IN VITRO ANTIMICROBIAL RESISTANCE OF INTESTINAL *ESCHERICHIA COLI* AND ENTEROCOCCI IN CLINICALLY HEALTHY DOGS IN ESTONIA

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Abstract. The aim of this study was to estimate the antimicrobial resistance of intestinal *Escherichia coli* and enterococci and identify the risk factors that are associated with resistance of enteric microflora in clinically healthy dogs.

Fecal samples were collected from 86 clinically healthy dogs. Antibacterial susceptibility of *E. coli* and enterococci was determined using the disc diffusion assay on Mueller–Hinton agar.

E. coli was isolated in 68 of 86 (79.1 %) fecal samples, and *Enterococcus spp*. was isolated in 66 (76.7 %) cases. The resistance to at least one antimicrobial agent was found among 10.3 % (n = 7) of *E. coli* and 60.6 % (n = 40) of *Enterococcus* spp. isolates. No cefotaxime resistant *E. coli* and vancomycin resistant enterococci were found. The isolated enterococci were resistant to tetracycline (45.5 %) and ciprofloxacin (21. 2 %). Previous antibiotic treatment, dog age, bodyweight, living environment and travelling were not associated with the resistance of *E. coli* and enterococci.

This was the first study addressed to the issue of the resistance of indicator bacteria in dogs in Estonia. Although significant resistance to antibiotics was not detected and suspected risk factors did not influence the antimicrobial resistance, the potential transmission of resistant bacteria between animals and humans needs to be considered and investigated in future studies.

Keywords: antimicrobial resistance, intestinal microflora, dog

Introduction

Antimicrobial resistance is the most disturbing health problem in humans and veterinary medicine (Barton and Hart, 2001). The number of companion animals has been increasing steadily and the contact between humans and animals may promote the transmission of antimicrobial-resistant bacteria to humans (Tamang et al., 2012). Intestinal commensal bacteria, such as *Escherichia coli* (*E. coli*) and enterococci, are part of the normal enteric microflora. These bacteria act as indicators of antimicrobial selection pressure, and they may harbour a reservoir of antimicrobial resistance genes for pathogenic or zoonotic bacteria.

The resistance patterns of enteric bacteria change in response to increased antibiotic exposure (Houndt and Ochman, 2000; Maddison, et al., 2008). In dogs, after antibiotic treatment, the majority of fecal coliform rapidly developed a high level of antimicrobial resistance to enrofloxacin and amoxicillin (Boothe et al., 2011). The most active resistance among enterococci isolated from dogs and cats treated with antibiotics was observed for erythromycin and oxytetracycline, and considerable resistance was found to lincomycin, gentamycin and kanamycin (Kataoka et al., 2013). Therefore, the intestinal tract may be the basic site in organisms for the development and spread of resistant microbes (Harmoinen et al., 2004, Skurnik et al., 2006).

Antimicrobial resistance of indicator bacteria in clinically healthy animals has been studied primarily in pigs, cattle and poultry; most often during national antimicrobial monitoring programs. The antimicrobial resistance of normal microflora in healthy dogs is not often studied. The prevalence of resistant *E. coli* and enterococci is low in Finland and Canada (Rantala et al., 2004; Murphy et al., 2009), but a recent study from Portugal showed high resistance against several antibiotics (Leite-Martins et al., 2014). The colonization of healthy companion animals with different types of extended spectrum betalactamase (ESBL)-producing *Enterobacteriaceae* was found in some studies.. The frequency of isolation of ESBL ranged between 2.6 % and 3.8 % in the United Kingdom and Portugal to 12.2 % and 22 % in Tunisia and Kenya respectively (Costa et al., 2008; Wedley et al., 2011; Albrechtova et al., 2012; Sallem et al., 2013).

This study estimated the antimicrobial resistance of intestinal *E. coli* and enterococci in clinically healthy dogs in Estonia and identified the risk factors that are associated with the resistance of enteric microflora in healthy dogs.

Materials and methods

Data collection

The study was performed in clinically healthy dogs in Estonia. The dogs were selected randomly, with permission and interest of dog owners. The dogs were selected from those brought to veterinary clinics for vaccination or veterinary consultation. Only one (the oldest) dog from the same household was selected. The first inclusion criterion was that dogs were not treated with antimicrobials during the last three months. The data on the dogs' health, living environment and travelling history were collected from the dog owners. The information about the previous (last two years) antibiotic treatments was collected from the databases of veterinary clinics.

