# Prevalence and antimicrobial resistance of Campylobacter isolates from poultry offals

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Summary

The aim of the study was to determine the presence of Campylobacter strains in poultry by-products and define antimicrobial resistance of isolates. In total, 400 samples were tested among which 300 included the liver, heart and stomach, and 100 samples represented the contents of the cecum. The samples were taken from chickens and turkeys in the slaughterhouse after evisceration. The prevalence of Campylobacter in chicken samples was 100% with regards to the contents of cecum and offal. The turkey origin Campylobacter strains were noted in 76% of the livers, 78% hearts and 82% gizzards. The samples of cecum contents were positive in 60%. Species analysis of the strains isolated showed C. jejuni as dominant. The estimation of sensitivity to antibiotics showed that Campylobacter strains were most frequently resistant to quinolones and tetracyclines. Resistance to ciprofloxacin was detected among 52.7% and 52.5% chicken and turkey origin strains. The same was noted regarding nalidixic acid, resistance to which was shown in 56% and 58.5% isolates, respectively. Regarding tetracyclines, the highest resistance of the strains from chicken and turkey was detected to doxycyclinum in 61.3% and 53.3% of isolates, respectively. However the highest sensitivity was showed to erythromycin, gentamicin and chloramphenicol. Only one C. coli strain from turkey offal was resistant to gentamicin. Simultaneously multi drug resistance was defined. The aimed studies showed that 62% of C. jejuni and 53.8% of C. coli strains from chicken offal were resistant to two or more agents. In turkey origin isolates MDR was detected in 54.7% of C. jejuni and 53.3% of C. coli strains.

Keywords: Campylobacter, poultry, offal, contamination, antimicrobial resistance

In recent years, attention has been paid to the growing number of gastrointestinal diseases in humans caused by *Campylobacter* strains. Poultry plays an important role in transmission of this foodborne pathogen. In particular, chicken and turkey carcasses have been reported to be contaminated with rates up to 100% and 62.1%, respectively (16, 24). Contamination and subsequent colonization of poultry flocks on the farm often lead to transmission of *Campylobacter* along the whole production chain (19). Contamination of carcasses and internal organs in poultry occur during slaughter and post-slaughter processing (20). People become infected mainly by eating raw or undercooked meat and poultry products. However, the contamination can occur even during handling or preparation of raw meat. Horrocks et al. (11) and Frederick and Huda (9) point out that not only poultry meat, but also by-products represent important sources of food poisoning microorganisms such as *Campylobacter* spp., *Salmonella* spp. and

*Listeria* spp. The infective dose required to cause illness is as low as 500 organisms (10). Therefore, human infections occur relatively easily and often. The report of the European Food Safety Authority (EFSA) shows, that the number of confirmed cases of human campylobacteriosis in 2015 was 229 213 with a notification rate of 65.5 per 100 000 population (7). *Campylobacter* infects the small intestine and colon, causing an illness generally characterized by diarrhea, abdominal cramps and fever (12). Symptoms of campylobacteriosis are usually limited and disappear within 3-10 days. Most cases do not require special treatment except from replenishing fluids and electrolytes. However, antibiotic treatment is recommended in case of long-term, severe diarrhea, high fever and confirmation of the presence of *Campylobacter* spp. in the blood. In terms of the widespread use of antibiotics in animal production, it is necessary to be aware of the increasing number of strains resistant to chemotherapy. The

problem of *Campylobacter* resistance to commonly used antibiotics affects not only public health but also animal husbandry and emerges as a global problem (15). It is worth noting that in 2015 31.2% infected persons required hospitalization due to *Campylobacter* infections, and the mortality rate reached 0.03% (7).

The aim of the present work was to determine the presence of *Campylobacter* strains in poultry by-products and define antimicrobial resistance of isolates.

