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Antimicrobial resistance of *Escherichia coli* isolated from healthy dogs in Lithuania

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ABSTRACT

The objective of this study was to establish the prevalence and antimicrobial resistant patterns of commensal *Escherichia coli* isolated from healthy dogs. In total, 55 rectal swabs from clinically healthy dogs, kept outside (n = 20), and inside a house (n = 20) and from dogs kept at an animal shelter (n = 15), were collected for isolation of *Escherichia coli*. Resistance patterns to 11 antimicrobial agents were tested using E-test (Epsilometer test) to determine the MIC (Minimum Inhibitory Concentration). Multiplex polymerase chain reaction (M-PCR) amplification was used to detect selected genes conferring resistance to beta-lactams, tetracycline, aminoglycoside, sulphanilamide, quinolone, and phenicol classes of antimicrobial agents. Forty-eight *E. coli* strains were isolated from 55 (87.3%) dogs. Multi-drug resistance was present in 38% of resistant isolates. *E. coli* isolates showed the highest resistance rates to streptomycin (85.1%), ampicillin (77.1%), sulfamethoxazole (70.8%) and tetracycline (64.6%). The isolates were most sensitive to enrofloxacin (87.5%) and chloramphenicol (72.9%). Bacterial resistance genes were determined to tetracycline *(tet)* (9.7%) trimethoprim/sulfamethoxazole *(dfrA1)* (16.7%,), and chloramphenicol *(catA1)* (5.5%). In general, the prevalence of antimicrobial resistance in *E. coli* isolates from shelter dogs population was higher than in those from dogs kept inside and outside ($P<0.05$). Companion animals in Lithuania are important reservoirs of resistant *Escherichia coli* strains. Only appropriate use of antimicrobials can minimize the spread of resistant bacteria among healthy and diseased animals and humans.

Key words: antimicrobial resistance; commensal *E. coli*; healthy dogs

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Introduction

Bacterial resistance to antibiotics is a world-wide problem in human and veterinary medicine. It is generally accepted that the main risk factor for the increase in the antibiotic resistance is extensive use of antibiotics. In fact, for the last 50 years, high levels of antibiotics have been commonly used for treatment and prevention of infectious diseases in humans and animals. This has led to the emergence and dissemination of resistant bacteria and resistance genes in wild populations (BOUROUNI et al., 2012).

Companion animals, such as dogs, play an important role in the exchanging of antimicrobial resistance determinants in bacterial populations, since they are exposed to antimicrobial agents (for treatment), similar to those used for humans (TAMANG et al., 2012). Through the years, the relationship between companion animals and humans has radically changed, and now cats and dogs are more and more in close contact with humans (MOYAERT et al., 2006). There have been several studies about outbreaks of multi-drug resistant bacterial infection, where a veterinary facility or animal shelter was the only common exposure for infected persons. These studies demonstrate that infected animals brought to veterinary clinics and animal shelters can be foci for nosocomial transmission to other animals, and for zoonotic transmission to humans (DAMBORG et al., 2016).

Escherichia coli (E. coli) are usually a commensal bacterium of humans and animals. Pathogenic variants can cause intestinal and extra intestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicaemia in both humans and animals (TADESSE et al., 2012). For this reason, faecal *E. coli* is considered to be a very good indicator for selection pressure in antimicrobial use and expected resistance problems in pathogens (COSTA et al., 2008).

The prevalence of antimicrobial resistance for *E. coli* strains in food producing and companion animals has been documented (SAWANT et al., 2007; AHMED et al., 2010, TADESSE et al., 2012; ÖSTERBERG et al., 2016). Antimicrobial resistant bacteria have been isolated from cattle, pigs, birds, horses, dogs, and cats (GUARDABASSI et al., 2004; MOYAERT et al., 2006; SAWANT et al., 2007; AHMED et al., 2010, LEONARD et al., 2012; TADESSE et al., 2012; ÖSTERBERG et al., 2016, GHOSH et al., 2017). The possibility of transmission of resistant *Escherichia coli* strains between companion animals and humans has been described (PLATELL et al., 2011). In Lithuania there are studies about antimicrobial resistance in *E. coli* isolates recovered from human clinical samples (POVILONIS et al., 2010) and clinical samples of poultry, calves and pigs (POVILONIS et al., 2010; ŠEPUTIENĖ et al., 2010), but to our knowledge, no study has been carried out on healthy pets.

The objective of this study was to establish the prevalence and antimicrobial resistant patterns of commensal *Escherichia coli* isolated from healthy dogs.