Description of the study group

A total of 86 dogs (53 females and 33 males) of 39 different breeds were included in the study. One dog was excluded due to fever (39.8ºC). Twenty-six dogs were of mixed breeds. The average age of dogs in the study group was 5.7 years (min. 3 month; max. 15 years), and the average bodyweight was 23.4 kg (min. 3.5 kg; max. 58 kg).

Clinical examination of dogs and collection of fecal samples

All dogs were examined clinically before fecal sample collection. Only clinically healthy dogs were included in the study: body temperature less <39.0ºC, heart rate <120/min, respiratory rate <30/min, and no visible enlargement of the main lymph nodes (Rijnberk et al., 2009). Five grams of fecal sample was collected immediately after defecation using a sterile spoon and collection tube. Fecal samples were placed in the refrigerator (+2-4 ºC) initially and thereafter stored at -80ºC. All collected fecal samples were sent to the Estonian Veterinary and Food Laboratory for the bacteriological analysis.

Laboratory analysis

The identification of *E. coli* and enterococci was performed according to accredited methods in the Estonian Veterinary and Food Laboratory.

The material for identification of *E. coli* was inoculated directly to eosin methylene blue (EMB) agar. The colonies of *E. coli* were identified based on the occurrence of a green-metallic sheen that appears on the surface of the bacterial colonies after incubation at 37°C overnight.

For identification of *Enterococcus faecalis/faecium,* an enrichment broth agar (6.5% NaCl Brain Heart Infusion (BHI)) incubated at 37°C overnight was used for the stabilization of enterococci before cultivation of into Slanetz–Bartley agar.

Up to four colonies with typical morphology of *E. coli*, *E. faecalis* / *E. faecium* were sub-cultivated on blood agar. Colonies were identified according to laboratory protocols. All pure isolates of *E. coli*, *E. faecium* and *E. faecalis* were stored (-80°C) for antimicrobial susceptibility testing.

Antimicrobial susceptibility

Antibacterial susceptibility was determined using the disc diffusion assay on Mueller–Hinton agar. Testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute protocols M02-A11 and VET01-A4. Quality control strains of *E. coli* ATCC® 25922 and *Enterococcus faecalis* ATCC 29212 were included with each batch of isolates tested. Epidemiological cut-off values (ECOFF) issued by the EUCAST (http://www.escmid.org) were used to interpret the results of susceptibility testing of indicator bacteria (*E. coli* and enterococci). The clinical breakpoints recommended for animal pathogens by CLSI VET01-S2 and M100-S22 were considered in the absence of EUCAST-issued ECOFFs.

The antimicrobial susceptibility of *E. coli* was tested for ampicillin, gentamycin, streptomycin, kanamycin, trimethoprim, sulfomethoxasole, tetracycline, nalidic acid, ciprofloxacin, cefotaxime and ceftazidime. The antimicrobial susceptibility of enterococci was tested for ampicillin, erythromycin, gentamycin, tetracycline, chloramphenicol, vancomycin, ciprofloxacin and linezolid.

The criteria for the interpretation of zone diameter used in this study are described in Table 1. Isolates with phenotypically identified acquired resistance to three or more antimicrobial classes were defined as multiresistant (Schwarz et al., 2010).

Data analysis

Stata 10.0 (StataCorp, Texas, USA) software was used for statistical analyses. A logistic regression model with backward elimination procedure was used to identify associations between the resistance of enterococci and *E. coli* and different risk factors. The resistance of enterococci and *E. coli* was an outcome variable. Estimated (anticipated) risk factors were categorized before statistical analyses. The full model includes the following parameters: dog age as a 4-level category variable (less than 1 years, 1–5 years, 5–10 years, more than 10 years), dog bodyweight as a 4-level category variable (less than 10 kg, 10–25 kg, 25–40 kg, 40–60 kg), living environment as a two-level variable (living inside but going out; living only outside), visiting another country in the last year (yes, no) and visit to veterinary clinics (yes, no). The Wald test was used to evaluate the overall significance of the categorical variables with more than two levels. Odds ratios (OR) with 95 % confidence intervals (95 % CI) were calculated. Statistical significance was set at $p \leq 0.05$.

Results

Occurrence of antimicrobial resistance

E. coli was isolated in 68 of the 86 (79.1 %) fecal samples, and *Enterococcus spp*. was isolated in 66 (76.7 %) cases. Resistance to at least one antimicrobial agent was found among 10.3 % (n = 7) of *E. coli* and 60.6 % (n = 40) of *Enterococcus* spp. isolates. Two *E. coli* and two *Enterococcus spp*. isolates were multiresistant.

