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Selection of CMY-2 producing *Escherichia coli* in the faecal flora of dogs treated with cephalixin

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28 Abstract

29 Cephalexin is a first generation cephalosporin commonly used in dogs for treatment of pyoderma.
 30 The objective of this study was to evaluate the *in vivo* effects of cephalexin on selection of
 31 *Escherichia coli* resistant to extended-spectrum cephalosporins. A cohort study was conducted on
 32 13 dogs presenting clinical signs of pyoderma and treated with cephalexin and 22 healthy dogs that
 33 had not been treated with antibiotics during the previous six months. Selective plating of faeces on
 34 MacConkey agar plates containing cefotaxime (CTX) yielded growth of CTX-resistant *E. coli* for
 35 eight of the 13 treated dogs (62%), whereas no growth was observed for any of the control dogs
 36 (Fisher Exact test, $P < 0.001$). PCR and sequence analysis identified *bla*_{CMY-2} in all eight dogs.
 37 PCR-based replicon typing and restriction fragment length polymorphism (RFLP) of *E. coli*
 38 transformants revealed location of *bla*_{CMY-2} on indistinguishable IncI1 plasmids in five of the eight
 39 dogs. One representative of these five epidemiologically-related IncI1 plasmids was further
 40 characterized as sequence type (ST2) by plasmid multilocus sequence typing (pMLST). *E. coli* from
 41 the remaining three dogs harboured *bla*_{CMY-2} on distinct plasmids with non-typeable replicons. A
 42 single isolate was classified as an extraintestinal pathogenic *E. coli* (ExPEC) due to the presence of
 43 *iutA*, *papC* and *sfa/foc*. The results provide a strong indication that cephalexin selects for *E. coli*
 44 producing plasmid-borne CMY-2 β -lactamase. The isolation of a specific IncI1 plasmid carrying
 45 *bla*_{CMY-2} from five epidemiologically unrelated dogs suggests that cephalexin use may contribute to
 46 the spread of this plasmid lineage among Danish dogs.

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48

49 Keywords: antibiotic resistance, antibiotic therapy, AmpC, β -lactams, companion animals

1. Introduction

A steady increase in usage of antimicrobial agents for companion animals, in particular for dogs, has been observed in Europe over the last years. In the UK, the total sales (kg of active ingredient) of antimicrobials registered for dogs increased by 250 % in the period from 2004 to 2009 (Goodyear, 2010). Data from Denmark on total sales of antimicrobial agents for pet animals show a similar trend (Danmap, 2004 & 2009). Much of this increase can be attributed to the frequent usage of cephalosporins and fluoroquinolones. In Sweden, prescription of these two antimicrobial classes in 1998-2005 has increased by 91% and 39%, respectively (SVARM, 2005). Along with the increasing use of antibiotics, several important multi-resistant pathogens have emerged in dogs. Amongst Gram-negative species, various types of extended-spectrum β -lactamase genes have been described in *Escherichia coli* of canine origin, mainly *bla*_{CTX-M} (Carattoli et al., 2005b) and *bla*_{CMY} (Sanchez et al., 2002).

In veterinary dermatology, cephalexin has been used for many years as the first choice in the management of pyoderma, one of the most frequent bacterial infections in the dog. According to a study in Finland (Rantala et al., 2004), cephalexin accounts for approximately 50% of all antibiotic prescriptions in dogs. On the contrary, the use of this first generation cephalosporin has been gradually abandoned in human medicine because of the limited antimicrobial activity against Gram-negative species. As a consequence, its impact on selection of extended-spectrum cephalosporin resistance in Gram-negative bacteria is largely unknown.

The present manuscript describes a cohort study designed to evaluate the possible selection of *E. coli* resistant to extended-spectrum cephalosporins in dogs treated with cephalexin. Treated and control dogs were screened for the occurrence of *E. coli* resistant to a third generation cephalosporin (cefotaxime), and the specific genes and plasmids mediating cephalosporin resistance were identified and characterized.

2. Materials and Methods

2.1 Study design and sampling

Four small animal hospitals in the Greater Copenhagen area agreed to participate in the cohort study. Dogs attending these hospitals between February 2007 and March 2008 were considered eligible to the treatment group if they presented clinical signs of pyoderma, were prescribed minimum 14 days of standard oral treatment with cephalexin (Cefaseptin® Vet., 25mg/kg, BID) and did not receive any other medicine during treatment. Healthy dogs admitted to the same clinics for vaccination were enrolled in the control group if they had not received any antibiotics within 6 months prior to the study. Owners were asked to fill out a written consent of participation and send one fresh faecal sample per dog by ordinary mail to our laboratory upon hospital discharge. For the exposed dogs, samples were collected and sent on the last day of cephalexin treatment (day 14-28), whereas samples from control dogs were sent at the owners convenience in the week following vaccination.

