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Original article

PREVALENCE OF RESISTANCE TO SOME BETA-LACTAMS AMONG COMMENSAL CANINE *E. COLI* ISOLATES

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Summary

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The sensitivity of 80 *E. coli* strains isolated from canine rectal swabs to antimicrobial drugs was tested in this study. The results showed 47.5% resistance to ampicillin, 18.7% to the combination amoxicillin/clavulanic acid and 6.2% to cephalothin. The percentage of *E. coli* isolates resistant to tetracycline was 26.2%, to ciprofloxacin 12.5%, and to gentamicin 10%. The resistance to cefotaxime and ceftazidime was the lowest (1.2% and 2.5% respectively). Determined MIC_{90} of ampicillin were 16 µg/mL, and of amoxicillin/clavulanic acid and cephalothin 8 µg/mL. The main resistance genotype of isolates to tested beta-lactams was associated with presence of bla_{TEM}.

Key words: commensal *E.coli*, dogs, resistance to beta-lactams

INTRODUCTION

Antimicrobial resistance is an important problem with serious public health impact. The spread of resistant microbial pathogens and resident microorganisms leads to therapeutic failures along with dramatic economic losses in livestock husbandry. On the other hand, the uncontrolled use of antimicrobial drugs could result in selection of multiresistant bacterial strains (DANMAP, 2009).

Similarly to livestock species, the oral use of antimicrobial drugs in dogs exerts a selective pressure on commensal *E. coli* bacteria, which subsequently may cause the emergence of multiresistant strains. This could be realised by mutations in bacterial chromosome or genetic transfer from other representatives of intestinal microflora or transient bacteria. In this sense, resident *E. coli* are an interesting reservoir of various genetic factors conferring resistance to different groups of chemotherapeutics (Guardabassi *et al.*, 2004). Commensal multiresistant *E. coli* bacteria in the intestinal tract are an excellent indicator in studies on the spread of genes of antimicrobial resistance (Enne *et al*., 2008).

The resistance to beta-lactam antibiotics, respectively third- and fourth-generation cephalosporins, cefotaxime, ceftazidime, ceftiofur etc. in pathogenic and

resident *E. coli* producing extended-spectrum beta-lactamases (ESBL) is among the most extensively investigated mechanisms of resistance during the last years (Livermore, 2008). Recently, the spread of ESBL-producing enterobacteria causing nosocomial and out-hospital infections has increased (Pitout, 2005). Harada *et al*. (2014) reported a similar tendency in *E. coli* isolates from dogs as well. The spread of plasmid-mediated resistance to beta-lactams, expressed by AmpC enzymes and the resistance to beta-lactam inhibitors are problems discussed not only by human medicine, but also by livestock and pet medicine, in particular as *E. coli* isolates from dogs are concerned (Philippon *et al*., 2002; Carattoli *et al.,* 2005).

Although more rarely, data about resistance to antimicrobial drugs in canine *E. coli* isolates are reported in some European countries (SVARM, 2006; Costa *et al*., 2008), and results are interpreted from ecological point of view including also risks related to antimicrobial drugs sales. That is why, in the Norwegian National Strategy Against Antibiotic Resistance 2015-2020 the government lays down steps for reducing resistance by 39% in pet bacterial isolates. Such a crucial step is not possible without analysis of objective information for antimicrobial drugs sales and the spread of resistance among bacteria (Anonymous, 2015).

In Bulgaria, such an analysis could be extremely risky provided the lack of information on both aspects, e.g. the lack of objective information on sales volumes of antibiotics and for specific mechanisms of resistance in *E. coli* bacteria of public health significance.

This study aimed to investigate the prevalence of resistance in commensal canine *E. coli* isolates to different groups of antimicrobial drugs, with emphasis on some beta-lactam antibiotics. That is why the information for phenotypic expression of resistance to some beta-lactams was supplemented with genetic studies on the presence of resistance genes bla_{TEM}, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-1}}$.

MATERIALS AND METHODS

The study conducted between 2015 and 2017 and included 80 *E. coli* isolates from canine rectal swabs. Strains were isolated from patients of small animal clinics in Stara Zagora (n=36), Varna (n=20) and Burgas (n=24).

Isolation and identification of *E. coli* were performed by conventional microbiological methods and kits for identification of intestinal and non-fermenting bacteria (BBL). Results were interpreted by means of the semi-automated Crystal system (BBL).

The sensitivity of *E. coli* isolates to antimicrobial drugs was evaluated by the disk diffusion method and MIC determination (EUCAST, 2015). The antibiotic disks used for evaluation of sensitivity of strains were loaded with: ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), cephalothin (30 μg), ceftazidime (10 μg), cefotaxime (5 μg), gentamicin (10 μg), tetracycline (30 μg), and ciprofloxacin (5 μg). They were produced by Emapol (Poland). ESBL production was determined with antibiotic disks loaded with beta-lactamase inhibitor clavulanic acid (20/10 μg) in combination with disks loaded with oxyimino-cephalosporins ceftazidime (30 μg) and cefotaxime (30 μg), Emapol (Poland).

