Incidence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* in broiler flocks in the Czech Republic

G. BORILOVA* and I. STEINHAUSEROVA

Faculty of Veterinary hygiene and Ecology / Department of Meat Hygiene and Technology, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho1/3, 612 42 Brno, Czech republic
*Corresponding author: gborilova@vfu.cz

*Campylobacter jejuni* is now recognized as one of the main causes of bacterial foodborne disease in the Czech republic with *Campylobacter coli* less frequently implicated. Foods of animal origin, in particular poultry, have been identified as significant sources of this enteropathogen as a result of infection and contamination at the pre-harvest and harvest levels. In the period from March 2005 to January 2006, fourteen different broiler flocks from the commercial farms with extensional rearing technology from the Czech republic were examined. The methods of cultivation and identification of the isolated strains were used in accordance with the effective ISO guidelines. Confirmation was made by PCR/RFLP technique. Of the total of 674 samples, 78.33 % (n = 528) were positive for the presence of thermophilic *Campylobacter* spp. Ratio of *C. jejuni* and *C. coli* was 91.67 % (n = 484) and 9.09 % (n = 48), respectively. Four samples were evaluated as mixed samples. In seven farms only *C. jejuni* and in six farms both *C. jejuni* and *C. coli* were found. Only one of fourteen conventional broiler farms was *Campylobacter* spp. negative. Antimicrobial resistance was examined by reference agar dilution method in 247 strains and minimal inhibition concentrations (MICś) were determined. High level resistance was detected to nalidixic acid, ciprofloxacin and ampicillin. All isolated strains were sensitive to gentamicin and chloramphenicol. PCR and PCR-RFLP techniques were used for identification of mutations of gyrA gene (resistance to quinolones), two point mutations in 23S rRNA gene (resistance to erythromycin) and for detection of tet genes (resistance to tetracycline). Our results showed that molecular biology methods are usable only for strains with high and middle levels of MICś.

**Keywords**: *Campylobacter* spp.; antimicrobial resistance; agar dilution method; PCR; PCR RFLP

**Introduction**

*Campylobacter* spp. is recognised as one of the main cause of bacterial foodborne disease in the Czech republic. In particular, *Campylobacter jejuni* and *Campylobacter coli* are detected when humans are infected. Foods of animal origin, in particular poultry, have been identified as significant sources of this enteropathogen as a result of infection and contamination at the preharvest and harvest levels (Moore *et al.*, 2005).

Many factors have contributed to the emergence of antimicrobial resistant *Campylobacter* spp. The increasing number of human infections by antimicrobial resistant strains of *C. jejuni* makes the clinical management of campylobacteriosis very difficult. Resistance to antimicrobial drugs can prolong illness and complicate the treatment of patients with bacteremia (Snelling *et al.*, 2005a).
Materials and Methods

The samples were taken in the period from March 2005 to January 2006. The testing was focused on the caecum of broiler chickens from commercial broiler farms with extensional rearing technology. Samples of the digestive tract (duodenum-cloaca) were collected in the slaughterhouse at the slaughter line from each carcass following the slaughtering, degutting and the veterinary inspection at regular intervals once a week. Individual samples were placed to sterile PE bags, put to a cooling box and shipped to the laboratory where the caecum was cut aseptically and 1 g of caecal content for Campylobacter prevalence was investigated.

The samples were transferred to tubes containing 10 ml of saline solution and homogenised in the Stomacher. 10 µl of initial dilution of each sample were spread onto modified charcoal cefoperazone deoxycolate agar plates (Campylobacter Blood-Free Selective Agar Base CM0739, Oxoid, UK, CCDA Selective supplement SR0155, Oxoid, UK) and incubated for 48 hours at 42°C in microaerobic atmosphere (Gas Generating Kit Campylobacter system, Dixo-Oxoid, CZ). Typical greyish – white colonies with trailing growth were selected for the next confirmation.

