# **Routine antibiotic therapy in dogs increases the detection of antimicrobial resistant faecal *Escherichia coli***

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# Abstract

**Background:** Antimicrobial Resistance (AMR) is a critical health problem, with systemic antimicrobial therapy driving development of AMR across the host spectrum.

**Objectives:** This study compares longitudinal carriage, at multiple time-points, of AMR faecal *Escherichia coli* in dogs undergoing routine antimicrobial treatment.

**Patients and Method**s: Faecal samples (n=457) from dogs (n=127) were examined pre-treatment, immediately after, and one- and three-months post-treatment with one of five antimicrobials*.* Isolateswere tested for susceptibility to a range of antimicrobials using disc-diffusion for each treatment-group at different time-points; the presence/absence of corresponding resistance genes were investigated using PCR assays. The impact of treatment-group/time-point and other risk factors on the presence of resistance (MDR, ciprofloxacin-resistance, third-generation-cephalosporin-resistance (3GCR) and ESBL- and AmpC-production) was investigated using multilevel modelling. Samples with at least one AMR *E. coli* from selective/non-selective agar were classed as positive. Resistance was also assessed at the isolate level, determining the abundance of AMR from non-selective culture.

**Results:** Treatment with beta-lactams or fluoroquinolones was significantly associated with the detection of 3GCR, AmpC-producing, MDR and/or ciprofloxacin-resistant *E. coli,* but not ESBL-producing *E. coli* immediately after treatment. However, one-month post-treatment, amoxicillin/clavulanate only, was significantly associated with the detection of 3GCR; there was no significant difference at three-months post-treatment for any antimicrobial compared to pre-treatment samples.

**Conclusions:** Our findings demonstrated that beta-lactam and fluoroquinolone antibiotic usage is associated with increased detection of important phenotypic and genotypic AMR faecal *E. coli* following routine therapy in vet-visiting dogs. This has important implications for veterinary and public health in terms of antimicrobial prescribing and biosecurity protocols, and dog waste disposal.

# Running Title

Antibiotics select for AMR canine faecal *E. coli*

# **Introduction**

The gastrointestinal tract is an important reservoir for Antimicrobial Resistant (AMR) Gram-negative organisms. 1, 2 MDR (resistance to three or more antimicrobial classes),3 ESBL- and AmpC-producing faecal *Escherichia coli* carried by dogs are of particular concern. They may act as a reservoir for self-infection including for further transmission of resistance genes, as well as pathogens and resistance genes potentially being transferred into other hosts including people, other pets and the environment.4-6 Systemic antimicrobial therapy selects for AMR Gram-negative bacteria, so increased use compounds AMR issues. In humans, even short-term therapy with ciprofloxacin, cephalosporin or clindamycin can lead to long-term disturbance of commensal bacterial populations and prolonged carriage of AMR Enterobacteriaceaeor anaerobic bacteria.7, 8

There are a limited number of, mostly broad-spectrum, antimicrobials authorised for use in companion animals in the United Kingdom. Amongst these, beta-lactams and fluoroquinolones are commonly utilised and critically important for the treatment of bacterial infections.9 Beta-lactam antimicrobials include oral cephalexin, oral amoxicillin/clavulanate, the most commonly prescribed, for dogs, by first-opinion veterinarians,10, 11 and injectable cefovecin (administered subcutaneously every 14 days). Enrofloxacin and marbofloxacin are oral second generation fluoroquinolones and clindamycin is an oral lincosamide antimicrobial.7, 12

The overall effect of antimicrobial treatment on human commensal bacterial populations has been shown to depend on the pharmacokinetics, spectrum of activity, dose and treatment duration, and the levels of AMR bacteria present before treatment.7 In dogs, a number of studies have shown that treatment with either beta-lactam or fluoroquinolone antimicrobials may positively select for intestinal/faecal AMR *E. coli* for variable periods of time.13-20 This study aimed to compare the extent and characteristics of AMR *E. coli* carriage in the faeces of community-dogs before- and after-treatment with five different antimicrobials.

# Materials and methods

## Study population

Dogs attending veterinary consultations at three centres, including first opinion and referral practice in the North-West of England between June 2011 and September 2012 were recruited. Inclusion criteria were dogs diagnosed with a bacterial infection (skin, soft tissue, urinary tract, dental, respiratory tract, orthopaedic, gastrointestinal, ocular) requiring systemic antimicrobial therapy with one of five antimicrobials, authorised for use including cephalexin, amoxicillin/clavulanate, cefovecin, clindamycin, or a fluoroquinolone (enrofloxacin or marbofloxacin). Exclusion criteria included antimicrobial therapy or veterinary admission within the previous three months and dogs aged less than 12 months due to fluoroquinolone contraindication. Dogs were excluded if they were prescribed systemic antimicrobials during the follow-up period. The veterinarian in charge of the case selected and implemented the treatment plan (antimicrobial, dose, frequency and duration) according to clinical need. Before enrolment, all dog owners read the study outline and gave written informed consent. The University’s Veterinary Science Ethics Committee approved the study protocol in June 2011.

## Detection and characterisation of faecal *E. coli*

### *E. coli* isolation

Owners were asked to provide a fresh faecal sample from their dog pre-treatment, immediately after treatment, and at one- and three-months post-treatment. Samples were delivered in person or by first-class pre-paid return-post. Faecal samples were refrigerated and processed on delivery (within 24-72 hours of collection).

All faecal samples were processed by selective and non-selective methods, as previously reported.21-24 In brief, an equal volume of faeces (5 grams) and brain heart infusion broth with 5% glycerol (BHI-G) (5mL) were homogenised before streaking onto plain eosin methylene blue agar (EMBA), EMBA impregnated with third generation cephalosporins (1μg/mL ceftazidime and 1 μg/mL cefotaxime) and spread-plating onto plain EMBA with antimicrobial discs (10μg ampicillin, 30μg amoxicillin/clavulanate, 1μg ciprofloxacin, 30μg chloramphenicol, 30μg nalidixic acid, 30μg tetracycline and 2.5μg trimethoprim)22. Following overnight aerobic incubation at 37°C, when present, ten random colonies, morphologically resembling *E. coli*, were selected from plain EMBA and one colony from each (ceftazidime/cefotaxime) impregnated EMBA plate and/or growing within the inhibition zone around each antimicrobial disc were selected for further investigation; this methodology was used to investigate if there is a reduction in diversity and/or emergence of low-prevalence AMR clones following antimicrobial selective pressure.4, 22, 25 It was therefore possible to select a maximum of 19 isolates from each faecal sample. Selected colonies were sub-cultured onto nutrient agar for pure growth and incubated aerobically overnight at 37°C before gram-stain, biochemical analysis (catalase production, lack of oxidase, lactose fermentation, indole production and inability to use citrate as a carbon source). PCR assays for *uid*A gene26 confirmed isolates as *E. coli*. All antimicrobial discs were obtained from MAST Group Ltd., Liverpool, UK, and media from LabM Ltd, Bury, UK; cephalosporin powder was from Sigma-Aldrich Company Ltd, Gillingham, UK.

