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SHORT COMMUNICATION

Monitoring of Virulence Genes, Drug-Resistance in *Campylobacter coli* Isolated from Golden Retrievers

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Abstract

The investigation was performed on 75 of Golden Retriever puppies. Faecal samples were collected on the 42 day of the puppies life (control). Probiotic preparation was administered on 43 day of the puppies life and 10 days after the application of the probiotic, faecal samples were collected again (on 53 day of puppies life). All isolates of *Campylobacter coli* isolated prior to the administration of the probiotic were found to contain the *cad*F gene responsible for adhesion, as well as, the *fla*A gene influencing motility of the examined bacteria. Significant differences ($P < 0.05$) were recorded only in the case of enrofloxacin.

Key words: *Campylobacter coli*, dogs microflora, drug-resistance, virulence genes

Campylobacter coli is the most seldom reported *Campylobacter* spp. in the majority of dog populations sampled (Acke *et al*., 2009). However, some studies have found *Campylobacter jejuni* to be the most commonly isolated species in dogs, particularly outside of Europe (Tsai *et al*., 2007). Other species of *Campylobacter* such as *C. coli* and *Campylobacter lari* have also been isolated from dogs on occasion, but these species are usually of very low prevalence (Rossi *et al*., 2008). At the moment, it is believed that the following genes are responsible for the pathogenicity of *Campylobacter* spp.: *fla*A gene conditioning motility, *cad*F – affecting adhesion, *cdt*B – responsible for toxin production and *iam* – determining invasiveness (Krutkiewicz, 2008). The European Food Safety Administration (EFSA) and the European Centre for Disease Control (ECDC) published the second joint report concerning antibiotic resistance of pathogenic bacteria infecting people, animals and food articles. Campylobacteriosis is the most frequently recorded animal-born infection in humans. High resistance of *Campylobacter* spp. strains to some antimicrobiological substances, including ciprofloxacin constitutes a growing problem in EU countries (EFSA, 2014). The aim of this study was to show varied shares of virulence genetic markers among strains isolated from dogs with diarrhea. The aim of the second stage of experiments was to presents impact of two commonly applied methods on changes in drug – resistance of the obtained isolates.

The investigation was performed on 75 of Golden Retriever puppies. *Campylobacter* sp. isolates were obtained from the rectum using swab kits with transport substrate (Euro Tubo Collection Swab Rubi, Spain). Faecal samples were collected on the 42 days of puppies life (control), (moment of weaning). Probiotic preparation was administered on 43 days of puppies life and 10 days after the application of the probiotic, faecal samples were collected again (on 53 day of puppies life to examine possible changes in *C. coli* drug-resistance).

The applied preparation BioProtect 200 mg (Vet-Expert) contained 5×10^6 CFU of *Lactobacillus acidophilus*, *Enterococcus faecium*, *Bifidobacterium longum*, *Lactobacillus rhamnosus*, and manno-oligosaccharides, fructooligosaccharides. The probiotic was administered 2 capsules daily for 10 days.

Campylobacter isolates were cultured at 42 ± 1°C in Campy Selective Agar Base Preston (Neogen) for 48 h in an atmosphere composed of 6% oxygen, 10% carbon dioxide and 84% nitrogen. *Campylobacter* spp. identification was performed using PCR for the detection of *C. jejuni* and *C. coli*. The following positive strains: *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were

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also included. All strains were preserved in 20% glycerol at –70°C. Extraction of DNA (Andrzejewska *et al*., 2011) was performed using CHELEX-100 chelating resin (Bio-Rad). Bacterial colonies were suspended in 100 µl Tris buffer and 45 µl 20% CHELEX and boiled for 10 min. Samples were then immediately placed on ice for 1 min and centrifuged at 13.000 g for 10 min at room temperature. The supernatant (2 µl) was used in PCR. The purity and concentration of DNA were estimated using spectrophotometry at 260 and 280 nm. The presence of the *cad*F, *fla*A, *cdt*B and *iam* genes was determined with the primers given by Nachamkin *et al.* (1993), Konkel *et al*. (1999), Carvalho *et al*. (2001) and Bang *et al*. (2001). All PCR amplifications (Andrzejewska *et al.*, 2011) were performed in a mixture (25 µl) containing: 2.5 µl of the PCR buffer (10 × concentrated), 2.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM), 1 µl of each primer (100 µM), 0.5 µl (1 U) of the *Taq* thermostable DNA polymerase (Promega Corporation), 2 µl of the bacterial template DNA and 15 µl nuclease free water. The PCR products were analyzed by electrophoresis in 1.5% agarose gel. The size of the PCR amplicons was compared to the 100 bp DNA marker (Promega Corporation).

