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1 **Monitoring of Antimicrobial Resistance in Healthy**
2 **Dogs: First Report of Canine Ampicillin-Resistant**
3 ***Enterococcus faecium* Clonal Complex 17**

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5
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8 **Abstract**

9 National surveillance programs on antimicrobial usage and antimicrobial resistance in animals have
10 been established in various countries but few of them include bacteria from pets. The objectives of
11 this study were to assess the prevalence of antimicrobial resistance in healthy dogs and to search for
12 resistance phenotypes of clinical relevance. *Escherichia coli* and *Enterococcus* spp. were isolated
13 from faecal swabs obtained from 127 dogs. Disk diffusion was used to measure antimicrobial
14 susceptibility in 117 *E. coli*, 10 *E. faecium* and 51 *E. faecalis* of canine origin. Resistance was
15 relatively low compared with food animal species in Denmark. All *E. coli* isolates were susceptible
16 to broad-spectrum aminopenicillins, third generation cephalosporins and fluoroquinolones. Despite
17 the low prevalence of resistance, statistical analysis of questionnaire data revealed a significant
18 association ($p=0.02$) between recent antimicrobial treatment and resistance in *E. coli*. Interestingly,
19 two dogs were found to shed *E. faecium* resistant to ampicillin. Multilocus sequence typing of these
20 isolates indicated that the two isolates belonged to sequence types associated with human
21 nosocomial infections, and one (ST-192) was genetically related to human epidemic clonal complex
22 17. The detection of ampicillin-resistant *E. faecium* warrants further studies on the prevalence of
23 these bacteria in dogs and on the possible implications to both animal and human health. The results
24 suggest that distinct methods for detection and assessment of antimicrobial resistance in animals
25 should be considered depending on the target animal species and the purposes of the study.

26

27

Key words: antibiotic resistance, canine, enterococci

28 **1. Introduction**

29 In 1999, the European Union adopted the directive 2003/99/EC with the purpose to ensure that
30 zoonoses and antimicrobial resistance in animals are properly monitored. Since then, national
31 surveillance programs on antimicrobial consumption and resistance have been established for
32 humans and production animals in various European countries. Pet animals are rarely included in
33 these surveillance programs and when they are, bacteria are often obtained from diseased animals
34 (Norm-Vet, 2004; SVARM, 2005; Grobbel et al., 2007). Reports from Denmark, Norway and
35 Sweden have described an increased antimicrobial use in pet animals, particularly broad-spectrum
36 agents such as fluoroquinolones, cephalosporins and aminopenicillins with clavulanic acid (Heuer
37 et al., 2005; SVARM, 2006; Odensvik et al., 2001). Although not supported by published data, a
38 similar trend in the patterns of antimicrobial prescription has occurred worldwide in small animal
39 practice. The consequences of this increase in the use of antimicrobials are unknown but various
40 emerging resistance phenotypes of clinical relevance have been reported in pets during the last
41 years (Guardabassi et al., 2004). Some of them, such as methicillin-resistant *Staphylococcus aureus*
42 (Loeffler et al., 2005), vancomycin-resistant enterococci (Torres et al., 2003) and *Escherichia coli*
43 producing extended-spectrum beta-lactamases (Moreno et al., 2007) are potentially hazardous to
44 both the colonised animals and the humans living in contact with them. The aim of the present study
45 was to investigate the levels of antimicrobial resistance in intestinal flora of healthy dogs and to
46 search for resistance phenotypes of particular concern to animal and human health. The levels of
47 resistance were monitored in the indicator bacteria *Escherichia coli*, *Enterococcus faecium* and
48 *Enterococcus faecalis*, and compared with those reported in other domestic animals and humans in
49 Denmark. Possible associations with antimicrobial use were investigated by a questionnaire study
50 and selected bacterial isolates displaying clinically relevant resistance phenotypes were
51 characterized by genotypic methods.

52 **2. Materials and methods**

53 *2.1. Sampling and collection of data on antimicrobial treatment*

54 Veterinary staff from 12 randomly selected small animal hospitals in Denmark was asked to take
55 faecal swabs from dogs during June to August 2006. Dogs selected for the study were clinically
56 healthy, i.e. dogs admitted to the hospitals for vaccination or elective surgery and showing no signs
57 of bacterial infection or diarrhoea. Swabs were kept in Stuarts Media (SSI Diagnostika, Hillerød,
58 Denmark) and sent by ordinary mail to our laboratory. Information on prescription of antimicrobial
59 agents for the participating dogs until 6 months prior to sampling was provided when possible.