MIC were determined by Liofilchem Test Strips (Italy). The strips displaying MIC scales were loaded with respective concentrations of cefotaxime (0.25–16 μg/mL) and ceftazidime (0.5– 32 μg/mL).

For determination of inhibitory effect of clavulanic acid, strips loaded with ceftazidime+clavulanic acid (0.064–4 μg/mL) and cefotaxime+clavulanic acid (0.016–1 μg/mL) were used. The same tests were used for evaluation of sensitivity of *E. coli* strains, MIC of ampicillin, and amoxicillin/clavulanic acid. MIC of cephalothin was assayed by micro-broth dilution test and Muller-Hinton broth, Emapol (Poland). MIC methods were controlled with a reference strain *Escherichia coli* ATTC 25922.

The statistical processing of the data involved the determination of the confidence intervals with Graph Pad InStat 3.

DNA was extracted by means of DNeasy Blood Tissue kit (Qiagen, Germany). The following primers were used in bla_{TEM} (850 bp) amplification protocol (Arlet *et al*., 1995): OT3: 5′ ATGAGT ATTCAACATTTCCG 3′ and OT4: 5′ CCAATGCTTAATCAGTGAGG 3′ . The thermal cycle of the PCR reaction comprised: initial activation step (94 °C, 5 min); denaturation (94 °C, 60 s); annealing ×30 cycles (55 °C, 60 s); extension (72 °C, 60 s) and final extension (72 °C, 10 min).

Amplification protocol of the bla_{OXA-1} gene used the following sequences of primers (Steward *et al*., 2001): F-5′ACA CAATACATATCAACTTCGC-3′ and R-5′AGTGTGTTTAGAATGGTG ATC-3′. The thermal profile of the reaction included the following steps: initial activation step (96 °C, 5 min); denaturation (96 °C, 60 s); annealing ×35 cycles (61 °C, 60 s); extension (72 °C, 2 min) and final extension (72 °C, 10 min).

Positive control used in determination of bla_{TEM} and bla_{OXA-1} genes was *E. coli* ATCC 35218.

Amplifications of $bla_{CTX-M-1}$ were done in a STRATAGENE Mx3000P system. Ready microbial DNA assay kits (Qiagen, Germany) containing master mix, specific primer pairs and TaqMan probe loaded with FAM at the 5` end for the respective sequences of resistance genes, were used.

Apart the reaction components, kits contained also positive DNA control and internal amplification control. The standard protocol required the following amounts of components: 2× qPCR master mix – 12.5 μL; qPCR primer pair and TaqMan probe – 1 μL; extracted DNA – 5 μL; DNA-free sterile water – 6.5 μL. Total reaction volume was 25 μL.

The temperature regime of amplification included an initial activation step of PCR at 95 °C for 10 min. The second stage comprised two steps of 40 cycles of denaturation and annealing/extension at 95 °C for 15 sec; annealing/extension at 60 °C for 2 min. Positive DNA control had values $C_T \leq 34$, and positive amplification control: $C_T = 22 \pm 2$.

RESULTS

The results on sensitivity of *E. coli* isolates from dogs to antimicrobial drugs demonstrated 47.5% resistance to ampicillin, 18.7% to amoxicillin/clavulanic acid and 6.2 % to cephalothin. The percentage of resistant *E. coli* strains to tetracycline was 26.2%, to ciprofloxacin – 12.5%, and to gentamicin – 10%. The resistance rates to cefotaxime and ceftazidime were the lowest – 1.2% and 2.5% respectively (Table 1).

MIC_{90} values of ampicillin were 16 μg/mL, and for both amoxicillin/clavulanic acid and cephalothin – 8 μg/mL. The main genotypic profile of resistance to tested beta-lactams involved the presence of bla_{TEM} (Tables 2, 3; Fig. 1).

Table 1. Prevalence of resistant *E. coli* isolates from dogs (n=80)

Antimicrobial drugs	Resistant strains number (%)	95% confidence limits
Ampicillin	38 (47.5%)	36.7÷58.4
Amoxicillin/clavulanic acid	15 (18.7%)	10.9÷27.9
Cephalothin	5 (6.2%)	2.0÷12.4
Cefotaxime	1 (1.2 %)	0÷4.7
Ceftazidime	2 (2.5 %)	0.2÷7.0
Gentamicin	8 (10.0%)	4.4÷17.5
Tetracycline	21 (26.2 %)	17.2÷36.3
Ciprofloxacin	10 (12.5 %)	6.2÷20.6

Table 2. Distribution of MIC of beta-lactam antibiotics in commensal canine *E. coli* isolates (n=80)

Antimicro-bial drugs	Cumulative MIC, μg/mL														
	≤0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	126	≥256
Amoxicillin						10.5	30.1	45.7	62.0	62.0	81.7	94.5	100		
Amoxicillin/ clavulanic acid						24.2	58.1	74.0	81.2	92.5	100				
Cephalothin						20.0	40.0	40.0	80.0	93.8	100				

