GENERAL REVIEW

UDC: 579.61/.62:615.015.8]:616.34-002-022
DOI : 10.2298/VSP1302200T

Frequency of antimicrobial resistance in thermophilic *Campylobacter* strains from humans, poultry and pigs

Učestalost antimikrobne rezistencije termofilnih *Campylobacter* sojeva poreklom od ljudi, živine i svinja

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Key words:
campylobacter; anti-bacterial agents; drug resistance; humans; animals; poultry.

Ključne reči:
campylobacter; antibiotici; lekovi, rezistencija; ljudi; životinje; živina.

Introduction

Campylobacteriosis is classified as zooanthroponosis. It is an infection caused mainly by thermophilic campylobacters: *Campylobacter jejuni, Campylobacter coli, Campylobacter lari, Campylobacter upsaliensis. Campylobacter jejuni* and *Campylobacter coli* are causing the most important bacterial intestinal infections in modern era, with 400 000 000 patients in the world every year. A very important factor in intestinal campylobacteriosis development is a very low infective dosis of only 500 bacteria [1].

Humans get infected by this bacteria consumming insufficiently thermally processed meat, mostly poultry meat, pork and beef [2, 3], consumming unpasteurised milk and contaminated water [4], and beeing in contact with domestic pets [5]. Important role of poultry in human infections is demonstrated in Belgium during the Dioxin crisis in 1999, when, due to high levels of poison dioxin detected, domestic poultry and eggs were withdrawn from the market, resulting in lowering number of campylobacteriosis cases by 40% [2].

Thermophilic *Campylobacter* spp. mostly produce intestinal disorders, but could also produce extraintestinal disorders. Gut lesions in intestinal campylobacteriosis, similar to infections due to *Salmonella* and *Shigella* genera, are manifested as inflammatory infiltrates in lamina propria and abscesses in crypts [6]. The most frequent extraintestinal forms of disease are: meningitis, endocarditis, septic arthritis, osteomyelitis and neonatal sepsis. Several cases of myocarditis as a complication of *Campylobacter jejuni* infection were reported.

Secondary diseases reported by various authors [5, 7] as a consequence of thermophilic *Campylobacter* spp. primary infection, are Guillain-Barré syndrom (GBS) and Reiter's syndrom. Arthritis, GBS and Miller-Fisher's syndrom (a form of GBS) are possible complications in campylobacteriosis. Campylobacteriosis is generally a mild and self-limiting disorder. In patients with more severe and prolonged forms, an antibiotic treatment is recommended [8].

Although a significant percentage of animals is colonized, they rarely develop a disease, but they are reservoirs of infection for humans. Poultry aged 2–3 weeks are in 50%–90% of cases colonized by thermophilic *Campylobacter* spp. [3]. Swines are less than poultry colonized by the same bacteria. Tambur et al. [9] demonstrated that 80.88% of poultry and 77.27% of swines are contaminated by thermophilic *Campylobacter* spp.

After inoculation to newborn calves thermophilic *Campylobacter* spp. produce a mild and self-limiting enteritis and bacteriemia. *Campylobacter* spp. can produce dysentery in cattle and swine [7]. *Campylobacter jejuni* produces abortions in sheeps, acute enteritis in calves, dogs and cats, and hepatitis in poultry. Clinical symptoms of hepatitis in poultry are somnolence, weakness, diarrhoea and eggs-laying disorders [10].

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Treatment of campylobacteriosis and investigation of susceptibility to antibiotics

Drugs, generally used in human campylobacteriosis treatment are: erythromycin, quinolones, tetracyclin, ampicillin, chloramphenicol and gentamycin. Disk-diffusion test, E-test with strips and agar dilution test are used in investigations of susceptibility to antibiotics.

Different results were obtained by the three methods applied in investigation of susceptibility [2].

Antimicrobial susceptibility testing in *Campylobacter* spp. and methodology standardization

At present, several methods have been employed for *Campylobacter* susceptibility testing. Agar dilution is recommended by many authors [8, 11], but it is time consuming and not suitable for routine laboratory work. The E-test, a diffusion method with MIC determination, gives results faster than agar dilution, but its cost and need for standardization can be limitating [12]. Also, some authors recommended broth dilution method [13] (microbroth dilution by Trek, VetMic etc.) as suitable for routine use. Although disc diffusion is the simplest method, absence of available standards limits its application in clinical laboratories.