## Material and methods

**Bacterial isolation.** Material consisted of both internal organs (liver, heart and gizzard) and ceca contents, collected in the chicken and turkey abattoir after evisceration. A total of 400 samples were analyzed. From chickens, 50 samples of ceca, livers, hearts and gizzards were obtained. The same amount of samples were taken from turkeys. Cecum and offals were collected from the same carcasses. Collected by-products were not subjected to any technological processes. Each sample was put in a sterile bag and transported to the laboratory in a bag with a cooling pack. Then the samples were homogenized and 10 g of each homogenized sample was placed in stomacher bag with 90 ml buffered peptone water and mixed for 2 minutes in stomacher in order to prepare 10-fold dilutions. Aliquots (0.1 ml) of these dilutions were spread onto Karmali agar (Oxoid) and mCCDA (charcoal cefoperazone deoxycholate modified agar, Oxoid). After incubation at 42°C for 24-48 h under microaerophilic conditions (5% $O_2$, 10% $CO_2$ and 85% $N_2$), the plates were examined for morphologically typical *Campylobacter* colonies. Confirmation included microscopic morphology, motility, microaerobic growth at 25°C and the presence of oxidase. *Campylobacter* isolates selected for further analyses were subcultured only once in order to minimize cultural changes and then stored at –80°C in defibrinated horse blood (Oxoid) with the addition of glycerol (80: 20 v/v).

**Identification.** Identification of species of *Campylobacter* isolates was carried out using PCR method. Bacteria grown on agar medium were suspended in 1 ml of redistilled water and centrifuged at 13 000 × g for 1 minute. The precipitate was suspended in 100 µl of Tris buffer, and then DNA was isolated with the use of Genomic Mini Kit (A&A Biotechnology), according to the manufacturer's instruction. Purity and concentration of DNA obtained was determined spectrophotometrically; next, after appropriate dilution, it was used for the PCR assays. The primers enabling amplification of gene fragments mapA (specific for *C. jejuni*), ceuE (the sequence characteristic for *C. coli*) and 16S rRNA (specific for *Campylobacter* spp.) were used. The sequence of the primers used for amplification are shown in Table 1.

Amplification was performed in a reaction mixture containing: 5 µl of enzymatic buffer, 5 µl of dNTPs (final concentration 200 mM), 0.5 ml of each primer (final concentration 0.1 mM), 10 µl (5 mM) of MgCl₂, 2 µl of the thermostable Taq polymerase, 5 µl of

template DNA and deionized water free of DNA and RNA to a final volume of 50 µl. All PCR reactions were carried out using the following parameters: initial denaturation at 94°C for 5 min., then 30 cycles: denaturation for 1 min. at 95°C, primers annealing for 1 min at the temperature appropriate for a given primer pair, the strand elongation for 1 min. at 72°C. The final elongation step was carried out at 72°C for 5 min. The resulting amplification products were identified on 2% agarose gel stained with ethidium bromide at a concentration of 5 µg/ml. The size of the amplification products obtained were compared with the 100 bp molecular weight marker.

**Antimicrobial resistance.** Disc diffusion methodology based on the National Committee for Clinical Laboratory Standards (NCCLS) was used in the study. The disc content was as follows: ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg) and tetracycline (30 µg). In order to obtain pure culture, each selected Campylobacter spp. colony was suspended in Brucella broth so as to obtain a suspension with a density of 1.0 McFarland. The resulting culture was spread on the surface of Mueller-Hinton agar supplemented with 5% sheep blood and then antibiotic-impregnated discs were applied. The plates were incubated at 37°C for 44-48 h in microaerophilic atmosphere. After incubation, the zone of inhibition was read.

**Statistical analysis.** Statistical differences in the presence of *Campylobacter* strains isolated from poultry offals were determined with the use of Tukey test (Statistica, Poland). Chi-square test (Statistica, Poland) was used to determine statistical differences in the presence of antimicrobial resistance. The level of significance was set at $P < 0.05$.