Materials and methods

Samples collection and bacterial isolation. In this study 55 clinically healthy dogs were investigated according to the requirements of the Law of the Republic of Lithuania on Animal Welfare Regulations (No. B1-866, 2012; Nr. XI-2271, 2012) which was approved by the Lithuanian Committee of Veterinary Medicine and Zootechnics Sciences (Protocol No.09/2012). The rectal swabs were collected by veterinarians using sterile cotton swabs. The swab was inserted approximately 1 cm into the rectum of pets then placed in Amies transport medium (Amies, Liofilchem, Italy) and stored at +4 °C until processing within 24 h.

Forty of the investigated dogs were kept inside or outside a house, and fifteen of the dogs were living in an animal shelter. The rectal samples were collected from clinically healthy dogs of different ages, sexes and breeds (Table 1). None of the investigated animals had taken antimicrobials during the four months prior to the sampling.

Table 1. The characteristics of the clinically healthy dogs examined

	Age				Sex		Bread	
Day time location	≤1	1-5	6-10	≥10	Male	Female	Purebred	Mixed-breed
Outside the house (n = 20)	1	12	6	1	8	12	15	5
Inside the house (n = 20)	4	10	4	2	10	10	16	4
Animal shelter (n = 15)	2	11	2	0	9	6	6	9

Rectal swabs were streaked onto MacConkey's agar (Liofilchem, Italy) and incubated at +37 °C for 18 to 24 h. Rose pink colonies were observed after incubation, then a single colony was streaked onto TBX agar (Liofilchem, Italy) and incubated at +37 °C for 24 h. The next day blue/green colonies were considered as *E. coli* and were identified by biochemical testing with an API 20E system (BioMérieux, La Balme Les Grottes, France).

Antimicrobial susceptibility testing. Antimicrobial minimum inhibitory concentrations (MICs) were determined using an E-test system (Epsilometer test) in accordance with the manufacturer's instructions (MIC Test strip, Liofilchem, Italy).

E. coli isolates were sub-cultured onto Nutrient agar (Liofilchem, Italy) and incubated at +37 °C temperature for 24 h. The next day at least three colonies of each isolate were selected and re-suspended in Mueller Hinton broth (Liofilchem, Italy) until the 0.5 standard was reached (DEN-1 McFarland Densitometer, Biosan, Latvia). The suspension was swabbed onto Mueller-Hinton agar (Liofilchem, Italy) and the plates were dried in a bio hood at room temperature. Two E-test strips containing a predefined gradient of the antimicrobials were placed on each plate. The E-test strips were placed

onto inoculated agar surfaces with the minimum inhibitory concentration (MIC) scales facing upwards. The plates were then incubated in aerobic conditions at +35 ± 2 °C for 18 to 24 h. The MICs were read according to the E-test reading guide: where the edge of the inhibition ellipse intersected the side of the strip (AB Biodisk, Solna, Sweden). Eleven antimicrobial agents were tested in this study: ampicillin, tetracycline, streptomycin, sulfamethoxazole, chloramphenicol, polymyxin B, trimethoprim/sulfamethoxazole, enrofloxacin, nitrofurantoin, gentamicin, and amoxicillin/clavulanic acid (Trek Diagnostic Systems, Cleveland, OH). The results of these investigations were used to categorise the isolates as susceptible, intermediate or resistant to antimicrobials, according to the MIC breakpoints reported by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017). Susceptibility to chloramphenicol, polymyxin B and tetracycline was calculated using breakpoints derived from human data, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2015). A reference strain of *E. coli* ATCC 25922 was used for quality control for antimicrobial susceptibility testing.

Table 2. Sequences of oligonucleotide primers used in PCR assays for identification of antimicrobial resistance genes in *E. coli* isolates

Antimicrobial agent	Targe gene	Nucleotide Sequence	Product length (base pairs)	Reference
Aminoglycoside	*aadA1*	TATCCAGCTAAGCGCGAACT ATTTGCCGACTACCTTGGTC	447	Van et al., 2008
	aac(3)-IV	CTTCAGGATGGCAAGTTGGT TCATCTCGTTCTCCGCTCAT		
Tetracycline	*tet(A)*	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	577	Randall et al., 2004
	tet(B)	CCTCAGCTTCTCAACGCGTG GCACCTTGCTGATGACTCTT	634	Randall et al., 2004
Trimethoprim/ sulfamethoxazole	*dfrA1*	GGAGTGCCAAAGGTGAACAGC GAGGCGAAGTCTTGGGTAAAAAC	367	Toro et al., 2005
Quinolones	*qnrA*	GGGTATGGATATTATTGATAAAG CTAATCCGGCAGCACTATTTA	670	Mammeri et al., 2005
Sulphanilamide	*sul1*	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	822	Van et al., 2008
Beta-lactams	bla_{SHV}	TCGCCTGTGTATTATCTCCC CGCAGATAAATCACCACAATG	768	Van et al., 2008
	bla_{CMY}	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	Van et al., 2008
Phenicol	*catA1*	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	547	Van et al., 2008
	cmlA	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	698	Van et al., 2008