Genomic DNA was isolated using commercial DNeasy® Tissue Kit (Qiagen, UK). Each Campylobacter spp. strain was identified to the species level by using biochemical tests and polymerase chain reaction (PCR). Biochemical profiling system was based on the detection of hippurate hydrolysis and on the detection of cytochrome oxidase according to manufacturers instructions for MIKRO/-LA-TEST® (PLIVA – Lachema, CZ). The PCR technique involved the amplification of a 491 bp ampiclon of a highly polymorphic part of the 23S rRNA gene and further species differentiation was accomplished by digestion of the PCR product using two restriction enzymes, AluI and Tsp509I, resulting in characteristic restriction fragments for each species (Fermér and Engvall, 1999).

Antimicrobial resistance testing was done using agar dilution method in accordance with the NCCLS guideline (M11-A6, 2004) and using molecular biology methods. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration that achieved inhibition of visible growth of Campylobacter spp. strains.

Seven antimicrobials were tested: ampicillin (Sigma-Aldrich, CZ), tetracycline (Sigma-Aldrich, CZ), nalidixic acid (Sigma-Aldrich, CZ), chloramphenicol (Sigma-Aldrich, CZ), erythromycin (Sigma-Aldrich, CZ), ciprofloxacin (Ciprinol 100 mg / 50 ml, Krka, PL) and gentamicin (Sigma-Aldrich, CZ). The MIC’s determination of the antimicrobial agents was carried out on Mueller–Hinton agar (Dixo-Oxoid, CZ) supplemented with 5 % sheep blood (Bioveta, CZ) and mixed with the relevant dilution of antimicrobial drug. The inoculum was prepared from 48-hour culture in Brain Heart Infusion (Dixo-Oxoid, CZ) and suspension was adjusted to 0.5 McFarland (the final cell number 10⁵ / ml). Samples were incubated at 42°C in micro-aerobic conditions (Gas Generating Kit Campylobacter system, Dixo-Oxoid, CZ) for 48 hours. Susceptibility categorisation was determined according to the NCCLS guideline (M11-A6, 2004) and the statement of the Antibiogram Committee of the French Society for Microbiology (1999). The reference strains Campylobacter jejuni subsp. jejuni ATCC 33560 (CCM, CZ) and Campylobacter coli ATCC 43478 (CCM, CZ) were used as the control.

The resistance to (fluoro-) chinolones was also investigated by the mismatch amplification mutation assay (MAMA) PCR (Zirnstein et al., 1999, Zirnstein et al., 2000). Three specific primers were chosen for detection of point mutation (Thr-86 -Ile) in quinolone resistance determining region (QRDR) of the gyrA gene in C. jejuni and C. coli isolated strains.

For confirmation of resistance to erythromycin two point mutations at positions 2074 and 2075 on the 23S rRNA gene in domain V were detected using the PCR – restriction fragment lenght polymorphism (RFLP) technique with BsaI and BceAI enzymes. This method was published by Vacher et al. (2003).

The resistance to tetracyclines was monitored by PCR technique developed by Gibreel et al. (2004). Using specific primers the presence of tet(O) and tet(M) genes asscocited with tetracycline resistance in Campylobacter spp were detected.
Results and discussion

Incidence of Campylobacter spp. in broiler chicken

In the period of March 2005 to January 2006, fourteen different broiler flocks from the commercial farms with extensional rearing technology from the Czech Republic were examined. The number of 674 taken samples were investigated for the presence of thermotolerant Campylobacter spp. and 78.33 % (n = 528) were confirmed as campylobacter-positive. Higher percentage was found during the summer and autumn (100 %) compared to the samples from winter (60 %). Our results indicate high prevalence of Campylobacter spp. in broiler flocks in the Czech Republic. Similar data were also reported in studies from France, Italy, Geat Britain and Switzerland (Snelling et al, 2005b; Padungton et al., 2003; Refregier-Petton et al., 2001), Atanassova and Ring (1999), while reports from eastern Europe and Scandinavian countries show much lower contamination (Padungton et al., 2003).

