Extended Spectrum Beta-Lactamase/ AmpC-Producing *E Coli* in Dogs Treated with Antimicrobials in Surgical Wards

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ABSTRACT

The aim of this study is to investigate the prevalence and carriage of Extended Spectrum Beta-Lactamase (ESBL) and AmpC-producing strains of *E. coli* and *Klebsiella spp* in hospitalized dogs treated with antimicrobials. Tissue and fecal samples from 66 dogs were analyzed for presence of AmpC or ESBL producing bacteria. The dogs had to have been admitted to the surgical ward for at least 24 hours and have received antimicrobial treatment. Samples were plated onto bovine blood agar and after incubation for 24 + 24 h, five colonies morphologically consistent with *E.coli* and *Klebsiella spp*, were selected and recultured onto media containing antimicrobials. Dogs carrying ESBL/AmpC- producing bacteria were retested for rectal colonisation at 3-6 months intervals for up to 16 months. Five (7.6%) dogs carried bacterial strains positive for ESBL/AmpC- producing- genes in feces. All tissue samples were negative. One dog, previously positive for bla_{CMY-2}, carried ESBL genotype bla_{TEM-52}, in the second sample. Four dogs remained positive throughout the testing. None of the dogs had signs of infection or symptoms associated with the carriage of ESBL or plasmid mediated-AmpC- producing bacteria. Seven unique MLVA-types were identified. The results from this study show fecal carriage for as long as 16 months of ESBL/ AmpC- producing *E.coli* in dogs treated with antimicrobials. Although clonal spread could not be verified in this study, the risk of dissemination of multiresistant bacteria in animal hospitals and in the community must be considered.

INTRODUCTION

Antimicrobial resistance is a large and increasing problem in both human (Coque et al 2008) and veterinary medicine (Wieler et al 2011). The use and misuse of antibiotics has played a role in the emergence and spread of resistant bacteria in animal and human hospitals and in the community (Rantala et al 2004, Dietrix et al 2004). The Infectious Diseases Society of America has listed Escherichia coli and *Klebsiella spp* as two out of six pathogens for which new drugs are urgently needed in order to combat resistance development (Talbot et al 2006). Resistance to extended spectrum cephalosporins is particularly worrying, as it is considered by the World Health Organization (WHO) to be a critically important antibiotic for human medicine (WHO 2007). The production of extended-spectrum beta-lactamases (ESBL) and AmpC enzymes results in resistance to the majority of the commonly used beta-lactam antimicrobials, including third generation cephalosporins (Pitout 2010). ESBL is frequently plasmid-mediated and AmpC beta lactamase activity is chromosomal (cAmpC)- or plasmid-mediated (pAmpC) (Peter-Getzlaff et al 2011). The location of genes in plasmids means that they can be transferred via horizontal gene transfer between bacteria within and between bacterial species (Brolund et al 2013). Another clinical challenge is that ESBL/AmpC- producing isolates are commonly multi-drug resistant resulting in that the numbers of treatment options often are few.

European studies show noticeable high numbers of ESBL/AmpC- producing E.coli in clinical samples from horses and dogs primarily with diarrhea (Dietrix et al 2012, Swedres-SVARM 2012, Hordijk et al 2013), as well as in samples from healthy dogs (Hordijk et al 2013). In Scandinavia there are only a limited number of studies describing the prevalence of ESBL/AmpC in dogs. The Swedish Veterinary Institute (SVA) screened 84 healthy dogs for ESBL/AmpC- producing E.coli in feces in 2012, and one isolate of pAmpC was detected (1%) (Swedres-SVARM 2012). Of the 15 resistant E.coli isolates from urogenital tracts or wounds referred to SVA during 2011, 12 isolates were ESBL/pAmpC- producing (Swedres-SVARM 2012).

The main purpose of this study was to investigate the presence of ESBL/AmpC-producing strains of *E. coli* and *Klebsiella spp* in surgical sites and feces in hospitalized surgically treated dogs, treated with antimicrobials and the fecal carriage over time.

MATERIALS AND METHODS

Animal welfare

Ethical approval for this study was granted by the Uppsala Ethical Committee, Uppsala, Sweden. The board of agriculture approved the use of client-owned dogs. All dog owners had to provide written consent before their dogs were enrolled in the study.

Sampling of patients

Two Swedish animal hospitals participated in the study: A University Animal Hospital and a Referral Animal Hospital. To be enrolled in the study, dogs were required to meet the inclusion criteria; admitted to the animal hospital for at least 24 hours and have received antimicrobial treatment for at least 24 hours within the last month. The criteria were based on the identified risk factors for ESBL compiled by the Swedish National Board of Health (STRAMA) where factors such as hospitalization and use of antibiotics, in particular cephalosporins and fluoroquinolones, are considered to contribute to the development of resistance (STRAMA 2007).