The isolates were examined for resistance genes using multiplex polymerase chain reaction (M-PGR) according to MOMTAZ et al. (2012). Beta-lactams-resistant isolates were investigated for the presence of *blaSHV* and *blaCMY* genes. Tetracycline-resistant isolates were examined for *tetA* and *tetB* genes. Strains resistant to trimethoprim/sulfamethoxazole were investigated for *dfrA1*. Aminoglycoside-resistant isolates were examined for *aadA1* and *aac(3)-IV* genes. Strains resistant to sulfamethoxazole were investigated for the *sul1* gene, and strains resistant to enrofloxacin - for the *qnrA* gene. Chloramphenicol-resistant strains were investigated for the *catA1* and *cmlA* genes. The primers used in M-PGR are described in Table 2 (MOMTAZ et al., 2012).

Bacterial DNA from *E. coli* was extracted with a Chelex 100 (Sigma, USA) according to the manufacturer's instructions. The colony of each *E. coli* strains from Nutrient agar (Liofilchem, Italy) was picked and placed into a separate sterile Eppendorf tube, containing 200 µL of 5% Chelex solution. The suspension was heated at +80 °C for 25 min and boiled at +95 °C for 10 min. The heated solution was then centrifuged for 3 min at 10 000 rpm. The supernatants were transferred to new sterile Eppendorf tubes and used as template DNA in PCR.

The PCR amplifications were performed in a final volume of 25 µL including 10 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100; MBI, Ferment), 1.5 mM $MgCl_2$, 200 µm of each dNTP (MBI, Fermentas), 1 IU of Taq DNA polymerase (MBI, Ferment), 1 µL of each of the oligonucleotides and 5 µL of template DNA. The amplifications were performed on a PTC-100 programmable thermal controller (MJ Research Inc., USA) under the following conditions: initial denaturation at +95 °C for 3 min, followed by thirty-five cycles of +94 °C for 1 min, ~55 °C for 90 s, +72 °C for 1 min and a final extension step of +72 °C for 10 min. Electrophoresis of PCR products was performed in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), at 100 V for 60 min. PCR products were analysed in 1% Top Vision LE GQ Agarose gels (MBI, Fermentas) with 1.5% ethidium bromide under a UV lamp. The GeneRuler TM 100 bp DNA Ladder (MBI, Fermentas) was used to evaluate the sizes of PCR products. Strains of *E. coli* ATCC 25922 as the positive control, and sterile distilled water as the negative control were used in the PCR.

Statistical analysis. Descriptive statistical analyses were calculated using the SPSS 13.0 statistical package for Windows (2004). The Chi-squared ($\chi 2$) test was used to examine the association of the prevalence of *E. coli* with the dogs' age, sex, breed, and housing situation. The Kruskal-Wallis test was used to evaluate the correlation between antimicrobial resistances of *E. coli* with the housing conditions and the ages of the pets. The P values $P<0.05$, $P<0.01$ and $P<0.001$ were considered statistically significant.

Results

To assess the prevalence of *E. coli* in clinically healthy dogs, 55 faecal samples were investigated. A total of 48 *E. coli* strains were isolated from 55 (87.3%) dogs. Eighteen (37.5%) bacterial isolates were retrieved from dogs kept inside a house, 20 (41.7%) from dogs kept outside, and 10 (20.8%) - from dogs kept in an animal shelter. The pets' housing situation had a significant influence (P<0.01) on the occurrence of *E. coli* in the dogs. A higher prevalence of these bacteria was obtained from dogs kept outside a house than from dogs kept inside and in a shelter.

The prevalence of *E. coli* was found to be higher in female dogs than in male dogs. *E. coli* more commonly colonized purebred dogs (Table 3). Regarding the age of animals, it was found that dogs in the age groups from 1 to 5 years old and 6 to 10 year-old age ranges showed a rather high occurrence of these bacteria, as compared to the age groups of less than one and greater than 10. However, none of these differences were statistically significant (P>0.05).