All *E. coli* isolates were susceptible to cefotaxime and ceftazidime. Three (4.4 %) *E. coli* isolates were resistant to ampicillin and streptomycin, and two (2.9 %) of the isolates showed resistance against tetracycline, ciprofloxacin and sulfamethoxazole. In total, 45.5 % (n =

30) of enterococci were resistant to tetracycline, 21.2 % (n = 14) to ciprofloxacin and 10.6 % (n = 7) to erythromycin. None of isolated enterococci were resistant to vancomycin.

Risk factors of antimicrobial resistance

Sixteen of the 86 dogs (18.6 %) lived only outdoors, and 70 (81.4 %) lived indoors but walked outside regularly. Out of the 86 dog owners, 28 % (n = 24) had visited other countries during the last year. The main regions visited were Scandinavia and western parts of Europe. During last three years, 76.7 % (n = 66) of dogs have visited veterinary clinics, and 66.7 % (n = 44) of these dogs were treated with antibiotics. Health records and information on antibiotic treatment were available on 36 (87.8 %) dogs. The main purpose for antimicrobial treatment was traumas and urogenital tract infections (19.4 %), followed by an equal proportion (13.9 %) of ear and skin infections and respiratory infection. The most frequently used antibiotics were amoxicillin in combination with clavulane acid (83.3 %) and cephalosporins (19.4 %). The antibiotic resistance and anticipated risk factors are presented in Table 2.

Table 1. **Zone diameter interpretive criteria**

Concentration of AB in disc (µg)	*E. coli*		*Enterococcus* spp.	
	R < mm	R ≤ mm	R < mm	R ≤ mm
	EUCAST ECOFF	CLSI	EUCAST ECOFF	CLSI
Ampicillin 2 µg	–	–	10	–
Ampicillin 10 µg	14	–	–	–
Erythromycin 15 µg	–	–	–	13
Gentamycin 10 µg	16	–	–	–
Gentamycin 30 µg	–	–	8	–
Kanamycin 30 µg	–	13	–	–
Chloramphenicol 30 µg	17	–	–	12
Linezolid10 µg	–	–	19	–
Nalidic acid 30 µg	19	–	–	–
Streptomycin 10 µg	–	11	–	–
Sulfamethoxazole 250 mg	–	12	–	–
Tetracycline 30 µg	–	11	–	14
Trimethoprim 5 µg	20	–	–	–
Cefotaxime 5 µg	23	–	–	–
Ceftazidime 10 µg	22	–	–	–
Ciprofloxacin 5 µg	25	–	–	15
Vancomycin 5 µg	–	–	12	–

Table 2. **The anticipated risk factors and antibiotic resistance of isolated enterococci** (n=66)

	Number (%) of resistant isolates	p-value	Wald-test p-value
Total number of isolated enterococci (n=66)	40 (60.6)		
Previous antibiotic treatment			
No (n=35)	22 (62.9)		
Yes (n=31)	18 (58.1)	0.93	
Visit abroad			
No (n=47)	30 (63.3)		
Yes (n=19)	10 (52.6)	0.44	
Dog weight			0.91
<10 kg (n=17)	10 (58.8)		
>10-25 kg (n=20)	13 (65)	0.94	
>25-40 kg (n=19)	10 (52.6)	0.68	
>40-60 kg (n=10)	7 (70)	0.74	
Age of dogs			0.73
< 3 year (n=16)	11 (68.8)		
>3-5 year (n=21)	14 (66.7)	0.74	
>6-10 year (n=19)	10 (52.6)	0.42	
>11-15 year (n=10)	5 (50)	0.32	
Dog living environment			
Inside, walking outside (n=51)	31(60.8)	0.73	
Only outside (n=15)	9(60)		

We did not find any significant associations between resistance of enterococci and *E. coli* and anticipated risk factors. Only seven (10.3 %) *E. coli* isolates out of 68 were resistant to antibiotics and any of estimated risk factors did not associate ($p < 0.05$) with antimicrobial resistance of *E. coli.*

Discussion

The present study investigated the antimicrobial resistance of normal enteric microflora in clinically healthy dogs. The number of microbial isolates is quite small and the results of that investigation do not represent the antimicrobial resistance situation in dog's population in Estonia, but it gives a preliminary standpoint for future discussion and investigations. The antimicrobial resistance of *E.coli* was generally low in our study, which is consistent with other studies (Costa et al., 2008), but resistance among enterococci was prevalent. *E.coli* strains developed resistance against ampicillin, streptomycin and trimethoprim, and fecal enterococci were primarily resistant to tetracycline, erythromycin and ciprofloxacin. No cefotaxime-resistant *E. coli* or vancomycin-resistant enterococci were found in this study. The prevalence of cephalosporin-resistant *E. coli* varies between countries: 12 % in Canada (Murphy et al., 2009); 6 % in the USA (Shaheen et al. 2011); 5 % in Finland (Jalava et al. 2012); and 40.9 % in Croatia (Šeol et al. 2011). Other published studies reported that the prevalence of VRE in dogs in Spain was 17 % and 26 % in the Netherlands (Herrero et al., 2004; Van Belkum et al., 1996). Despite the fact that we did not find ESBL or VRE in this study, a great attention should be paid to these pathogens in future resistance monitoring.