In order to assess the resistance of the isolates, the disc method was employed using the following antibiotics (Oxoid): ciprofloxacin (5 µg), enrofloxacin (5 µg), erythromycin (15 µg) and tetracycline (30 µg) Culturing was conducted on nutrient broth (NB Merck) which, after 18 hours of incubation at the temperature of 37°C, was diluted at 1:10.000 in sterile physiological liquid. The suspension (500 µl each) was screened onto plates with Mueller-Hinton (Oxoid) substrate and discs with antibiotics were placed on the agar surface. Following 18-hour incubation at 37°C, inhibition zone diameters were determined. The control of antibiotic activity was carried out with the assistance of the *C. coli* ATCC 33559 reference strain.

E test strips were used in accordance with the manufacturer's instructions. They were removed from –20°C storage and brought to room temperature prior to use. Mueller – Hinton agar plates supplemented with defibrinated 5% sheep blood (Oxoid) were inoculated by swabbing evenly in three directions with a 0.5 McFarland standard of the test organism. Four E test strips were applied to the surface of the plate in an equidistance radial manner, with the lowest concentration toward the centre. Plates were incubated under the same condition as for disc diffusion. MICs were read directly from the test strip at the point where the zone of inhibition intersected the MIC scale on the strip. National Committee for Clinical Laboratory Standards were used for interpretation of the results (Murat *et al*., 2005).

Results of investigations regarding numbers of microorganisms were subjected to statistical analysis using the *glm* procedure of the SAS (2012) program and the significance of differences was verified by Duncan's test.

All isolates of *C. coli* isolated prior to the administration of the probiotic were found to contain the *cad*F gene responsible for adhesion, as well as, the *fla*A gene influencing motility of the examined bacteria. The *ctd*B gene, involved in preconditioning the development of CDT toxin, was identified in 48% of the isolates, whereas gene *iam*, affecting invasiveness – in 49.3%. The examination of the isolates obtained after the administration of the probiotic failed to reveal any significant influence of the probiotic on the frequency of occurrence of the above – mentioned genes (Table I). Table II presents the results of the comparison of two methods of determination of resistance of isolates (obtained prior to probiotic administration) to selected antibiotics. Two tests (disc diffusion test and E Test strips) which were used to determine quantities of isolates sensitive (S) and resistant (R) were compared to the applied antibiotic. In case of ciprofloxacin, erythromycin, and tetracycline, the results of the two tests did not differ significantly in terms of statistic. Significant differences ($P < 0.05$) were recorded only in the case of enrofloxacin. *C. coli* isolates obtained prior to the administration of the probiotic revealed the highest resistance in relation to ciprofloxacin and enrofloxacin. Numerical data towards susceptible and resistant isolates turned out to be similar to the results obtained in the control group.

As mentioned above, *Campylobacter* spp. are among the most frequently reported bacterial cause of human gastroenteritis worldwide (CDC, 2008). The invasive ability of a *Campylobacter* is strongly affected by its motility, provided by the flagellum. This has been demonstrated by studies that inactivated the *fla*A gene (encodes for the filament of the flagella) or generated mutant bacteria, and found that this affected mobility and thus invasiveness (Konkel *et al*., 1999). In the carried studies, this gene was identified in 100% of the

Table I
Numbers and percentages of virulence genes in *C. coli* (PCR)

Isolate group	Number of positive isolates			
	*cad*F	*fla*A	*cdt*B	*iam*
Dogs (control) *C. coli* (n = 75)	75 (100%)	75 (100%)	36 (48%)	37 (49.3%)
Dogs (diet with probiotic) *C. coli* (n = 75)	75 (100%)	75 (100%)	38 (50.7%)	37 (49.3%)

Table II
Results of susceptibility testing of 75 isolates *C. coli* by disc diffusion and E test methods for four antibiotics (control)

Antimicrobial agents	E Test	Disc diffusion
Ciprofloxacin (5 μg)	38S (*MIC ≤ 1 μg ml^{-1})	38S (≥ 21 mm)
	37R (MIC ≥ 4 μg ml^{-1})	37R (≤ 15 mm)
Enrofloxacin (5 μg)	43S (MIC ≤ 1 μg ml^{-1})a	60S (≥ 23 mm)b
	32R (MIC ≤ 2 μg ml^{-1})a	15R (≤ 16 mm)b
Erythromycin (15 μg)	72S (MIC ≤ 0.5 μg ml^{-1})	73S (≥ 23 mm)
	3R (MIC ≥ 8 μg ml^{-1})	2R (≤ 13 mm)
Tetracycline (30 μg)	74S (MIC ≥ 4 μg ml^{-1})	74S (≥ 19 mm)
	1R (MIC ≥ 16 μg ml^{-1})	1R (≤ 14 mm)

