Antimicrobial Sensitivity of Campylobacter jejuni Poultry Isolates from the Republic of Bulgaria

Valentina URUMOVA1*, Todor STOYANCHEV2, Mihni LYUTSKANOV1, Hristo DASKALOV3, Ivan VASHIN2, Atanas MARAMSKI3

1Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, 6000, Stara Zagora, Bulgaria
2Department of Food Safety, Veterinary Legislation and Management, Faculty of Veterinary Medicine, Trakia University, 6000, Stara Zagora, Bulgaria
3Head of National Reference Centre of Food Safety, Sofia, Bulgaria

*Corresponding Author: Valentina URUMOVA Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, 6000, Stara Zagora, Bulgaria

e-mail: valentina_62@abv.bg

ABSTRACT

A total of 110 caecal samples were collected from broiler chickens originating from 6 small poultry farms in Bulgaria during the first half of 2009, and (45) Campylobacter jejuni strains were isolated. Twenty four (53.3%) of the C. jejuni isolates were determined as resistant to several antibiotics. The highest percentage of resistance was observed against (tetracycline) – 22.2%, (enrofloxacin) – 13.4% and (ampicillin) – 11.1%. Two strains (4.4%) were resistant to erythromycin. The distribution of isolates on the basis of Minimal Inhibition Concentration revealed the highest value of 64 μg/ml to tetracycline in one of the strains. Four isolates resistant against ampicillin were with MIC of 32 μg/ml, and another one – with MIC 16 μg/ml.

Key Words: Campylobacter jejuni, poultry, antibacterial sensitivity

ÖZET

BULGARİSTAN'DA KANATLI KÖKENLI CAMPYLOBACTER JEJUNI İZOLATLARININ ANTİBİYOTİK DUYARLILIKLARI

2009 yılının ilk yarısında Bulgaristan'da 6 farklı küçük tavuk çiğnengage bulunan etlik piliçlerden toplam 110 sekum örneği toplandı ve 45 adet Campylobacter jejuni suşu izole edildi. C. jejuni izolatlarının yirmi dördünün (%53,3) çeşitli antibiyotiklere karşı dirençli olduğu belirlendi. Yüksek oranlarda tetrasiyklin (%22,2), enroflokksasin (%13,4) ve ampisilin (%11,1)'e karşı dirençtı tüctü. İki suş (%4,4) eritromisin dirençli bulundu. Minimum inhibisyon konsantrasyonuna bağlı olarak, izole edilen suşların tetraksikline karşı en yüksek değerinin 64 μg/ml olduğu belirlendi. Ampisiline dirençli dört izolatın MİK değeri 32 μg/ml ve birinin MİK değeri 16 μg/ml olarak saptandı.

Anahtar Kelimeler: Campylobacter jejuni, kanatlı, antibakteriyel duyarlılık
Introduction

Many authors believe that Campylobacter spp. and C. jejuni in particular are among the most important etiological agents of human bacterial diarrhoea (Allos, 2001). As representatives of the transient resident microflora, Campylobacter spp. could be detected in the gut of many warm-blooded animals. In various stress conditions, mostly of alimentary origin, campylobacterias could provoke infections and especially, diseases with diarrhoeic syndrome. Shedding, more commonly incidental occurs with feces, and the infection of susceptible species, including men, occurs by the fecal-oral route.

Domestic fowl are of special importance for the epidemiology of Campylobacter infections as reservoir hosts because of the significant carrier rates in their gastrointestinal tract and gallbladders. Diseases, etiologically related to Campylobacter spp., are rarely observed in poultry, but cases of hepatitis, caused by C. jejuni, are occasionally observed.

The importance of poultry as a source of human intestinal campylobacteriosis is much higher. The reason is the possibility for contamination during the slaughter of birds and carcass processing with all related risks.

On the other hand, the treatment of chickens with chemotherapeutics often results in selection of resistant strains, including Campylobacter spp. isolates against one or multiple antimicrobial drugs. That is why poultry and poultry products pose also a hazard for potential transfer of (multi)resistant strains to human (Endberg et al., 2001). In this connection, the resistance of Campylobacter spp. to fluoroquinolones and particularly to enrofloxacin, is especially important (Hoge et al., 1998; Mc Dermot et al., 2002; Saenz et al., 2000; Van Den Bogaard and Stobberingh, 1999; Van Looveren et al., 2001).

There is evidence that apart intestinal infections in men, campylobacterias could also provoke bacteriaemia, reactive arthritis, abortions as well as that they are involved in the Guillain-Barre syndrome (Leonard et al., 2004; Skirrow, 1991; Yuki, 2001).

So far, a purposeful investigation on the resistance of poultry Campylobacter spp. isolates in the Republic of Bulgaria has not been performed despite the extensive use of both fluoro quinolone and macrolides, especially in broiler chickens production systems.

Objective of the study was to determine the resistance pattern of Campylobacter spp. isolates from poultry to antimicrobial agents.