## Results and discussion

**Bacterial isolation.** Analysis of ceca contents from chicken showed the presence of bacteria from genus *Campylobacter* in all tested samples, at an average level of log 6.23 cfu/g. Simultaneously the contamination of all chicken offals was confirmed and was up to log 1.71 cfu/g, log 1.77 cfu/g and 1.89 cfu/g, in heart, liver and gizzard samples, respectively. While bacteriological examination of ceca contents from turkey indicated the prevalence of *Campylobacter* spp. at the level log 4.56 cfu/g in 30 of 50 (60%) of the samples analyzed. The prevalence of *Campylobacter* contamination was detected in 76%, 78% and 82% of livers, hearts and gizzards, respectively. The contamination was at an average level of 1.01 log cfu/g in liver to

**Tab. 1. Primers used in PCR assay for identification of *Campylobacter* spp.**

| rget Gene | Sequences (5'-3') | PCR product size (bp) | Annealing temperature (°C) | References |
|---|---|---|---|---|
| *16S rRNA* for *Campylobacter* spp. | F – ATCTAATGGCTTAACCATTAAAC<br>R – GGACGGTAACTAGTTTAGTATT | 857 | 58 | Begum et al. (3) |
| *mapa* for *C. jejuni* | F – CTATTTTATTTTTGAGTGCTTGTG<br>R – GCTTTATTTGCCATTTGTTTTATTA | 589 | 58 | Begum et al. (3) |
| *ceuE* for *C. coli* | F – AATTGAAAATTGCTCCAACTATG<br>R – TGATTTTATTATTTGTAGCAGCG | 462 | 58 | Begum et al. (3) |
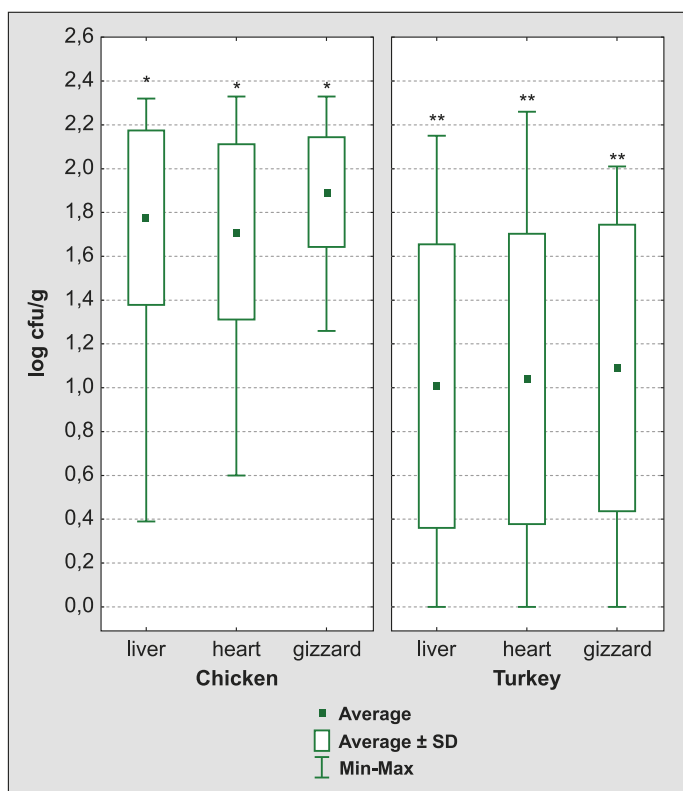
**Fig. 1. Number of *Campylobacter* spp. isolated from chicken and turkey offals**
Explanation: Asterisks indicate the significant differences obtained by Tukey test (P < 0.05).

