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ESBL/AmpC-Producing Enterobacteriaceae Fecal Colonization in Dogs after Elective Surgery

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Abstract: The purpose of this study was to evaluate the presence and load of ESBL/AmpC-producing Enterobacteriaceae fecal carriage in healthy dogs. Fecal samples were collected from dogs submitted to surgical procedures (n = 25). Fecal samples were collected before surgery (BS) and after surgery (AS). β-lactamases were detected by PCR. Statistical analyses were performed with SAS software (v.9.4); a p value $\leq$ 0.05 was considered statistically significant. The ESBL/AmpC-producing Enterobacteriaceae bacteria species detected in this study were *E. coli*, *K. pneumoniae* and *E. cloacae*. TEM, and CTX-M-1 group genes were the most frequent β-lactamases detected. The number of dogs colonized with 3GC-resistant Enterobacteriaceae bacteria was significantly higher in the AS (63.6%, n = 14/22) group compared to in the BS group (20.0%, n = 5/25, p = 0.0033). The ESBL/AmpC-producing bacteria fecal load was significantly higher in the AS group compared to in the BS (p = 0.025) group. This study shows that 3GC-resistant Enterobacteriaceae and ESBLs/AmpC producers in the veterinary clinical practice are a concern and highlights the need to implement preventive measures to minimize their spread.

Keywords: veterinary hospitals; antimicrobial prophylactic use; ESBLs; pAmpC; third generation cephalosporin resistance; gut colonization

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1. Introduction

The European Medicine Agency has reviewed the public health risks associated with the transfer of antimicrobial resistance from companion animals and has identified the major microbiological hazards coming from companion animals to humans, including third-generation cephalosporin-resistant bacteria [1]. The presence of antimicrobial-resistant bacteria in companion animals and their close contact with humans provides opportunities for interspecies transmission [1]. In veterinary hospitals, infections acquired during hospitalization caused by resistant bacteria are an increasing problem [2,3]. Antimicrobials are regularly used for the prevention and control of infections in companion animals, and many of the antimicrobials used are the same or closely related to those used in the treatment of bacterial infections in humans [4,5].

β-lactams are among the most important antimicrobials used in veterinary medicine. Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and the monobactam aztreonam except for cephamycins and carbapenems [6]. In addition to ESBLs, Enterobacteriaceae can acquire plasmid-encoded ampC genes (pAmpC) as an important resistance mechanism against β-lactams. AmpC β-lactamases hydrolyze several β-lactam antibiotics, including cephamycins, oxyimino cephalosporins, and monobactam aztreonam [7]. ESBL/AmpC-encoding genes are located on mobile genetic elements, and many are plasmid-mediated and transferable between bacteria of different species. The first ESBLs were described

in Europe in the 1980s, and since then, ESBLs have been reported worldwide as a major source of antimicrobial resistance in Gram-negative pathogens [6,8]. The most frequent ESBL producers are *Escherichia coli* and *Klebsiella pneumoniae*, which are the main source of community- and hospital-acquired infections in human and veterinary medicine [6,8,9].

Prophylactic antimicrobial use involves the administration of the antimicrobial in the absence of infection, with the aim of preventing it, for example in the perioperative period [5]. Ideally, the antimicrobial prophylaxis scheme should be selected and prescribed to minimize the possible impact on the normal bacterial flora of the patient and on the microbiologic ecology of the hospital [10]. Inappropriate prophylaxis may promote the selection of antimicrobial-resistant bacteria [5].

The gastrointestinal tract is one of the main reservoirs for the emergence and dissemination of antimicrobial-resistant bacteria. Dog feces are a recognized source of resistant bacteria that can be transmitted to humans through direct contact or through shared (domestic and public) environments [1,10–16]. Antimicrobial resistance to third generation cephalosporins (3GC) has been previously detected in bacteria from canine fecal samples in recent years [14,15,17–20]. However, to the best of our knowledge, this is the first study to focus on the dynamics of ESBLs/AmpC producing- Enterobacteriaceae in the intestinal tracts of healthy dogs that were admitted to perform elective surgical procedures. The purpose of this study was to evaluate the presence and load of ESBLs/AmpC-producing Enterobacteriaceae fecal carriages in healthy dogs undergoing surgery.