Materials and Methods

Bacterial isolates: A total of 110 caecal samples were obtained from broiler chickens originating from 6 small poultry farms in the Southern region of Bulgaria during the first half of 2009. Caecal samples were collected during slaughter and were transported in a plastic bag at room temperature (≤ 20°C) and cultivation in selective medium was done at the same day.

Nutrient media and culturing: For isolation of Campylobacter spp., from caecal samples Campylobacter enrichment broth medium (Merck), containing antimicrobial supplements (trimethoprim - 5 mg/L), polymyxin B- (5 UI/mL), vancomycin (10 mg/L), (Campylobacter Selective Supplement, product number SR0069, Oxoid) was used. Pre-enrichment started with a resuscitation procedure that consists of 4h incubation at 37°C after which the pre-enrichment broths were transferred to 42°C for 48h. For subcultivation of bacteria, Campylobacter agar (Merck), supplemented with vancomycin (10 mg/L), polymyxin B (2,500 UI/L) and trimethoprim
(5 mg/L), (Campylobacter Selective Supplement, product number SR0069, Oxoid) was used. To the basic agar medium, 10% defibrinated horse blood (product number SR0050, Oxoid) was added. Streaked plates were incubated at 42°C in anaerobic jar under a microaerophilic atmosphere produced from gas generating sachets (Campy- Gen, Oxoid, 5% O₂, 10% CO₂ & 85% N₂) for 48 h.

Identification: Suspected colonies on each plate were sub-cultured on Columbia agar (Oxoid) with 5% sheep blood and incubated in a microaerophilic environment at 42°C for 24 to 36 h. Suspect colonies were noted for absence of hemolysis on blood agar, shiny, convex, colorless to grayish colony characteristics with irregular or round edged nature. Microscopy (wet mount) was done to see characteristic darting motility with iris diaphragm closed effectively to contrast the field. Gram stained morphology showed a Gram-negative organism with “S”-shaped appearance.

The phenotypic determination of isolates was performed by the some basic classical phenotypic characteristics of the most important thermophilic Campylobacter species, like sensitivity to nalidix acid (30 μg) and cefalotin (30 μg), production of catalase and oxidase, hydrolysis of hippurate, hydrolysis of indoxyl acetate, aerobic growth at 41.5 °C, microaerophilic growth at 25 °C, in conditions according proposal tests for identification of Campylobacter spp. by Winn et al. (2006) and OIE Terrestrial Manual (2008). The additional identification of the species of isolates was performed with API Campy (Biomérieux, France).

Antimicrobial drug susceptibility testing: For determination of MIC, twofold serial dilutions of chemotherapeutics in agar were carried out according to CLSI (2006) standards. Muller-Hinton agar (Oxoid) supplemented with 5% defibrinated sheep blood (mechanically defibrinated aseptically collected sheep blood) was used. The sensitivity to ampicillin, tetracycline, erythromycin (National Center of Infectious and Parasitic Diseases-BULBIO, Bulgaria) and enrofloxacin (Bayer, Leverkusen, Germany) was tested.

Standardized bacterial suspensions were preliminary prepared in sterile physiological saline, equivalent to 0.5 McFarland density units with final concentrations of 10⁴ cfu/ml. The cultivation was performed at 42°C for 24 h, at microaerophilic conditions (Campy-Gen, Oxoid, 5% O₂, 10% CO₂, 82% N₂). Campylobacter jejuni ATCC 33560 was used as the quality control organisms.

Isolates were scored as sensitive (S), intermediate (I) and resistant (R) for MICs based on CLSI criteria for animal bacterial isolates – CLSI, (2006), Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Sixteenth Informational supplement (M100-S16) and Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, Tentative guideline M31-A3-Third Edition, Report of the Task Force on Zoonoses Data Collection including a proposal for a harmonized monitoring scheme of antimicrobial resistance in Salmonella in fowl, turkeys and pigs and Campylobacter jejuni and Campylobacter coli in broilers, EFSA (Anonymous, 2007). (Table 1).

Results

A total of 110 caecal samples were obtained from broiler chickens for bacteriological analysis and (45) Campylobacter jejuni strains were isolated. The results from testing the sensitivity of Campylobacter jejuni isolates through their MICs to antimicrobial drugs are presented in (Table 2).

The lowest MICs (0.25 μg/ml) were observed against erythromycin and enrofloxacin although in few isolates – 13.3% (6) and 4.4% (2), respectively. The major part of strains exhibited MICs of 0.5 μg/ml – 80.0% (36) against erythromycin and 82.2% (37) against enrofloxacin. With respect to tetracycline however, there were isolates with MICs within 16–64 μg/ml, and to ampicillin – in the range 16–32 μg/ml; in the first case more than 1/5 (9) of isolates were concerned and in the second – more than 10% (5).
Valentina Uramova, Todor Stoyanchev, Milhni Lyutskanov, Hristo Daskalov, Ivan Vashin, Atanas Maramski

Table 1. MICs value of tested chemotherapeutics among *Campylobacter jejuni* isolates.