60

61 *2.2. Bacterial isolation and identification*

62 On the day of arrival, faecal swabs were streaked directly on MacConkey agar (Oxoid, Basingstoke,
63 UK), Slanetz-Bartley agar (Oxoid), and Slanetz-Bartley agar supplemented with 20 µg/ml
64 vancomycin. Plates were incubated 1-2 days at 37°C, followed by subculture and storage of one
65 colony from each culture-positive plate. Putative *E. coli* from MacConkey agar were speciated
66 using the IMViC tests (indole, methyl red, Voges Proskauer and citrate) and red colonies obtained
67 from Slanetz-Bartley agar were identified as *Enterococcus faecium*, *Enterococcus faecalis* or
68 *Enterococcus* spp. by a multiplex PCR method (Dutka-Malen et al., 1995).

69

70 *2.3. Antimicrobial susceptibility*

71 Antimicrobial susceptibility was tested on pure cultures by the disk diffusion method. Inhibition
72 zone diameters were interpreted according to the Clinical Laboratories Standards Institute (CLSI,
73 formerly NCCLS) breakpoints (NCCLS, 2002) and, when breakpoints were unavailable for bacteria
74 of animal origin, according to the human CLSI breakpoints (CLSI, 2005). Florfenicol resistance in
75 enterococci was defined tentatively based on histograms of the inhibition zone diameters. For *E.*

76 *coli*, the following antimicrobial discs (Oxoid) were used: amoxicillin + clavulanic acid (20/10 µg),
77 ampicillin (10 µg), ceftiofur (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), florfenicol (30 µg),
78 gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulfonamide (300 µg), tetracycline
79 (30 µg) and trimethoprim (5 µg). *E. faecium* and *E. faecalis* were tested against: ampicillin (10 µg),
80 chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), high-level
81 gentamicin (120 µg), linezolid (30 µg), rifampicin (5 µg), tetracycline (30 µg) and vancomycin (30
82 µg). *E. faecium* were furthermore tested for streptogramin resistance using discs containing 15 µg
83 quinopristin and dalfopristin.

84

85 2.4. Multilocus sequence typing

86 Ampicillin-resistant *E. faecium* were subjected to multilocus sequence typing according to a
87 previously described protocol (Homan et al., 2002). Alleles and sequence types were assigned by
88 using the software available on www.mlst.net.

89

90 3. Results

91 3.1. Prevalence of bacterial indicators

92 Among a total of 127 dogs tested, we isolated 118 *E. coli* (92%), 51 *E. faecalis* (40%), 10 *E.*
93 *faecium* (11%) and 22 *Enterococcus* spp. (17%). No colonies were detected on Slanetz-Bartley agar
94 supplemented with vancomycin. Fourteen dogs (11%) had been treated at least once with an
95 antimicrobial agent within 6 months prior to sampling. The antimicrobial classes used for these
96 dogs included broad-spectrum aminopenicillins, cephalosporins, fluoroquinolones, fusidic acid,
97 lincosamides, macrolides and nitroimidazole derivatives. Sixty-five dogs (51%) were untreated
98 during the same period. No information on antimicrobial treatment was provided for the remaining
99 48 dogs.

100 3.2. Resistance in *E. coli*

101 Among *E. coli* isolates, the highest prevalences of antimicrobial resistance were observed for
102 streptomycin (9%), ampicillin (9%) and sulfonamide (8%) (Table 1). All isolates were susceptible
103 or showed intermediate resistance to amoxicillin with clavulanic acid, ceftiofur, ciprofloxacin,
104 florfenicol, gentamicin and nalidixic acid. Five different multi-resistance phenotypes were present
105 (Table 2). The most common of these, comprising resistance to five different antimicrobial classes
106 (ampicillin, streptomycin, sulfonamide, tetracycline and trimethoprim), was displayed by four
107 isolates. Prescription data provided by the veterinary staff showed that *E. coli* isolated from dogs
108 treated with antimicrobial agents within 6 months prior to sampling were significantly associated
109 with resistance to one or more antimicrobials in comparison with untreated dogs (Fisher Exact test,
110 $P=0.02$). There was no specific correlation between the antimicrobial classes used and the
111 resistance patterns observed in *E. coli* (data not shown).