C. jejuni dominated among the Campylobacter strains isolated from chicken. The number of 484 (91.67 %) positive strains were phenotyped as C. jejuni according to the PCR-RFLP. Many studies showed, that the PCR-RFLP method offers more accurate results for species identification than the hippurate test, that could give false negative reactions. The hippurate test is, however, still often used for differentiation between C. jejuni and C. coli, even though it could be uncertain (Rönner et al., 2004, Engvall et al., 2002). Of the isolated strains only 9.09 % (48) were confirmed as Campylobacter coli. Four samples (0.8 %) were evaluated as mixed samples, because they showed the presence of both. Our results correspond with the data from the other European countries, where C. jejuni represents the most frequently isolated species (70 – 90 %) than other thermotolerant campylobacters from poultry (Avrain et al., 2003; Snelling et al., 2005b).

Antimicrobial resistance

The incidence of antimicrobial resistance of 207 C. jejuni strains and 40 C. coli strains tested using agar dilution metod is shown in Table 1.

### Table 1 Antimicrobial resistance of 247 Campylobacter spp. strains isolated from broiler chicken

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>C. jejuni</th>
<th>C. coli</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N(^a)</td>
<td>n/N(^a)</td>
<td>n/N(^a)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>86/207 (41.5)</td>
<td>38/40 (95)</td>
<td>124/247 (50.2)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>95/207 (45.9)</td>
<td>38/40 (95)</td>
<td>133/247 (53.8)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>30/207 (14.5)</td>
<td>5/40 (12.5)</td>
<td>35/247 (14.2)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>13/207 (6.3)</td>
<td>3/40 (7.5)</td>
<td>16/247 (6.5)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50/207 (24.1)</td>
<td>39/40 (97.5)</td>
<td>89/247 (36)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0/207 (0)</td>
<td>0/40 (0)</td>
<td>0/247 (0)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0/207 (0)</td>
<td>0/40 (0)</td>
<td>0/247 (0)</td>
</tr>
<tr>
<td>Drug resistance(^b)</td>
<td>101/207 (48.8)</td>
<td>39/40 (97.5)</td>
<td>140/247 (56.7)</td>
</tr>
<tr>
<td>Multiresistance(^c)</td>
<td>22/207 (10.6)</td>
<td>7/40 (17.5)</td>
<td>29/247 (11.7)</td>
</tr>
</tbody>
</table>

\(^a\) The number of resistant strains/the number of tested strains  
\(^b\) Resistance to one or more antimicrobial drugs  
\(^c\) Resistance to four or more antimicrobial drugs

In our study, higher percentage of C. jejuni strains (41.2 %) was susceptible to all antibiotic tested, in comparison with C. coli strains (2.5 %). The resistance to one or more antibiotic was overall 56.7 % and there is also significant difference between C. jejuni (48.8 %) and C. coli (97.5 %). Studies from Slovenia, France and Brasil showed similar results (Kurinčič et al., 2005; Avrain et al., 2003; De Moura Oliveira et al., 2006).
Multiresistance (resistance to four or more antibiotics) reduces therapy of human patients by limiting choice of antibiotics. Our results showed the multiresistance profile 11.7 % overall, for *C. coli* 17.5 % and for *C. jejuni* 10.6 %. Our data are comparable with the results of multiresistance studies from Slovenia (overall 10.9 %) (Kurinčič et al., 2005), but is much higher than the profile presented from Ireland (0.8 % for *C. jejuni* and 0 % for *C. coli*).

The percentages of strains resistant to nalidixic acid, ciprofloxacin, tetracycline and ampicillin were higher for *C. coli* than for *C. jejuni*. No *Campylobacter* spp. strains resistant to gentamicin and chloramphenicol were found. Sensitivity to gentamicin and chloramphenicol showed also the studies from France (Avrain et al., 2003) and Sweden (Rönner et al., 2004).

