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# Antimicrobial resistance rates of *Campylobacter* species isolated from clinical samples during 2010–2011 and 2023–2024 in Serbia

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Received: July 22, 2025 • Accepted: August 18, 2025
Published online: September 25, 2025

Acta Microbiologica et Immunologica Hungarica

72 (2025) 3, 256–260

DOI:
10.1556/030.2025.02685


RESEARCH ARTICLE

**ABSTRACT**

Campylobacteriosis is the most frequent zoonosis in Europe and its most important human pathogens are *Campylobacter jejuni* and *Campylobacter coli*. Resistance of *Campylobacter* to fluoroquinolones and tetracyclines, previously recommended for treatment of prolonged or complicated disease, has been emerging. We examined phenotypic resistance to erythromycin, tetracycline, ciprofloxacin, gentamicin, amoxicillin-clavulanic acid and imipenem in 150 *Campylobacter* isolates from human stool during 2010–2011 and the same number of strains during 2023–2024, using the antimicrobial gradient method in both periods, to determine minimum inhibitory concentration (MIC). Comparing the two periods, resistance rate of *C. jejuni* increased by 35% and 38.43% to tetracyclin and ciprofloxacin, respectively and decreased by 0.83% to erythromycin. Prevalence of resistance in *C. coli* increased by 42.59% and 42.01% against tetracyclin and ciprofloxacin, respectively and all isolates were sensitive to erythromycin. Resistance of *C. jejuni* to amoxicillin-clavulanic acid decreased by 0.96% and increased by 3.70% in *C. coli*. All isolates showed sensitivity for imipenem in both periods, while only 2% of *C. jejuni* strains were resistant to gentamicin in 2023–2024. The difference of mean MICs for all antibiotics was statistically significant in two examined periods, with exception of those for erythromycin both in *C. jejuni* and *C. coli*. In 2023–2024, 79 isolates of *C. jejuni* and 22 isolates of *C. coli* were resistant against two and more antibiotics, most frequently tetracycline and ciprofloxacin. The present study provides evidence that in *Campylobacter* spp. the antimicrobial resistance to ciprofloxacin and tetracycline is on rise in Serbia. Our findings are in accordance with recent reports from countries geographically close to Serbia.

**KEYWORDS**

antimicrobial susceptibility testing, *Campylobacter jejuni*, *Campylobacter coli*, human strains, phenotypic resistance

## INTRODUCTION

Campylobacteriosis is the most prevalent zoonosis in Europe and its most frequent causative agents isolated from humans are *Campylobacter jejuni* and *Campylobacter coli* [1]. Usually, human campylobacteriosis is a self-limiting gastroenteritis that resolves spontaneously. However, long-term disease with extraintestinal complications requires antimicrobial therapy. Antimicrobials of relevance for clinical treatment are fluoroquinolones, macrolides, aminoglycosides and tetracyclines. For invasive *Campylobacter* infections aminoglycosides or carbapenems are recommended [1]. Antimicrobial resistance to fluoroquinolones and tetracyclines is emerging worldwide [2–6], causing the necessity of antimicrobial susceptibility testing (AST) for *Campylobacter* spp. So far, AST is not routinely performed by huge number of clinical laboratories.

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AST plays an important role in the choice of therapy and to monitor resistance [7]. Therefore, the use of reliable and simple method of AST in routine work is very important, for reducing the dependence on empirical medication and literature recommendations when treating campylobacteriosis [8]. *In vitro* AST requires a measurement of the antimicrobial's activity using phenotypic methods, inhibition zone diameter or minimum inhibitory concentration (MIC) [7]. Several methods are currently available, including disk diffusion, E test®, and broth and agar dilution methods [2]. The method of choice for Campylobacters recommended by the Clinical and Laboratory Standards Institute (CLSI) is agar dilution or broth microdilution. The European Committee for Antimicrobial and Susceptibility Testing (EUCAST) proposed MIC determination by broth microdilution method according to ISO standard 20776-1 and standardised disk diffusion test, as well as epidemiological cut-offs (ECOFFs) for macrolides, tetracycline and fluoroquinolones for the two main *Campylobacter* species *C. jejuni* and *C. coli* [9]. There is an opinion either agar or broth dilution techniques are not convenient for routine performance [7].