### Antimicrobial susceptibility testing

All confirmed *E. coli* isolates underwent antimicrobial susceptibility disc-diffusion testing and interpretation as previously reported24 according to BSAC guidelines (Version 11.1 May 2012)27 with the same panel of seven antimicrobial discs as used above. *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on nutrient agar at 37°C was the control.

### ESBL- and AmpC-producing *E. coli*

Isolates selected from third generation cephalosporin impregnated EMBA and isolates from other selective and non-selective agar with phenotypic resistance to ampicillin or amoxicillin/clavulanate were further screened for third-generation-cephalosporin-resistance (3GCR) (cefpodoxime 10 μg) and phenotypic ESBL- and AmpC- production, according to manufacturer instructions (Extended Spectrum Beta-Lactamase Set D52C, MAST Group Ltd., Liverpool, UK and AmpC detection set D69C, MAST Group Ltd., Liverpool, UK).28, 29 *E. coli* ATCC® 25922 (LGC Standards, Teddington, UK) cultured overnight on nutrient agar at 37°C was the control. Isolates with phenotypic ESBL- or AmpC-production were further tested for the presence of *bla*CTX-M,30 *bla*SHV, *bla*TEM*, bla*OXA31 genes and *bla*AmpCgenes,32 including *bla*CIT-M as a screen for *bla*CMY (the most common AmpC–gene in the UK).24, 33 If positive for *bla*CTX-M, isolates were tested for the presence of CTX-M group 1, 2 and 9 genes,34, 35 as these are reported to be the most common CTX-M group genes amongst animals in the UK.24, 36

## Statistical Analyses

### Sample-level prevalence of antimicrobial resistance over time

All isolates (from selective and non-selective agar) were included in the analysis. To account for multiple isolates per sample, microbiological data were collapsed to sample-level, such that a sample with at least one resistant isolate was classed as resistant. Five resistance outcomes were considered: ciprofloxacin resistance (FQR), 3GCR, phenotypic ESBL- or AmpC-producing and MDR. The percentage of samples with each of the five resistance outcomes were calculated (including 95% confidence intervals), for each treatment-group/time-point~~.~~

#### Isolate-level prevalence of antimicrobial resistance over time

To quantify the abundance of antimicrobial resistant *E. coli* for each treatment group/time point, ten random isolates (if available) from non-selective EMBA were tested from each sample (n=3897 isolates). The percentage of isolates (including 95% confidence intervals) with resistance to each tested antimicrobial or MDR was determined for each treatment-group/time point.

### Risk factors for antimicrobial resistant faecal *E. coli*

#### Questionnaire data

A questionnaire investigating potential risk factors for AMR bacteria was completed by owners at the start of the study and at each faecal collection. The attending veterinary surgeon completed a one-page questionnaire detailing diagnosis, treatment regime, and previous antimicrobial treatment within the last 12 months. All questionnaire-derived information was available as potential explanatory variables for inclusion in multivariable modelling of antimicrobial resistance outcomes. Except for age, all variables were categorical. Collinearity between explanatory variables was assessed using two-by-two tables and Pearson’s chi-square test for independence or Fisher’s exact tests if N<5. A one-way between-groups analysis of variance (ANOVA) was used to investigate differences between animals of different ages (normal distribution) within treatment groups pre-treatment (Table 1). To investigate significant pre-treatment differences between treatment groups, for each AMR outcome (FQR, 3GCR, MDR, ESBL- and AmpC-producing *E. coli*) and questionnaire-derived variables, simple univariable and multivariable logistic regression analysis with a binomial distribution and logit link function were used. All questionnaire data analyses were undertaken using SPSS software package (SPSS 20.0 for Mac, SPSS Inc, Chicago, Illinois).

#### Multilevel models

Multilevel multivariable logistic regression modelling was used to examine differences between treatment-groups/time-points, including dog as a random effect term (level-two unit, due to repeated measurements in dogs). Faecal samples were the level-one unit of interest. At enrolment all dogs were classed as ‘untreated’. The different combinations of time (n=3) and treatment group (n=5) provided 15 categories for analyses.

Univariable analyses were initially performed; all variables showing some association with the resistance outcome (*P*-value<0.25)37 were considered for incorporation into the final multivariable model. Models were constructed using backwards stepwise procedures where variables with a Wald *P*-value <0.05 were retained; treatment group/time-point were always retained. Once a final multivariable model was generated, all variables significantly (*P*<0.05) different between treatment groups at baseline were forced into the multilevel model to ensure that there was no confounding effect on remaining variables.

Univariable and multivariable calculations utilised penalised quasi-likelihood estimates (second-order PQL for all outcomes other than phenotypic ESBL which was first-order MQL due to lack of model convergence)38 were performed. First-order interaction terms were tested for all variables remaining in final models. The residuals +/-1.96 SD x rank (caterpillar plots) were calculated and graphed for each dog to check for outliers. Multilevel models were analysed using the MLwiN statistical software package (MLwiN Version 2.28 Centre for Multilevel Modelling, University of Bristol).

# Results

## Study population

One hundred and twenty-seven dogs were enrolled from three centres (Supplementary Table S1). All dogs provided samples pre-treatment and at treatment-end, 105 dogs provided samples at one-month post-treatment and 98 dogs provided samples at three-month post-treatment. Information regarding sample time-points and the reasons for missing samples are described in the Supplementary Data.