Disc diffusion and agar dilution are often compared in order to obtain diameter zone for application in routine work. With respect to these methods, Gaudreau and Gilbert [14] reported complete agreement for tetracycline and ciprofloxacin, with only minor differences for erythromycin but poor correlation coefficient for ampicillin. Similarly, Luangtongum et al. [15], revealed an excellent correlation between the agar dilution and the disk diffusion for aminoglycosidesand, quinolone/fluoroquinolones; a high-level correlation for erythromycin, clindamycin, and tetracycline, and a weak correlation for ampicillin. They suggested setting the MIC breakpoint for erythromycin-susceptible *Campylobacter* strains at ≤ 2 μg/mL and ≥ 8 μg/mL for resistant isolates and the zone diameter breakpoints of the disk diffusion method at ≥ 23 mm for susceptible isolates and ≤ 18 mm for resistant isolates. Also, they recommended the MIC breakpoints for clindamycin to be ≤ 2 μg/ml for susceptible isolates and ≥ 8 μg/mL for resistant strains and the zone diameter breakpoints ≥ 17 mm for susceptible isolates and ≤ 12 mm for resistant ones. Proposed values for the zone diameter breakpoints for tetracycline are ≥ 28 mm for susceptible strains and ≤ 8 mm for resistant strains. Authors also suggested that the disk diffusion method can be used as a reliable alternative method for susceptibility testing of thermophilic *Campylobacter* to several classes of antimicrobial agents, particularly to quinolone/fluoroquinolones and aminoglycosides.

Gaudreau et al. [16] recommended zone diameters of 6 mm and ≥ 20 mm around the erythromycin disk as resistant and susceptible breakpoints of *C. jejuni* isolates. Also, for ciprofloxacin susceptibility testing of *C. jejuni* isolates, zone diameters of ≤ 17 mm and ≥ 21 mm around the ciprofloxacin disk and the absence or the presence of an inhibition zone around the nalidixic acid disk are suggested as breakpoints for resistance and susceptibility, respectively.

With disk diffusion, the following zone diameters were proposed to be resistant and susceptible breakpoints, respectively, for susceptibility testing of *Campylobacter* coli: no inhibition zone and ≥ 15 mm for erythromycin, and ≤ 20 mm and ≥ 25 mm for ciprofloxacin, in the absence or presence of an inhibition zone around the nalidixic acid disk. For susceptibility testing of *C. coli* and *C. jejuni*, diameter zones ≤ 20 mm and ≥ 26 mm for tetracycline were recommended [17].

A recommendation, followed by these findings, is given that disk diffusion could be used to detect *C. jejuni* and *C. coli* isolates with reduced susceptibilities to ciprofloxacin and erythromycin in clinical laboratories [18].

Up to date, The Clinical and Laboratory Standards Institute (CLSI), has established minimal inhibitory concentration (MIC) breakpoints for agar dilution for erythromycin, ciprofloxacin, tetracycline and doxycycline. In addition, for disc diffusion, zone diameter is given only for erythromycin and ciprofloxacin [19]. EUCAST (the European Committee on Antimicrobial Susceptibility Testing) is still working on standards and epidemiological cut off is proposed for *C. jejuni* and *C. coli* for erythromycin, ciprofloxacin, tetracycline, streptomycin, gentamicin, chloramphenicol, and nalidixic acid [20].

Molecular techniques, also, can be applied for resistance determination as the Mismatch Amplification Mutation Assay (MAMA-PCR) [21], and the Lightcycler mutation assay [22] for the detection of ciprofloxacin-resistant *C. jejuni* and *C. coli* isolates. However, these and similar techniques can be applied only if prior knowledge about genetic basis for resistance exist. Usually, they cannot be refered to a routine resistance detection, and may not detect resistance if a new resistance mechanism emerge [22]. Some authors consider that combination of phenotypic and genotypic methods in resistance detection should be more convenient [23].

Mechanisms of erythromycin resistance in campylobacters

Erythromycin and other macrolide antibiotics bind to the subunit 50S of bacterial ribosome and restrict elongation of polypeptide chain [24]. Sites for macrolide action are parts of subunits 23S rRNA, and ribosomal proteins L4 and L22. Proteins L4 and L22 form parts of exit channel for polypeptide in bacterial ribosome 70S and they are described in several bacterial species [25]. Eyithromycin resistance can be mediated by enzymatic inactivation, can evolve through target modification by mutation or methylation, and by active efluxe [26]. In *Campylobacter*, resistance to macrolides confer to gene mutation with change of target site for drug binding to bacterial ribosome [27]. Other mechanism that confer resistance is active effluxe [28]. Resistance occurs as synergy between gene modification and efflux pumpe CmeABC activity [29]. Two types of resistance to macrolides are described: resistance to high levels of drug concentration (high level resistance - HLR) [25] and resistance to lower drug concentration (low level resistance - LLR) [28]. In HLR, MICs for erythro-

mycin are higher than128 mg/L, and in LLR, MICs are in range from 8–16 mg/L [25, 30]. In *C. jejuni* and *C. coli* strains, HLR is a consequence of mutation in 23S rRNA V domen in target gene at the positions 2074 and 2075. LLR can be a result of effluxe pumpe activity [31]. Also, it is recognized that modifications of L4 and L22 contribute to low level Ery resistance in *C. jejuni* [32].