Samples were taken from wound or diseased tissue and from feces (carrier sample) between January 2012 and June 2014 with a Copan swab in Aimes medium (Copan, Italy). Samples from the University Animal Hospital were cultured within 24 hours and samples from the Referral Animal Hospital, within 36 hours.

From dogs shown to carry ESBL or AmpC producing- *E. coli* or Klebsiella spp, three new samples were collected from feces

with 3-6 months intervals, to evaluate if the dog was still a carrier of the bacteria. When it was impossible for the owners to bring in their animals to the clinic for follow-up testing, they were given the option of swabbing the feces of their animals themselves and then sending it in immediately for culturing.

Bacterial isolation and phenotypic identification

Tissue samples were plated onto bovine blood agar (National Veterinary Institute, Uppsala, Sweden) and MacConkey agar supplemented with cefotaxime 1mg/L (National Veterinary Institute, Uppsala, Sweden) and incubated at 37° for 24 hours and then another 24 hours. Fecal samples were plated onto MacConkey agar supplemented with cefotaxime 1mg/L (National Veterinary Institute, Uppsala, Sweden) and incubated at 37° for 24 hours and then a further 24 hours. After plating, fecal swabs were placed in MacConkey broth supplemented with cefotaxime 1mg/L (National Veterinary Institute, Uppsala, Sweden) and incubated for 24 hours at 37°C. Twenty µL were then spread onto MacConkey agar supplemented with cefoxitime 1mg/L. Plates were incubated aerobically for 24+24 hours. If the direct plating culture were positive, the cultures from pre-enrichments were disregarded. Five colonies from primary culture or enrichment culture, morphologically consistent with E.coli and Klebsiella spp, were selected and recultured onto bovine blood agar plates (SVA, Uppsala, Sweden) and purple agar with lactose (SVA, Uppsala, Sweden) and incubated for 24 hours. Oxidase-negative (Becton, Dickinson and Company (BD), USA) strains were confirmed as E.coli or *Klebsiella spp* with API20E (Bio Merieux, France).

Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed using broth micro dilution (Vet-MICGN-moE-395103, National Veterinary Institute, Uppsala, Sweden), and control strain CCUG 17620 (ATCC 25922).

On strains resistant to ampicillin (breakpoint >16mg/L) and cefotaxime (breakpoint >0,25mg/L), ESBL-detection testing according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2010 CLSI document M100-S16 (ISBN 1-56238-588-7)) and AmpC detection testing according to RAF (http://www.srga.org/rafmetod/betamas.htm) were performed. Negative control strain CCUG 17620 (ATCC 25922) and positive control strain CCUG 45421 (ATCC 700603) were used.

Genotypic characterization

E. coli isolates confirmed to be producing pAmpC or ESBL were screened for the presence of gene groups bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-1}, and genes encoding pAmpC (Fang et al 2008, Perez-Perez et al 2002 and Woodford et al 2006). Isolates PCR-positive for any of the genes were subjected to sequencing as previously described (Egervärn et al 2014). To establish a possible clonal relationship, ESBL and pAmpC- producing *E. coli* isolates were subjected to Multiple-Locus Variable number tandem repeat Analysis (MLVA) (Lobersli et al 2012).

Statistical analysis

All results are presented as mean±standard deviation. A one-way ANOVA was used for analyzing the difference between the positive and negative groups, using JMP 10 (SAS Institute, Cary, NC, USA). A P-value of 0.05 was considered significant.

RESULTS

Sixty-six dogs of various breeds were included in the study. All the dogs tested were admitted to the surgical wards for at least 24 hours because of a variety of surgical diseases. The median age of the dogs was 7 (1-13) years old. Of the included dogs, five (henceforth referred to as dogs A-E) were positive for plasmid or chromosomal AmpC-producing *E. coli* (7.6 %) showing AmpC phenotype, in the first fecal sample (Table 1). 6 % of the included dogs were positive for plasmid AmpC. No ESBL/AmpC- producing E.coli or Klebsiella spp. could be identified in samples from surgical sites, a variety of other bacteria were found in the surgical sites and were probably the source

Table 1. *AmpC- genes of positive tested dogs*
Antimicrobial: enro = enrofloxacin, amox=amoxicillin, cepha=cephalosporin 1:st generation
Ssi = surgical site infection