Table 3. The prevalence of *E. coli* at rectum of dogs according to the main dog's characteristic

Animal characteristic	Day time location								
	Outside only (n = 20)			Inside the house (n = 18)			Animal shelter (n = 10)		
	n	%	P-value	n	%	P-value	n	%	P-value
Sex of dogs									
Female	12	60	1	9	50	1	7	70	0.329
Male	8	40		9	50		3	30	
Age of dogs									
≤ 1	1	5	1	3	16.7	0.763	1	10	0.912
1-5	12	60		9	50		7	70	
6-10	6	30		4	22.2		2	20	
≥10	1	5		2	11.1		0	0	
Bread of dogs									
Pure	16	80	0.426	14	77.8	0.217	5	50	1
Mixed	4	20		4	22.2		5	50	

P-value considered statistically significant at P<0.05

The susceptibility of *E. coli* isolates to eleven antimicrobial agents is shown in Table 4.

Overall, of the 48 *E. coli* tested by E-test, 24 (49.5%) isolates were resistant to at least one antimicrobial agent. High resistance rates were observed to streptomycin (85.1%), ampicillin (77.1%), sulfamethoxazole (70.8%), and tetracycline (64.6%). Moderate resistance rates were found to nitrofurantoin (47.9%), gentamicin and polymyxin B (41.7% each), amoxicillin/clavulanic acid (39.6%), and trimethoprim/sulfamethoxazole (37.5%). Lower resistance rates were observed for enrofloxacin (27.1%) and chloramphenicol (12.5%). Resistance to ampicillin, amoxicillin/clavulanic acid, streptomycin, gentamicin, trimethoprim/sulfamethoxazole, tetracycline and sulfamethoxazole was the most common combination in the *E. coli* isolates.

Resistance to three or more antimicrobials were observed in 37.5% (18/48) of the *E. coli* tested. *E. coli* isolates from shelter dogs (70%) had a significantly higher prevalence of multi-drug resistance to antimicrobial agents than that observed for *E. coli* isolates from dogs kept inside and outside a house (40%; $P<0.05$).

Resistance to gentamicin (90%) and polymyxin B (100%) was determined more often in the isolates from shelter dogs than in those isolated from dogs kept inside and outside ($P<0.05$). Isolates from the population of shelter dogs were more frequently resistant to chloramphenicol (40%) and enrofloxacin (50%) than isolates obtained from dogs kept outside ($P<0.05$). Isolates from dogs kept outside were more frequently resistant to enrofloxacin (38.9%) comparable with isolates from dogs kept inside (5%) ($P<0.05$). The antimicrobial resistance patterns of the 48 *E. coli* isolates against antimicrobial agents are shown in Table 5.

The age of dogs has a significant influence on the occurrence of *E. coli* resistance in dogs kept inside and outside a house ($P<0.05$; $P<0.01$). Isolates obtained from 1 to 5 year old dogs kept outside a house and from 5 to 10 year old dogs kept inside were more resistant to streptomycin, chloramphenicol and amoxicillin/clavulanic acid ($P<0.05$) than bacteria isolated from the remaining age groups of the same dog populations. Resistance to sulfamethoxazole and enrofloxacin ($P<0.01$) was more often determined in bacteria isolated from 5 to 10 year old dogs kept outside a house than in other isolates of *E. coli.*

A total of 31 tetracycline-resistant *E. coli* isolates were analysed by multiplex PCR for *tetA* and *tetB* gene identification. Ten percent (3/31) of these isolates were positive for *tet* genes: the *tetA* gene was detected in 6.4% and *tet*B in 3.2% of *E. coli.* The *catA1* gene was identified in 16.7% (1/6) of the chloramphenicol-resistant isolates. Only 5.5% (1/18) trimethoprim/sulfamethoxazole-resistant isolate had *dfrA1* gene encoding resistant to trimethoprim.

Thirty-seven of the *E. coli* isolates showed resistance to ampicillin and nineteen to amoxicillin/clavulanic acid. No genes encoding resistance to beta lactams antibiotics (bla_{SHV} and bla_{CMY}) were detected in resistant strains. No genes encoding resistance to aminoglycosides (*aac(3)-IV* and *aadA1),* fluoroquinolone (*qnrA*), sulfamethoxazole *(sul1)* and trimethoprim/sulfamethoxazole *(cml*A) were detected by PCR in any isolates.