Mechanisms of fluoroquinolones resistance in campylobacters

Fluoroquinolones inhibit the activity of DNA gyrase due to mutations in the DNA gyrase and DNA topoisomerase IV genes in most bacterial species [8]. Enzyme DNA gyrase is composed of two pairs of subunits, GyrA and GyrB, while topoizomerase IV consists of ParC and ParE [33]. Resistance to fluoroquinolones is a result of aminoacid changes in topoisomerase as well in gyrase. In *Campylobacter* strains, resistance to fluoroquinolones is a consequence of mutation in gene gyrA which encodes GyrA subunit of DNA gyrase [8]. Up to date, no mutations in DNA gyrase B have been associated with FQ resistance in *Campylobacter* [34]. The most frequently observed mutation in fluoroquinolones resistant isolates of *Campylobacter* is the point mutation Thr-86-Ile in gyrA gene [35] which leads to the T86I substitution in the gyrase and confers HLR to fluoroquinolones [33]. Other reported mutations of gyrA in C. *jejuni* include Thr-86-Ala (HLR to nalidixic acid and LLR to ciprofloxacin), Ala-70-Thr, Thr-86-Lys, Asp-90-Asn, and Pro-104-Ser [35, 36]. Double point mutations of gyrA have also been reported [35].

In *C. jejuni* and *C. coli*, a unique modification in the GyrA subunit is sufficient to confer a fluoroquinolone-resistant phenotype. Also, decrease in permeability of outer membrane and activity of effluxe system confer the fluoroquinolones resistance [37]. In *Campylobacter jejuni/coli* strains, apart of the mutations in GyrA, the multidrug efflux pump, CmeABC, also contributes to fluoroquinolones resistance by reducing the accumulation of the agents in *Campylobacter* cells [38]. Thus, CmeABC functions synergistically with the gyrA mutations in mediating fluoroquinolones resistance [39].

To understand the roles of multidrug efflux transporters in the pathobiology of *C. jejuni*, Jean et al. [40] characterized the function of an MFS transporter (Cj1375) designated CmeG. The results indicated that CmeG functions as a multidrug efflux transporter contributing to antibiotic resistance especially to fluoroquinolones and oxidative defense in *Campylobacter*.

Mechanisms of tetracyclines resistance in campylobacters

Tetracyclines, (e.g. tetracycline, chlortetracycline, and minocycline) bind to the ribosome and inhibit accommodation of the aminoacyl-tRNA (aa-tRNA) into the ribosomal A site and, therefore, prevent the elongation phase of protein synthesis [41]. Tetracycline resistance can be mediated by different mechanisms: efflux, the enzymatic degradation of drug, protection of the ribosomal binding site and mutations in 16S rDNA [42]. In *C. coli* and *C. jejuni*, genes for tetracycline resistance are located on self-transmissible plasmids. They have been identified as a ribosomal protection gene and designated *tet*(O) [43]. These genes are widely present in *Campylobacter* isolates recovered from various animal species [23]. They encode ribosomal protection proteins (RPPs) [41]. *Tet(*O) confers resistance by binding to the ribosome inducing a conformational change with subsequent release of the bound tetracycline molecule and its displacing from its primary binding site, such that the aa-tRNA can bind to the ribosomal A site and protein synthesis can continue [44].

The presence of *tet*(O) in different Gram-positive bacteria [45] suggest the origin of the resistance genes and their sharing between species. In *C. jejuni, tet*(O) was first cloned from a transferable plasmid pUA466 [46]. Sequencing of two tetracycline-resistance plasmids, one from *C. jejuni* strain 81–176 [47], and other from *C. coli* strain CC31, revealed a high level of sequence identity and genomic organization despite their temporal and spatial distance [48].

Although, in most strains, the *tet*(O) gene is plasmid-encoded, it can be located on the chromosome, which is reported for 33% of tetracycline-resistant *C. jejuni* isolates from Alberta, Canada [49] and 76% of tetracycline-resistant isolates from Australia Pratt, Korolik [50]. On *tet*(O)-carrying plasmids it is described the presence of an insertion element IS607 and therefore it is possible that mobile genetic elements other than transmissible plasmids may be involved in the acquisition and dissemination of tet(O) [51].