1.09 log cfu/g in gizzard. The incidence rate and the level of *Campylobacter* contamination of offals between chicken and turkey origin isolates was significantly statistically different (P < 0.05) (Fig. 1 and Fig. 2). Available literature confirms the results obtained. The scientific report of EFSA (5) published in 2010 showed that the mean level of the prevalence of *Campylobacter* in broiler flocks was 71.2%. However the Member State prevalence varied from 2% to 100% for cecal contents. Meade et al. (14) notice that broilers can carry high cecal *Campylobacter* loads between $10^6$ and $10^8$ cfu/g feces. In turn, among turkey flocks prevalence of *Campylobacter* is less frequent, about 30% (18), with contamination levels of ceca ranging from $10^2$ to $10^7$ cfu/g (4). Such a high level of prevalence of *Campylobacter* in ceca contents lead to contamination of poultry meat. Skarp et al. (19) stress that transport has only a limited effect on the contamination of carcasses, whereas the slaughter process, especially plucking and evisceration, lead to contamination of carcasses and offals.

An equally high level of contamination of chicken livers has been

indicated by Wang et al. (22), which confirmed 76% of samples tested to be *Campylobacter* positive. Also Fernandez and Pison (8) found *Campylobacter* in a large number of tested samples of frozen poultry livers. They isolated *Campylobacter* in 92.9% of all samples and *C. coli* was dominant (78.6%) followed by *C. jejuni* in 21.4% of the samples. However, it was the prevalence of *C. jejuni* that was the highest in the aimed study. It was identified in 90% of liver samples, while *C. coli* was found in the remaining 10%. More or less positive results have been demonstrated in the research conducted by Atanossova et al. (1), confirming the occurrence of *Campylobacter* only in 9.7% of the livers tested, at a level of contamination reaching from log 1.6 to log 4.0 cfu/g. In contrast to these results our findings revealed 100% *Campylobacter* positive samples of livers, with a contamination rate from 0.39 to log 2.26 cfu/g.

The study conducted by Hope et al. (10) points out that consumption of poultry livers can lead to infection due to the potentially high infectious dose of *Campylobacter*. In addition, these authors oobserved a shorter incubation period in infected people because of high levels of *Campylobacter* present in livers. Moreover, it should be noted that most of the outbreaks were caused by inadequate cooking of poultry livers (10, 27). The internal temperature achieved during preparation of the liver dish was not adequate to kill *Campylobacter*. Poultry liver should be cooked for 2 to 3 minutes after they reach an internal temperature of 70°C (23).

With regard to contamination of heart and gizzard the aimed studies confirmed the prevalence of *Campylobacter* in 100% chicken origin offals. Among turkey offals this pathogen was found in 78% positive heart samples and 82% positive gizzard samples. Comparing the above results, Bagherpour et al. (2)
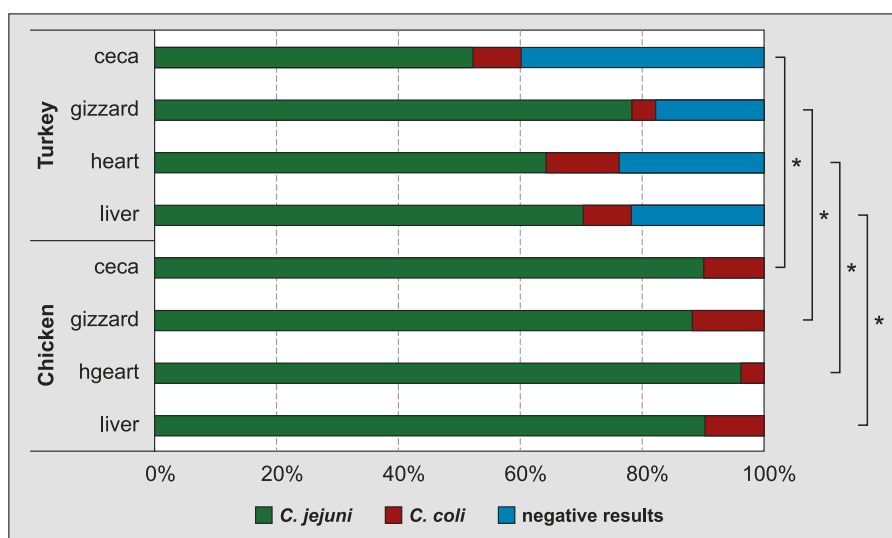


**Fig. 2. Prevalence (%) *C. jejuni* and *C. coli* among chicken and turkey isolates**
Explanation: Asterisks indicate the significant differences between contamination of chicken and turkey offals and ceca by *Campylobacter* species obtained by Chi-square test (P < 0.05).

reported the rate of contamination in 40% and 45% of hearts and gizzards, respectively, while Suzuki and Yammamto (21) found the presence of *Campylobacter* in 62.2% gizzard and 33.3% heart samples analyzed.