Table 4. The activity of antimicrobial agents against 48 *E. coli* strains isolated from clinically healthy dogs

Antimicrobial agents	MIC vealue (µg/mL)											
	0.095	0.19	0.38	0.5	0.75	1	1.5	2	3	4	6	8
Ampicillin	-	-	-	-	-	-	-	-	1	4	3	3
Amoxicillin/ Clavulanic acid	-	-	-	-	-	4	-	3	6	2	9	5
Chloramphenicol[1]	-	-	-	2	1	4	-	1	3	11	7	7
Enrofloxacin	3	12	9	10	1	-	-	1	-	-	1	4
Gentamicin	-	-	-	2	2	-	12	-	12	2	3	2
Nitrofurantoin	-	-	-	-	-	1	4	-	2	2	-	-
Polymyxin B[1]	-	-	2	5	3	7	5	6		2		2
Trimethoprim/ Sulfamethoxazole	5	10	8	5	-	2	-	-	-	1	-	7
Tetracycline[1]	-	-	-	-	-	-	-	1	5	2	8	1
Sulfamethoxazole	-	-	-	3	-	2	-	1	8	2	-	-
Streptomycin	-	-	-	1	-	1	-	-	5	-	9	-

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Table 4. The activity of antimicrobial agents against 48 *E. coli* strains isolated from clinically healthy dogs (continued)

Antimicrobial agents	MIC vealue (µg/mL)										MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
	12	16	24	32	64	96	128	192	256	512			
Ampicillin	3	6	-	12	16	-	-	-	-	-	3-64	≥24	≥32
Amoxicillin/ clavulanic acid	3	4	9	2	1	-	-	-	-	-	1-64	6	≥24
Chloramphenicol[1]	-	2	4	1	2	3	-	-	-	-	0.5-96	4	24
Enrofloxacin	-	2	-	5	-	-	-	-	-	-	0.095-32	0.38	16
Gentamicin	5	4	-	4	-	-	-	-	-		0.5-32	≥3	≥16
Nitrofurantoin	6	3	2	3	2	2	11	3	6	1	1-512	≥32	192
Polymyxin B[1]	4	5	1	-	6	-	-	-	-	-	0.38-64	1.5	≥32
Trimethoprim/ Sulfamethoxazole[1]	5	5	-	-	-	-	-	-	-	-	0.095-16	0.38	12
Tetracycline[1]	-	25	3	3	-	-	-	-	-	-	2-32	≥12	16
Sulfamethoxazole	-	6	-	6	3	9	3	-	5	-	0.05-256	16	128
Streptomycin	-	6	5	6	5	-	7	-	3	-	0.5-256	≥16	≥96

MIC_{50}, lowest concentration that inhibit at least 50% of the isolates; MIC_{90}, lowest concentration that inhibit at least 90% of the isolates. [1]Indicates breakpoints derived from human breakpoints used (CLSI, 2015).

Table 5. Antimicrobial susceptibility profiles among the *E. coli* strains isolated from different populations of dogs

Antimicrobial agents	Day time location	Resistant		Susceptible		P-value
		n	%	n	%	
Amoxicillin/ Clavulanic acid	Inside the house	6	33.3	12	66.7	P>0.05
	Outside	7	35	13	65	
	Animal shelter	6	60	4	40	
Ampicillin	Inside the house	13	72.2	5	27.8	P>0.05
	Outside	14	70	6	30	
	Animal shelter	10	100	0	0	
Gentamicin	Inside the house	6	33.3	12	66.6	P<0.01
	Outside	5	25	15	75	
	Animal shelter	9	90	1	10	
Enrofloxacin	Inside the house	7	38.9	11	61.2	P<0.01
	Outside	1	5	19	95	
	Animal shelter	5	50	5	50	
Nitrofurantoin	Inside the house	8	44.4	10	55.6	P>0.05
	Outside	8	40	12	60	
	Animal shelter	7	70	3	30	
Chloramphenicol	Inside the house	0	0	18	100	P<0.05
	Outside	2	10	18	90	
	Animal shelter	4	40	6	60	
Streptomycin	Inside the house	15	83	3	16.7	P>0.05
	Outside	16	80	4	20	
	Animal shelter	10	100	0	0	
Polymyxin B	Inside the house	5	27.8	13	72.2	P<0.001
	Outside	5	25	15	75	
	Animal shelter	10	100	0	0	
Sulfamethoxazole	Inside the house	13	72.2	5	25.7	P>0.05
	Outside	13	65	7	35	
	Animal shelter	8	80	2	20	
Trimethoprim/ Sulfamethoxazole	Inside the house	8	44.4	10	55.6	P>0.05
	Outside	5	25	15	75	
	Animal shelter	5	50	5	50	
Tetracycline	Inside the house	11	61.1	7	38.9	P>0.05
	Outside	11	55	9	45	
	Animal shelter	9	90	1	10	

P-value considered statistically significant at P<0.001, P<0.01 and P<0.05.

Discussion

Resistance of commensal *E. coli* to many frequently used antimicrobials is a global problem in the chemotherapy of common bacterial diseases, including pyoderma, ear and wound infections, gastroenteritis and urinary tract infections (PEDERSEN et al., 2007). Members of most classes of antimicrobials, such as tetracyclines, macrolides, lincosamides, chloramphenicols, aminoglycosides, penicillins and cephalosporins, have been used for long periods in both human and veterinary medicine, and the corresponding resistance genes have been identified in bacteria from humans and pet animals. The occurrence of the same resistance gene in bacteria from different sources suggests the transfer of the resistance gene (GUARDABASSI et al., 2004).

