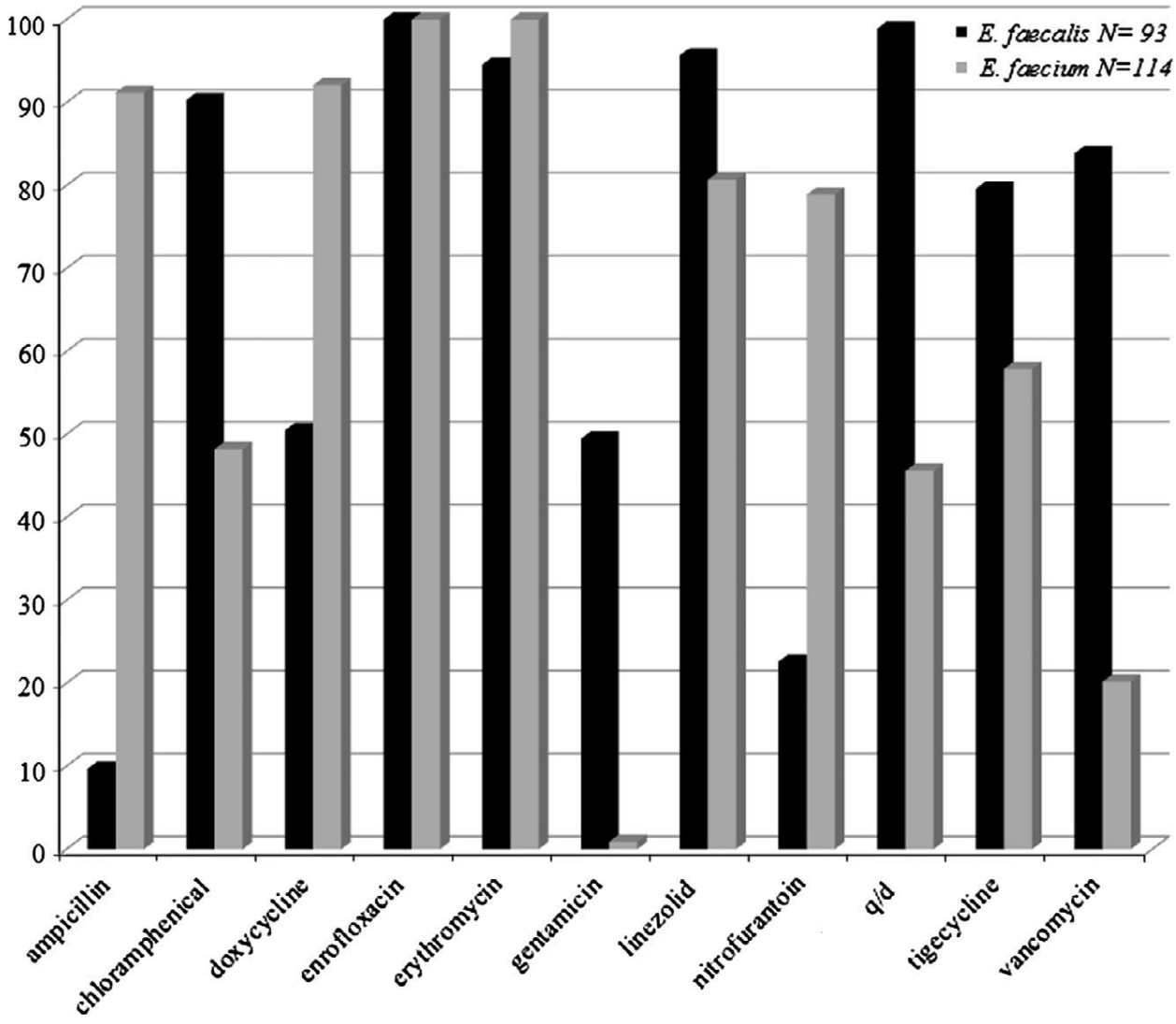

Table 1. Sample preparation for the determination of cefovecin concentration in plasma and plasma standards.

Step	
1	Add 0.1 mL plasma or plasma standard to microcentrifuge tube
2	Add 0.4 mL methanol containing cephalexin 0.5 µg/mL to microcentrifuge tube
3	Vortex microcentrifuge tube for 5 s
4	Centrifuge microcentrifuge tube at 15,000 *g* for 5 min
5	Transfer 0.2 mL supernatant to injection vial

Table 2. Mobile phase for the determination of cefovecin in plasma and plasma standards. A C18 column (Supelco Discovery, 50 x 2.1 mm, 5µM, Sigma) achieved separation while maintained at 40 °C.

	Mobile Phase	
Time (min)	A = Acetonitrile	B = 0.1% formic acid in water
0	5%	95%
1	20%	80%
3.5	5%	95%
5.5	5%	95%

Table 3. Mass spectrometry settings for the determination of cefovecin concentration in plasma using cephalexin as the internal standard. The accuracy and coefficient of variation were determined on replicates of three at each of the following concentrations: 0.05, 1, and 50 µg/mL.

	Qualifying ion (*m/z*)	Quantifying ion (*m/z*)	Accuracy	Coefficient of variation	Linear standard curve range
Cefovecin	454.08	241.00	99.6%	3.8%	0.05-50 µg/mL
Cephalexin	348.09	158.00	N/A	N/A	N/A

N/A not applicable

Table 4. MIC range and MIC_{90} values for tested antimicrobials among cefovecin-susceptible and cefovecin-resistant *E. coli* collected on days 0, 7, and 28.

	Day 0 Cef-S N=26	Day 0 Cef-R N=44	Day 7 Cef-S N=6	Day 7 Cef-R N=64	Day 28 Cef-S N=4	Day 28 Cef-R N=66
Cefovecin	Range <0.625-	Range <0.625-	Range 1.25-	Range 1.25->200	Range 60->200	Range 20->200

	10 MIC_{90} 10	200 MIC_{90} 60	>200 MIC_{90} N/A	MIC_{90} >200	MIC_{90} N/A	MIC_{90} >200
Ceftriaxone	Range <0.625-5 MIC_{90} 5	Range <0.625-5 MIC_{90} 5	Range 1.25-2.5 MIC_{90} N/A	Range 0.25->200 MIC_{90} >200	Range 1.25-10 MIC_{90} N/A	Range 1.25-20 MIC_{90} 20
Enrofloxacin	Range 0.5-16 MIC_{90} 2	Range 0.5-16 MIC_{90} 16	Range 2-16 MIC_{90} N/A	Range 0.5->200 MIC_{90} >200	Range 0.5-2 MIC_{90} N/A	Range <0.5-100 MIC_{90} 16
Gentamicin	Range 5-20 MIC_{90} 20	Range 5-20 MIC_{90} 20	Range 10-20 MIC_{90} N/A	Range 10->200 MIC_{90} >200	Range 5-10 MIC_{90} N/A	Range 2.5-20 MIC_{90} 10
Imipenem	Range <0.625- 20 MIC_{90} 5	Range <0.625-5 MIC_{90} 5	Range 1.25-20 MIC_{90} N/A	Range 1.25->200 MIC_{90} >200	Range <0.625- 1.25 MIC_{90} N/A	Range 0.625-20 MIC_{90} 10

Cef-S, cefovecin-susceptible based on disc diffusion; Cef-R, cefovecin-resistant based on disc diffusion; N/A, not applicable