In search of a simple, accurate method of performing AST, several authors evaluated MIC gradient strip technology (E test), comparing MICs obtained by this test and by broth microdilution method [2, 8, 10]. They found a highly significant correlation between these methods.

In Serbia, antimicrobial gradient method has been used for *Campylobacter* spp for fifteen years. The aims of this study were to update antibiotic resistance rates among *Campylobacter* isolates detected in two periods (2010–2011 and 2023–2024) and to identify drug resistance trends for several antimicrobials of clinical importance for treating campylobacteriosis.

## MATERIALS AND METHODS

### Sample collection

*Campylobacter* strains were collected at National Reference Laboratory for *Campylobacter* and *Helicobacter* (Institute of Public Health of Niš, Serbia) during 2010–2011 and at Belgrade City Institute of Public Health (Serbia) during 2023–2024. All strains were isolated from diarrhoeal stools of patients presented with gastroenteritis. In previous period, species identification was performed by classical microbiological methodology (catalase production, growth at 25 and 43 °C, indoxyl acetate hydrolysis, urea and hippurate hydrolysis and $H_2S$ production) and in recent period by matrix-assisted laser desorption ionization time of flight mass spectrometry MALDI-TOF (Vitek MS, bio Merieux, France). In both periods, 100 strains of *C. jejuni* and 50 strains of *C. coli* were randomly chosen for *in vitro* AST.

### *In vitro* antibiotic susceptibility test (AST)

AST was performed by antimicrobial gradient method, in 2010–2011 by E test (AB Biodisk, Solna, Sweden) and in 2023–2024 by Ezy MIC Strip (Hi-media, Mumbai, India). For performing the test, 0.1 ml prepared bacterial suspension adjusted to 1.0 McFarland was plated on a Mueller-Hinton agar plate supplemented with 5% lysed sheep blood. After drying the surface, impregnated strip with an increasing concentration gradient of the antimicrobial agent across its length was deposited on each plate. Six antimicrobial agents were tested – erythromycin (ERY), tetracycline (TET), ciprofloxacin (CIP), gentamicin (GEN), amoxicillin-clavulanic acid (AMC) and imipenem (IMI). The plates were incubated at 42 °C for 48 h under microaerophilic conditions. MICs were read directly from the strip, according to the instructions of the manufacturer. Elliptical zone of inhibition intersected with the MIC scale on the strip. Breakpoints were adopted according to Eucast Clinical Breakpoints for *Campylobacter* and *Enterobacteriales* for MICs in microbroth dilution method (9) and are summarized in Table 1. Quality control for AST was done according to manufactures guidelines using *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559.

### Statistical analysis

All statistical analyzes were performed in GraphPad Prism 8.0. The Kolmogorov-Smirnov test was used to determine whether the data distribution followed a normal distribution. After normality distribution check, we used the Mann Whitney test that compares the medians of two independent samples whose data do not match normal (Gaussian) distribution, and compared individual MIC values for all isolates between the two test periods. The statistical significances were designated as follows: * if $P < 0.05$, ** if $P < 0.01$, *** if $P < 0.001$ and **** if $P < 0.0001$.

## RESULTS

Resistant rates of *C. jejuni* and *C. coli* strains in two periods are shown in the Table 2.

*Table 1.* Breakpoints for resistance of *C. jejuni* and *C. coli* against selected antibiotic

| Antibiotic agent | MIC breakpoints (μg mL$^{-1}$) | Reference guideline |
|---|---|---|
| ERY | *C. jejuni* >4 *C. coli* >8 | EUCAST* |
| TET | >2 | EUCAST* |
| CIP | >0.5 | EUCAST* |
| AMC | >8 | EUCAST** |
| GEN | >2 | EUCAST** |
| IMI | >4 | EUCAST** |

MIC (minimum inhibitory concentration).
* Eucast Clinical Breakpoint Tables for *Campylobacter* v. 14.0, valid from 2024-01-01.
**Eucast Clinical Breakpoint Tables for *Enterobacteriales* v. 14.0, valid from 2024-01-01.