2. Materials and Methods

2.1. Sampling Procedure and Collection of Data

From February to July 2014, fecal samples were obtained from 25 healthy dogs (without signs of gastrointestinal disease in the previous week) that went to a veterinary teaching hospital to undergo a surgical procedure. The surgery study group was divided: (i) before surgery (BS) upon admission to the Veterinary Hospital and (ii) after the surgical procedure (AS). Animals were excluded if they had been treated with an antimicrobial agent in the previous month. The surgical procedures considered for this study were soft tissue and orthopedic surgery. Fecal samples were collected at two different time points, namely before (BS) and after surgery (AS). A total of 25 animals were included in the BS group. The follow up samples included in the AS group were collected one week after surgery. However, in three animals, the follow-up was not possible and, therefore, 22 samples were studied. The dog owners were questioned verbally, and all replied to the questions regarding: age, gender, hospitalization, and antimicrobial treatment within the last year, cohabitation with other animals, street access, shelter/hotel access, and surgery type (soft tissue, orthopedic) and surgery reason (elective surgery or non-elective). The fecal sample collection was conducted by the owners using non-invasive methods. Owners were given specific instructions about the collection method to avoid sample contamination through contact with the ground. The instructions also included the fecal collection into sterile containers and the use of gloves.

2.2. Bacteria Isolation, Identification and DNA Extraction

One gram of feces was diluted in sterile saline solution (NaCl, 0.85%-Merck—Germany). Once homogenized, 10 µL was directly cultured on MacConkey agar plates (Scharlau, Barcelona, Spain) supplemented with 2.0 µg/mL of cefotaxime (CTX) (Sigma–Aldrich, St. Louis, MO, USA) and incubated overnight at 37 °C. CTX-resistant Enterobacteriaceae bacteria were then quantified by counting the colony-forming units (CFU) per gram of feces. Positive samples were screened for the presence of different colony morphologies of CTX-resistant Enterobacteriaceae. One isolate of each unique morphology was selected from each positive sample for further study. The bacterial species were determined using an API 20E kit, the software APIWEB (BioMérieux, Marcy-l'Étoile, France) and by species-specific PCR [21,22]. DNA extraction was conducted using a boiling method [23].

2.3. Escherichia coli Phylogenetic Typing

Phylogenetic typing was performed in all *E. coli* isolates to determine the main phylogenetic groups (A, B1, B2 and D) according to the amplification of *chu*A and *yja*A genes, and TspE4C2 fragment) by multiplex PCR [24].

2.4. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing and interpretation were performed using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [25]. The following antimicrobial disks (Oxoid, Basingstoke, Hampshire, UK) were used: 10 μg ampicillin (AMP), 30 μg amoxicillin-clavulanate (AMC), 30 μg cephalothin (KF), 30 μg cefotaxime (CTX), 30 μg ceftazidime (CAZ) and 30 μg cefoxitin (FOX). ESBL production was confirmed by the double-disk synergy test according to CLSI standards and isolates were classified as susceptible or resistant according to CLSI criteria [25]. The reference strain *E. coli* ATCC 25922 was used for quality control testing [25].

