PREVALENCE OF MUTATION IN *gyrA* GENE AT THR-86 POSITION IN *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* ISOLATED FROM POULTRY AND HUMANS

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Abstract

The aim of the following research was to analyse the presence of mutation in *gyrA* gene in *C. jejuni* and *C. coli* strains isolated from broilers, laying hens, turkeys collected between 1994-1996 and 2005-2008, and isolated from humans in 2006. The results of the research demonstrated that the highest percentage (100%) of the isolates showing mutation in *gyrA* gene was observed in *Campylobacter coli* strains isolated from turkeys between 2005 and 2008.

**Key words:** poultry, humans, *Campylobacter*, gyrA gene mutation, fluoroquinolones.

Campylobacteriosis is a large epidemiological problem in Poland and other European countries. According to the European Food Safety Authority (EFSA) report from 2007, the number of verified cases of salmonellosis in humans was amounting to 152 cases per 100,000 peoples, while the number of documented cases of campylobacteriosis in humans was calculated at 200 per 100,000 (3). *Campylobacter* genus consists of many different species; however both *Campylobacter jejuni* and *Campylobacter coli* are the major cause of bacterial gastroenteritis in humans (6). Beside increasing percentage of campylobacteriosis cases in humans, additional threat is caused by a growing *Campylobacter* resistance to chemotherapeutics, mainly fluoroquinolones. Resistant strains may be transmitted to humans by poultry products and they may result in difficult to treat infections, which is an important epidemiological issue.

Resistance to fluoroquinolones is commonly due to point mutations in the quinolone resistance determining region (QRDR) of the gyrase A (*gyrA*) gene, involving the Thr-86 for high, and the Asp-90 and Ala-70 amino acid residues for lower-level resistance (4). The most popular mutation resulting in fluoroquinolone resistance is nucleotide substitution in codon 86 (Thr-86→Ile), which causes the change of threonine to isoleucine amino acid. PCR-restriction fragment length polymorphism (PCR-RFLP) method is commonly used for the mutation detection. Substitution of threonine to lysine (Thr-86→Lys) or alanine (Thr-86→Ala) in codon 86 or replacement of alanine to threonine in codon 70 (Ala-70→Thr) may be another reason for growing resistance to fluoroquinolones of *Campylobacter* strains, although, there are other mutations responsible for it. Mutations in codon 90 (Asp-90→Asn) and in codon 104 (Pro-104→Ser) are less frequent and their role in the process of growing resistance is still not clear (16).

The aim of the presented study was to analyse the prevalence of *Campylobacter* sp. strains with *gyrA* gene mutation in isolates from the poultry and humans.

Material and Methods

**Bacterial isolates.** The research material included 314 *Campylobacter* sp. DNA isolates from broilers, turkeys, and laying hens collected between 2005 and 2008, 43 *Campylobacter jejuni* DNA isolates from humans collected in 2006 and obtained from the Department of Bacteriology, National Institute of Public Health – National Institute of Hygiene in Warsaw, and
73 Campylobacter sp. DNA isolates from the museum collection at the Bird Disease Department, Wroclaw University of Environmental and Life Sciences. Museum strains were isolated from broilers and laying hens between 1994 and 1996. The bacterial strains were stored in BHI broth (Oxoid, UK) containing 17% glycerol at -70°C. Template DNA was extracted with commercially available Genomic - Mini® kit (A&A Biotechnology, Poland), according to the manufacturer’s instructions.

Multiplex PCR. Species identification was confirmed by multiplex PCR reaction with specific primers for C. jejuni and C. coli. The test was performed in 25 µl of reaction mixture under conditions described earlier (26). Amplified products were separated by electrophoresis in 2% agarose gel. DNA bands were stained with ethidium bromide, visualised with an ultraviolet transilluminator. The PCR amplicon of 589 bp was specific to C. jejuni species, while 462 bp corresponded to C. coli species.

