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# IMPACT OF THE PRIOBIOTIC ON THE PRESENCE OF SELECTED VIRULENCE GENES AND DRUG-RESISTANCE AMONG *CAMPYLOBACTER COLI* ISOLATED FROM PIGLETS

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## Abstract

The investigations comprised 100 piglets of crossbreed Polish Landrace x Large White Polish breed. Faeces samples were collected on the 2nd d of piglets' life (control). On the 5th d of life of the piglets, probiotic paste was applied and 7 d later, faecal samples were collected again. The material included 100 isolates of *Campylobacter* sp. obtained from healthy piglets. All isolates were assigned to the *Campylobacter coli* species. The occurrence of virulence genes was determined by the PCR method. Drug-resistance of the obtained isolates was determined using diffusion tests and E-test strips. All isolates deriving from the control group piglets were found to contain the *cadF* gene responsible for adhesion, as well as, gene *flaA* influencing motility of the examined bacteria. In piglets fed diets supplemented with probiotics, the *cadF* gene occurred in 100% isolates and gene *flaA* – in 99% isolates. *Campylobacter coli* isolates obtained from piglets from the control group exhibited the highest resistance with respect to ciprofloxacin and enrofloxacin. The similar results were recorded in the case of isolates obtained after the probiotic application. The majority of the isolates generated α type haemolysis (91%-92%). No significant differences were recorded in the capability of generating haemolysis between isolates obtained before probiotic administration and the isolates obtained after the application of the experimental probiotic.

**Key words**: piglets, *Campylobacter coli*, drug-resistance, virulence genes.

*Campylobacter* sp. is represented by over two hundred species and subspecies, including frequently isolated *Campylobacter jejuni* and *Campylobacter coli*. *Campylobacter* sp. rods are among the most frequently isolated aetiologic factors of food poisoning of animals and humans. The infection takes place during the contact of a man with contaminated poultry meat, water, or unpasteurised milk (9). Factors preconditioning pathogenicity of *Campylobacter* sp. include: motivity and chemotaxis, as well as, adhesion and invasiveness (14). At the moment, it is believed that the following genes are responsible for the pathogenicity of *Campylobacter* sp.: *flaA* gene conditioning motivity, *cadF* – affecting adhesion, *cdtB* – responsible for toxin production, and *iam* – determining invasiveness (7). 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-borne infection in humans. A high resistance of *Campylobacter* sp. strains to some antimicrobiological substances, including cyprofloxacine, constitutes ~~a~~ an increasing problem in the EU countries (4).

The objective of the performed investigations was to demonstrate the probable effect of probiotic paste on variations in the proportions of genetic virulence markers among strains isolated from healthy piglets with no symptoms of campylobacteriosis. The aim of the second stage of experiments was to present the impact of the applied probiotic on changes in drug-resistance of the obtained isolates, using two commonly applied methods. In addition, the capability of *Campylobacter coli* causing haemolysis was also determined.

## Material and Methods

**Animals**. The investigation was performed on 100 piglets of Polish Landrace x Large White Polish crossbreed. *Campylobacter coli* isolates were obtained from the rectum using swab kits with transport substrate (Euro Tubo Collection Swab Rubi, Spain). Faecal samples were collected on the 2nd d of piglets' life (control). Probiotic paste was administered on the 5th d

of piglets' life and on 7th d after the application of the probiotic, faecal samples were collected again (on 12th d of piglets' life).

**Probiotic**. The applied iron (III) paste LACTIFERM A $D_3$ E $Fe^{++}$ (Fodder Preparations Plant Polmass S.A) contained 2 x $10^9$ CFU of *Enterococcus faecium* M 74 bacteria, vitamins: A – 2000 j.m., $D_3$ – 200 j.m., E – 10 mg, as well as an addition of available iron (100 mg) in fumaran form. The paste was administered once in the form of one application dose.

**Isolates**. *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* sp. identification was performed using an API Campy Test (BioMerieux) and multiplex PCR for the simultaneous detection of *Campylobacter jejuni* and *Campylobacter coli*. The positive strains of *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were also included. All strains were preserved in 20% glycerol at -70˚C.

DNA extraction (1) was performed using CHELEX-100 chelating resin (Bio-Rad). Bacterial colonies were suspended in 100 µl of TRIS and 45 µl of 20% CHELEX and boiled for 10 min. Then, the samples were 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 *cadF, flaA, cdtB,* and *iam* genes was determined using primers listed in Table 1. All PCR amplifications (1) were performed in a mixture (25 µl) containing: 2.5 µl of the PCR buffer (10 x 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 (1U) of the Taq thermostable DNA polymerase (Promega Corporation), 2 µl of the DNA bacterial template, and 15 µl nuclease free water. The PCR products were analysed by electrophoresis in 1.5% agarose gel. The size of the PCR amplicons was compared with the 100 bp DNA marker (Promega Corporation).

**Disc diffusion test.** 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 h of incubation at 37°C, was diluted at 1:10,000 in sterile physiological saline. 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-h incubation at 37°C, inhibition zone diameters were determined. The control of antibiotic activity was carried out with the assistance of the *Campylobacter coli* ATCC 33559 reference strain.