2.5. ß-Lactamase Resistance Genes

3GC-resistant Enterobacteriacea isolates were screened by PCR for the presence of bla_{SHV}, $bla_{\text{OXA-1}}$, bla_{TEM} β-lactamase genes; $bla_{\text{CTX-M}}$, $bla_{\text{CTX-M-1group}}$ and $bla_{\text{CTX-M-9group}}$ ESBLs [26–28] and $bla_{\text{MOX-1}}$, $bla_{MOX\text{-}2}$, $bla_{\text{CMY-1}}$, $bla_{\text{CMY-8}}$ to $bla_{\text{CMY-11}}$, $bla_{\text{LAT-1}}$ to $bla_{\text{LAT-4}}$, $bla_{\text{CMY-2}}$ to $bla_{\text{CMY-7}}$, $bla_{\text{BIL-1}}$, $bla_{\text{DHA-1}}$ to $bla_{\text{DHA-2}}$, bla_{ACC}, $bla_{\text{MIR-1T}}$, $bla_{\text{ACT-1}}$ and $bla_{\text{FOX-1}}$ to $_{\text{FOX-5}}$ pAmpC encoding genes [29]. Negative and previously sequenced positive controls were included in all PCR reactions. Negative controls were PCR mixtures with the addition of water in place of template DNA.

2.6. Statistical Analysis

Statistical analysis was performed using SAS statistical software package for Windows, version 9.4 (SAS Institute, Cary, NC, USA). For the categorical variables, proportions were compared using Fisher's exact test and General linear model (GLM) procedures were used to perform descriptive statistics of ESBL/AmpC-producing Enterobacteriaceae load. The results were considered statistically significant when $p < 0.05$.

3. Results

Thirty six percent (n = 9/25) of fecal samples were obtained from female dogs and 64.0% (n = 16/25) from males, with a median age of 7 years (ranging from 0.2–13 years). All the animals belonged to private owners. Previous hospitalization in last year was observed in 60.0% (n = 15/25) of the dogs and 50.0% (n = 12/24) had been treated with an antimicrobial agent within the last year. All the animals had access to the street, 68.0% (n = 17/25) and 12.0% (n = 3/25) of the dogs cohabited with other animals and had been in shelters/hotels, respectively (Table 1).

About 76.0% (n = 19/25) of the animals were submitted to soft tissues surgery and 24.0% (n = 6/25) to orthopedic surgery. Regarding prophylactic antimicrobial treatment, 92.0% (n = 23/25) of the dogs received prophylactic antimicrobial treatment through oral administration by different antimicrobials before and after surgery (Table 1). About 52.0% (n = 12/23) of the dogs and 73.9% (n = 17/23) were administered AMC, before surgery and after surgery, respectively. One dog received AMC and a second-generation cephalosporin (2GC) after surgery; three dogs received other antimicrobials (2GC, n = 1; AMC and metronidazole, n = 1; metronidazole and macrolides n = 1) in both time points.

Table 1. Descriptive statistics of healthy dogs sampled (n = 25) in this study.

Dogs Sampled		**% (*n*)**
Gender	Female	36.0 (9)
	Male	64.0 (16)
Origin	Private owner	100.0 (25)
Antimicrobial treatment in last year	Yes	50.0 (12)
	No	46.0 (11)
	No data	4.0 (1)
Hospitalization in last year	Yes	60.0 (15)
	No	40.0 (10)
Cohabitation with other animals	Yes	12.0 (3)
	No	88.0 (22)
Street access	Yes	68.0 (17)
	No	32.0 (8)
Shelter/hotel access	Yes	12.0 (3)
	No	88.0 (22)
Surgery reason	Soft tissues	76.0 (19)
	Orthopaedic	24.0 (6)
Antimicrobial prophylactic treatment	Yes	92.0 (23)
	No	8.0 (2)

In this study, 20.0% (n = 5/25) of the dogs were colonized with ESBL-producing Enterobacteriaceae at the time of hospital admission, four of which were *E. coli* and one was *Klebsiella pneumoniae* (Table 2). The most common antimicrobial resistance phenotype of *E. coli* isolates was AMP^R-AMC^R-KF^R-CTX^R-FOX^R-CAZ^R (60.0%, n = 3/4). Two *E. coli* harbored the bla_{TEM} gene (n = 2/4), one harbored the bla_{SHV} gene (n = 1/4) and one $bla_{CTX\text{-}M\text{-}1group}$ (n = 1/4) (Table 2).