Table 3. Phenotypic and genotypic profiles of resistance to beta-lactams in commensal canine *E. coli* isolates (n=80)

Phenotypic profiles of resistance to beta-lactams			Genotypic profiles of resistance to beta-lactams		
	number (%)	95% confidence limits	*bla* $_{\text{TEM}}$	*bla* $_{\text{OXA-1/}}$	*bla* $_{\text{CTX-M-1}}$
A	38 (47.5%)	37.7 ÷58.4	*bla* $_{\text{TEM}}$	–	–
A CF	5 (6.2%)	2.0÷12.4	*bla* $_{\text{TEM}}$	–	–
A AMC	15 (18.7%)	10.9÷27.9	*bla* $_{\text{TEM}}$	–	–

Legend: A – ampicillin; CF – cephalothin; AMC – amoxicillin/clavulanic acid.

DISCUSSION

Investigations on resident *E. coli* bacteria place a particular emphasis on their resistance to fluorinated quinolones and novel generations of cephalosporins. Guardabassi *et al.* (2004) affirm that the selective pressure from the extensive use of antimicrobial drugs in pet medicine results mostly from the increasing application of aminopenicillins, as well as some second- and third-generation cephalosporins.

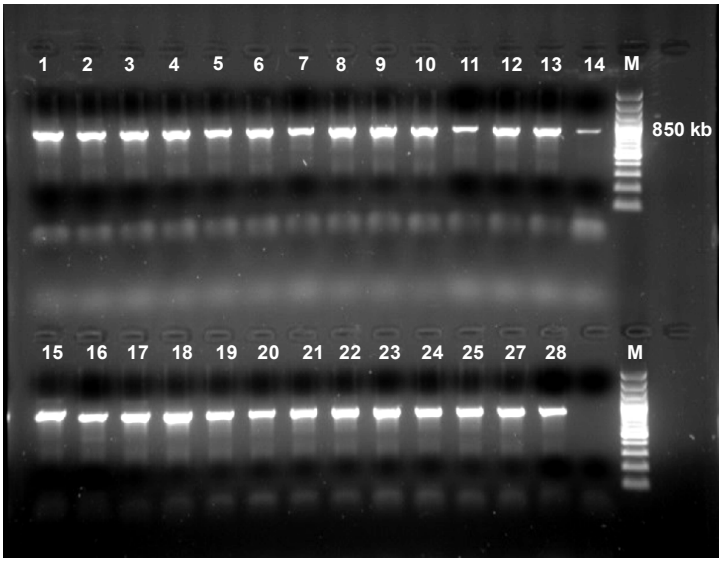

Fig. 1. Electrophoretic profiles of 850 bp amplification products for *bla* $_{TEM}$ gene: lanes 1–26 are positive for *bla* $_{TEM}$ gene; lane 27: positive control; lane 28: negative control; M: 100 bp DNA ladder.

The data on the spread of commensal *E. coli* isolates from dogs and cats, resistant to cephalosporins are still few compared to similar data in livestock species. In France, Haenni *et al*. (2014) reported a higher (18.5%) prevalence of resident canine *E. coli* strains with plasmids harbouring $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CMY\text{-}2}$ genes, conferring production of extended-spectrum beta-lactamases, in comparison with other EC countries. In an earlier study from Portugal, Costa *et al*. (2008) reported 12% resistance to aminopenicillins but no resistance to ceftazidime in commensal canine and feline *E. coli* isolates. The authors detected the bla_{TEM} gene in 70% of amoxicillin-resistant strains. Only two isolates from a single patient were resistant to cefotaxime and positive for the $bla_{CTX\text{-}M\text{-}1}$ gene. Our results about antimicrobial resistance rates, respectively the genotype of commensal *E. coli* strains resistant to beta-lactams are similar to those published by Costa *et al*. (2008), as we also observed high percent of amoxicillin-resistant strains possessing bla_{TEM} gene (100%), but no $bla_{CTX\text{-}M\text{-}1}$ in isolates resistant to cefotaxime and ceftazidime. Several years later in the Netherlands, Hordijk *et al*. (2013) found out that 45% of commensal *E. coli* isolates from dogs were resistant to cefotaxime and that the main genotype of this resistance pattern was associated with presence of the $bla_{CTX\text{-}M\text{-}1}$ gene. In Brazil, Carvalho *et al*. (2016) discussed the broad spread of bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ in resident *E. coli* strains detected in dogs and their owners.

Ljungquist *et al*. (2016) established similar clones of resistant *E. coli* isolates from dogs and their owners, producing plasmid-mediated expended-spectrum beta-lactamases (bla_{AMC}, $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}27}$). It should be kept in mind that integrons conferring multiresistance to antimicrobial drugs in enterobacteria have identical gene cassettes. In the different regions of the world, their sequences were

identical (Ochoa *et al*., 2016). It should be therefore affirmed that the animal population is a possible primary reservoir of integrons characterised with ubiquitous spread and possibility for transfer to species from the resident and pathogenic microflora.

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