## Detection and characterisation of faecal *E. coli*

*E. coli*was detected in 95% (434/457) of faecal samples. 3GCR was detected in 158 samples from 60% of dogs, phenotypic ESBL-producing *E. coli* were detected in 59 samples from 31% of dogs and AmpC-producing *E. coli* detected in 138 samples from 60% of dogs. Table 2 shows the number and percentage of samples with at least one faecal *E. coli* with ESBL- and/or AmpC-producing genes for each time-point/treatment group. Carriage of *bla*CIT-M, was detected in the faecal samples of 50% of dogs during the full study period; *bla*DHA-1/*bla*DHA-2 and *bla*MOX were detected from only one dog each in addition to *bla*CIT-M. The most commonly detected *bla*CTX-M genes belonged to group 1 (13% of dogs) followed by group 9 (2% dogs); *bla*CTX-M group 2 genes were detected in *E. coli* from a single dog at one-month post-fluoroquinolone treatment.

## Sample-level prevalence of antimicrobial resistance

Generally there were an increased percentage of samples with MDR, ESBL- or AmpC-producing *E. coli* following treatment with cephalexin, amoxicillin/clavulanate and cefovecin and an increased percentage of samples with FQR *E. coli* following cephalexin, cefovecin and fluoroquinolone (FQ) treatment. However, the percentage of samples with resistance had generally declined by three-month post-treatment (Figure 1).

## Prevalence of antimicrobial resistance at the isolate level

During the full study-period, isolates (n=3897: pre-treatment n=1097, immediately post-treatment n=1011, one-month post-treatment n=911 and three-month post-treatment n=878) were randomly selected from non-selective agar. For all treatment groups, the percentage of isolates with resistance to each tested antimicrobial and MDR increased immediately post-treatment compared to D0 (pre-treatment), but declined by three-months post-treatment (Figure 2); of note was the increased detection MDR and a lack of fully susceptible isolates immediately after fluoroquinolone treatment (Figure 2).

## Risk factors for antimicrobial resistant faecal *E. coli*

When compared to all pre-treatment samples, MDR *E. coli* was significantly more likely to be detected following treatment with amoxicillin/clavulanate or cefovecin (Table 3). The risk of detecting 3GCR *E. coli* was more likely following treatment with amoxicillin/clavulanate, cephalexin or cefovecin; for AmpC-producing *E. coli*, cephalexin or cefovecin therapies increased the risk of detection (Table 4). Finally, ciprofloxacin resistant *E. coli* was more likely to be detected following treatment with cephalexin, a fluoroquinolone or cefovecin (Table 3). At one-month post-amoxicillin/clavulanate, the risk of detecting 3GCR *E. coli* increased compared to pre-treatment samples (Table 4), otherwise no other significant differences were detected at one- or three-month post-treatment compared to pre-treatment.

The final models also showed that there were positive associations between: living in a multi-dog household and 3GCR or ESBL-producing *E. coli*, recruitment from referral consultations and AmpC-producing *E. coli* (compared to first-opinion), eating animal stools and ciprofloxacin resistance, owner working in healthcare and MDR, a ‘diagnosis of pyoderma’ and ESBL-producing *E. coli*, and body weight and AmpC-producing *E. coli* (dogs of small to medium weight were less likely than large dogs to have resistance).

# Discussion

This study used a prospective, longitudinal design to examine the effect of different antimicrobials, on the selection and carriage of AMR amongst faecal *E. coli* in a large cohort of vet-visiting dogs. Faecal samples were collected before, and at multiple time-points, including three-months, after completing therapy. Resistance to critically important antimicrobials was investigated (including third-generation cephalosporins and fluoroquinolones)*.* Our findings suggest that single courses of systemic antimicrobials select for resistance immediately after treatment, but effects then wane. In particular, beta-lactams selected for 3GCR and/or MDR, and cephalosporins and fluoroquinolones selected for FQR. This suggests that broad spectrum antimicrobials authorised for the treatment of bacterial infections in dogs, create a reservoir of AMR *E. coli* and potentially transmissible resistance genes within the canine gastrointestinal tract. Both can provide a source of environmental contamination, be transmitted to other hosts, including owners, or influence re-infection.

## Selection of 3GCR including AmpC

Treatment with cephalexin and cefovecin significantly increased the risk of detecting 3GCR and in particular, AmpC-producing *E. coli*. These results confirm those of Damborg *et al*,39 who examined cephalexin-only treatment in a small number of community dogs compared to untreated controls and also found an increase in AmpC-producing *E. coli.* Similarly, Lawrence *et al*20 reported increased AmpC-producing faecal *E. coli* 28-days-post-cefovecin-injection treatment in a small number of laboratory Beagles.

## Impact of amoxicillin/clavulanate

It was surprising that amoxicillin/clavulanate (aminopenicillin plus beta-lactamase inhibitor combination), the other beta-lactam antimicrobial investigated in this study, did not significantly select for AmpC-producing *E. coli*, as did the cephalosporins. Treatment with amoxicillin/clavulanate, is expected to select for 3GCR due to Amp-C production, but not necessarily ESBL-mediated resistance. Clavulanate, a beta-lactamase inhibitor, is less effective against AmpC-beta-lactamases, so could select for these enzymes over other beta-lactamases, including ESBL-variants.40, 41 Gibson *et al*16 also reported that unexpectedly, they did not detect treatment with beta-lactams or potentiated-beta-lactams as risks for MDR AmpC-producing *E. coli* in hospitalised dogs; however the findings of other studies supportthe selection of beta-lactam resistance following treatment with amoxicillin without clavulanate.15, 18 Although the amoxicillin/clavulanate treatment group was of similar size to other treatment groups, apart from the fluoroquinolones, we cannot exclude a sample size effect and further larger studies are required to investigate these findings further; other factors such as differing antimicrobial excretion and concentration within the intestinal tract should be investigated.

## Selection for ESBL, MDR and FQR

The detection of ESBL-producing *E. coli* was not associated with use of any antimicrobials administered in this study, as previously reported.14, 42, 43 The percentage of samples positive for ESBL-producing *E. coli* was lower overall than other resistance outcomes. This may have reduced the power to detect significant associations, particularly as cephalexin and cefovecin were found to be a risk for 3GCR, an outcome including phenotypic ESBL- and AmpC-producing *E. coli.* Furthermore, previous studies in both healthy and hospitalised dogs23, 24, 44 have reported a higher prevalence of canine faecal AmpC-producing compared to ESBL-producing *E. coli*.