*Table 2.* Resistant rates of *C. jejuni* and *C. coli* strains against six common antibiotics in two periods (2010–2011 and 2023–2024)

| Antibiotics | 2010–2011 | | 2023–2024 | |
|---|---|---|---|---|
| | *C. jejuni* ($n$ = 100) | *C. coli* ($n$ = 50) | *C. jejuni* ($n$ = 100) | *C. coli* ($n$ = 50) |
| AMC | 4.96% | 0 | 4% | 3.70% |
| IMI | 0 | 0 | 0 | 0 |
| GEN | 0 | 0 | 2% | 0 |
| TET | 14% | 16.67% | 49% | 59.26% |
| ERY | 1.83% | 4% | 1% | 0 |
| CIP | 45.57% | 46.88% | 84% | 88.89% |

$n$ – number of isolates.

Comparing the two periods, resistance rate of *C. jejuni* increased by 35% and 38.43% to tetracyclin and ciprofloxacin, respectively and decreased by 0.83% to erythromycin. Prevalence of resistance in *C. coli* increased by 42.59% and 42.01% to tetracyclin and ciprofloxacin, respectively and all isolates were sensitive to erythromycin. Resistance of *C. jejuni* to amoxicillin-clavulanic acid decreased by 0.96% and increased by 3.70% in *C. coli.* All isolates showed sensitivity for imipenem in both periods, while only 2% of *C. jejuni* strains were resistant to gentamicin in 2023–2024. The difference of mean MICs for all antibiotics was statistically significant in two examined periods, with exception of those for erythromycin both in *C. jejuni* and *C. coli* (Tables 3 and 4).

In 2023–2024, 79 isolates of *C. jejuni* and 22 isolates of *C. coli* were resistant against two and more antibiotics, most frequently tetracycline and ciprofloxacin (Table 5).

*Table 3.* Distribution of MIC range, $MIC_{50}$ and $MIC_{90}$ of *Campylobacter jejuni* against six common antibiotics in two periods (2010–2011 and 2023–2024)

| Period / Antibiotics | | 2010–2011 μg mL$^{-1}$ | 2023–2024 μg mL$^{-1}$ | $P$* |
|---|---|---|---|---|
| AMC | $MIC_{50}$ | 0.38 | 0.23 | <0.0001 |
| | $MIC_{90}$ | 1.5 | 1.5 | |
| | MIC range | 0.016 - ≥ 256 | 0.016 - ≥ 256 | |
| IMI | $MIC_{50}$ | 0.094 | 0.023 | <0.0001 |
| | $MIC_{90}$ | 0.23 | 0.064 | |
| | MIC range | 0.002–4 | 0.002–4 | |
| GEN | $MIC_{50}$ | 0.38 | 0.19 | <0.0001 |
| | $MIC_{90}$ | 0.75 | 0.5 | |
| | MIC range | 0.016–8 | 0.047–19 | |
| TET | $MIC_{50}$ | 0.25 | 1.5 | <0.0001 |
| | $MIC_{90}$ | 32 | 256 | |
| | MIC range | 0.032- ≥ 256 | 0.019 - ≥ 256 | |
| ERY | $MIC_{50}$ | 0.19 | 0.23 | 0.0865 |
| | $MIC_{90}$ | 0.75 | 0.5 | (NS) |
| | MIC range | 0.016- ≥ 256 | 0.016 - ≥ 256 | |
| CIP | $MIC_{50}$ | 3 | 256 | <0.0001 |
| | $MIC_{90}$ | ≥32 | 256 | |
| | MIC range | 0.002- ≥ 32 | 0.002 - ≥ 256 | |

*The $P$ value represents a Mann Whitney comparison of the individual MIC values for all isolates in two periods, NS – not significant.

## DISCUSSION

In our previous work we reviewed studies on *Campylobacter* resistance from 2007 to 2020 in Serbia [11]. Data evaluated from 2011 to 2020 were regarded to *Campylobacter* strains isolated from chickens and pigs. In this work, we performed AST on *C. jejuni* and *C. coli* isolated from human stool during 2010–2011 and during 2023–2024, in aim to compare findings of *Campylobacter* resistance to selected antibiotics. In both periods we used the same method of AST (e.g. MIC gradient strip test), since we had found this technique easy-to-perform and more affordable comparing to other tests.