**E test strips**. 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. Standards of the National Committee for Clinical Laboratory Standards were used for interpretation of the results (10).

**Table 1**
PCR primers used in the study

| Primers | Sequence (5'→3') | Product bp | References |
|---|---|---|---|
| *cadF*-F | TGGAGGGTAATTTAGATATTG | 400 | (6) |
| *cadF*-R | CTAATACCTAAAGTTGAAAC | | |
| *flaA*-F | GGATTTCGTATTAACACAAATGGTGC | 1728 | (11) |
| *flaA*-R | CTGTAGTAATCTTAAAACATTTTG | | |
| *cdtB*-F | GTTAAAATCCCCTGCTATCAACCA | 495 | (2) |
| *cdtB*-R | GTTGGCACTTGGAATTTGCAAGGC | | |
| *iam*-F | GCGCAAAATATTATCACCC | 518 | (3) |
| *iam*-R | TTCACGACTACTATGCGG | | |

F – forward primers, R – reverse primers

**Haemolysis**. The ability of the bacteria to haemolysis was assessed on an agar substrate supplemented with 5% sheep blood. The incubation was carried out for 18-24 h at 37°C. Occurrence of a lighter zone surrounding the colony was considered as a positive result.

**Statistics**. Results of investigations regarding numbers of microorganisms were subjected to the statistical analysis using the *glm* procedure of the SAS programme (16) and the significance of differences was verified by Duncan's test.

## Results

All isolates of *Campylobacter coli* isolated prior to the administration of the probiotic were found to contain the *cadF* gene responsible for adhesion, as well as, the *flaA* gene influencing motility of the examined bacteria. The *ctdB* gene, involved in preconditioning the development of CDT toxin, was identified in 53% of the isolates, whereas gene *iam,* affecting invasiveness – in 12%. 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 2).

Table 3 presents the results of the comparison of two methods of determination of virulence and resistance of isolates (obtained prior to probiotic administration) to selected antibiotics. Two tests were compared: disc diffusion test and E test strips, which were used to determine quantities of isolates sensitive (S) and resistant (R) to the applied antibiotic. In case of ciprofloxacin, erythromycin, and tetracycline, the results of the two tests did not differ significantly in terms of statistics. Significant differences ($P<0.05$) were recorded only in the case of enrofloxacin. *Campylobacter coli* isolates obtained prior to the administration of the probiotic revealed the highest resistance in relation to ciprofloxacin and enrofloxacin.

The results referring to isolates collected after the administration of the probiotic are presented in Table 4. Numerical data regarding susceptible and resistant isolates turned out to be similar to the results obtained in the control group.

Table 5 presents the results associated with the capability of *Campylobacter coli* to cause hydrolysis. No significant differences in the haemolytic capacity of the isolates obtained prior to the administration of the probiotic were observed when compared with the isolates obtained after the application of the preparation. The majority of the isolates caused α-type haemolysis (91%-92%).

**Table 2**
Number of virulence genes in *Campylobacter coli* (PCR)

| Isolate group | Number of positive isolates | | | |
|---|---|---|---|---|
| | *cadF* | *flaA* | *cdtB* | *iam* |
| Piglets (control) *C. coli* (n=100) | 100 | 100 | 53 | 12 |
| Piglets (diet with probiotic) *C. coli* (n=100) | 100 | 99 | 51 | 13 |

**Table 3**
Results of susceptibility testing of 100 *Campylobacter coli* isolates by disc diffusion and E test methods to four antibiotics (control)

| Antimicrobial agents | E Test | Disc diffusion |
|---|---|---|
| Ciprofloxacin (5 μg) | 51S (*MIC≤1μg mL$^{-1}$) | 50S (≥21 mm) |
| | 49R (MIC≥4 μg mL$^{-1}$) | 50R (≤15 mm) |
| Enrofloxacin (5 μg) | 62S (MIC≤1μg mL$^{-1}$)a | 73S (≥23 mm)b |
| | 38R (MIC≤2μg mL$^{-1}$)a | 27R (≤16 mm)b |
| Erythromycin (15 μg) | 84 S (MIC≤0.5μg mL$^{-1}$) | 85S (≥23 mm) |
| | 16R (MIC≥8 μg mL$^{-1}$) | 15R (≤13 mm) |
| Tetracycline (30 μg) | 93S (MIC≥4 μg mL$^{-1}$) | 92S (≥19 mm) |
| | 7R (MIC≥16 μg mL$^{-1}$) | 8R (≤14 mm) |

* Minimum inhibitory concentration (MIC) specified by the National Committee for Clinical Laboratory Standards (12); S - susceptible, R - resistant; a, b – means in rows designated with the same letters do not differ significantly.