113 3.3. Resistance in enterococci

114 Resistance to rifampicin was predominant amongst both *E. faecium* (60%) and *E. faecalis* (65%),
115 and tetracycline resistance occurred in approximately one third of both enterococcal species (Table
116 1). Considering the most clinically relevant antimicrobials, enterococci were either susceptible
117 (74%) or showed intermediate resistance (26%) to vancomycin, and one *E. faecalis* (2%) and two *E.*
118 *faecium* (20%) were resistant to gentamicin and ampicillin, respectively. Intermediate resistance to
119 linezolid and streptogramins was also detected (Table 1). Multi-resistance patterns are displayed in
120 Table 2. No significant associations were observed between recent antimicrobial treatment and
121 occurrence of resistance in enterococci.

123 3.4. Multilocus sequence typing

124 The two ampicillin-resistant *E. faecium* were characterized as sequence types (ST) 192 and 266,
125 respectively. Both ST's have been previously associated with human nosocomial infections and ST-
126 192 belongs to a specific genogroup, labeled clonal complex 17 (CC17) (Top et al., 2008).

127

128 **4. Discussion**

129 Generally low levels of resistance were observed in indicator bacteria from healthy dogs, and all *E.*
130 *coli* were susceptible to fluoroquinolones as well as broad-spectrum aminopenicillins- and
131 cephalosporins. However, resistance phenotypes of clinical interest were observed among
132 enterococcal isolates. Remarkably, we reported for the first time the occurrence of ampicillin-
133 resistant *E. faecium* (AREF) CC17 in dogs. This genotype has been described only once in animals,
134 namely in a pig isolate (Leener et al., 2005). Epidemic AREF CC17 are important nosocomial
135 pathogens in humans and their prevalence seems to have increased in European countries such as
136 Denmark and the Netherlands (Top et al., 2008; pers. comm., Camilla H. Lester, Statens Serum
137 institut, Denmark). The great concern associated with AREF is that penicillins alone or in
138 combination with gentamicin are one of few treatment options for life-threatening enterococcal
139 infections such a bacteraemia and endocarditis. Vancomycin is the last choice for treating human
140 infections caused by AREF. Apart from the possible zoonotic implications associated with the
141 occurrence of AREF in dogs, a veterinary perspective should also be kept in mind. *E. faecium* is a
142 cause of canine urinary tract infections (UTI) and AREF are usually resistant to all antimicrobial
143 agents commonly used in dogs, including ampicillin, amoxicillin combined with clavulanic acid,
144 first generation cephalosporins, potentiated sulfonamides and fluoroquinolones. The fact that canine
145 UTI are often associated with multiple bacterial species, may further complicate treatment of
146 AREF. Indeed, a difficult case of canine UTI associated with AREF and *E. coli* was recently
147 recorded at our diagnostic laboratory.

148 The overall low levels of resistance observed in healthy dogs are similar to those reported by
149 previous studies in other countries. The Swedish Veterinary Antimicrobial Resistance Monitoring
150 Program (SVARM, 2006) investigated resistance in 257 *E. coli* from healthy dogs and found
151 comparable or lower levels of resistance. In a study in Portugal (Costa et al., 2008), *E. coli* from 39
152 dogs were generally susceptible to most antimicrobials, including those characterised by broad-
153 spectrum of activity. Based on the current knowledge, it appears that resistance phenotypes of
154 clinical relevance are more common in enterococci than in *E. coli* isolated from dogs. For example,
155 relatively high resistance levels were detected by Poeta et al. (2006) among enterococci isolated
156 from healthy dogs in Portugal, including resistance to clinically relevant drugs such as gentamicin
157 (13%). Although at lower frequency (2%), gentamicin resistance was also detected in the present
158 study. Rifampicin resistance has not been investigated in studies on canine enterococci, and
159 consequently the high frequency of rifampicin resistance observed in this study in both *E. faecium*
160 (65%) and *E. faecalis* (60%) was noteworthy. Rifampicin resistance is clinically important because
161 this antibiotic can be used as a second-line drug for treatment of enterococcal infections in humans
162 (Cetinkaya et al., 2000). High-level resistance to vancomycin was not detected but intermediately
163 resistant isolates appeared to be relatively common (26%). Intermediate resistance was also
164 observed for other clinically relevant antimicrobials such as linezolid and streptogramins, which are
165 second-line treatment options for human enterococcal infections. It should be noted that the CLSI
166 interpretive zone diameters used for defining susceptibility and intermediate resistance to these
167 antibiotics are very close (e.g. 2 mm for vancomycin). Further analysis by determination of
168 minimum inhibitory concentrations and/or detection of resistance genes would be needed to exclude
169 that these results were not due to inaccuracy of the disc diffusion method.