Regarding samples collected after surgery (AS), about 64.0% (n = 14/22) of dogs were colonized with 3GC-resistant Enterobacteriaceae. Around 45.0% (n = 10/22) of the fecal samples were *E. coli* positive, followed by *K. pneumoniae* (18.2%, n = 4/22) and *E. cloacae* (13.6%, n = 3/22). Interestingly, in one of the fecal samples (FMVS3), several ESBL producers were found, along with one *E. coli* isolate and three *Klebsiella pneumoniae* isolates with different antimicrobial phenotypes and genotypes (Table 2).

Among the *E. coli* isolates, the most common antimicrobial resistance phenotype was AMP^R-AMC^R-KF^R-CTX^R-FOX^R-CAZ^R (n = 8/10), while among *K. pneumonia* and *E. cloacae* the most common was AMP^R-AMC^R-KF^R-CTX^R-CAZ^R (n = 2/4) and AMP^R-KF^R-CTX^R-FOX^R-CAZ^R (n = 3/3), respectively. Furthermore, 70.0% of the *E. coli* isolates (n = 7/10) harbored the bla_{TEM} gene, and the remaining isolates harbored the $bla_{CTX\text{-}M\text{-}1group}$ gene (n = 1/10), bla_{SHV} gene (n = 1/10) and one carried the combination of $bla_{OXA\text{-}1}$+bla_{TEM} genes (Table 3). Regarding *K. pneumoniae*, all isolates were positive for $bla_{OXA\text{-}1}$+bla_{TEM}+$bla_{CTX\text{-}M\text{-}1group}$ genes (Table 2). In addition to the increase in 3GC-resistant Enterobacteriaceae in fecal samples after surgery (AS), most *E. coli* isolates belonged to commensal phylogenetic groups (group-A, n = 4/10; group-B1, n = 3/10). Pathogenic phylogenetic groups were also detected (group-B2, n = 2/10; group-D, n = 1/10) (Table 2). However, there was no statistically significant difference between BS and AS regarding pathogenic *E. coli* phylogenetic groups.

In this study, the number of dogs colonized with ESBL/AmpC-producing Enterobacteriaceae was significantly higher in the AS group (63.6%, n = 14/22) than in the BS group (20.0%, n = 5/25, p = 0.0033) (Table 2). Moreover, the ESBL/AmpC-producing Enterobacteriaceae load mean after surgery was $1.74 \times 10^6 \pm 5.33 \times 10^6$ CFU/g of feces, and before surgery it was $1.10 \times 10^2 \pm 4.51 \times 10^2$ CFU/g of feces. The CTX-resistant bacteria fecal load was statistically significantly higher in the AS group than in the BS group (p = 0.025) (Table 3).

Table 2. Genotypic and phenotypic traits of ESBL/AmpC-producing Enterobacteriaceae from healthy dogs before surgery and after surgery (AS).

Animal Group	ESBLs (%)	*p* Value	Isolates ID	Bacteria	Antimicrobial Resistance Phenotype	β-Lactamases	*E. coli* Phylogroup
Before surgery (*n* = 25)	20.0	0.0033	FMVS1	*E. coli*	AMP KF CTX	$bla_{\text{CTX-M-1group}}$	B1
			FMVS2	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	D
			FMVS14	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{SHV}	B1
			FMVS18	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	B1
			FMVS20	*K. pneumoniae* *	AMP AMC KF CTX FOX	bla_{SHV}	-
After surgery (AS) (*n* = 22)	63.6		FMVS1	*K. pneumoniae* *	AMP AMC KF CTX CAZ	$bla_{\text{OXA-1}}$, bla_{TEM}, $bla_{\text{CTX-M-1group}}$	-
			FMVS2	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	D
			FMVS3a	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	B2
			FMVS3b	*K. pneumoniae* *	AMP AMC KF CTX CAZ	$bla_{\text{OXA-1}}$, bla_{TEM}, $bla_{\text{CTX-M-1group}}$	-
			FMVS3c	*K. pneumoniae* *	AMP AMC KF CTX FOX CAZ	bla_{TEM}, $bla_{\text{CTX-M-1group}}$	-
			FMVS3d	*K. pneumoniae* *	AMP AMC KF CTX	$bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-1group}}$	-
			FMVS4	*E. cloacae* **	AMP KF CTX FOX CAZ	nd	-
			FMVS6	*E. cloacae* **	AMP KF CTX FOX CAZ	nd	-
			FMVS7	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	B1
			FMVS9	*E. coli*	AMP KF CTX	$bla_{\text{CTX-M-1group}}$	B2
			FMVS11	*E. coli*	AMP AMC KF CTX FOX CAZ	$bla_{\text{OXA-1}}$, bla_{TEM}	A
			FMVS12	*E. cloacae* **	AMP KF CTX FOX CAZ	nd	-
			FMVS13	*E. coli*	AMP KF CTX CAZ	bla_{SHV}	B1
			FMVS16	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	A
			FMVS17	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	A
			FMVS21	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	B1
			FMVS25	*E. coli*	AMP AMC KF CTX FOX CAZ	bla_{TEM}	A