The determination of mutation in Campylobacter sp. strains using PCR-RFLP method. PCR reaction with the usage of primers such as CjgyrF 5’-AAA TCA GCC GTG ATA GTG GGT GCT GTT TAT CAC CCA CAC ATG GAG GT-3’ and CjgyrR 5’-TCA GTA TAA CGC ATC GCA GC-3’ was performed at the first step of this study. Primers were designed for gyrA gene. PCR amplification was performed in the mini-MJ-Biorad thermocycler. The reaction solution of 25 µl contained: 0.5 µl of dNTP 10 mM (Fermentas, Lithuania), 1 µl of primers (Oligo, Poland) with 25 pM concentration, 1 µl of Red-Taq (Sigma-Aldrich, Germany) polymerase with 1 U/µL activity, 2.5 µl of 10x concentrated buffer to Red-Taq polymerase (Sigma-Aldrich, Germany), 18 µl of bidistilled and deionised water, and 1 µl of genomic DNA (about 30 ng/reaction). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, then 35 cycles of: 30 s of denaturation at 94°C, annealing 1 min at 55°C, complementary DNA synthesis 1 min at 72°C. Amplification products were separated by electrophoresis in 2% agarose gel. DNA was stained with ethidium bromide. A 100 bp ladder (Gene Ruler 100bp DNA Ladder 0.5 µg/µL, Fermentas, Lithuania) was used as a molecular size standard.

The PCR product of 179 bp size was digested by restriction enzyme RsaI (Fermentas, Lithuania) with an activity of 10 U/µL, at 37°C for 2 h. Twenty microlitres of the reactive mixture comprised the following ingredients: 1 µl of restriction enzyme RsaI, 2 µl of 10x concentrated buffer Tango (Fermentas, Lithuania), 7 µl of bidistilled water, and 10 µl of the PCR product.

Products of enzymatic digestion were subjected to electrophoresis in 2% agarose gel with ethidium bromide. Fermentas mass marker (Gene Ruler 100 bp DNA Ladder 0.5 µg/µL, Lithuania) was used for the determination of amplification products. C. jejuni and C. coli strains, obtained from the National Institute of Public Health – National Institute of Hygiene in Warsaw, were used as a positive control. In case of the mutation in gyrA gene, one band was visible in electrophoresis separation. The lack of the mutation was indicated by two bands: 54 and 125 bp as a result of PCR product digestion with restriction enzyme RsaI.

Statistical analysis. The percentage of Campylobacter sp. strains carrying mutation in gyrA gene at Thr-86 position was calculated using Fisher’s test. All the calculations were done in the R statistical packet. The significance level was measured at P<0.05.

Results

Multiplex PCR. Multiplex PCR results showed that 221 (57.1%) strains isolated from poultry were determined as C. jejuni species, while 166 (42.9%) as C. coli. Detailed results regarding particular technological groups are presented in Table 1.

<table>
<thead>
<tr>
<th>Technological group</th>
<th>Sampling period</th>
<th>Number (%) of Campylobacter jejuni</th>
<th>Number (%) of Campylobacter coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broilers</td>
<td>1994-1996</td>
<td>36 (65.5)</td>
<td>19 (34.5)</td>
</tr>
<tr>
<td>Laying hens</td>
<td></td>
<td>14 (77.8)</td>
<td>4 (22.2)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>50 (68.5)</td>
<td>23 (31.5)</td>
</tr>
<tr>
<td>Broilers</td>
<td>2005-2008</td>
<td>84 (43.8)</td>
<td>108 (56.2)</td>
</tr>
<tr>
<td>Laying hens</td>
<td></td>
<td>9 (39.1)</td>
<td>14 (60.9)</td>
</tr>
<tr>
<td>Turkeys</td>
<td></td>
<td>78 (78.8)</td>
<td>21 (21.2)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>171 (54.5)</td>
<td>143 (45.5)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>221 (57.1)</td>
<td>166 (42.9)</td>
</tr>
</tbody>
</table>
Presence of the gyrA gene mutation. Table 2 presents the results concerning the presence of mutations in the gene gyrA among isolates obtained from broilers, laying hens, turkeys in 1994-1996 and 2005-2008, as well as from humans in 2006.

In comparison to the isolates collected in 1994-1996, an increase in the percentage of gyrA mutated isolates obtained from broiler flocks between 2005 and 2008 was found to be statistically significant (P<0.05). Among the isolates collected from broilers between 1994 and 1996, the percentage of isolates with gyrA gene mutation was 23.6%, while the mutation occurred in 78.6% of strains isolated between 2005 and 2008. An increase in the percentage of gyrA gene mutated strains was observed also in the isolates obtained from laying hens. Between 1994 and 1996 the percentage was amounting to 44.4%, while between 2005 and 2008 it reached 65.2%.