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Dilutions line (µg/ml)</th>
<th>Sensitive (µg/ml)</th>
<th>Intermediate (µg/ml)</th>
<th>Resistant (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.03-64</td>
<td>≤8</td>
<td>16</td>
<td>≥32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.03-64</td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.03-64</td>
<td>≤4</td>
<td>8</td>
<td>≥16</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.03-8</td>
<td>&lt;0.5</td>
<td>-</td>
<td>≥1</td>
</tr>
</tbody>
</table>

*Campylobacter jejuni* ATCC 33560-A: 2 µg/ml; T: 0.5 µg/ml; E: 0.25 µg/ml; Enr- 0.25 µg/ml

Table 2. Distribution of MICs value of antimicrobial agents among *Campylobacter jejuni*.

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Percentage of isolates, according MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.03</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>-</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. Frequency of resistance (%) against tested antibacterial agents among *Campylobacter jejuni* isolates.

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>39</td>
<td>86.7</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>35</td>
<td>77.8</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>43</td>
<td>95.6</td>
<td>1</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>39</td>
<td>86.6</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3 shows the frequency of resistant pattern among *Campylobacter jejuni* isolates to tested antimicrobial drugs. Increasing rates of erythromycin (95.6%) sensitivity of *Campylobacter spp.* were observed followed by sensitivity to ampicillin and enrofloxacin (86.6%). Most commonly, strains were resistant to tetracycline (22.2%). Intermediate sensitivity was shown with regard to ampicillin.

As multiresistance was concerned, a small percentage of isolates (4.4%) showed a resistance to enrofloxacin and tetracycline, whereas 2.2% (1) of *Campylobacter spp* were resistant to both erythromycin and tetracycline and another 2.2% (1) to both ampicillin and enrofloxacin.

Twenty-four (53.3%) of all tested *Campylobacter jejuni* strains were resistant to one or several of tested antimicrobial drugs. The highest resistance was exhibited against tetracycline (22.2%), followed by enrofloxacin (13.4%) and ampicillin – 11.1%. Two strains (4.4%) only were resistant to erythromycin.
**Discussion**

The isolation of resistant *Campylobacter jejuni* strains from poultry is not surprising, especially with respect to tetracycline’s. They are frequently used in poultry husbandry in Bulgaria. Resistance to tetracyclines was reported in other countries too. Chen et al. (2010), documented a high percent of resistant *C. jejuni* poultry isolates in different regions of China both to tetracycline (100%) and enrofloxacin (over 98.0%) and at a lower extent, to erythromycin (26.7%). The reported occurrence of multiresistance is also significant – in about 90% of isolates. Having monitored the resistance pattern in poultry, porcine and human *Campylobacteria* spp. isolates within 1999 – 2004, a high resistance to tetracycline/doxycycline in poultry isolates (40.6%) for 2004, was also observed by Gallay et al. (2007). Similar data were published by Tetsuo et al. (2007) in Japan. The authors analyzed the increasing resistance to tetracyclines and quinolones in Japan with regard to the increasing use of these groups of chemotherapeutics in productive animals.

In many European countries, monitoring programs for control of drug resistance and policies for restriction of the use of antibiotics and chemotherapeutics for prevention purposes are implemented. This is especially valid for birds. Nevertheless, the levels of (multi)resistance are high. The result of such a monitoring program in Denmark, concerning a higher percentage of resistance to ciprofloxacin and nalidixic acid in broiler chicken and human *C. jejuni* isolates, despite the ban on the use of fluoroquinolones in the country since 2003, were presented by Skjot-Rasmussen et al. (2009). Similar to our findings, the highest observed resistance of isolates was against tetracycline. High levels of resistance to ciprofloxacin (99.0%) in poultry *Campylobacteria* spp. isolates were reported in Spain by Saenz et al. (2000), as far as 1997-1999.

In the first monitoring on bacterial resistance among *Campylobacteria* spp. strains isolated from broiler chickens and men in the Czech Republic (2006-2007), a higher resistance in animal isolates compared to human ones also was established by Bardon et al. (2009).

Unlike all cited studies, Norstrom et al. (2007), detected a low resistance of *Campylobacter* spp. strains isolated from the environment of broiler chickens in Norway, including to oxytetracycline and quinolones (1.3%), and the resistance to ampicillin was (4%). The authors attributed the results to the rare use of antibiotics in the national livestock husbandry.

Despite the numerous specific data about the different regions of the country with respect to the resistance of poultry campylobacteria to commonly used antimicrobial drugs, the high resistance to tetracyclines and the increasing resistance to fluoroquinolones pose a significant risk and indicate a need for revision and reevaluation of their use. This is also valid for our country, where the monitoring of bacterial resistance is just beginning.

**REFERENCES**


CLSI. 2006. Clinical and Laboratory Standards Institute. Performance Standards for...


