ANTIMICROBIAL SUSCEPTIBILITY OF CAMPYLOBACTER ISOLATES IN THE CAPITAL OF NORTH MACEDONIA

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ABSTRACT

Background: Campylobacter infections are typically self-limited, but in cases with severe enteritis, immuno-compromised system and bacteremia, an appropriate antimicrobial treatment is demanding. Our study aim was to determine the isolation rate of Campylobacter among patients with acute enteritis in the capital of North Macedonia and its antimicrobial susceptibility.

Material and methods: A total number of 3820 patients clinically diagnosed as acute enteritis, were included in the study. Stool samples were collected and Campylobacter was isolated and identified by classical microbiological methods. Antimicrobial susceptibility of all isolates to Ceftriaxone, Amoxicillin-clavulonic acid, Erythromycin, Ciprofloxacin, Tetracycline and Gentamicin was determined by disc-diffusion technique. Additionally, minimal inhibitory concentrations of all Campylobacter isolates against erythromycin, ciprofloxacin and tetracycline were determined by Epsilon gradient tests.

Results: Campylobacter species was isolated in 97 patients. Although the mean isolation rate of Campylobacter spp. during the whole study period was 2.53%, a statistically significant increase was detected in 2016 and 2017, in comparison with the data from previous four years of the study. The isolation rate of Campylobacter spp. didn’t reveal statistically significant difference between males and females (p > 0.05). 46.4 % of patients with Campylobacter enteritis were children at the age under 15 years. Forty-three C. jejuni isolates were susceptible to all six antibiotics, but the remaining 44 isolates revealed resistance to at least one antibiotic. C. coli isolates were resistant to 3 antibiotics simultaneously. Two C. coli isolates only, were susceptible to all 6 antibiotics. 40.90% of C. jejuni and 50% of C. coli isolates were resistant to beta-lactams, fluoroquinolones and tetracyclines, simultaneously.

Conclusion: The increase of the isolation rate of Campylobacter from patients with acute enteritis indicates the need for permanent isolation and identification of Campylobacter from every clinically diagnosed patient, as acute enteritis. Erythromycin is the most effective antibiotic for treatment of Campylobacter enteritis in our patients. The high level of Campylobacter resistance to beta-lactams, fluoroquinolones and tetracyclines requires more rational approach in the treatment of Campylobacter enteritis.

Keywords: acute enteritis, Campylobacter, antimicrobial susceptibility
INTRODUCTION

Campylobacter is one of the most common causes of bacterial enteritis in developed and developing countries. It is responsible for more than 500 million cases of diarrhea worldwide, every year [1]. The number of reported laboratory confirmed cases of human campylobacteriosis in the USA and European Union was 13.02 and 50.28 per 100000 population, respectively in 2011. However, many causes left undiagnosed or unreported [2, 3]. Campylobacter infections are typically self-limited, but in cases with severe enteritis, immuno-compromised system and bacteremia, an antimicrobial treatment is demanding. In many developing countries, acute enteritis due to Campylobacter species is treated empirically with cephalosporines, macrolides and fluoroquinolones [4]. But, the rate of Campylobacter resistance to these drugs is increasing all around the world, especially in developing countries.

The objective of this study was to determine the isolation rate of Campylobacter among patients with acute enteritis in the capital of North Macedonia and its antimicrobial susceptibility.

MATERIAL AND METHODS

A cross-sectional study was conducted at the Institute for Microbiology and parasitology, Medical Faculty in Skopje, from May 2016 to August 2017. In the study 3820 patients were included clinically diagnosed as acute enteritis, according to World Health Organization (WHO) [5] at the age between 1 and 77 years. Stool samples were collected from every patient and were inoculated on Skirrow’s selective medium and incubated for 48 h at 420C in a microaerophilic atmosphere. Campylobacter spp. was identified by its typical growth, characteristic micromorphology and positive oxidase reaction. Differentiation of Campylobacter jejuni, Campylobacter coli and Campylobacter lari was made according the hippurat hydrolysis and indoxil acetate reactions.

Antimicrobial susceptibility testing of the isolates was determined by disc-diffusion technique. 0.5 McFarland turbidity standard equivalent bacteria suspension was prepared and inoculated on Muller-Hinton agar supplemented with 5% sheep blood. Antimicrobial discs (Oxoid Ltd. Company, UK) were applied at every single plate and they were incubated at the same conditions as for the primary isolation. For that purpose the following antimicrobials were used with their respective concentrations: Ceftriaxone (CRO, 30µg), Amoxicillin-clavulonic acid (AMC, 20µg/10µg), Erythromycin (ERY, 15 µg), Ciprofloxacin (CIP, 5 µg), Tetracycline (TET, 30 µg), Gentamicin (GEN, 10 µg). The length of the zones of inhibition around the discs were measured and interpreted on the bases of EUCAST interpretive criteria [6]. Additionally, minimal inhibitory concentrations (MICs) of all Campylobacter isolates against erythromycin, ciprofloxacin and tetracycline were determined by Epsilon gradient tests (E-tests) with their corresponding antimicrobial concentrations of 0.016-256, 0.002-32 and 0.016-256, respectively. The antimicrobial concentration ranges of Campylobacter jejuni (ATCC 700819) and E. coli (ATCC 25922) were used as control strains.