The highest percentage (100%) of the isolates demonstrating mutation in gyrA gene was observed in Campylobacter coli strains isolated from turkeys between 2005 and 2008. Similar results were reported among the isolates from broilers and laying hens collected at the same time. There was a high percentage (91.7%) of gyrA gene mutated isolates in C. coli obtained from broilers, while the percentage of C. coli with the mutation of gyrA gene isolated from laying hens was 71.4%. Equally high percentage (62.8%) of gyrA gene mutation frequency was found in C. jejuni isolates obtained from humans.

C. jejuni strains isolated in 1994-1996 from the broilers and laying hens was characterised by a higher proportion of isolates with a mutation in the gene gyrA, in contrast to the strains of C. coli. The percentage of C. jejuni strains with mutation obtained from the broilers was 30.6%, while the percentage of C. coli strains with mutation was only 15.8%. Among isolates obtained from laying hens, the percentage of C. jejuni with mutation in the gene gyrA was up to 57.1%, and among strains of C. coli, the mutation was not detected.

Discussion

The obtained results showed that the percentage of Campylobacter sp. strains with mutation in gyrA gene isolated from poultry is increasing and this creates a great problem. It can be an effect of uncontrolled usage of chemotherapeutics for the purpose of treatment. This may cause a danger to consumers for whom meat infected with microorganisms such as Campylobacter sp. may be a source of bacterial infection. Smith et al. (22) and Guevermont et al. (7) have proved this statement. They reported the presence of the same C. jejuni (with the same genotype) resistant to fluoroquinolones first in poultry and then in humans.
In the presented research, it was demonstrated that more than 60% of strains of *C. jejuni* with *gyrA* gene mutation were isolated from humans. Such a high percentage may be an indirect result of broad and frequent usage of fluoroquinolones in humans and veterinary medicine, which facilitates creation of this mutation in susceptible strains and selection of the resistant strains. Up-to-date records (with the following one) show massive usage of enrofloxacin (chemotherapeutic from the same pharmacological group) in poultry production as one of the main reasons for growing resistance in *Campylobacter* sp. strains (18, 24). In Finland, Sweden, and Australia, the percentage of susceptible to fluoroquinolones *Campylobacter* bacteria isolated from humans is still high (9, 11, 19, 23), which may directly result from the lack of enrofloxacin usage in poultry production limiting transmission of the resistant strains.

The number of reports concerning the resistance of *Campylobacter* sp. isolated from humans to antibacterial chemotherapeutics shows a great interest of scientific centres in this problem. In England, Wilson *et al.* (25) claimed that the percentage of *C. jejuni* strains isolated from humans and resistant to ciprofloxacin has increased from 3% in 1991 to 12% in 1997. In the United States, the percentage of strains resistant to fluoroquinolones has increased from none in 1989 to 13% in 1997 and 19% in 2001 (8). According to the European Centre for Disease Prevention and Control report from 2008, resistance to ciprofloxacin of *C. jejuni* isolated from humans was amounting to 89.6% (2). In some countries, the percentage of *C. jejuni* strains resistant to fluoroquinolones was as high as 80% (10, 17, 21).

Comparing the results obtained in earlier studies (26) on the resistance to fluoroquinolones determined by MIC indicator with molecular method (PCR-RFLP), a higher percentage of strains resistance was reported using MIC indicator. Studies on the resistance of *Campylobacter* sp. strains with MIC indicator were performed on the same group of strains as in the present study. The percentage of isolates resistant to fluoroquinolones collected between 1994 and 1996 from broilers was almost twofold higher than the percentage of strains with the gene *gyrA* mutation. Similar relation was reported during the analysis of strains collected between 2005 and 2008. The difference between the percentage of resistant strains with gene *gyrA* mutation may result, among other things, from other mechanisms responsible for *Campylobacter* sp. fluoroquinolone resistance, not included in the research, such as efflux pumps located in *Campylobacter* sp. cell membrane or mutation in *gyrB*, *parC*, or *parH* genes (7, 12).

It should be stated that membrane pumps play the role of additional defence mechanisms, protecting bacteria from adverse substances, which along with the *gyrA* gene mutation attribute to higher value of the MIC indicator for particular *Campylobacter* sp. strains. Additionally, *Campylobacter* sp. demonstrates high vulnerability to *gyrA* gene mutation, which is revealed during the usage of chemotherapeutics (13, 15).

In summary, the presented study shows a high percentage of *Campylobacter* sp. strains with the mutation of *gyrA* gene, which may be an indirect cause of growing resistance to fluoroquinolones. Therefore, it is very important to use chemotherapeutics rationally and monitor the susceptibility of zoonotic strains such as *Campylobacter* to chemotherapeutics broadly used in poultry production.

### References