In this study, the administration of both amoxicillin/clavulanate and cefovecin increased the risk of detecting MDR *E. coli* post-treatment. These results uphold previous work using a small number of dogs, where selection for MDR faecal *E.* *coli* followed treatment with ampicillin, amoxicillin, enrofloxacin or cefovecin;15, 18, 20, 45 retrospective risk analysis also identified cephalexin as a risk for MDR *E. coli* rectal carriage during hospitalisation.16 Cefovecin was also a risk for the detection of AmpC-producing *E. coli*, that are oftenMDR40.

The use of cephalosporins and fluoroquinolone antimicrobials increased the risk of detecting FQR post-treatment in this study. This upholds results from Boothe *et al1*8 (selection of MDR FQR *E. coli* following treatment with enrofloxacin in two laboratory dogs) and Lawrence *et al*20 (increased detection of enrofloxacin resistant faecal *E. coli* after administration of cefovecin). As *E. coli* strains with high-level fluoroquinolone resistance are commonly resistant to cephalosporins,46 this suggests co-selection of resistance.2 However, we did not find an association between fluoroquinolone therapy and MDR or 3GCR at treatment-end, possibly due to a small sample size in this treatment group.

## antimicrobial exposure recovery period

Chronic antimicrobial therapy likely maintains MDR amongst canine commensal *E. coli*.47 Our study aimed to report AMR prevalence and risk factors at time-points post-treatment, but in the absence of repeated antimicrobial prescriptions. We found that after amoxicillin/clavulanate treatment, the risk of detecting 3GCR *E. coli* increased and remained higher at one-month post-treatment, but at three-months it had returned to pre-treatment levels. The length of time for which significantly different levels of resistance were evident is longer than that suggested in previous work examining amoxicillin without clavulanate; Gronvold *et al*15 and Boothe *et al*18 showed recovery at two-weeks post-treatment. This may mean that whilst amoxicillin/clavulanate may have less overall impact on the selection of 3GCR than cephalosporins, the treatment effects are longer lasting. Further larger studies are required to corroborate and investigate the basis of these findings. For example amoxicillin/clavulanate may have differing effects on other members of the microbiome (such as anaerobic bacteria), compared to cephalosporins where higher generation drugs have increasing activity against Gram-negative bacteria. For other treatment groups (cephalexin, cefovecin, clindamycin and fluoroquinolones) there was no association with resistance at one-month post-treatment. Previous work, however, identified resistance at 21 and between 17 and 37 days after enrofloxacin (Boothe *et al*18 and Trott *et al*,13 respectively) and resistance at day 28 after cefovecin.20

## length of antimicrobial treatment

The length of antimicrobial treatment has been investigated particularly for human patients, and shorter courses have been shown to reduce antimicrobial use, costs, adverse events and exposure to commensal organisms (and thereby AMR selection), without increasing morbidity or mortality.48 The results of this study, however, did not suggest an association between treatment length and resistance, particularly given the shorter treatment length for amoxicillin/clavulanate; however, sample sizes were small in some groups, reducing the likelihood of detecting associations between variables. Overall, selection and persistence of resistance within the gastrointestinal tract is likely to be influenced by multiple factors including antimicrobial class (broad- or narrow-spectrum), resistance type (MDR or not) and mechanism of resistance (transmissible or chromosomal),18 pharmacokinetics/pharmacodynamics, the level of resistance present before therapy7 and bacterial virulence/fitness.49

## Magnitude of resistance

Quantifying resistance (assessing the number of isolates with an AMR trait) would be a better measure to detect changes over time than analysis at the sample level (sample classed as AMR if at least one isolate is AMR), particularly where there is high pre-treatment AMR prevalence. High pre-treatment prevalence can make it difficult to detect change following therapy, increase the risk of AMR in the following sample and influence recovery time. This study limitation was offset by selecting ten random isolates from non-selective agar for each sample for analysis at the isolate level at each time-point/treatment group. At the sample level, this study did not identify an association between cephalexin or fluoroquinolone therapy with MDR *E. coli*, however there was an increase immediately at treatment-end compared to pre-treatment when examined at the isolate level; this concurs with our expectations and the findings of previous authors.13, 18, 20, 45 Concordantly, also at the isolate level, fluoroquinolone and cefovecin therapy appeared to have the most effect on fully-susceptible *E.* *coli*; this was also noted at the sample level where *E. coli* were not detected in over one third of dogs directly after fluoroquinolone treatment. Both Lawrence *et al*20 and Trott *et al*13 reported significant inhibition of faecal *E. coli* and/or coliforms during and beyond treatment with cefovecin and enrofloxacin. Inhibition of susceptible isolates may create a vacant niche in the gastrointestinal tract for colonisation with resistant or pathogenic bacteria.

## Study implications

Antimicrobial therapy selects for MDR *E. coli* within the gastrointestinal tract of humans and dogs. These bacteria may be then shared between hosts (including between humans or between pets and between humans and pets) within households4, 5 and health-care settings; carriage isolates may cause extra-intestinal infections.16, 50 Antimicrobial therapy is paramount to the successful treatment of many patients. Implementation of veterinary hospital prescribing guidelines can reduce overall use and misuse of important antimicrobials,51 reducing selection pressure for AMR bacteria. This study provides important information on both the effect and the timescale of the effect following routine antimicrobial therapy in dogs. This information can be used to design biosecurity guidelines that limit transfer of such bacteria to in-contact individuals or to the environment, including barrier nursing,52, 53 appropriate disposal of dog waste6 and strict hand hygiene.54, 55

## Conclusions

Antimicrobials impact not just the pathogens they are designed to target, but also the commensal microbiota. Our results suggest that treatment with many commonly used systemic antimicrobials (particularly beta-lactams and fluoroquinolones) affects the commensal faecal flora of dogs, causing a shift towards a more resistant bacterial population of *E. coli*. There is up to a one-month window, following the end of therapy where treated dogs are more likely to carry AMR faecal *E. coli*. Proactive strategies such as prudent antimicrobial-prescribing and hospital biosecurity programs are urgently needed to limit development and dissemination of antimicrobial resistance. In particular, policies for antimicrobial use during specific clinical conditions, alongside utilisation of culture and susceptibility testing, could help reduce misuse and overuse of important antimicrobials. Full genome sequencing e.g. deep sequencing of shotgun metagenomics of the microbiome could help to elucidate the overall impact of therapy with different antimicrobials.