Tetracycline resistance in *C. jejuni* is also associated with the CmeABC multidrug efflux pump [52].

Resistance of thermophilic *Campylobacter* strains isolated from humans, poultry and swines to erythromycin

Alarming is the rise of resistance to erythromycin, the first choice drug for treatment of campylobacteriosis. Detection of the resistant strains started with the use of macrolides, generally thylosine in veterinary practice, mostly in swine farming [8, 13, 53].

An investigation [53] detected 12.5% *Campylobacter* strains isolated from humans resistant to erythromycin. These results are in accordance with the results of other authors [54–56]. Lower levels of resistance to erythromycin, ranging from 3.4% to 9.1% are reported by the authors in Brasil, Australia, USA and India [5, 57–59].

A tendency of rising frequency of resistant *Campylobacter* to erythromycin is evident. For example, in Canada there were 3% *Campylobacter jejuni/coli* resistant strains in 1998, but the percentage increased to 12% in 2001 [60].

A high percentage of *Campylobacter jejuni/coli* strains isolated from broilers was found [61] contrary to the fact that erythromycin has not been used in poultry farming. A low level of resistance to erythromycin in thermophilic *Campylobacter* strains was recorded in Great Britain (0–8%) [62], USA (3.1%) [63] and Czech Republic (6%) [64]. A high percentage of *Campylobacter coli* strains resistant to erythromycin isolated from broilers and eggs-laying hens (25% and 40%)

was found in Japan [65]. Authors in Italy reported a high level of resistance to erythromycin, up to 45%, in *Campylobacter coli* strains isolated from poultry faeces [55]. In Africa, high erythromycin resistance levels were observed in human clinical isolates, but low resistance rate to this antibiotic were noticed in *C. jejuni* and *C. coli* isolated from husbandry animals [66]. Reports from Asia describe low resistance of C. *jejuni* to macrolides, but higher resistance of *C. coli* strains [67]. Also, increased resistance to macrolides was observed among *C. coli* isolates from pigs in Australia [68].

Macrolides are widely used in swine farming and, as a consequence of intensive pressure of drugs included in this thylosine group, an increase of *Campylobacter* strains resistant to erythromycin originating from swines occured.

The investigation detected that even 40% of thermophilic *Campylobacter* spp. strains isolated from swines were resistant to erythromycin [61]. According to data from Spain, percentage of resistant *Campylobacter coli* was 81%, in Denmark percentage of resistant *Campylobacter jejuni* was 33% and of Campylobacter coli 74% [69].

Resistance of thermophilic *Campylobacter* strains isolated from humans, poultry and swines to quinolones

A rising frequency of thermophilic *Campylobacter* spp. originating from humans resistant to quinolones, drugs most frequently used in campylobacteriosis treatment [61, 70] is alarming. Emergence of the resistant strains coincided with the beginning of quinolones use in veterinary practice [8, 71].

Thermophilic *Campylobacter* spp. strains resistant to quinolones were produced diarrhea of mean duration 13.2 days, contrary to susceptible strains with mean duration of diarrhea of 10.3 days [72].

Investigation of resistance to ciprofloxacin of *Campylobacter* strains isolated from humans in Serbia, detected 50% resistance [73]. This results are in accordance to the results of others [5, 55, 58, 74–76]. In Chile the resistance of *Campylobacter jejuni/coli* to ciprofloxacin has not been recorded [77]. Fifty percent of thermophilic *Campylobacter* spp. originating from humans were characterized as resistant to ciprofloxacin in a controlled investigation of susceptibility to antibiotics [73]. A high level of resistance to ciprofloxacin (71.4%) was demonstrated in *Campylobacter jejuni/coli* isolated in India from humans, generally children in rural areas [58]. A high level of resistance to ciprofloxacin was registered in Spain, too. Resistance to this antibiotic was found in 75% *Campylobacter jejuni* and 70.7% *Campylobacter coli* strains [56].