A high percentage was detected of resistance of the faecal *E. coli* isolated from clinically healthy dogs to tetracycline, sulfamethoxazole, ampicillin, and streptomycin (64.6-85.1%). This is not surprising as these antimicrobial agents are antimicrobials commonly used in small animal veterinary practices in Lithuania. Similar resistance rates to these antimicrobial agents have been reported for *E. coli* isolates from pets, and food-producing animals in Ireland (KARCZMARCZYK et al., 2011), as well for isolates from clinically healthy and diarrheic pets in Brazil (PAULA et al., 2008). High percentages of resistance to streptomycin (96.4%), tetracycline (53%) and aminopenicillins (70.2%) have been reported for *E. coli* isolated from diseased dogs and cats in Poland (RZEWUSKA et al., 2015). Resistance to similar antimicrobial agents was reported for *E. coli* isolated from clinical specimens from humans and food animals in the United States. *E. coli* isolates from humans were most often resistant to sulphonamide (19.9%), followed by tetracycline (18%) and ampicillin (16.5%). High resistance rates to tetracycline (71.1%), streptomycin (59%), sulphonamide (57.7%), and to ampicillin (34.1%) have been recorded for *E. coli* isolates from animal sources (TADESSE et al., 2012). The explanation of the high level of resistance among *E. coli* isolates to these antimicrobial agents may be the widespread use of broad-spectrum antimicrobials. Ampicillin is often used as a first-line antibiotic for empirical treatment of bacterial diseases in animals. Other generally effective antibiotics used empirically for prophylaxis and treatment are potentiated sulphonamides, tetracycline, chloramphenicol or cephalosporins. These are bacteriostatic agents, which generally demonstrate good efficacy against Gram-positive and/or negative bacteria (TADESSE et al., 2012; CHANG et al., 2015).

In the present study, a moderate level (37.5-41.7%) of resistance was identified of faecal *E. coli* isolates from healthy dogs to amoxicillin/clavulanic acid, gentamicin and trimethoprim/sulfamethoxazole, but it was higher than the rates reported (2-3%) in Canada (MURPHY et al., 2009) or (7.3-25.2%) in Denmark (PEDERSEN et al., 2007). In the United States, low percentages of resistance (9.1-15.6%) to amoxicillin/clavulanic acid, gentamicin and trimethoprim/sulfamethoxazole have been detected for *E. coli*

isolated from humans (0.1-2.4%) and food producing animals (TADESSE et al., 2012). An increase in resistance to these antimicrobial agents was determined in *E. coli* isolates from humans in Switzerland (BLAETTLER et al., 2009). Common use of antimicrobials may explain the rather high resistance rates for amoxicillin/clavulanic acid, gentamicin and trimethoprim/sulfamethoxazole. The results indicate that these antimicrobial agents should be used only if the susceptibility of the bacteria is confirmed by *in vitro* study.

In this study, *E. coli* isolated from healthy pets showed a moderate level (47.9% - 41.7%) of resistance to nitrofurantoin and polymyxin B. In small animal practice, the use of nitrofurantoin has been gradually abandoned due to its higher toxicity and lower pharmacokinetic performance (MAALAND and GUARDABASSI, 2011). The increased level of resistance to nitrofurantoin may be explained by the fact that in veterinary clinical practice the use of nitrofurantoin may be indicated for treatment of urinary tract infection caused by multidrug-resistant *E. coli* bacteria or methicillin-resistant staphylococcus, which are otherwise difficult to treat using conventional veterinary antimicrobial products (GUAY, 2001). The same experience of using these antimicrobials is applied in Lithuanian small animal veterinary clinics. The use of polymyxin s is similar to nitrofurantoin, and these polycationic antimicrobial peptides are currently the last-resort antibiotics for treatment of infection caused by multidrug-resistant Gram-negative bacteria (FALAGAS et al., 2005). The possibility that polymyxin -resistant, community-acquired strains exist increases the challenge of treating patients with infections caused by such organisms. It is important to mention that 37.5% of our isolated *E. coli* strains showed multi-drug resistance (MDR). MDR bacteria have been isolated from many animal species, including pigs, cattle, chickens, turkeys, dogs, cats, rodents (HO et al., 2011; CUNHA et al., 2014; RZEWUSKA et al., 2015) and humans (BAILEY et al., 2010). In companion animals, a moderate level of multi-drug resistance of *E. coli* has been found: 28.9% in the United States (SHAHEEN et al., 2010), and 43.3% in Japan (HO et al., 2010). Our findings suggest that inadequate selection and use of antimicrobials may lead to antimicrobial resistance in these bacteria, and make the treatment of bacterial infections more difficult.