Broth microdilution method of AST is recommended by both EUCAST and CLSI. In the last two decades, most of the authors published that they had used commercial microdilution tests for MIC determination [2, 5, 8, 10, 12]. During this period, resistance to antibiotics of choice for treating serious *Campylobacter* infection emerged, so panels of antimicrobials used in these studies do not meet current requirements completely.

Although MIC gradient strip test (E test) is not recommended by EUCAST and CLSI, several authors compared accuracy of this simpler AST method with the referent broth microdilution technique in the last twenty years. Sifré et al. [7] stated that E test was acceptable method for AST compared to the agar or broth dilution methods, especially for clinical laboratories that do not receive large numbers of isolates. In addition, the authors advice was to keep in mind that MICs determined by E test for *Campylobacter* are usually a bit lower that those determined by agar dilution. Van der Beek et al. [10] found that MIC values, as determined by E test, differed on average two-fold from the MIC values established by broth microdilution, but classification on resistance was identical using both methods. Azrad et al. [2] had similar findings as van der Beek et al. [10] and the authors claimed that both broth microdilution and the E test method are reliable, rapid and easy-to-perform techniques. Ge et al. [8] showed that E test method results exhibited high agreement with those of broth dilution method according to CLSI criteria for erythromycin, ciprofloxacin and tetracycline. Luber et al. [12] found that agreement between broth microdilution test and E test was 95.9% for erythromycin, 95.9% for tetracycline, 94.6% for gentamycin and slightly lower for ciprofloxacin (91.5%). They stated that, with the exception of erythromycin, the broth microdilution method always tended to give slightly higher MICs. Ge et al. (8) found that E test method results showed high concordance with those of broth microdilution method, 94.6%,

*Table 4.* Distribution of MIC range, $MIC_{50}$ and $MIC_{90}$ of *Campylobacter coli* against six common antibiotics in two periods (2010–2011 and 2023–2024)

| Period<br>Antibiotics | | 2010–2011<br>μg mL$^{-1}$ | 2023–2024<br>μg mL$^{-1}$ | *P** |
|---|---|---|---|---|
| AMC | $MIC_{50}$ | 0.5 | 1 | 0.0019 |
| | $MIC_{90}$ | 2.00 | 6 | |
| | MIC range | 0.094–2 | 0.032 - ≥ 256 | |
| IMI | $MIC_{50}$ | 0.5 | 0.047 | <0.0001 |
| | $MIC_{90}$ | 0.75 | 0.094 | |
| | MIC range | 0.032–1 | 0.016–0.47 | |
| GEN | $MIC_{50}$ | 0.75 | 0.19 | <0.0001 |
| | $MIC_{90}$ | 1.50 | 0.5 | |
| | MIC range | 0.38–2 | 0.016–0.47 | |
| TET | $MIC_{50}$ | 0.25 | 256 | 0.0004 |
| | $MIC_{90}$ | 64 | 256 | |
| | MIC range | 0.047- ≥ 256 | 0.016 - ≥ 256 | |
| ERY | $MIC_{50}$ | 0.19 | 0.25 | 0.4483 |
| | $MIC_{90}$ | 1 | 0.75 | (NS) |
| | MIC range | 0.032- ≥ 256 | 0.023–0.75 | |
| CIP | $MIC_{50}$ | 2 | 64 | <0.0001 |
| | $MIC_{90}$ | ≥32 | 256 | |
| | MIC range | 0.012- ≥ 32 | 0.047 - ≥ 256 | |

*The *P* value represents a Mann Whitney comparison of the individual MIC values for all isolates in two periods, NS – not significant

*Table 5.* Resistance against two and more antibiotics in 2023–2024

| Antibiotics | *C. jejuni* | *C. coli* | Total |
|---|---|---|---|
| CIP, ERY | 1 | 0 | 1 |
| CIP, TET | 71 | 16 | 87 |
| ERY, TET | 2 | 0 | 2 |
| CIP, AMC | 2 | 2 | 4 |
| CIP, TET, AMC | 2 | 2 | 4 |
| CIP, ERY, TET | 1 | 2 | 3 |
| Total | 79 | 22 | 101 |

94.6% and 98.6% for erythromycin, ciprofloxacin and tetracycline respectively.

