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

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28 **Abstract**

29 Cephalixin 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 cephalixin 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 cephalixin 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 cephalixin 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 cephalixin use may contribute to
46 the spread of this plasmid lineage among Danish dogs.

47

48

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

50 1. Introduction

51 A steady increase in usage of antimicrobial agents for companion animals, in
52 particular for dogs, has been observed in Europe over the last years. In the UK, the total sales (kg of
53 active ingredient) of antimicrobials registered for dogs increased by 250 % in the period from 2004
54 to 2009 (Goodyear, 2010). Data from Denmark on total sales of antimicrobial agents for pet animals
55 show a similar trend (Danmap, 2004 & 2009). Much of this increase can be attributed to the
56 frequent usage of cephalosporins and fluoroquinolones. In Sweden, prescription of these two
57 antimicrobial classes in 1998-2005 has increased by 91% and 39%, respectively (SVARM, 2005).
58 Along with the increasing use of antibiotics, several important multi-resistant pathogens have
59 emerged in dogs. Amongst Gram-negative species, various types of extended-spectrum β -lactamase
60 genes have been described in *Escherichia coli* of canine origin, mainly *bla*_{CTX-M} (Carattoli et al.,
61 2005b) and *bla*_{CMY} (Sanchez et al., 2002).

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

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

74 **2. Materials and Methods**

75 *2.1 Study design and sampling*

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

88 *2.2 Bacterial isolation and identification*

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

97

98 2.3 Antimicrobial susceptibility

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

108

109 2.4 Identification of β -lactamase genes

110 PCR-based screening for the presence of *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, and plasmid-borne genes
111 encoding AmpC β -lactamase was done as previously described (Perez-Perez and Hanson, 2002;
112 Hasman et al., 2005). Isolates positive for the AmpC phylogenetic group CIT were subjected to a
113 specific PCR targeting the entire *bla*_{CMY-2} gene (Pérez-Pérez and Hanson, 2002; Jørgensen et al.,
114 2010). Amplicons were purified and submitted for sequencing to Macrogen Inc. (Seoul, Korea).
115 The resulting nucleotide sequences were analysed using the basic local alignment search tool
116 (BLAST) available on the homepage of the National Center for Biotechnology Information
117 (<http://www.ncbi.nlm.nih.gov/>).

118

119 2.5 Plasmid typing

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

122 Taastrup, Denmark). Transformants were selected on BHI agar plates supplemented with 2ug/ml
123 CTX, and presence of β -lactamase genes was verified by colony PCR. Plasmid DNA from
124 transformants was used as template in PCR-based replicon typing (Carattoli et al., 2005a) and for
125 restriction fragment length polymorphism (RFLP) with the FastDigest[®] PstI enzyme (Fermentas,
126 Helsingborg, Sweden). One IncI1 plasmid representing the unique RFLP pattern found was
127 subjected to plasmid multilocus sequence typing (pMLST) as described by García-Fernández et al.
128 (2008).

129

130 2.6 Phylotyping and virulence gene profiling

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

136

137 3. Results

138 3.1 Description of dogs in the treatment and control group

139 A total of 13 dogs suffering from pyoderma and subjected to cephalexin treatment were included in
140 the treatment group. These dogs represented 10 breeds and were 1-11 years old (average 4.8 years).
141 Seven of the dogs were male and six were bitches. All dogs had been treated daily with cephalexin
142 for 14-28 days prior to sampling, and six of the dogs had been subjected to antimicrobial treatment
143 with either cephalexin (n=4) or amoxicillin-clavulanate (n=2) during the last year (Table 1).
144 Twenty-two dogs were enrolled in the control group. They represented 15 breeds and were 1-7
145 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

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

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

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

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

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

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

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

248

249 **5. Conclusion**

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

254

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262

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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 cephalalexin 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.