170 When comparing overall resistance levels to those in indicator *E. coli* of Danish humans and
171 production animals (DANMAP, 2006) (Table 1), the situation in dogs closely resembled what has

172 been recorded for cattle. Higher levels of resistance have been reported in *E. coli* from humans, pigs
173 and broilers. For example, the prevalence of ciprofloxacin-resistant *E. coli* was significantly higher
174 in humans ($p=0.044$) and broilers ($p=0.010$) than in dogs (Table 1). For *E. faecalis*, pig isolates
175 were more frequently resistant to erythromycin ($p<0.001$) and tetracycline ($p<0.001$), which are two
176 antibiotics commonly used in pig production.

177 The low prevalence of resistance to beta-lactams and fluoroquinolones in canine *E. coli* was
178 surprising given the relatively common use of these antimicrobial classes in small animal practice
179 compared with human medicine and other veterinary practices in Denmark (Guardabassi et al.,
180 2004; Heuer et al, 2005). However, it should be noted that only 14 of 79 dogs in the current study
181 had been treated with antimicrobial agents within six months prior to sampling. Of these, only four
182 dogs were treated with amoxicillin and only two with either a cephalosporin or a fluoroquinolone.
183 Thus, the relatively low antimicrobial selective pressure exerted on the study population could
184 explain the low levels of resistance, particularly towards broad-spectrum antimicrobials.

185 An interesting outcome of the study was the finding that dogs subjected to treatment were more
186 likely ($p=0.02$) to harbour *E. coli* resistant to one or more antimicrobials. In fact, three of the four *E.*
187 *coli* displaying the penta-resistance phenotype ampicillin-streptomycin-sulfonamide-tetracycline-
188 trimethoprim (Table 2) were isolated from dogs exposed to antimicrobial treatment. This is a likely
189 example of selection or co-selection of multi-resistance following antimicrobial usage. Selection of
190 resistant bacteria upon antimicrobial treatment is well-recognized and has been documented in dogs
191 following changes in treatment regimes (Cooke et al., 2002). The consequences of such selection
192 may be treatment failure and zoonotic transmission of multi-resistant strains. Prudent use of
193 antimicrobial agents should be practiced by veterinary practitioners to alleviate this problem. It
194 would be a good practice to submit selected samples to laboratory analysis in order to confirm
195 diagnosis, to monitor the efficacy of antimicrobial therapy as well as to evaluate the effects of

196 antimicrobial policies. When empirical therapy is needed, clinical signs, cytology and local data on
197 antimicrobial susceptibility could be usefully employed to predict the resistance profile of the
198 pathogen involved and to select the most appropriate antimicrobial drug for treatment. Following
199 these rules will maximize the clinical efficacy of important antimicrobial agents in small animal
200 medicine by limiting development of resistance and emergence of clinically relevant phenotypes
201 such as AREF, multi-resistant *E. coli* or staphylococci (Guardabassi et al., 2008).

202 The results of this study were generated by measuring antimicrobial resistance in one *E. coli* and
203 one *Enterococcus* strain randomly isolated from each dog. Similar random, non-selective isolation
204 strategies are employed by DANMAP and other national surveillance systems in Europe. This
205 isolation method provides a good overview of the predominant types of resistance in the bacterial
206 populations of the faecal samples under study. However, clinically important resistance phenotypes
207 such as AREF or VRE are less likely to be detected because they may be present at low numbers in
208 the faecal flora. Accordingly, antimicrobial selective isolation methods should be considered as they
209 allow higher sensitivity by improving detection limits. To exemplify this concept, preliminary
210 studies at our laboratory indicate that AREF can be detected more easily in faecal samples when
211 using agar media supplemented with ampicillin.