Obtained differences in the presence of *Campylobacter* among samples of liver, heart and gizzard may be largely due to varying degrees of contamination of slaughtered poultry, as well as differences in methods of killing or sampling method (2).

**Identification.** Identification of species of *Campylobacter* strains showed that *C. jejuni* was dominant (Fig. 2). In regard to 50 *Campylobacter* isolates from chicken ceca, 45 (90%) were recognized as *C. jejuni* and 5 (10%) as *C. coli*. In turn *C. jejuni* from chicken offals was isolated from 90% livers, 96% of hearts and 88% of gizzards. In relation to the samples taken from turkey *C. jejuni* and *C. coli* were detected in 52% and 8% samples of ceca, respectively. Contamination of turkey offals is similar and the results are as follows: *C. jejuni* was detected among 70%, 64% and 78% and *C. coli* among 10%, 12% and 4% of livers, hearts and gizzards, respectively. These results correspond with data obtained by other authors, who revealed mostly *C. jejuni* in the samples obtained from poultry (26). However, the analysis of *Campylobacter* strains carried out by Wieczorek and Osek (25) showed that the majority were *C. coli* (58.9%) positive while *C. jejuni* was detected in the remaining 41.1% of poultry meat samples. According to Skarp et al. (19) a flock is either colonized by one strain only, which has been

shown in own research, however at farms with less stringent biosecurity, multiple strains can colonize the same flock simultaneously.

**Antimicrobial resistance.** The aassessment of resistance to commonly used antibiotics of all isolated *Campylobacter* strains was conducted. The results of antimicrobial resistance of chicken and turkey isolates are summarized in Table 2 and Table 3, respectively.

None of *Campylobacter* strains isolated from the samples obtained from chicken and turkey offals were resistant to chloramphenicol and erythromycin. According to Wieczorek and Osek (25) chloramphenicol resistance among *Campylobacter* isolates is rare. This is mainly due to the ban on the use of this anti-

**Tab. 2. Prevalence (%) of antimicrobial resistance among *C. jejuni* and *C. coli* strains from chicken**

| Antibiotics | | No. of resistant strains (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | offals | | | ceca | | |
| | | *C. jejuni* (n = 137) | *C. coli* (n = 13) | Total (n = 150) | *C. jejuni* (n = 45) | *C. coli* (n = 5) | Total (n = 50) |
| Quinolones | Ciprofloxacin | 74 (54.1%) | 5 (38.5%) | 79 (52.7%) | 24 (52%) | 2 (40%) | 26 (52%) |
| | Nalidixic acid | 78 (56.3%) | 6 (46.1%) | 84 (56%) | 23 (51.1%) | 2 (40%) | 25 (50%) |
| Tetracyclines | Tetracycline | 37 (27%) | 3 (23%) | 40 (26.7%) | 12 (26.6%) | 1 (20%) | 13 (26%) |
| | Doksycyclinum | 83 (60.5%) | 9 (69.2%) | 92 (61.3%) | 27 (60%) | 3 (60%) | 30 (60%) |
| Macrolides | Erythromycin | 0 | 0 | 0 | 0 | 0 | 0 |
| Aminoglicosides | Gentamicin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Streptomycin | 17 (12.4%) | 1 (7.7%) | 18 (12%) | 6 (13.3%) | 1 (20%) | 7 (14%) |
| Amphenicols | Chloramphenicol | 0 | 0 | 0 | 0 | 0 | 0 |
| β-lactam antibiotics | Ampicillin | 41 (29.9%) | 3 (23%) | 44 (29.3%) | 13 (28.9%) | 1 (20%) | 14 (28%) |