2.2 Bacterial isolation and identification

Upon receipt in the laboratory, one gram of faeces was mixed with 700 µl 50% glycerol and 300 µl brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and stored at -80°C until processing. Samples were thawed and vortexed, and cefotaxime (CTX)-resistant coliforms were quantified by plate dilution. Briefly, 100 µl from 10-fold dilutions made in Milli Q water was spread on MacConkey agar plates (Oxoid) supplemented with 2 µg/ml CTX. Following overnight incubation at 37°C, lactose-positive colonies were counted, and one colony was randomly selected from each dog and subjected to the ImVIC phenotypic tests (indole, methyl red, Voges Proskauer and citrate) for *E. coli* identification.

2.3 Antimicrobial susceptibility

Antimicrobial susceptibility testing of CTX-resistant *E. coli* was done by disk diffusion according to the Clinical Laboratories Standards Institute (CLSI, 2008a). The following discs (Oxoid) were used: amikacin (30 µg), amoxicillin-clavulanic acid (20/10 µg), ampicillin (10 µg), aztreonam (30 µg), cefepime (30 µg), cefixime (5 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), cephalothin (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), sulfamethoxazole-trimethoprim 19:1 (25 µg), and tetracycline (30 µg). Susceptibility towards the combination of cefotaxime and clavulanic acid (30/10 µg) was measured using BD BBL Sensi-Discs (Becton, Dickinson and Company, USA). Human CLSI breakpoints (CLSI, 2008b) were used for compounds without established breakpoints for bacteria of animal origin.

2.4 Identification of β -lactamase genes

PCR-based screening for the presence of *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, and plasmid-borne genes encoding AmpC β -lactamase was done as previously described (Perez-Perez and Hanson, 2002; Hasman et al., 2005). Isolates positive for the AmpC phylogenetic group CIT were subjected to a specific PCR targeting the entire *bla*_{CMY-2} gene (Pérez-Pérez and Hanson, 2002; Jørgensen et al., 2010). Amplicons were purified and submitted for sequencing to Macrogen Inc. (Seoul, Korea). The resulting nucleotide sequences were analysed using the basic local alignment search tool (BLAST) available on the homepage of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

2.5 Plasmid typing

Plasmid DNA (Plasmid Midi Kit, Qiagen Inc., Venlo, The Netherlands) was obtained from all strains producing β -lactamase and transformed into electrocompetent Genehog *E. coli* (Invitrogen,

Taastrup, Denmark). Transformants were selected on BHI agar plates supplemented with 2ug/ml CTX, and presence of β -lactamase genes was verified by colony PCR. Plasmid DNA from transformants was used as template in PCR-based replicon typing (Carattoli et al., 2005a) and for restriction fragment length polymorphism (RFLP) with the FastDigest[®] PstI enzyme (Fermentas, Helsingborg, Sweden). One IncI1 plasmid representing the unique RFLP pattern found was subjected to plasmid multilocus sequence typing (pMLST) as described by García-Fernández et al. (2008).

2.6 Phylotyping and virulence gene profiling

The phylotypes of *E. coli* isolates were determined by multiplex PCR (Clermont et al., 2001). Another multiplex PCR was used to screen for the occurrence of virulence genes associated with extraintestinal pathogenic *E. coli* (ExPEC) (Johnson et al., 2003). Isolates were defined as ExPEC if they were PCR positive for at least two of the following genes: *papA* and/or *papC*, *sfa/foc*, *afa/dra*, *kpsM II* and *iutA* (Johnson et al., 2003)

3. Results

3.1 Description of dogs in the treatment and control group

A total of 13 dogs suffering from pyoderma and subjected to cephalexin treatment were included in the treatment group. These dogs represented 10 breeds and were 1-11 years old (average 4.8 years). Seven of the dogs were male and six were bitches. All dogs had been treated daily with cephalexin for 14-28 days prior to sampling, and six of the dogs had been subjected to antimicrobial treatment with either cephalexin (n=4) or amoxicillin-clavulanate (n=2) during the last year (Table 1). Twenty-two dogs were enrolled in the control group. They represented 15 breeds and were 1-7 years old (average 4.0 years). The control group included 11 males and 11 bitches.

146

147 *3.2 Quantification and identification of CTX-resistant isolates*

148 CTX-resistant *E. coli* were isolated from eight dogs treated with cephalexin (62 %), whereas no
 149 growth was obtained from dogs in the control group (Fisher exact test, $P < 0.001$). Amongst the
 150 positive dogs, counts of CTX-resistant coliforms ranged from 4×10^7 to 4×10^{10} CFU/g faeces
 151 (average 7×10^9 CFU/g). The eight selected isolates, one for each positive dog, displayed
 152 susceptibility profiles indicating presumptive production of AmpC-type β -lactamase (i.e. inactivity
 153 of clavulanic acid and resistance to all β -lactams except for cefepime and imipenem). All isolates
 154 were susceptible to amikacin, enrofloxacin and gentamicin. Three isolates displayed either
 155 intermediate or full resistance to sulfamethoxazole-trimethoprim, and one isolate was resistant to
 156 tetracycline (Table 1).