Dog	diagnosis	length of hospitalization	antimicrobial	length of treatment	1:st sample		2:nd sample 3-5 months		3:rd sample 11-16 months	
A	abscess	3 days	enro	2 days	bla_{CMY-2}		bla_{CMY-2}	bla_{TEM-1}		
B	ectopic ureters	1 day	amox	9 days	bla_{CMY-2}		negative		negative	
C	ssi	6 days	amox	4 days	bla_{CMY-2}	bla_{TEM-1}	bla_{CMY-2}		bla_{CMY-2}	bla_{TEM-1}
D	ssi	6 days	cepha	19 days	Neg		Neg		Neg	
E	bite wound	3 days	amox	6 days	bla_{CMY-2}		bla_{TEM-52}		bla_{CMY-2}	

of infection. None of the dogs showed signs of infection or symptoms related to their carriage of ESBL/pAmpC- producing *E. coli* such as wound dehiscence or prolonged healing time. The *E. coli* isolates collected from dogs A-C and E were described to carry the blaCMY-2, due to the plasmid AmpC. The isolate from dog D was negative for all genes tested, indicating a chromosomal AmpC. No ESBL/AmpC- producing Klebsiella bacteria were found. The bacterial strains from dog A-E showed resistance to ampicillin and to the third-generation cephalosporines cefotaxime and ceftazidime (table 2). The first isolate from dog A was also resistant to sulfamethoxazole and trimethoprim and the second isolate from dog E was additionally resistant to ciprofloxacin and nalidixic acid (table 2).

At retesting after 3-5 months, *E. coli* isolates carrying bla_{CMY-2} were found in fecal samples in dogs A, C, and E, while dog B was negative. Further testing of dog A after five months was not possible due to euthanasia because of degenerative joint disease. The second retesting took place 11-16 months after initial testing and at this time dogs C and D remained positive for phenotypically AmpC- producing *E. coli*, but no plasmid-associated genes were detected. The primary sample on dog E revealed two phenotypically different *E. coli* colonies, lactose fermenting and non-fermenting, and both isolates carried bla_{CMY-2}. On the first retesting of dog E three months after initial testing, *E. coli* carrying bla_{CMY-2} could no longer be detected. However, dog E was on this occasion positive for ESBL- producing E.coli carrying bla_{TEM-52} . On the second retesting of dog E 16 months after primary sampling it was positive for *E. coli* carrying bla_{CMY-2} but isolates carrying bla_{TEM-52} could no longer be found.

For the nine *E. coli* isolates carrying bla_{CMY-2} (table 1), seven unique MLVA types differing in two or more alleles were described. The isolates sharing identical MLVA types were isolated from dog C eleven months apart (table 3).

The dogs in the study had all been treated with various antibiotics, the most common being amoxicillin (Table 1). The length of the antibiotic treatment in the group where isolates of ESBL, pAmpC or cAmpC was found was 8±6.7 days. In the group where no ESBL, pAmpC or cAmpC was found, the length was 7.5±8.7 days. In the ESBL, pAmpC or cAmpC group the average time of hospitalization was 3.8±2.2 days; in the other group it was 2.8±1.5 days. There was no significant difference between the groups regarding length of treatment (P=0.88) or hospitalization (P=0.36).

DISCUSSION

In Sweden the occurrence of clinical isolates of ESBL and pAmpC- producing *Enterobacteriaceae* among dogs appears to be uncommon. Only 15 isolates resistant to 3rd-generation cephalosporins were submitted from dogs to the National Veterinary Institute for further analysis during 2012, and twelve were found to be producing ESBL or pAmpC (Swedres-SVARM 2012).

Table 2: *Resistant charts over plasmid-mediated AmpC producing ESBL from dogs in surgical wards treated with antibiotics. Dogs A, B, C and E, resistant isolates in bold*

Sample	Am >8µg/ml	Ci >0.06µg/ml	Nal >16µg/ml	Gm >2µg/ml	Sm >16µg/ml	Tc >8µg/ml	Ff >16µg/ml	Cs >2µg/ml	Su >256µg/ml	Tm >2µg/ml	Cm >16µg/ml	Km >8µg/ml	Ctx >0.25µg/ml	Caz >0.5µg/ml	Imi-penem >1 mg/L
1 A1	>128	0,06	4	1	8	2	8	<0,5	>1024	16	8	<8	>2	>16	0,125
2, A2	>128	0,06	4	1	8	2	8	<0,5	16	0,5	4	<8	>2	16	0,25
3, B1	>128	0,03	4	0,5	8	2	8	<0,5	16	0,5	8	<8	>2	16	0,19
4, C1	>128	0,06	2	2	8	2	8	<0,5	<8	0,5	4	<8	>2	16	0,19
5, C3	128	0,06	<1	4	8	<1	<4	<0,5	16	0,5	<2	16	>2	>16	0,125
6, C2	>128	0,06	2	2	16	<1	8	2	32	0,5	4	<8	>2	16	0,125
7, E1	>128	0,06	4	0,5	8	<1	8	<0,5	128	0,5	4	<8	>2	16	0,125
8, E2	>128	0,5	>128	1	8	2	16	<0,5	16	0,25	8	<8	>2	16	0,19