**Tab. 3. Prevalence (%) of antimicrobial resistance among *C. jejuni* and *C. coli* strains from turkey**

| Antibiotics | | No. of resistant strains (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | offals | | | ceca | | |
| | | *C. jejuni* (n = 106) | *C. coli* (n = 12) | Total (n = 118) | *C. jejuni* (n = 26) | *C. coli* (n = 4) | Total (n = 30) |
| Quinolones | Ciprofloxacin | 57 (53.8%) | 5 (41.7%) | 62 (52.5%) | 14 (53.8%) | 2 (50%) | 16 (53.3%) |
| | Nalidixic acid | 64 (60.3%) | 5 (41.7%) | 69 (58.5%) | 16 (61.5%) | 2 (50%) | 18 (60%) |
| Tetracyclines | Tetracycline | 44 (41.5%) | 4 (33.3%) | 48 (40.7%) | 11 (42.3%) | 1 (25%) | 12 (40%) |
| | Doksycyclinum | 56 (52.8%) | 7 (58.3%) | 63 (53.3%) | 14 (53.8%) | 2 (50%) | 16 (53.3%) |
| Macrolides | Erythromycin | 0 | 0 | 0 | 0 | 0 | 0 |
| Aminoglicosides | Gentamicin | 0 | 1 (8.3%) | 1 (0.8%) | 0 | 1 (25%) | 1 (3.3%) |
| | Streptomycin | 14 (13.2%) | 0 | 14 (11.9%) | 4 (15.4%) | 0 | 4 (13.3%) |
| Amphenicols | Chloramphenicol | 0 | 0 | 0 | 0 | 0 | 0 |
| β-lactam antibiotics | Ampicillin | 28 (26.4%) | 3 (25%) | 31 (26.3%) | 7 (26.9%) | 1 (25%) | 8 (26.7%) |

biotic in animals destined for food production because of the lack of a safe level of residues and associated inability to determine the withdrawal period. The susceptibility of all analyzed strains to erythromycin was confirmed in the studies conducted by Wieczorek (24). The results published by Rożynek et al. (17) and Woźniak and Wieliczko (26) also showed low resistance levels, not exceeding 1% to macrolides that are usually used to treat severe infections caused by *Campylobacter* spp.

Not only macrolides, but also quinolones, such as ciprofloxacin and nalidixic acid, are the primary antimicrobials used for the treatment of human *Campylobacter* infections (19). However, the pertinent studies have shown the resistance to ciprofloxacin in 52.7% chicken and 52.5% turkey isolates from offals. Whereas 56.0% and 58.5% of chicken and turkey isolates were resistant to nalidixic acid, respectively. Nevertheless, higher levels of resistance shown for *C. jejuni* than *C. coli* isolates did not result in statistically significant differences (P > 0.05). According to Wieczorek and Osek (25) resistance to quinolones requires only one point mutation in the *gyrA* gene and has increased rapidly among chicken and human *Campylobacter* isolates since the early 1990s. These high resistance rates may also be explained by the allowance of quinolones for therapeutic use in veterinary practice in Poland (24).

Tetracyclines are broad-spectrum agents, which exhibit their activity against a wide range of bacteria. Therefore, they have been extensively used in the therapy of human and animal infections. In the recent years the decreasing sensitivity of bacteria to these agents has been observed. In the aimed study the resistance rates to tetracycline and doxycyclinum among *Campylobacter* isolates from chicken offals were 26.7% (27.0% for *C. jejuni* and 23.0% for *C. coli*) and 61.3% (60.5% for *C. jejuni* and 69.2% for *C. coli*), respectively. Moreover, it was similar among *C. jejuni* and *C. coli* (P > 0.05). Among turkey origin strains from offals 41.5% *C. jejuni* and 33.3% *C. coli* shown to be resistant to tetracycline. Doxycyclinum resistant isolates were 52.8% of *C. jejuni* and 58.3% of *C. coli* strains. Altogether the resistance level of *Campylobacter* strains was 40.7% and 53.3% to tetracycline and doxycyclinum, respectively. A slightly higher resistance rate to tetracycline was observed by Maćkiw et al. (13), who demonstrated 64.3% resistant *Campylobacter* strains, including 62% for *C. coli* and 71.4% for *C. jejuni*. Moreover, the European Union Summary report on antimicrobial resistance points to significant differences in resistance among *C. coli* and *C. jejuni* strains, reaching up to 36.3% and 73.9%, respectively (6).