A low percentage of *E. coli* isolates resistant to enrofloxacin and chloramphenicol was found in the present study. Fluorquinolones are available for treatment of companion animals and humans in many European countries (BLAETTLER et al., 2009; RZEWUSKA et al., 2015). Over the years, the prevalence of fluorquinolone resistance has increased in bacteria isolated both from human patients and animals (BLAETTLER et al., 2009; GIBSON et al., 2010). The chloramphenicol resistance level found in this study was higher than those reported from healthy dogs in Denmark (0.6-3.9%) and Canada (0.5-5%) (PEDERSEN et al., 2007; MURPHY et al., 2009). However, in this study, enrofloxacin and chloramphenicol demonstrated a low level of *in vitro* resistance, which suggests that they may be used as adequate alternative therapy for animal infections, if other appropriate antimicrobial agents are not effective.

Tetracycline resistance in the *E. coli* isolates described in this study was associated with *tetA* (6.4%) and *tet*B (3.2%) genes. The most common resistance mechanism found in Gram-negative bacteria obtained from humans (WILKERSON et al., 2004) and animals (WILKERSON et al., 2004; COSTA et al., 2008; AHMED et al., 2010) is the energy-dependent efflux pump system (efflux genes), encoded by *tetA, tetB, tetC, tetD*, and *tetG*, with *tetA* and *tetB* genes being the most frequently described. Efflux genes are normally associated with large plasmids, which often carry other antibiotics resistance genes, heavy metal resistance genes, and/or other pathogenic factors, such as toxins (DIARRASSOUBA et al., 2007; OLOWE et al., 2013). Ribosomal protection mechanisms are more common among Gram-positive bacteria (OLOWE et al., 2013). The results of our study suggest the possibility that *E. coli* from companion animals may be a reservoir for tetracycline resistance. Regarding other isolates which were phenotypically resistant against tetracycline, and did not carry *tetA* or *tetB* genes, the resistance was most likely due to the presence of other tetracycline resistance genes eg., *tetE* or *tetD* (OLOWE et al., 2013).

A small number (5.5%) of trimethoprim/sulfamethoxazole-resistant isolates were positive to the *dfrA1* in the present study. Epidemiological studies indicate that the *dfrA1* gene is very common among trimethoprim-resistant *E. coli* isolates of animal and human origin (COSTA et al., 2008; AHMED et al., 2010). It has been proven that the *dfrA1* gene spreads rapidly on the transposon *Tn7* and is the most prevalent gene responsible for trimethoprim resistance in *E. coli* isolates (TOWNER et al., 1994; COSTA et al., 2008; AHMED et al., 2010). We suppose that other *E. coli* isolates which do not carry the *dfrA1*gene, may be resistant to trimethoprim through the prevalence of *dfrA14, dfrA7, dfrA1, dfrA8, dfrA13, dfrA5, dfrA12, dfrA 9* or *dfrA 17* genes (LEE et al., 2001; KANG et al., 2005; AHMED et al., 2010). A low percentage (16.1%) of *catA1* gene encoding the synthesis of chloramphenicol acetyltransferase and the absence of the *cmlA* gene was found in the chloramphenicol resistant *E. coli* isolates in the present study. However, the small number of the *catA1* gene identified by PCR suggests that the observed chloramphenicol resistance was possibly present due to the different types of *cat* genes (NG et al., 2014). No beta-lactam resistance encoded genes (bla_{SHV} and bla_{CMY}) were detected in the ampicillin and amoxicillin/clavulanic acid resistant *E. coli* isolates in this study. There are several hundred types of beta-lactamase enzymes, encoded by different types of *bla* (beta-lactamase) genes. Several studies have reported that TEM beta-lactamase genes (i.e. TEM-1 beta-lactamase gene) are the most prevalent in ampicillin resistant *E. coli* of animal origin, as well as being commonly reported in human *E. coli* isolates of hospital origin (AHMED et al., 2010). Additional investigation of the TEM-1 genes may be necessary to identify the mechanism leading the resistance against beta-lactamic antimicrobials.