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# Transparency Declarations

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# Author’s contributions

Conceived and designed the experiments: VS, GP, TN, NM, SD, NJW; collected samples: VS, TN, NM; performed the experiments: VS; collated, analysed and interpreted the data: VS, GP, NJW and drafted and reviewed the manuscript: VS, GP, KMM, NJW. All authors read and approved the final manuscript.

# Supplementary Data

Figures S1 is available as Supplementary data at JAC Online (http:// jac.oxfordjournals.org/).

Table 1: The pre-treatment (D0) variables considered for inclusion in the final multivariable model, with the number and percentage (%) of dogs in each treatment-group and variable category.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Variable | CFX  (n=32) | AC  (n=28) | CVN  (n=24) | CD  (n=29) | FQ  (n=14) | Total  (n=127) | *P*-value |
| Mean agea (months) | 44 | 50 | 68 | 79 | 83 | 62 | **0.002** |
| Weight |  |  |  |  |  |  | **0.002** |
| Small (< 11 kg) | 1 (5) | 2 (10) | 3 (15) | 10 (50) | 4 (20) | 20 (16) |  |
| Medium (11-20 kg) | 4 (25) | 3 (19) | 0 | 7 (44) | 2 (13) | 16 (13) |  |
| Large (> 20 kg) REF | 27 (30) | 23 (25) | 21 (23) | 12 (13) | 8 (9) | 91 (72) |  |
| Gender |  |  |  |  |  |  | 0.8 |
| Male REF | 19 (25) | 17 (23) | 12 (16) | 17 (23) | 10 (13) | 75 (59) |  |
| Female | 13 (25) | 11 (21) | 12 (23) | 12 (23) | 4 (8) | 52 (41) |  |
| Treatment duration |  |  |  |  |  |  | **0.001** |
| 1 week REF | 6 (18) | 16 (47) | 0 | 10 (29) | 2 (6) | 34 (27) |  |
| > 1 or < 3 weeks | 12 (26) | 9 (19) | 9 (19) | 11 (23) | 6 (13) | 47 (37) |  |
| > 3 weeks | 14 (30) | 3 (7) | 15 (33) | 8 (17) | 6 (13) | 46 (36) |  |
| Recruitment site |  |  |  |  |  |  | **0.001** |
| First opinion practice REF | 24 (33) | 27 (37) | 4 (17) | 17 (23) | 1 (1) | 73 (57) |  |
| Referral consultation | 8 (15) | 1 (2) | 20 (83) | 12 (22) | 13 (24) | 54 (43) |  |
| Diagnosis of pyoderma at enrolment1 | 28 (35) | 3 (4) | 23 (28) | 16 (20) | 11 (14) | 81 (64) | **0.001** |
| Previous systemic antimicrobial treatment2 | 16 (26) | 10 (16) | 17 (28) | 10 (16) | 8 (13) | 61 (48) | **0.048** |
| Previous beta-lactam antimicrobial treatment2 | 11 (28) | 7 (18) | 9 (23) | 7 (18) | 6 (15) | 40 (31) | 0.41 |
| Previous hospital admission2 | 17 (42) | 12 (29) | 5 (12) | 6 (15) | 1 (2) | 41 (32) | **0.007** |
| In-contact human or pet received antimicrobials3 | 7 (26) | 5 (19) | 4 (15) | 7 (26) | 4 (15) | 27 (21) | 0.9 |
| In-contact human or pet admitted to hospital or veterinary premises3 | 4 (15) | 3 (12) | 6 (23) | 8 (31) | 5 (19) | 26 (20) | 0.2 |
| Owner works in healthcare | 5 (21) | 2 (8) | 4 (17) | 10 (42) | 3 (13) | 24 (19) | 0.08 |
| Multi-dog household | 18 (15) | 15 (26) | 12 (21) | 8 (14) | 4 (7) | 57 (45) | 0.1 |
| Enrolled dog regularly eats animal stools | 7 (18) | 5 (13) | 8 (21) | 11 (29) | 7 (18) | 38 (30) | 0.08 |

CFX=cephalexin; AC=amoxicillin/clavulanate; CVN=cefovecin; CD=clindamycin; FQ=fluoroquinolone. aAge was the only continuous value and is represented by the mean age of dogs in each treatment group; REF = the reference category for non-dichotomous variables; 1Other infections (n=46) include urinary tract/prostate (n=11), abscess/bite wound (n=11), dental (n=10) and post-operative (n=8). 2Within 12-months but more than three months as per enrolment criteria; 3Within 12-months of enrolment; significant if *P* <0.05 (Pearson’s chi-square or aANOVA)