In our country, campylobacteriosis is the second most common gastroenteral infectious disease, after salmonellosis, with incidence rate 7.11 of 100.000 inhabitants in 2023 [13]. In this study, we considered that MIC gradient strip test was convenient for monitoring antimicrobial resistance of *Campylobacter* in Serbia, like the authors from the other countries reported [3, 14].

Comparing the two periods, we found statistically significant difference of mean MICs for all examined antibiotics, except for erythromycin. Resistance rate of erythromycin was 1.83% and 4% for *C. jejuni* and *C. coli* respectively in 2010–2011 and 1% and 0% for *C. jejuni* and *C. coli* respectively in 2023–2024. In Annual Epidemiological Report of European Centre for Disease Control (ECDC) for 2022, for macrolides, resistance was detected in only 0.9% of *C. jejuni*, but in 7.8% of *C. coli*. Decreasing trends in macrolide resistance were observed in six out of 18 countries for *C. jejuni* and in four out of 13 for *C. coli*. [15].

We observed evolution of resistance against ciprofloxacin and tetracycline from 2010 to 2024 in Serbia. For *C. jejuni*, prevalence of resistance to ciprofloxacin increased by from 45.57% to 84% and for *C. coli* from 46.88% to 88.89%. For *C. jejuni*, prevalence of resistance to tetracycline increased by from 14% to 49% and for *C. coli* from 16.67 % to 59.26 %. Reports from countries geographically close to Serbia are similar to our findings in 2023–2024. We made comparison with data from Hungary, Italy and Slovenia. Antimicrobial resistance in these countries ranged from 75.2 to 88.9% for ciprofloxacin and 46.4%–57.1% for tetracycline in *C. jejuni* human isolates. In *C. coli* human isolates resistance rates ranged from 79.4 to 86% for ciprofloxacin and 48.9 – 64.7 % for tetracyclin [15].

According to the report [15], resistance against gentamicin in *C. jejuni* and *C. coli* from humans was 0.7% and 2.4% on average for *C. jejuni* and *C. coli,* respectively. Our findings for this antibiotic showed that all examined strains of both *Campylobacter* species were sensitive in 2010–2011, and fenotipic resistance was revealed in only 2% of *C. jejuni* isolates in 2023–2024. Tramuta et al. [5] reported resistance rate of 5.4% for aminoglycosides in Italy during 2020–2023. Studies from South America showed that resistance to gentamicin was infrequently, but is on rise [6].

Most *Campylobacter* species are inherently resistant to many beta-lactams. Post et al. [4] reported that no resistance to amoxicillin-clavulanic acid and meropenem was detected in strains collected until 2014. But recent data showed that resistance to amoxicillin-clavulanic acid is increasing [6, 15]. Resistance to amoxicillin-clavulanic acid was reported by only eight member states of EU (15). Prevalence was 6.6% and 9.2% on average for *C. jejuni* and *C. coli,* respectively. We found lower resistance rate (3.70%) in *C. coli* than in *C. jejuni* (4%). In our study, prevalence of resistance in 2023–2024 for both species was lower than average in EU member states.

Multidrug resistance (MDR) of *Campylobacter*, e.g. resistance to at least three or more antimicrobial classes, is emerging worldwide [11]. In 2023–2024, we found that 87 out of 150 all examined isolates (58%) were resistant to ciprofloxacin and tetracycline and 7 out of 150 (4.6%) exhibited MDR. In EU, 1% and 9.9 % of MDR strains were reported in *C. jejuni* and *C. coli* human isolates, respectively [15].

The present study provides evidence that antimicrobial resistance to antibiotics used in treatment of campylobacteriosis is on rise in Serbia, especially against ciprofloxacin and tetracycline. Sensitivity to erythromycin is still high, so macrolides could be antimicrobials of the first choice. MIC gradient strip test is quantitate and is convenient either for monitoring resistance or for therapeutically purpose. AST should be performed in patients with prolonged and severe gastroenteritis or extraintestinal infections.

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