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

Peter Damborg*, Anne H. Sørensen and Luca Guardabassi

Department of Veterinary Pathobiology, Faculty of Life Sciences, University of Copenhagen, Stigbøjlen 4, 1870 Frederiksberg C., Denmark

*Corresponding author:

Peter Damborg

Department of Veterinary Pathobiology, Faculty of Life Sciences, University of Copenhagen, Stigbøjlen 4, 1870 Frederiksberg C., Denmark

Tlf: +45 35332725; fax: +45 35332755; e-mail: peda@life.ku.dk

Abstract

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

Key words: antibiotic resistance, canine, enterococci

1. Introduction

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

2. Materials and methods

2.1. Sampling and collection of data on antimicrobial treatment

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

2.2. Bacterial isolation and identification

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

2.3. Antimicrobial susceptibility

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

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

2.4. Multilocus sequence typing

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

3. Results

3.1. Prevalence of bacterial indicators

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

3.2. Resistance in E. coli

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

3.3. Resistance in enterococci

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

3.4. Multilocus sequence typing

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

4. Discussion

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

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

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

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

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

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

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

[a]R, resistance; I, intermediate resistance (categorized as susceptible in DANMAP 2006); -, data not available

[b]Prevalence of resistance significantly ($p<0.05$) different from that recorded in canine bacteria

230

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

Bacterial species	Resistance pattern	No. of isolates
E. coli	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
E. faecalis	No resistance	19
	Rif	24
	Tet	3
	Ery, Tet	2
	Rif, Tet	1
	Ery, Gen, Tet	1
	Chl, Ery, Rif, Tet	1
E. faecium	No resistance	2
	Rif	2
	Tet	1
	Amp, Ery	1
	Cip, Rif	2

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

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