**Table 4**
Results of susceptibility testing of 100 *Campylobacter coli* isolates by disc diffusion and E test methods to four antibiotics (diet with probiotic)

| Antimicrobial agents | E Test | Disc diffusion |
|---|---|---|
| Ciprofloxacin (5 μg) | 53S (MIC≤1 μg mL$^{-1}$) | 52S (≥21 mm) |
| | 47R (MIC≥4 μg mL$^{-1}$) | 48R (≤15 mm) |
| Enrofloxacin (5 μg) | 63S (MIC≤1 μg mL$^{-1}$)a | 75S (≥23 mm)b |
| | 37R (MIC≤2 μg mL$^{-1}$)a | 25R (≤16 mm)b |
| Erythromycin (15 μg) | 85S (MIC≤0.5 μg mL$^{-1}$) | 85S (≥23 mm) |
| | 15R (MIC≥8 μg mL$^{-1}$) | 15R (≤13 mm) |
| Tetracycline (30 μg) | 94S (MIC≥4 μg mL$^{-1}$) | 92S (≥19 mm) |
| | 6R (MIC≥16 μg mL$^{-1}$) | 8R (≤14 mm) |

**Table 5**
Number of *Campylobacter coli* isolates with ability for haemolysis

| Isolate group | Type of haemolysis | | |
|---|---|---|---|
| | α | ß | γ |
| Piglets (control) *C. coli* (n=100) | 92 | 3 | 5 |
| Piglets (diet with probiotic) *C. coli* (n=100) | 91 | 4 | 5 |

## Discussion

*Campylobacter* sp. is capable of colonising the gastrointestinal tract of all animal species; nevertheless, it is isolated most frequently from birds (15). In pigs, the most frequently isolated species is *Campylobacter coli*, which occurs as a component of ordinary intestinal flora but can cause pathologic changes in the small intestine (13). Investigations, which have been carried out demonstrated differences in the share of virulence genetic markers, as well as, genotype variations in *Campylobacter* sp. strains isolated from animals and people.

*Campylobacter* sp. virulence is influenced by motility and chemotaxis. Its filaments contain two proteins – FlaA and FlaB coded by *flaA* and *flaB* genes (7). In the experiments, the *flaA* gene was found to be present in all the examined isolates in the control group as well as in 99% of the isolates from the group fed diets containing the experimental probiotic.

The next factor affecting virulence is gene *cadF*, which is responsible for production of adesines. In the carried studies, this gene was identified in 100% of the isolates from both experimental groups. According to investigations by Andrzejewska *et al*. (1) on *Campylobacter coli* occurrence in people, dogs, and cats, the presence of the *cadF* gene in all the examined isolates was also recorded. Some researchers attribute a high importance of the *cadF* gene causing campylobacteriosis in humans (18).

All the examined isolates were carriers of the *cdtB* gene coding cytolethal distending toxin (CDT) protein of toxic properties. This toxin can cause DNA degradation of the host (8).

In the carried studies, the *iam* gene, responsible for invasiveness, was found to occur on a similar level (12%-12%). Carvahlo *et al.* (3) reported that the *iam* gene occurred more frequently in *Campylobacter jejuni* strains and less frequently in *C. coli*.

According to the EFSA report (4), antibiotics, which are most commonly used in the treatment of people, as well as, in the veterinary medicine, to eliminate microorganisms causing infections are: fluorochinons (*e.g*. cyprophloxin), third generation cephalosporins (*e.g*. cephotaxim), and macrolides (including erythromycin).

*Campylobacter* sp. strains exhibit a considerable resistance to ciproflaxin. According to Kurtkiewicz (7), approximately 55.9% to 58% 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 47%-50%. High levels of resistance to ciprofloxacin may be caused by a mutation in the gyrase-coding gene, which leads to changes in this protein and reduces affinity to fluorochinolons (7).

The two tests, which were used to determine drug-resistance to selected chemotherapeutics failed to demonstrate any differences in the amount of antibiotics to which *Campylobacter coli* bacteria were sensitive. The results of the two tests were similar. Tambur *et al.* (17) compared the E-TEST strips and disc diffusion methods and observed a distinct increase in the determined antibiotics to which *Campylobacter coli* and *C. jejuni* were resistant.

Bacteria of *Campylobacter* sp. are capable of producing many toxins and proteolitic enzymes, which can injure red blood cells. Many of these toxins/haemolysins are considered as virulence factors because of their ability to increase the availability of iron to the pathogen through the process of infection *via*

lysis of erythrocytes and subsequent release of haeme from haemoglobin (5).

To conclude, it should be emphasised that *Campylobacter* sp. can be a perpetrator of a greater amount of infections of the gastrointestinal tract of animals and humans in comparison with the bacteria from the *Salmonella* genus. The examined animals were symptomless carriers of these rods. However, a great abundance of *cadF* and *flaA* genes, as well as, smaller of *cdtB* and *iam* genes in the isolated strains can pose a threat associated with an increase in their pathogenicity. With regard to the report published by EFSM concerning antibiotic resistance of animal transmitted bacteria, it turns out that campylobacteriosis appears to be the most common infection among the EU residents. In 2010, there were 200,000 cases of the disease-. Many *Campylobacter* strains exhibited a high resistance to cyprofloxacine, amphicilin, and tetracycline and low resistance to erythromycin. The resistance to ciproflaxin was also determined in strains derived from animals (in particular, from chickens, pigs, and cattle), as well as, from food articles.

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