157

158 *3.3 Genotypic characterization of CTX-resistant E. coli*

159 All eight isolates carried *bla*_{CMY-2}. Replicon typing showed that this gene was present on an IncI1
 160 plasmid in five of the eight isolates, whereas the remaining three isolates carried the gene on
 161 plasmids that were non-typeable by replicon PCR. The IncI1 plasmids were indistinguishable by
 162 RFLP analysis. One representative IncI1 plasmid from dog R7 (Table 1) was identified as sequence
 163 type (ST) 2 by pMLST analysis. The extended-spectrum β -lactamase (ESBL)-encoding genes
 164 *bla*_{CTX-M} and *bla*_{SHV} were not detected, whereas *bla*_{TEM-1} was present in one isolate (Table 1). All
 165 isolates belonged to phylotype D, and one of the isolates was characterized as an ExPEC due to
 166 simultaneous presence of the virulence genes *iutA*, *papC* and *sfa/foc*.

167

168

169 4. Discussion

170 The present study shows that oral treatment of dogs with cephalexin results in high
 171 numbers of *E. coli* producing plasmid-mediated CMY-2 β -lactamase at the end of the treatment
 172 period. This information is noteworthy considering the extensive use of first-generation
 173 cephalosporins in small animal practice. CMY-2 confers resistance towards all β -lactams licensed
 174 for use in dogs and therefore may pose a serious animal health problem due to the limited
 175 alternative options available for treatment of *E. coli* infections in veterinary medicine. Although all
 176 the strains described in this study remained susceptible to most non- β -lactams tested (Table 1),
 177 multidrug-resistant *E. coli* producing CMY β -lactamase have been described as a cause of
 178 opportunistic infections in dogs (Sanchez et al., 2002; Sidjabat et al., 2006). Plasmids carrying
 179 *bla*_{CMY-2} can easily acquire multiple resistance genes as suggested by frequent co-residence of
 180 different resistance genes (Winokur et al., 2001).

181 The results of this study suggest that dogs receiving prolonged treatment with first-
 182 generation cephalosporins may be an important reservoir of CMY β -lactamases. Sparse information
 183 available in the literature supports these findings. Murphy et al. (2009) isolated CMY-2-producing
 184 *E. coli* in 2 out of 180 healthy dogs attending veterinary hospitals in Canada. However, the
 185 prevalence of CMY-2 was likely underestimated as the study did not employ antimicrobial selective
 186 methods of isolation. In a recent study in the US (O'Keefe et al., 2010), CMY was detected in
 187 approximately one third of 150 canine and feline *E. coli* isolated from urinary tract infections and
 188 was by far the most common β -lactamase. Other studies have reported the occurrence of ESBL
 189 genes such as *bla*_{CTX-M-1}, *bla*_{SHV-12} or *bla*_{TEM-52} in *E. coli* isolated from dogs in Portugal and Italy
 190 (Costa et al., 2004; Carattoli et al., 2005b). The apparent geographical variation in the distribution
 191 of β -lactamase types in dogs may be influenced by the methodologies used in different studies but

could also reflect local patterns in the use of antimicrobials and in the epidemiology of β -lactamases in the human population.

Despite the sporadic occurrence of human infections caused by CMY-2-producing *E. coli* in Scandinavian countries (Jørgensen et al., 2010; Naseer et al., 2010), the presence of these bacteria in dogs may be of public health concern. One of the eight canine CMY-2-producing isolates described in this study (R6AC) was an ExPEC, which is the most common cause of urinary tract infections in humans (Johnson and Russo, 2001). Furthermore, all isolates belonged to phylotype D, which - together with phylotype B2 - is frequently shared between dogs and humans living in the same household (Damborg et al., 2009). In conjunction with our results, a strong association between CMY-2 and phylotype D has been recently demonstrated in *E. coli* infections in Spain (Oteo et al., 2010).