Macrolides are the agents of choice for treating *Campylobacter* infections. Resistance to erythromycin were mainly found in strains of animal origin, especially *C. coli* from pigs and *C. jejuni / C. coli* from chickens (Engberg et al., 2001). Our results presented the resistance to erythromycin (MIC > 8 µg /mL) in *Campylobacter* spp. 14.2 % (14.5 % for *C. jejuni* and 12.5 % for *C. coli*). Kurinčič et al. (2005) showed the similar percentage of erythromycine resistance strains (14.5 % overall), but De Moura Oliveira et al., (2006) published extremely high prevalence (80 %). On the other hand in Sweden no resistance to the macrolides was found (Rönner et al., 2004). Erythromycine-resistant strains were tested for presence of mutations at position 2074 and 2075 of 23S rRNA gene which are associated with macrolide resistance (Vacher et al., 2003). Our results showed that these point mutations were detected only in strains with high level of MIC (≥128 µg /mL). Low-level resistance is conditioned by efflux system (Gibreel et al., 2005)

The presence of the efflux system has been described as one of the mechanisms for the development of quinolone resistance. Zirnstein et al., (1999) described as the main resistance mechanism the mutations in the target topoisomerase, in particular mutation at position Thr-86-Ile in the quinolone resistance determining region (QRDR) of the gyrA gene. These mutations are more commonly associated with resistance to nalidixic acid and ciprofloxacin in *Campylobacter* spp. In this study, detection of point mutation at position 86 of the gyrA gene using MAMA PCR was compared with MIC determined by agar dilution method. We confirmed, that this gyrA mutation is significantly associated with resistance to nalidixic acid, but nonsignificantly with resistance to ciprofloxacin. The same results were presented by Jesse et al., (2006). Our monitoring showed that 53.8 % of the isolated strains were resistant to nalidixic acid (MIC ≥ 32 µg / mL) and 50.2 % to ciprofloxacin (MIC ≥ 4 µg / mL). The occurrence of resistance was much lower in *C. jejuni* (45.9 % to nalidixic acid and 41.5 % to ciprofloxacin) than in *C. coli* (95 % to nalidixic acid and 95 % to ciprofloxacin). The study from Belgium (Van Looveren et al., 2001) presented the similar high percentage of (fluoro-)quinolone resistant strains in broilers (42 % of *C. jejuni* and 62 % of *C. coli*). In opposite the reports from Sweden (Rönner et al., 2004) and from Spain (De Moura Oliveira et al., 2006) showed no increased quinolone resistance (2 % / 0 % to nalidixic acid and 2 % and 0 % to ciprofloxacin) in *Campylobacter* spp. strains from poultry.

The percentage of the strains resistant to tetracycline was 6.5 % (6.3 % for *C. jejuni* and 7.5 % to *C. coli*). Other researchers reported higher resistance rates (e.g. 100 % - De Moura Oliveira et al., (2006), 24.1 % Avrain et al., (2003). Tetracycline resistance are preliminary plasmid-mediated. Association of the tet(O) and tet(M) genes with tetracycline resistance was described by Gibreel et al., (2004). We monitored the presence of this genes using PCR techniques in strains which were detected as intermediate or resistant to tetracycline using agar dilution method. The results showed only the presence of tet(O) gene. The possible explanation may be that tetracyclines are used as therapeutic or preventer to feed additives for livestock and for poultry abroad but in the Czech republic is using of antibiotic drugs as feed additives suppressed.

Use of several antimicrobial agents, either growth promoters or as drugs seemed to reflect the resistance level of baterials. Modern food animal production depends on the use of large amounts of antibiotics for disease control. This provides favourable conditions for selection, spread and persistence of antimicrobial-resistant bacteria capable of causing infections in animals and humans (Moore et al., 2005). This has emphasized to need for global initiatives of monitoring system for determining the occurrence of resistance of foodborne pathogens in all countries.

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References