First column contain cut-off-values for E.coli
Amp=Ampicillin, Ci=Ciprofloxacin, Nal=Nalidixic acid, Gm=Gentamicin, Sm=Streptomycin, Tc=Tetracyklin, Ff=Florfenicol, Cs=Colistin, Su=Sulfamethoxazole, Tm=Trimethoprim, Cm=Chloramphenicol, Km= Kanamycin, Ctx=Cefotaxime, Caz=Ceftazidime

Table 3: *The different MLVA-genotypes identified in dog A, B, C and E positive for plasmidAmpC-producing ESBL, treated in surgical wards and with antimicrobials*

Dog/Case (Sampling time)	Allele									
	CVN001	CVN002	CVN003	CVN004	CVN007	CVN0014	CVN0015	CCR001	CVN0016	CVN0017
A1A007	6	7	-2	11	3	4	5	1	-2	-2
A2 A182	6	2	-2	11	3	11	5	1	1	-2
B1A025	6	2	-2	11	3	7	5	1	2	-2
C1A152	5	2	-2	13	3	7	5	1	12	-2
C3A039a	5	2	-2	13	3	7	5	1	12	-2
C2A210	5	0	-2	11	3	-2	-2	1	-2	-1
E1.1A033	7	2	-2	11	3	8	5	-2	-2	-2
E1.2A033B	6	2	-2	11	3	10	5	1	1	-2

Furthermore, a study during 2012, utilizing the same methodology as this study, describing intestinal carriage in healthy dogs found pAmpC- producing *E. coli* in only 1 % of the samples (Swedres-SVARM 2012). This differs from the current study were 6 % of Swedish dogs hospitalized in surgical wards and treated with antimicrobials for surgical diseases carried pAmpC- producing *E. coli* isolates. The results might therefore indicate that hospitalization in a surgical ward combined with antimicrobial therapy can increase the occurance of ESBL/pAmpC-producing *E. coli* in dogs. This assumption is also supported by previous studies for example one study by Damborg et al (2012) that studied the effect of antimicrobial prophylactic treatment on the emergence and maintenance of ESBL/AmpC-producing coliforms in a longitudinal study in horses. They described the emergence of ESBL/AmpC- producing strains from day 1-2 after treatment until up to three weeks, when the last sample was performed. In addition a Korean study by Nam et al 2010 describing higher carriage of antibiotic resistant *E. coli* in hospitalized dogs compared to stray dogs. Despite the higher carriage in hospitalized dogs compared to healthy dogs the situation in Sweden is still favorable compared to international studies. In a Dutch study, intestinal carriage of pAmpC/ESBL- producing E.coli was reported in 45% of healthy dogs and in 55% of dogs with diarrhea (Hordijk et al 2013). In addition, in another Korean study, ESBL and pAmp-C-producing *E.coli* were found in 33,3 and 23,8%, respectively, of the isolates from hospitalized dogs (So et al 2012). In a Spanish study of healthy dogs, 2,65% of the dogs harboured fecal E.coli isolates with reduced susceptibility to cefotaxime (Costa et al 2008). However, that study used only non-selective cultivation; hence the real carriage of *E.coli* isolates with reduced susceptibility to cefotaxime was probably higher.

The *E. coli* carrying bla_{CMY-2} dominates in our study and among clinical submissions to the National Veterinary Institute during 2012 (Swedres-SVARM 2012). Only one of our isolates was confirmed to produce ESBL carrying bla_{TEM-52}. Interestingly, this isolate was identified in a retest of a dog previously positive for an isolate with a bla_{CMY-2}. However, since only a few colonies were chosen for analysis, there is the possibility that the bla_{TEM-52} were missed in the first sample and vice versa with the bla_{CMY-2} in the second sample. The bla_{TEM-52} ESBL- gene-type has been isolated from clinical samples from dogs in a Dutch study (Dietrikx et al 2012), and in the present study the isolate may have been acquired in the hospital. The pAmpC-genes are commonly found in clinical isolates from dogs around the globe, and mostly from urinary samples (Dietrikx et al 2012, Hordijk et al 2013, Wagner et al 2014, O'Keefe et al 2010). PAmpC can pose a further clinical challenge compared to ESBL due to the fact that the beta-lactamase inhibitors such as clavulanic acid are not available as a treatment option. However, isolates of pAmpC-type from urinary tract infections has been shown to have a reduced virulence genotype (Wagner et al, 2014). The difference in prevalence and occurrence of different genes between different studies can have several explanations. These include geographic differences, selection of animals, methods used for sampling, antibiotic usage and bacterial isolation (such as enrichment procedures and the media applied). In our study, one isolate (dog A, sample 2) came out positive for AmpC after enrichment and could not be found on direct plating.