E. coli resistance to gentamicin is most commonly mediated by aacC2, *aac*(3)-I, *aac*(3)-II, *aac*(3)-IV, *ant*(2)-I and aadB genes. The increasing frequency of genes encoding these enzymes in clinical isolates of human and animal origin has been reported by HO et al. (2010), KARCZMARCZYK et al. (2011), SOLEIMANI et al. (2014) and CHANG et al. (2015). No *aac*(3)-IV and *aadA* genes in gentamicin-resistant *E. coli* isolates were identified by multiplex PCR in the present study. We were not able to identify resistance to quinolones and sulphanilamides with *qnrA* and *sul1* genes. Therefore, further characterization of the mechanisms of resistance to those antimicrobial agents is needed.

The prevalence of antimicrobial resistance for the *E. coli* isolates from the shelter dog population was significantly higher than for isolates from populations of outdoor and indoor dogs. Several studies (GINGRICH et al., 2011; PROCTER et al., 2014) showed that animals in the shelter environment may be at increased risk of becoming colonized or diseased with a variety of infectious agents. These dogs may serve as reservoirs of resistant strains of bacteria for several reasons, including high animal density, the potential for nosocomial spread by staff members and volunteers, suboptimal cleaning and disinfectant routines, the unknown pathogen carriage status of the majority of animals, widespread use of antibiotics, and stress from the environment. DE GRAEF et al. (2004) reported that multi-drug resistance was more frequent in breeding kennel dogs than in individually owned dogs. These findings support the notion that close contact between dogs, whether living together or staying together for a large part of the day, may increase the likelihood of sharing antimicrobial resistant bacteria and resistance genes (PROCTER et al., 2014).

Our study may be influenced by the consequences of a previous study. In 2010, veterinarians from small animal clinics in city X were asked for information on the most commonly used antimicrobials for the treatment of Gram negative bacterial infections, including *E. coli*. According to the veterinarians' answers the most frequency used antimicrobial materials were selected for this study. At present, the use of antimicrobial agents is changing, and veterinarians often choose cephalosporins since the prevalence of antimicrobial resistance is increasing. In a future study, it is necessary to investigate the resistance of commensal flora to different generations of cephalosporins.

In conclusion, clinically healthy dogs are important reservoirs of antimicrobial resistance to commensal bacteria. A commensal strain may later cause infection in the same or another host, or transfer its resistance genes to other bacteria. To our knowledge, this is the first study in our country which has investigated the prevalence and antimicrobial resistant patterns of commensal *E. coli* strains in healthy dogs. Therefore, the findings of this study indicate that companion animals in Lithuania are important reservoirs of antimicrobial resistance to commensal bacterial strains, and deserve more detailed studies.

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SAŽETAK

Cilj ovoga istraživanja bio je utvrditi prevalenciju i antimikrobno rezistentne oblike komenzalne bakterije *Escherichia coli*, izolirane iz zdravih pasa. Za izolaciju bakterija *Escherichia coli* korišteno je ukupno 55 briseva rektuma zdravih pasa od kojih je jedna skupina držana na otvorenom (n = 20), jedna skupina u kući (n = 20), a jedna je skupina držana u skloništu za životinje (n = 15). Analiza oblika rezistencije na 11 antimikrobnih sredstava provedena je E-testom (test Epsilometar) za određivanje minimalne inhibitorne koncentracije (MIK). Multipleks reakcija lančanom polimerazom (M-PCR) upotrijebljena je za pronalaženje odabranih gena odgovornih za otpornost na beta-laktame, tetraciklin, aminoglikozid, sulfanilamid, kinolon i fenikol klase antimikrobnih sredstava. Od 55 pasa izolirano je 48 sojeva (87,3 %) *E. coli*. Višestruka otpornost na lijekove bila je prisutna u 38 % rezistentnih izolata. Izolati *E. coli* pokazali su najveću stopu rezistencije na streptomicin (85,1 %), ampicilin (77,1 %), sulfametoksazol (70,8 %) i tetraciklin (64,6 %). Najveća osjetljivost izolata utvrđena je na enrofloksacin (87,5 %) i kloramfenikol (72,9 %). Bakterijski geni za otpornost određeni su za tetraciklin (*tet*) (9,7 %) trimetoprim/sulfametoksazol (*dfrA1*) (16,7 %) i kloramfenikol (*catA1*) (5,5 %). Općenito, prevalencija antimikrobne rezistencije izolata *E. coli* iz skupine pasa držanih u skloništima bila je veća nego kod pasa držanih u kući ili na otvorenom ($P<0,05$). U Litvi su životinje za druženje važan rezervoar rezistentnih sojeva bakterije *Escherichia coli*. Samo prikladna uporaba antimikrobnih lijekova može smanjiti širenje otpornih bakterija između zdravih i bolesnih životinja odnosno ljudi.

Ključne riječi: antimikrobna rezistencija; komenzalna *Escherichia coli*; zdravi psi