Legend: AMP, Ampicillin; AMC, Amoxicillin/clavulanic acid; CTX, Cefotaxime; CAZ, Ceftazidime; FOX, Cefoxitin; KF, Cephalothin; nd, not detected. * *K. pneumoniae* is intrinsic resistant to AMP; ** *E. cloacae* is intrinsic resistant to AMP, AMC, first-generation cephalosporins, and FOX [25].

Table 3. Descriptive statistics of ESBL/AmpC-producing Enterobacteriaceae load (CFU/g of feces) per dog before surgery (*n* = 25) and after surgery (*n* = 22) by the General linear model procedure.

Animal Group	Mean (CFU/g)	SD	SE	Min (UFC/g)	Max (UFC/g)	*p* Value
Before surgery (*n* = 25)	1.10×10^{2} a	4.51×10^{2}	5.24×10^{5}	0.0	2.25×10^{3}	0.025
After surgery (*n* = 22)	1.74×10^{6} b	5.33×10^{6}	4.00×10^{5}	0.0	1.84×10^{7}	

Legend: SD, standard deviation; SE, standard error mean; Min, minimum; Max, maximum. Mean values with a character with different letters are statistically significant (*p* value = 0.025).

4. Discussion

In this study, about 20.0% of dogs before surgery and before entering the hospital were already colonized with ESBL/AmpC-producing Enterobacteriaceae. The results obtained here were similar to those previously published using samples from 2010–2011 from healthy dogs [16]. It is likely that this similarity is related to the fact that both studies were conducted in the same geographical area (metropolitan region of Lisbon). A significant increase in antimicrobial resistance was detected among bacteria causing UTI in companion animals from the Lisbon area between 1999 and 2014 [30]. Therefore, the apparent stable frequency of the CTX-resistant bacteria fecal carriage among heathy dogs is considered a positive outcome from this study. Nevertheless, the frequency of colonization by CTX-resistant bacteria reported here (20%) before surgery should not be neglected since the fecal carriage of ESBLs/AmpC-producing may be a risk factor for secondary infections by MDR bacteria in hospitalized patients, as also occurs in humans.

The ESBL/AmpC-producing Enterobacteriaceae detected in this study were *E. coli*, *K. pneumoniae* and *E. cloacae*. *Enterobacter cloacae* is ubiquitous in the environment, and it is commensal in the intestinal tract of humans and animals [31]. This species is also prone to contaminating various medical, intravenous, and other hospital devices contributing to skin/soft tissue infections, urinary tract, and intra-abdominal infections [32]. Furthermore, *E. cloacae* has an intrinsic resistance to ampicillin, amoxicillin, first-generation cephalosporins, and cefoxitin owing to the production of constitutive AmpC β-lactamase. Resistance of *Enterobacter* spp. to 3GC is, in most cases, caused by overproduction of AmpC β-lactamases [33–35].