In the present study we also showed that the dogs can carry *E. coli* with bla_{CMY-2}, for up to at least 16 months after the initial sampling. This is to our knowledge, the first study to describe prolonged carriage of AmpC-producing *E.coli* in dogs. Indicating that it is important to be aware that dogs once diagnosed as carrying ESBL/AmpC can pose a risk for transmission for several months in both the hospital and in the community. Maddox et al (2012) found that horses stabled together with recently hospitalized horses had increased risk for acquiring ESBL producing *E.coli*. Clonal spread of ESBL/AmpC producing *E.coli* in

veterinary hospitals has been suggested by So et al (2012). In our study we identified unique MLVA patterns for isolates from all dogs, indicating that the occurrence was not due to clonal spread. However, only a small number of isolates from every dog were analyzed and it is likely that a dog can carry several different *E. coli* encoding pAmpC/ESBL. We also found two phenotypically different E.coli carrying bla_{CMY-2} in the same sample in dog E, and different clones in dog C at the first and second testing. Furthermore, we cannot exclude the possibility that spread of one specific plasmid carrying bla_{CMY-2} has occurred. One should also be aware of the possibility that such a plasmid can transferred in the intestine of the dogs even without a selective pressure.

Antibiotic treatment within the last three months before sampling has been identified as a risk factor for colonization with ESBL-producing *Enterobactriaceae* in healthy dogs and cats (Gandolfi-Descristophoris et al 2010). Furthermore, Damborg et al showed that first generation cephalosporins and aminopenicillins with clavulanic acid might select for E.coli producing pAmpC (2011). In the present study dogs were treated with antibiotics within a month before sampling and the dogs harbouring the *E. coli* with bla_{CMY-2} had been treated with amoxicillin, a first generation cephalosporin, or enrofloxacin (table 2). However, the treatment and length of treatment of the dogs carrying *E. coli* with bla_{CMY-2} did not significantly differ from the negative group. First generation cephalosporins are widely used in veterinary medicine (Hughes et al 2013), but since 2006 there has been a decrease in prescription of cephalosporines (-70%) and aminopenicillin with clavulanic acid (-52%) in Sweden (Swedres-SVARM 2012). This decrease in prescription of cephalosporines is likely connected to emerging resistance problems in farm animals and companion animals leading to a larger awareness among veterinarians concerning this. It is not uncommon that the plasmids carrying the ESBL and pAmpC genes often carry genes encoding resistance against other antimicrobial groups, for example fluoroquinolones (Pitout 2010). Fluoroquinolones are frequently used in treating dogs (Guardabassi et al 2004), and in our study the ESBL- bla_{TEM-52} isolate (dog E) was fluoroquinolone resistant, all of the other AmpC-producing isolates were sensitive to fluoroquinolones. In 2014 a new law was implemented in Sweden which stated that fluoroquinolones are only available to veterinarians after an antimicrobial susceptibility test have shown that it is the only treatment option at hand to try and decrease the usage of that antimicrobial group.

The nosocomial spread of highly resistant bacteria emphasizes the importance of hygiene routines in veterinary clinics (Wieler et al 2011). Besides direct contact, the contamination of the environment poses a risk for spread of resistant bacteria between dogs and to human contact areas. The close contact between pets and humans implicates a risk for zoonotic transfer of resistance genes, and might constitute a public health risk. Dierikx et al (2012) found the same ESBL genotypes in isolates from dogs and from humans in the Netherlands and suggests possible transmission between companion animals and humans.

In conclusion, dissemination of plasmid-mediated AmpC/ESBL- producing *Enterobacteriaeae* in the hospital environment and the community, must be considered as a major threat to animal and human health and welfare and need to be carefully monitored in all surgical wards and intensive care units. The potential carriership of several months may contribute to community-acquired infections affecting other animals and humans. Strict hygiene should be maintained around these animals and in particular their feces to prevent spread of resistance genes in the environment.

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