Table 2: The number and percentage (%) of samples that harboured at least one faecal *E. coli* positive for ESBL- or AmpC-resistance genes at each time-point/treatment-group.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Treatment Group** | **Time point & total samples** | ***bla*CTX-M** | **CTX-M group 1** | **CTX-M group 9** | ***bla*CitM** | ***bla*CTX-M & *bla*CitM** | **APhenotypic ESBL with *bla*TEM&/or *bla*OXA** |
| **CFX** | D0 (n = 32) | 1 (3) | 1 (3) | 0 | 4 (13) | 0 | 0 |
|  | E (n = 32) | 4 (13) | 3 (9) | 0 | 19 (60) | 4 (13) | 1 (3) |
|  | M1 (n = 27) | 2 (7) | 2 (7) | 0 | 9 (33) | 0 | 1 (4) |
|  | M3 (n = 24) | 0 | 0 | 0 | 4 (17) | 0 | 0 |
| **AC** | D0 (n = 28) | 0 | 0 | 0 | 5 (18) | 0 | 0 |
|  | E (n = 28) | 1 (4) | 1 (4) | 0 | 9 (32) | 0 | 1 (4) |
|  | M1 (n = 26) | 1 (4) | 1 (4) | 0 | 7 (27) | 0 | 1 (4) |
|  | M3 (n = 25) | 1 (4) | 0 | 1 (4) | 5 (20) | 0 | 0 |
| **CVN** | D0 (n = 24) | 5 (21) | 5 (21) | 0 | 9 (38) | 3 (13) | 1 (4) |
|  | E (n = 24) | 6 (25) | 4 (17) | 1 (5) | 15 (63) | 4 (17) | 1 (4) |
|  | M1 (n = 19) | 5 (26) | 2 (11) | 2 (11) | 5 (26) | 3 (16) | 1 (5) |
|  | M3 (n = 18) | 1 (6) | 1 (6) | 0 | 7 (39) | 2 (11) | 1 (6) |
| **CD** | D0 (n = 29) | 0 | 0 | 0 | 5 (17) | 0 | 1 (4) |
|  | E (n = 29) | 1 (3) | 0 | 0 | 3 (10) | 0 | 1 (4) |
|  | M1 (n = 25) | 1 (4) | 1 (4) | 0 | 2 (8) | 0 | 0 |
|  | M3 (n = 23) | 0 | 0 | 0 | 1 (4) | 0 | 0 |
| **FQ** | D0 (n = 14) | 2 (14) | 2 (14) | 0 | 4 (29) | 1 (7) | 1 (7) |
|  | E (n = 14) | 3 (21) | 2 (14) | 0 | 3 (21) | 2 (14) | 1 (7) |
|  | M1 (n = 7) | 1 (14) | 1 (14) | 0 | 1 (14) | 0 | 0 |
|  | M3 (n = 8) | 1 (13) | 1 (13) | 0 | 2 (25) | 1 (13) | 0 |
| **Treatment Overall** | D0 (n = 127) | 8 (6) | 8 (6) | 0 | 27 (21) | 4 (3) | 3 (2) |
|  | E (n = 127) | 14 (11) | 10 (8) | 1 (1) | 49 (39) | 10 (8) | 5 (4) |
|  | M1 (n = 105) | 9 (9) | 7 (7) | 2 (2) | 24 (23) | 3 (3) | 4 (4) |
|  | M3 (n = 98) | 3 (3) | 2 (2) | 1 (1) | 19 (19) | 3 (3) | 1 (1) |
| **Total dogs** | **(N = 127)** | **22 (17)** | **16 (13)** | **3 (2)** | **63 (50)** | **13 (10)** | **13 (10)** |

CFX=cephalexin; AC= amoxicillin/clavulanate; CVN=cefovecin; CD=clindamycin; FQ=fluoroquinolone; Treatment Overall=all antibiotics; D0=pre-treatment; E= treatment-end; M1=one-month post- treatment; M3 = three-month post-treatment-end; percentage in parenthesis; Total Dogs=number and percentage of dogs with ESBL- or AmpC-genes during the full study period (dog was classed as positive if at least one isolate in one sample was positive); Asequencing was not performed to confirm carriage of genes *bla*TEM and *bla*OXA.

Table 3: Multilevel multivariable results for the outcomes: Ciprofloxacin-Resistance (CipR) and MDR in 457 faecal samples from 127 dogs.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Variables | CipR | | | MDR | | |
|  | OR | 95% CI | P-value | OR | 95% CI | P-value |
| Time D0 | REF | ⎯ | ⎯ | REF | ⎯ | ⎯ |
| Time End and CFX | **5.1** | **1.6-16.6** | **0.006** | 1.7 | 0.6-5.0 | 0.4 |
| Time End and AC | 1.4 | 0.3-7.1 | 0.72 | **5.0** | **1.5-16.0** | **0.007** |
| Time End and CVN | **7.0** | **1.9-25.5** | **0.003** | **8.0** | **2.1-30.6** | **0.002** |
| Time End and CD | 0.7 | 0.1-4.9 | 0.7 | 1.6 | 0.4-5.7 | 0.5 |
| Time End and FQ | 5.6 | 1.2-25.7 | 0.03 | 0.8 | 0.2-4.0 | 0.8 |
| Time M1 and CFX | 2.02 | 0.5-8.8 | 0.35 | 2.1 | 0.7-7.0 | 0.2 |
| Time M1 and AC | 2.1 | 0.5-9.3 | 0.34 | 0.6 | 0.1-2.5 | 0.5 |
| Time M1 and CVN | 0.8 | 0.1-5.7 | 0.82 | 1.6 | 0.3-7.3 | 0.6 |
| Time M1 and CD | 0.3 | 0.02-4.1 | 0.37 | 1.8 | 0.5-6.4 | 0.4 |
| Time M1 and FQ | 2.7 | 0.3-22.9 | 0.34 | 1.8 | 0.2-14.1 | 0.6 |
| Time M3 and CFX | 0.4 | 0.03-5.6 | 0.53 | 0.7 | 0.2-2.8 | 0.6 |
| Time M3 and AC | 1.0 | 0.2-6.9 | 0.99 | 2.0 | 0.6-7.2 | 0.3 |
| Time M3 and CVN | 2.5 | 0.5-12.4 | 0.25 | 1.2 | 0.3-6.1 | 0.8 |
| Time M3 and CD | 0.4 | 0.03-6.02 | 0.54 | 0.4 | 0.1-1.8 | 0.2 |
| Time M3 and FQ | 2.9 | 0.4-23.0 | 0.32 | 0.3 | 0.02-4.5 | 0.4 |
| Time treatment overall | ⎯ | ⎯ | **0.09** | ⎯ | ⎯ | **0.045** |
| Owner works in healthcare | ⎯ | ⎯ | ⎯ | **3.6** | **1.32-9.88** | **0.012** |
| Dog eats animal stools | **2.9** | **1.2-7.0** | **0.018** | ⎯ | ⎯ | ⎯ |
| Level 2 (dog) Variance [standard error] VPC (%) | 1.6 [0.6] 32% | ⎯ | ⎯ | 2.7 [0.7] 45% | ⎯ | ⎯ |

OR=odds ratio; 95% CI=95% confidence interval; VPC=variance partition coefficient; P values are from the Wald chi-squared test; CFX=cephalexin, AC=amoxicillin/clavulanate; CVN=cefovecin; CD=clindamycin; FQ=fluoroquinolone; Day 0=pre-treatment; End = treatment-end; M1=one-month post-treatment-end; M3=three-month post-treatment; significant if P <0.05 (bold text)

Figure 1: The percentage of samples with MDR (a), phenotypic ESBL-producing (b), ciprofloxacin resistant (c) and Amp-C-producing (d) *E. coli* at each time-point for each treatment-group and treatment-overall (95% CI).