In this study, the bla_{TEM} and $bla_{\text{CTX-M-1group}}$ were the most frequent β-lactam-resistance genes, which is in agreement with previous studies [15,18,34]. Hordijk et al. [19] analyzed healthy dogs and cats without contact with the hospital environment in the Netherlands and detected a high percentage (45%) of dogs colonized with Enterobacteriaceae-producing β-lactamases (ESBL/AmpCs). Procter et al. [35] reported that 12.7% of *E. coli* strains isolated from dogs, who attended parks in three cities in Canada, were resistant to β-lactam antimicrobials. Aslantas and Yilmaz [36] detected 22% of dogs were colonized by CTX-resistant *E. coli* in Turkey. The different frequencies of β-lactam-resistant bacteria detected in these studies may be related to differences among geographical regions or to differences between study designs. Nevertheless, it highlights the importance of reporting data from different geographical regions.

In this study, ESBL-producing Enterobacteriaceae significantly increased between AS and BS, and changes in fecal microbiota occurred, which could be in part explained by the prophylactic use of amoxicillin-clavulanate. Moreover, in addition to antimicrobial administration, the administration of other medications and also the type of food have been identified as factors influencing fecal bacteria flora [37–39]. Furthermore, dogs food is a vehicle of ESBL and AmpC-type resistance to last-resort antimicrobials thus positioning dog food as an important source of antibiotic resistance spread [38]. Yet, in this study, these two variables were not considered in the questionnaire, as they were a limitation of the study. In future studies, it will also be important to include the type of diet and other medications, in addition to antimicrobials in order to understand the changes in the fecal flora. Another limitation of this study is the sample size; it would be interesting to increase the sample size, to enable the detection of possible risk factors in the future. Furthermore, the findings presented here regarding dog colonization by CTX-resistant bacteria are of public health and veterinary interest.

In one recent study conducted in the Netherlands using whole genome sequencing, around 43% of owned dogs were found to be persistently colonized by ESBL-producing Enterobacteriaceae (6 months) [40]. It is important to notice that van den Bunt et al. used pre-enrichment media, unlike the study presented here [37]. Therefore, the high frequency of colonized dogs by CTX-resistant bacteria detected after surgery (64.0%) could be even higher.

An important finding from this study is not only that the number of colonized dogs by CTX-resistant bacteria increased significantly with the antimicrobial treatment, but also that there was a significant increase in the detected fecal load (UFC/g), achieving a mean value of 1.74×10^6 UFC/g. These two findings together further highlight the importance of dogs in the dissemination of resistant bacteria and emphasize the need for appropriate fecal disposal during antimicrobial prophylaxis or treatment. This finding is of great importance, not only because of the direct impact on patients, but also because resistant bacteria can be transmitted from companion animals to humans and disseminated into the environment [1,12,14,15].

Future longitudinal studies should be conducted to access the evolution of the fecal CTX-resistant bacteria load over time once the antimicrobial treatment is interrupted.

ESBL/AmpC-producing Enterobacteriaceae may also spread from patient to patient due to inadequate attention to infection control measures, especially hand washing. Infections caused by Enterobacteriaceae have features that are of particular concern. These organisms are highly efficient at up-regulating or acquiring genes that code for mechanisms of antimicrobial drug resistance, especially in the presence of antimicrobial selection pressure [41].

5. Conclusions

The findings of the current research showed that about 20% of dogs before surgery and before entering in the hospital were already colonized with ESBL/AmpC-producing Enterobacteriaceae, mainly harboring the bla_{TEM} and $bla_{\text{CTX-M-1group}}$ genes. After elective surgery, the number of dogs colonized with ESBL/AmpC-producing Enterobacteriaceae and the mean load of ESBL/AmpC-producing Enterobacteriaceae was significantly higher than before surgery. Furthermore, ESBL-producing Enterobacteriaceae significantly increased with antimicrobial prophylactic use, and changes in fecal microbiota occurred. European and national appropriate antimicrobial surgical prophylaxis guidelines are urgently needed for the compliance of antimicrobial stewardship principles in veterinary hospitals.

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