High efficiency has been confirmed to aminoglicosides, in this way underlining their importance in reduction of *Campylobacter* infections. All *Campylobacter* strains isolated from chicken offals were susceptible to gentamicin. Among the strains of turkey origin from offals, only one isolate (0.8%) was resistant to this agent. On the other hand, relatively low resistance of *Campylobacter* strains to streptomycin was noted. In broilers 12.4% of *C. jejuni* and 7.7% of *C. coli* isolates were resistant to this agent. Among turkey origin strains only *C. jejuni* isolates were resistant to streptomycin, showing resistance level of 11.9%. High sensitivity of bacteria from genus *Campylobacter* to aminoglicosides was showed in the studies conducted by other authors (13, 17, 24).

A slightly higher resistance level was observed to β-lactam antibiotics. The respective rates in chicken and turkey isolates from offals were 29.3% and 26.3%. Rożynek et al. (17) underlined increasing resistance of *Campylobacter* strains to this agent, noting from 2003 to 2005, the increase in the number of strains resistance to ampicillin from 5.8% to 30.4%.

The aimed study revealed that contamination of poultry offals occurs during slaughter processing. To a large extent this is due to the presence of *Campylobacter* in the digestive tract. The research conducted showed no statistically significant differences (P > 0.05) between resistance of *Campylobacter* strains isolated from ceca and offals to selected antibiotics. Among chicken isolates from ceca (Tab. 2) no resistance was observed to erythromycin, gentamicin and chloramphenicol. Several strains were resistant to tetracycline (26%), streptomycin (14%) and ampicillin (28%). The highest levels of resistance was noted to ciprofloxacin (52%), nalidixic acid (50%) and doksycyclinum (60%). The result of resistance of turkey isolates from ceca to tested antimicrobials showed that resistance to quinolones and tetracyclines was more frequent (Tab. 3). All of the isolates were susceptible to erythromycin and chloramphenicol.

Many authors stress an emerging trend of the growth of the number of multi-resistance strains (24, 26). The authors' studies showed that 62% *C. jejuni* and 53.8% *C. coli* strains from chicken offals were resistant to two or more classes of antibiotics. Regarding turkey origin isolates, multi-resistance was detected in 54.7% *C. jejuni* and 53.3% *C. coli* strains. Woźniak and Wieliczko (26) stress that the broad usage of chemotherapy in poultry and increasing use of antibiotics in human medicine contribute to the increase in the percentage of the strains resistant to one or several chemotherapeuticals.

The results obtained showed a high level of *Campylobacter* contamination from 78% positive heart samples of turkey origin to 100% positive of all chicken offals. The highest levels rate were log 2.26 cfu/g and log 2.33 cfu/g in turkey and chicken offals, respectively. Moreover, evaluation of antimicrobial resistance showed macrolides and aminoglicosides to be the first choice antibiotics in treatment of campylobacteriosis.

In conclusion, it should be noted that the consumption of raw or heat-treated at low temperatures

poultry meat or by-products can have a significant risk of *Campylobacter* infections. Poultry offals such as liver, heart and gizzard have many fans among various classes of society due to their low prices, high nutritional value and different and favorite tastes (2). Therefore, the microbiological quality of poultry products is crucial. Cross-contamination of the surface of by-products during processing by contents from the intestines is significant with respect to *Campylobacter* counts obtained from offal samples.

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