212 In conclusion, although low levels of resistance occurred in indicator bacteria isolated from Danish
213 dogs, significantly higher prevalences of resistant *E. coli* were recovered from dogs exposed to
214 recent antimicrobial treatment and clinically relevant resistance phenotypes were detected among
215 canine enterococci. The first detection of AREF CC17 in healthy dogs is of concern to both animal
216 and human health and further investigation on the prevalence of AREF in dogs should be conducted
217 using selective media to enhance their detection. More generally, methods for detection and
218 assessment of antimicrobial resistance in animals should be tailored to the animal species of interest
219 and the specific objectives of each study.

220 **Table 1.** Prevalence (%) of antimicrobial resistance observed in canine *E. coli*, *E. faecalis* and *E.*
 221 *faecium* and corresponding resistance patterns previously reported for Danish broilers, cattle, pigs
 222 and humans (DANMAP 2006).

	Dogs		Broilers	Cattle	Pigs	Humans
	R ^a	I ^a				
<i>E. coli</i>						
Tetracycline	6	0	7	10	28 ^b	15 ^b
Florfenicol	0	0	0	0	<1	0
Ampicillin	9	2	17	2	20 ^b	19
Amoxicillin+clavulanic acid	0	0	0	0	0	0
Cephalothin	2	36	3	2	3	6
Ceftiofur	0	0	0	0	0	0
Sulfonamide	8	0	9	12	26 ^b	21 ^b
Trimethoprim	5	0	2	3	14 ^b	14
Gentamicin	0	0	0	0	<1	2
Streptomycin	9	7	11	11	41 ^b	19
Ciprofloxacin	0	0	7 ^b	0	<1	6 ^b
Nalidixic Acid	0	0	7 ^b	0	<1	6 ^b
<i>E. faecalis</i>						
Ampicillin	0	0	0	- ^a	0	0
Chloramphenicol	2	0	2	-	11	0
Ciprofloxacin	0	65	-	-	-	-
Erythromycin	8	29	20	-	38 ^b	7
Florfenicol	0	0	0	-	0	0

Gentamicin	2	0	0	-	4	0
Linezolid	0	16	0	-	0	0
Rifampicin	65	22	-	-	-	-
Tetracycline	31	2	27	-	85 ^b	39
Vancomycin	0	29	0	-	0	0
<i>E. faecium</i>						
Ampicillin	20	0	0 ^b	-	0 ^b	4
Chloramphenicol	0	0	0	-	<1	0
Ciprofloxacin	20	60	-	-	-	-
Erythromycin	30	40	29	-	34	8
Florfenicol	0	0	0	-	0	0
Gentamicin	0	0	0	-	0	0
Linezolid	0	0	0	-	0	0
Quino-/dalfopristin	0	20	1	-	1	0
Rifampicin	60	10	-	-	-	-
Tetracycline	30	10	7	-	61	8
Vancomycin	0	10	0	-	3	0

223 ^aR, resistance; I, intermediate resistance (categorized as susceptible in DANMAP 2006); -, data not
 224 available

225 ^bPrevalence of resistance significantly ($p < 0.05$) different from that recorded in canine bacteria

226

227

228

229

230

231 **Table 2.** Patterns of antimicrobial resistance observed in *E. coli*, *E. faecalis* and *E. faecium*.

Bacterial species	Resistance pattern	No. of isolates
<i>E. coli</i>	No resistance	100
	Amp	2
	Cep	3
	Str	1
	Amp, Str, Sul	2
	Amp, Str, Tet	2
	Amp, Sul, Tet	1
	Str, Sul, Tri	2
	Amp, Str, Sul, Tet, Tri	4
<i>E. faecalis</i>	No resistance	19
	Rif	24
	Tet	3
	Ery, Tet	2
	Rif, Tet	1
	Ery, Gen, Tet	1
	Chl, Ery, Rif, Tet	1
<i>E. faecium</i>	No resistance	2
	Rif	2
	Tet	1
	Amp, Ery	1
	Cip, Rif	2

Ery, Rif, Tet	1
Amp, Ery, Rif, Tet	1

232

233

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