A permanent trend of resistance increase to fluoroquinolones is spread worldwide. Enrofloxacin is licenced in Netherlands for use in veterinary medicine in 1987. Resistant *Campylobacter jejuni/coli* strains isolated from humans represented 8% in 1998, 11% in 1989 and 29% in 1997. A similar trend is registered in Austria, Denmark, Finland, France, Italy, Spain, Thailand, Great Britain and USA [8]. In Canada there were no resistance to ciprofloxacin in 1985/86. In the following period, 1995/97, 12.7% resistant *Campylobacter jejuni/coli* strains were isolated from humans [60]. A high level of thermophilic *Campylobacter* spp. resistant to ciprofloxacin has been registered (50% to 60%) [55, 62, 78]. Cardinale et al. (2002) [70], citing several other authors, reportspercentages of *Campylobacter jejuni/coli* resistance to ciprofloxacin in several countries: Germany 46%, Japan 46%, USA 23-100%, Kenya 7.7%, Belgium and Spain up to 100%, Taiwan and Thailand 56–84% and Senegal 34%. In Switzerland a very low level of thermophilic *Campylobacter* spp. isolated from poultry meat resistant to fluoroquinolones is registered: only 0.5%. Resistance to ciprofloxacin in thermophilic *Campylobacter* spp. isolated from poultry in Norway was also low (2.7%). The reason for this results could be found in the fact that fluoroquinolones were not approved for use in broilers in Norway [78].

Fluoroquinolones have not been applied in such extent in swine farming as in poultry farming, this being the reason that the percentage of *Campylobacter jejuni/coli* strains resistant to fluoroquinolones is lower in swines than in poultry. Results of an investigation [79] demonstrated 26.7% resistant *Campylobacter* strains isolated from swines. Similar results were reported in Italy and Switzerland [55, 80]. A low level of resistance to fluoroquinolones, only 0.5%, was registered in *Campylobacter jejuni/coli* strains isolated from swines in USA [81]. Hart et al. [82] did not register a resistance to ciprofloxacin in *Campylobacter jejuni/coli* isolated from swines in Australia, due to the fact that quinolones are not approved for use in veterinary medicine.

Resistance of thermophilic *Campylobacter* strains isolated from humans, poultry and swines to tetracyclines

It was noted that tetracyclines were used in human medicine without appropriate control [83]. According to numerous authors in the world 30%–40% thermophilic *Campylobacter* strains isolated from humans are resistant to tetracycline [73, 74]. High percentage of resistant thermophilic *Campylobacter* strains isolated from humans, ranging from 43% to 85%, are reported in Spain, USA and Finland [54, 58, 75, 84]. A lower level of resistance to tetracycline, ranging from 12% to 16%, was reported in Australia, India and Turkey [57, 59, 76]. Very low level of thermophilic *Campylobacter* spp. isolated from human, resistant to tetracyclines, only 1.8%, was registered in Chile [77]. The trend of resistance increase to tetracycline in many countries is annoying [2, 53]. Many authors report higher percentages of resistance to tetracycline of thermophilic *Campylobacter* spp. strains isolated from poultry [4, 56, 61, 65] but some reported lower percentages of resistance [83, 85–89]. It was noted that as far as 80% strains of thermophilic *Campylobacter* spp. originating from swines were resistant to tetracycline [69, 82, 90], but some authors registered lower percentages of resistance [80, 81]. Aarestrup and Wegener [85] in Denmark, found a low resistance level to tetracyclin in *Campylobacter jejuni/coli* strains isolated from swines (1%).

Investigation of sensitivity to antibiotics of thermophilic *Campylobacter* spp. collected from humans, applying disc-diffusion test, detected 47.1% strains resistant to two antibiotics, and 11.8% strains resistant to three antibiotics [91].

Hakanen et al. [92] detected 22% *Campylobacter jejuni* strains resistant to three or more antibiotics. Multiresistance to antibiotics of thermophilic *Campylobacter* spp. strains in India was 30.6%, most fequently to erythromycin, tetracycline and ciprofloxacin [59].

It is necessary to emphasize recorded multiresistance of thermophilic *Campylobacter* isolated from poultry and swines [54, 70, 81].

Conclusion

Consuming of food contaminated with thermophilic *Campylobacter* spp. results in transmission of strains resistance to antibiotics and resistency genes from animals to humans. Humans infected with strains resistant to antibiotics, get illness with more severe symptomatology and with prolonged course. High level of resistance to antibiotics of thermophilic *Campylobacter* spp. collected from humans and animals, even in high industrialized countries, is a consequence of irregular use and misuse of antibiotics, predominantly in veterinary medicine and husbandry, the fact demonstrated in many investigations. It should be emphasized that the level of resistance of 12.5% to erythromycin of *Campylobacter* strains collected from humans and poultry was detected, contrary to the fact that erythromycin was not being used in poultry farming. Resistance to ciprofloxacin of *Campylobacter* strains collected from humans and broilers was 50% or more. It was demonstrated that 30% strains originating from humans and 80% strains originating from swines are resistant to tetracycline. A trend of resistance increase to antibiotics of campylobacters collected from humans and animals is extensively evident.

Acknowledgments

We would like to thank the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Received on February 24, 2011.
Revised on April, 7 2011.
Accepted on April 18, 2011.