The *bla*_{CMY-2} gene was located on IncI1 plasmids with identical RFLP patterns of which one representative belonged to ST2, in five of the eight *E. coli* isolates (Table 1). The predominance of one plasmid type was unexpected, since the dogs were epidemiologically-unrelated and had been sampled on distinct dates at four different hospitals. This finding suggests that treatment with cephalexin may contribute to the spread of this plasmid lineage among dogs in Denmark. Unfortunately, no information is available on the type of plasmids carrying *bla*_{CMY-2} in human and canine *E. coli* infections in this country. IncI1 is the predominant plasmid incompatibility group among CMY-2-producing *E. coli* collected from humans in Norway (Naseer et al., 2010). According to the publically available database (<http://pubmlst.org/plasmid>), IncI1 ST2 plasmids carrying the *bla*_{CMY-2} gene have been previously detected in *E. coli* from dogs in Italy and in *E. coli* and *Salmonella Heidelberg* from Canadian and UK citizens. Such a widespread appearance across hosts and continents indicates high mobility and broad host-range of this plasmid type. Likely transfer of *bla*_{CMY-2}-containing plasmids between bacterial species and across host

barriers has previously been hypothesized by Winokur et al. (2001). The gene has been also reported in other plasmid incompatibility groups, primarily A/C and K/B (Carattoli, 2009; Mataseje et al., 2010).

The observed association between cephalexin and CMY-2-producing *E. coli* could have been enhanced by prior antimicrobial treatment, since at least six of the eight *bla*_{CMY-2}-positive dogs had previously been treated with β -lactams, mainly cephalexin (Table 1). In contrast, only one of the five culture-negative dogs from the treatment group had received β -lactams before. Studies in humans have shown a long-term effect of antimicrobial treatment on resistance levels in the intestinal flora (Jernberg et al., 2007). In animals, studies have mainly focused on the short-term selective effects of antimicrobial treatment and their results are often controversial, maybe as a consequence of the different methodologies, animal species, antibiotics and resistant bacteria used for assessing the impact of antimicrobial treatment (Cavaco et al., 2008; Singer et al., 2008; Grønvold et al., 2010).

A limitation of the study was the initial storage of faecal samples in BHI and glycerol at -80°C. However, the high counts of CTX-resistant coliforms revealed that freezing did not affect bacterial survival, and even if there was an effect of storage, such an effect would have equally affected the bacterial counts in all samples. We can therefore conclude that this methodological factor did not influence the main outcome of the study. As we analysed a single CTX-resistant *E. coli* isolate per sample, *E. coli* producing other β -lactamases than CMY-2 may have gone undetected. However, isolation of multiple colonies from the same plate was unlikely to overcome this intrinsic methodological limitation of culture methods, since it has previously been shown that multiple *E. coli* isolates from the same faecal sample often represent one, dominant clone (McLellan et al., 2003).

Previous *in vivo* studies have shown that oral administration of β -lactams to dogs impacts the composition of the intestinal *E. coli* population in favour of resistant strains (Mentula et al., 2004; Grønvold et al., 2010). However, such studies assessed the effects of β -lactam agents other than cephalexin (ampicillin and amoxicillin, respectively) and did not investigate the genetic background of the resistant strains selected by antimicrobial treatment. More *in vivo* studies are needed to identify drug- and gene-specific associations between antimicrobial usage and antimicrobial resistance. This information is of crucial importance to understand how the evolution of antimicrobial resistance may be influenced by patterns of antimicrobial usage, and consequently to develop effective antimicrobial policies in veterinary hospitals.

5. Conclusion

Oral treatment of dogs with cephalexin selects for intestinal *E. coli* producing CMY-2 and may contribute to the dissemination of specific Inc11 plasmids encoding this β -lactamase. The intensive use of cephalexin during the last decades may have favoured local dissemination of *bla*_{CMY-2} in Danish dogs.

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Table 1. Description of dogs and CTX-resistant *E. coli* isolates

Dogs with CTX-resistant <i>E. coli</i>			Characterization of CTX-resistant <i>E. coli</i>					
Dog ID	Days of cephalexin treatment	Previous β -lactam treatment (mth's before sampling)	β -lactamase gene(s)	Plasmid replicon type	Plasmid RFLP pattern	Resistance to non- β -lactams ^b	Phylotype	ExPEC
R1	28	Cephalexin (8) Amoxicillin-clavulanate (1)	<i>bla</i> _{CMY-2}	Unknown	A	-	D	No
R2	21	Unknown	<i>bla</i> _{CMY-2}	IncI1	B	-	D	No
R3	17	Amoxicillin-clavulanate (9)	<i>bla</i> _{CMY-2}	IncI1	B	-	D	No
R4	14	Cephalexin (2)	<i>bla</i> _{CMY-2}	Unknown	C	TET	D	No
R5	14	None	<i>bla</i> _{CMY-2}	IncI1	B	SXT	D	No
R6	19	Cephalexin (9) Cephalexin (3)	<i>bla</i> _{CMY-2}	IncI1	B	-	D	Yes
R7	21	Unknown	<i>bla</i> _{CMY-2} <i>bla</i> _{TEM-1}	IncI1 ^a	B	SXT	D	No
R13	15	Unknown	<i>bla</i> _{CMY-2}	Unknown	D	SXT	D	No

^a plasmid multilocus sequence type 2^b TET, tetracycline; SXT, sulphamethoxazole-trimethoprim.