The disc diffusion method for *in vitro* antimicrobial susceptibility testing was used in this study. This method is widely used for determinations of susceptibility of animal pathogens, especially in clinical work to determine the correct antimicrobial treatment. The primary disadvantage of this method in monitoring resistance development is that outcomes are reported on a qualitative basis (sensitive, intermediate or resistant), and subtle changes in susceptibility may not be noticeable. Therefore, comparisons of the results of studies using different methods for susceptibility testing are not acceptable (Schwarz et al., 2010).

Due to low number of resistant *E .coli* isolates, an evaluation of estimated risk factors and resistance by logistic regression model was not possible. We did not find an association between previous antibiotic treatment and antimicrobial resistance of enterococci. Rantala et al. (2008) also did not report an association between antimicrobial treatment and the resistance of enterococci, but *E. coli* resistance against a combination of sulfonamide-trimethoprim, streptomycin and amoxicillin was higher in dogs treated with antibiotics, although these associations were not statistically significant. Another study demonstrated that the resistance to beta-lactams was more common in fecal *E. coli* strains isolated from cefovecin-treated dogs compared to untreated dogs, but the resistance of enterococci was not altered (Lawrence et al., 2013). Previous scientific publications confirmed that dogs with a history of antimicrobial therapy in the past year had a higher risk of being carriers of ESBL-producing and plasmidic AmpC betalactamase-producing *E. coli* (Belas, et al. 2014). The retrospective data in the present study showed that antibiotics were primarily prescribed after clinical diagnosis, but data on bacteriological investigations were missing. The most frequently chosen antibiotics were beta-lactams, but no conclusion can be reached because of the small sample size and large variation. Shea et al. (2012) showed that doxycycline was prescribed in 58.8 % of cases without a clinical diagnosis, antibiotics were prescribed without infection in 38.4 % of cases, and antibiotics were used after a documented bacteriological diagnosis only in 17.5 % of cases. These results confirm that antibiotic treatment should be prescribed only when bacterial infection is expected based on clinical examinations or bacterial infection is diagnosed in the laboratory.

The resistance of *E.coli* against tetracycline was low in our study, but we found high resistance among the enterococci isolated from dogs that were not treated with tetracycline. Many studies show a high tetracycline resistance of *E.coli* isolated from the intestines of healthy dogs (Costa et al., 2008; Leener et al., 2005; Türkyilmaz et al., 2010; Damborg et al., 2008; Jackson et al., 2009), but an association between tetracycline treatment in dogs and the development of resistance was not found (Damborg et al., 2008). Tetracycline resistance is encoded by the *tet*M gene, which has a wide area of distribution and occurs in both Gram positive and Gram negative bacteria (Roberts, 1996). This characteristic allows resistance to tetracycline to be transferred from one bacterial strain or animal species to another. One possible route of distribution is food contaminated with resistant bacteria or distribution via the environment (Wu et al., 2013). Possible links between tetracycline resistant environmental bacteria and resistance of normal enteric microflora of dogs should be studied in the future.

Dog age and bodyweight was not a significant risk factor for resistance of enterococci ($p > 0.05$). Rantala et al. (2004) also did not find a significant association between dog age and the development of resistance. In addition, we did not find an association between dog living environment and resistance of enteric microflora. Only a few previous studies confirmed that the resistance of normal gut microflora is higher in dogs that lived in the country compared to dogs that were kept in town (Monaghan et al., 1981). Procter (2012) showed that *E. coli* strains isolated from large half-breed dogs showed higher resistance compared to strains isolated from small purebred dogs, and the dogs' living environments may play a role here. Skurnik et al. (2006) found that 17 % of the *E. coli* isolates from wild animals living in a low human density area were resistant to at least one antibiotic versus 49 % of isolates from wild animals living in a higher human density area. The potential threat posed by animals or animal food products as sources for resistant isolates cannot be ignored, but the current research has not identified the extent that livestock and pets contribute to the spread of resistance in human microflora.

Conclusions

In this study, *E. coli* and enterococci as a part of the normal enteric microflora of dogs did show different resistance to antibiotics, but the association between antimicrobial resistance and suspected risk factors was not proven.

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Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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