(a)

(b)

(c)

(d)

Day 0=pre-treatment; End=treatment-end; M1=one-month post-treatment; M3=three-month post-treatment; CFX=cephalexin; AC=clavulanate-amoxicillin; CVN=cefovecin; CD=clindamycin; FQ=fluoroquinolone

Figure 2: The percentage of isolates with resistance or susceptibility in each treatment-group: treatment-overall (a), amoxicillin/clavulanate (b), cephalexin (c), cefovecin (d), clindamycin (e) and fluoroquinolones (f) at each-time point (95% CI).

(a)

(b)

(c)

(d)

(e)

(f)

Amp=ampicillin; AC=amoxicillin/clavulanate; Cip=ciprofloxacin; Chlor=chloramphenicol; Nal=nalidixic acid; Tet=tetracycline; TM=trimethoprim; MDR=multidrug-resistance; Susc=fully susceptible; Day 0=pre-treatment; End=treatment-end; M1=one-month post-treatment; M3=three-month post-treatment; All=all treatment groups; CFX=cephalexin; CVN=cefovecin; CD=clindamycin; FQ=fluoroquinolone

Table 4: Multilevel multivariable results for Third-Generation-Cephalosporin-Resistance (3GCR), presence or absence of phenotypic ESBL- or AmpC-producing *E.* *coli* in 457 faecal samples from 127 dogs.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Variables | 3GCR | | | ESBL | | | AmpC | | |
|  | **OR** | **95% CI** | ***P*-value** | **OR** | **95% CI** | ***P*-value** | **OR** | **95% CI** | ***P*-value** |
| Time D0 | REF | ⎯ | ⎯ | REF | ⎯ | ⎯ | REF | ⎯ | ⎯ |
| Time End and CFX | **8.7** | **2.9-25.9** | **<0.001** | 1.6 | 0.5-4.8 | 0.42 | **8.84** | **3.1-25.4** | **<0.001** |
| Time End and AC | **3.9** | **1.1-14.5** | **0.04** | 1.3 | 0.2-7.6 | 0.77 | **2.81** | **1.0-8.3** | **0.06** |
| Time End and CVN | **9.6** | **2.5-37.7** | **0.001** | 2.5 | 0.8-8.3 | 0.12 | **9.31** | **2.7-31.9** | **<0.001** |
| Time End and CD | 1.0 | 0.3-3.8 | 0.99 | 0.4 | 0.1-3.5 | 0.43 | 0.78 | 0.2-2.7 | 0.69 |
| Time End and FQ | 0.6 | 0.1-3.2 | 0.51 | 2.4 | 0.5-11.5 | 0.29 | 0.47 | 0.1-2.6 | 0.39 |
| Time M1 and CFX | 2.2 | 0.7-6.9 | 0.182 | 0.5 | 0.1-2.5 | 0.41 | 2.76 | 1.0-8.0 | 0.06 |
| Time M1 and AC | **5.3** | **1.5-19.7** | **0.013** | 3.0 | 0.7-13.1 | 0.14 | 1.59 | 0.5-4.9 | 0.42 |
| Time M1 and CVN | 1.3 | 0.3-5.2 | 0.71 | 2.1 | 0.6-7.7 | 0.28 | 1.85 | 0.6-6.1 | 0.31 |
| Time M1 and CD | 0.7 | 0.2-3.1 | 0.63 | 0.6 | 0.1-4.6 | 0.59 | 1.04 | 0.3-3.7 | 0.96 |
| Time M1 and FQ | 0.7 | 0.1-5.3 | 0.69 | 0.9 | 0.1-10.4 | 0.91 | 0.42 | 0.03-5.8 | 0.52 |
| Time M3 and CFX | 0.8 | 0.2-2.6 | 0.57 | 0.9 | 0.2-3.8 | 0.91 | 0.62 | 0.2-2.3 | 0.47 |
| Time M3 and AC | 1.6 | 0.4-6.3 | 0.54 | 0.7 | 0.1-6.5 | 0.73 | 0.42 | 0.1-1.7 | 0.23 |
| Time M3 and CVN | 2.9 | 0.7-11.8 | 0.14 | 0.8 | 0.1-4.4 | 0.76 | 1.83 | 0.5-6.2 | 0.33 |
| Time M3 and CD | 0.5 | 0.1-2.8 | 0.44 | 0.7 | 0.1-5.2 | 0.69 | 0.28 | 0.04-1.9 | 0.19 |
| Time M3 and FQ | 0.8 | 0.1-6.6 | 0.87 | 1.1 | 0.1-11.2 | 0.92 | 0.96 | 0.1-7.2 | 0.97 |
| Time treatment overall | ⎯ | ⎯ | **<0.001** | ⎯ | ⎯ | 0.77 | ⎯ | ⎯ | **<0.001** |
| Weight (large) | REF | ⎯ | ⎯ | REF | ⎯ | ⎯ | REF | ⎯ | ⎯ |
| Weight (small) | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | 0.5 | 0.2-1.4 | 0.17 |
| Weight (medium) | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | **0.1** | **0.03-0.5** | **0.004** |
| Weight overall | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | ⎯ | **0.009** |
| Diagnosis of pyoderma | 2.3 | 0.8-6.6 | 0.11 | **3.63** | **1.18-11.13** | **0.024** | ⎯ | ⎯ | ⎯ |
| First opinion | RE | ⎯ | ⎯ | REF | ⎯ | ⎯ | REF | ⎯ | ⎯ |
| Referral consultation | 2.1 | 0.9-5.2 | 0.11 | ⎯ | ⎯ | ⎯ | **2.2** | **1.1-4.6** | **0.035** |
| Multi-dog household | **3.8** | **1.6-8.7** | **0.002** | **2.71** | **1.24-5.93** | **0.012** | ⎯ | ⎯ | ⎯ |
| Level 2 (dog) Variance [standard error] VPC (%) | 2.4 [0.6] 43% | ⎯ | ⎯ | 1.190 [0.5] 27% | ⎯ | ⎯ | 1.8 [0.5] 35% | ⎯ | ⎯ |

OR=odds ratio; 95% CI=95% confidence interval; VPC=variance partition coefficient; REF=reference category; *P* values are from the Wald chi-squared test; CFX=cephalexin, AC=amoxicillin/clavulanate; CVN=cefovecin; CD=clindamycin; FQ=fluoroquinolone; Day 0=pre-treatment; End= treatment-end; M1=one-month post-treatment; M3=three-month post-treatment; significant if *P* <0.05 (bold text)

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**Supplementary Data**

**Further Materials and Methods**

***Risk Factors for Antimicrobial Resistant Faecal E. coli: questionnaire***

About your dog.