* Minimum inhibitory concentration (MIC) specified by the National Committee for Clinical Laboratory Standards (2002); S – susceptible, R – resistant; a, b – means in rows designated with the same letters do not differ significantly at the level of $P < 0.05$

Table III
Results of susceptibility testing of 75 isolates *C. coli* by disc diffusion and E test methods for four antibiotics (diet with probiotic)

Antimicrobial agents	E Test	Disc diffusion
Ciprofloxacin (5 μg)	37S (*MIC ≤ 1 μg ml^{-1})	37S (≥ 21 mm)
	38R (MIC ≥ 4 μg ml^{-1})	38R (≤ 15 mm)
Enrofloxacin (5 μg)	45S (MIC ≤ 1 μg ml^{-1})a	61S (≥ 23 mm)b
	30R (MIC ≤ 2 μg ml^{-1})a	14R (≤ 16 mm)b
Erythromycin (15 μg)	74S (MIC ≤ 0.5 μg ml^{-1})	73S (≥ 23 mm)
	1R (MIC ≥ 8 μg ml^{-1})	2R (≤ 13 mm)
Tetracycline (30 μg)	73S (MIC ≥ 4 μg ml^{-1})	73S (≥ 19 mm)
	2R (MIC ≥ 16 μg ml^{-1})	2R (≤ 14 mm)

isolates from both experimental groups. According to the investigations by Andrzejewska *et al.* (2011) and Selwet and Galbas (2012a; 2012b) on *C. coli* occurrence in people, dogs, cats, and piglets, the presence of the *fla*A gene in all the examined isolates was also recorded (Selwet *et al.*, 2015). The next factor affecting virulence is gene *cad*F, which is responsible for production of adhesines. In the experiments, the *cad*F gene was found also in 100% of isolates from both study groups. Some researchers attribute a high importance of the *cad*F gene leading to campylobacteriosis in human (Selwet and Galbas, 2012a). Cytokines such as interleukin-8 (IL-8) are secreted by host cells in response to bacterial invasion, acting as early warning signs to the host immune system, and *Campylobacter* spp. flagellum and cytolethal distending toxin (CDT) are both thought to stimulate the secretion of IL-8 from host cells (Zheng *et al.*, 2008). In the carried studies, the *cdt*B gene was identified in 48–50.7% of the isolates. The *iam* gene responsible for invasiveness, was found to occur at a similar level (49.3%). Carvahlo *et al.* (2001) reported that the *iam* gene occured less frequently in *C. coli*. Referring to the EFSA report (2014), antibiotics which are most commonly used in the treatment of people, animals are fluorochinolons (*e.g.* ciprofloxacin) ability to mutation in the gyrase – coding gene, which leads to changes in this protein and reduces affinity to fluorochinolons. According to Krutkiewicz (2008) approximately 55.9% to 59% of *Campylobacter* sp. strains show resistance to ciprofloxacin. In the experiments, resistance to mentioned antibiotic was observed in both experimental groups at the level of 49.3–50.7%. Tambur *et al.* (2010) compared the E test strips and disc diffusion methods and observed a distinct increase of the determined antibiotics to which *C. coli* and *C. jejuni* were resistant. The two tests, which were used to investigate drug – resistance failed to show any differences in the amount of antibiotics to which *C. coli* were sensitive. *Campylobacter* sp. are able of producing many toxins, which can damage red blood cells. Many of these toxins/hemolysins are considered virulence factors because of their ability to increase the availability of iron to the pathogen throughout the process of infection *via* lyses of erythrocytes and subsequent release of heme from hemoglobin (Istivan *et al.*, 2008; Selwet and Galbas, 2012a). Summing up, the prevalence of *C. coli*

in dogs are low, suggesting that this population of dogs is unlikely to be a common source of *C. coli* infection for humans. The examined animals were symptomless carriers of these rods. However, great abundance of *cad*F and *fla*A genes as well as smaller of *cdt*B and *iam* genes in strains isolated from those puppies can pose a threat associated with increase of their pathogenicity. In our studies *Campylobacter* strains exhibited high resistance to ciprofloxacin, enrofloxacin and low resistance to erythromycin and tetracycline. Resistance to ciproflaxin was also determined in strains derived from animals (in particular, from chickens, pigs and cattle) as well as from food articles (EFSA, 2015).

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