1. Age Years Months Weeks  Don’t Know 
   1. Is this age Exact  Estimate
2. Breed Pedigree *(please specify)*

Cross *(please specify)*

1. Sex Male  Female  Neutered
2. How long have you owned him/her?

About your dog’s diet.

1. What is s/he fed? Tinned meat  Dry mixer

(*Tick all that apply)* Dry Complete  Raw chicken  Cooked chicken  Raw red meat

Cook red meat  Don’t Know

Other

1. Is s/he fed commercial dog treats? Never  Rarely  Sometimes  Often  Don’t know
2. Is s/he fed human titbits/ scraps? Never  Rarely  Sometimes  Often  Don’t know
3. Does your dog ever eat stools (faeces)? Never  Rarely  Sometimes  Often  Don’t know  If so what types of stools? *(Please tick all that apply)* Rabbit  Cat  Dog  Horse  Cow  Sheep  Badger  Other

About your household.

1. Are there any other dogs in the household?

Yes  No  Don’t Know  If yes, how many? 1  2  3 or more

1. Do you own any other animals (other than dogs)?

Yes  No  Don’t Know  If yes, what animals? *(Please tick all that apply)*

Cat  Bird  Rabbit

Rodent *(e.g. hamster)*  Reptile *(e.g. snake)*

Don’t know  Other

1. Does anyone in your household work with farm animals?

Yes  No  Don’t know  If yes, please state which species are worked with

1. Has anyone in your family (including other pets) to your knowledge in the last month taken antibiotics?

Yes  No  Don’t know

* 1. If yes, was this a Family Member  Pet
  2. Which antibiotic was prescribed *(if known)*

1. Does anyone in your household work in medical or veterinary healthcare?

Yes No  Don’t Know

* 1. If yes, in what setting? Hospital  Community Nursing

GP surgery  Nursing Home

Dentist  Veterinary practice

Don’t Know  Other

1. Has anyone in your household attended hospital in the last month?

Yes  No  Don’t Know

* 1. If yes, why? Admission to hospital  Visit

Outpatient appointment  Don’t Know

Other

***Risk Factors for Antimicrobial Resistant Faecal E. coli: questionnaire data***

Data included: patient signalment (age, breed, sex); diet; antimicrobial therapy and veterinary admission within the last 12 months (prior to the three-month study exclusion period); the presence, number and type of in-contact pets; previous antimicrobial therapy or hospitalisation of other household members; owners working with farm animals, or in a human healthcare setting. Treatment duration and body weight were included as three categories: ≤1 week, >1 week and ≤3 weeks, and >3weeksand small (<11kg), medium (11-20kg) and large (>20kg), respectively. Recruitment sites were divided into two categories (first-opinion or referral).

**Further Results**

***Study Population: time-points and missing samples***

Pre-treatment sample was always collected within 24 hours of starting therapy. The treatment-end sample was always collected at end of therapy, however the time from pre-treatment varied depending on the length of prescription. Due re-examination appointments and sample return, the one-month- and three-month post-treatment sample time-points varied. The one-month sample ranged from 21-60 days (mean=35.38 days) and the three-month sample ranged from 61-150 days (mean=100.97 days).

Missing samples from one-month post-treatment (n=22) were due to prescription of further antimicrobials (n=7), euthanasia due to unrelated reasons (n=3), or owner non-compliance (n=12). Missing samples from three-month post-treatment (n=29) were due to prescription of further antimicrobials (n=12), euthanasia due to unrelated reasons (n=4), re-homing (n=3) or owner non-compliance (n=10).The most common diagnosis for enrolment was pyoderma (n=81); other diagnoses included urinary tract/prostate infection (n=11), dog bite/other abscess (n=11), dental infections (n=10), post-operative infection (n=8) or infected tumour/nodule/ulcer (n=6).

**Supplementary Table S1**: The number of faecal samples provided at each time-point in each treatment-group.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Antimicrobial treatment group** | | | | | | |
| **Time point** | **CFX** | **AC** | **CVN** | **CD** | **FQ** | **Total** |
| D0 | 32 | 28 | 24 | 29 | 14 | 127 |
| End | 32 | 28 | 24 | 29 | 14 | 127 |
| M1 | 27 | 26 | 19 | 26 | 7 | 105 |
| M3 | 24 | 25 | 18 | 23 | 8 | 98 |
| Total | 115 | 107 | 85 | 107 | 43 | 457 |

CFX = cephalexin; AC = clavulanate-amoxicillin; CVN = cefovecin; CD = clindamycin; FQ = fluoroquinolone; D0 = pre-treatment; End = treatment-end; M1 = one month after treatment-end; M3 = three months after treatment-end

**Supplementary Table S2**: The number and percentage of samples negative for *Escherichia coli* in each treatment group at each time point during the study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Time point | CFX  (n = 115) | AC  (n = 85) | CVN  (n = 107) | CD  (n = 107) | FQ  (n = 43) |
| D0 | 2 | 1 | 0 | 0 | 1 |
| End | 2 | 2 | 0 | 2 | 5 |
| M1 | 0 | 0 | 4 | 1 | 0 |
| M3 | 1 | 0 | 0 | 0 | 1 |
| Total | 5 (4%) | 3 (4%) | 4 (4%) | 3 (3%) | 8 (19%) |

CFX=cephalexin; AC=clavulanate-amoxicillin; CVN=cefovecin; CD=clindamycin; FQ=fluoroquinolone; D0=baseline day zero; End=treatment end; M1=one month after treatment-end; M3=three months after treatment-end.