Chi-square and Fisher’s exact tests were used for testing the differences between proportions, and P value less than 0.05 was considered statistically significant.

RESULTS

Campylobacter species was isolated in 97 out of the total of 3820 patients with enteritis. Although the mean isolation rate of Campylobacter spp. during the whole period of the study was 2.53%, a statistically significant increase was detected in 2016 and 2017 in comparison with the data obtained in the previous four years of the study. (Graph. 1)

85 out of 97 (87.63%) isolates were C. jejuni, 12/97 (12.37%) were C. coli but C. lari was not detected. The isolation rate of Campylobacter spp. was not statistically different between males and females (p > 0.05). Campylobacter spp. was most frequently isolated (46.4%) in children under 15 years of age. In the remaining three age-groups
of patients: from 16 to 30 (19.6%), from 31 to 50 (17.5%) and above 50 years (16.5%), the isolation rate was very similar and it was twice lower than in children under 15.

Six antimicrobial agents were used for detection of antimicrobial susceptibility by disk-diffusion method in all 97 Campylobacter isolates. 52.57%, 35.05%, 32.99%, 18.55% and 8.24% of Campylobacter isolates were resistant to ceftriaxone, ciprofloxacin, tetracycline, amoxicillin-clavulanic acid and gentamicin, respectively. None of the isolates were resistant to erythromycin. The results are summarized in Table 1.

Fourtythree C. jejuni isolates were susceptible to all six antimicrobial agents, but the remaining 44 isolates revealed resistance to at least one antibiotic. Ten out of twelve C. coli isolates revealed resistance to at least 3 antibiotics and only two of them were susceptible to all six antibiotics included in the study (table 2).

Eighteen (40.88 %) and 23 (52.25 %) C. jejuni isolates were resistant to 2 and 3 antibiotics simultaneously. Three (6.81%) C. jejuni isolates were resistant to 1 antibiotic only. 7/10 (70%) C. coli isolates were resistant to 3 antibiotics simultaneously, but 1 (10 %) and 2 (20 %) C. coli isolates were resistant to 4 and 5 antibiotics, respectively (Table 2). Two C. coli isolates only, were susceptible to all 6 antibiotics. The last column of the table presents the rates of antimicrobial resistance of 44 C. jejuni and 10 C. coli isolates which revealed resistance to different number of antibiotics.

### Table 1. Antimicrobial susceptibility of C. jejuni and C. coli isolates

| Antimicrobial agents                | Number of Campylobacter isolates | % of resistant Campylobacter isolates |
|-------------------------------------|----------------------------------|-------------------------------------|
|                                     | S | I | R  |                                |                                    |
| Ceftriaxone (Cro)                   | 43/97 | 3/97 | 51/97 | 52.57                           |
| Ciprofloxacin (Cip)                 | 63/97 | / | 34/97 | 35.05                           |
| Tetracycline (Tet)                  | 65/97 | / | 32/97 | 32.99                           |
| Amoxicillin-clavulanic acid (Amc)   | 77/97 | 2/97 | 18/97 | 18.55                           |
| Gentamicin (Gen)                    | 89/97 | / | 8/97  | 8.24                            |
| Erythromycin (Ery)                  | 97/97 | / | /  | 0.0                             |

### Table 2. Antimicrobial resistance patterns of C. jejuni and C. coli isolates

| Campylobacter species | No. of antibiotics to which isolates are resistant | Antibacterial resistance profile | No. of isolates | Rate of resistance (%) | Total No. and (%) |
|-----------------------|--------------------------------------------------|---------------------------------|-----------------|------------------------|-------------------|
| C. jejuni             | 1                                                | Cro, Gen                        | 2               | 4.54                   | 3 (6.81)          |
|                       |                                                  |                                 | 1               | 2.27                   |                   |
| C. jejuni             | 2                                                | Amc, Cro, Amc, Cip, Cro, Cro, Tet, Cro, Gen, Gen, Tet | 7               | 15.90                  | 18 (40.88)        |
|                       |                                                  |                                 | 1               | 2.27                   |                   |
|                       |                                                  |                                 | 4               | 9.09                   |                   |
|                       |                                                  |                                 | 2               | 4.54                   |                   |
|                       |                                                  |                                 | 3               | 6.81                   |                   |
|                       |                                                  |                                 | 1               | 2.27                   |                   |
| C. jejuni             | 3                                                | Cro, Amc, Tet, Cro, Amc, Cip, Cro, Cip, Tet | 3               | 6.81                   | 23 (52.25)        |
|                       |                                                  |                                 | 2               | 4.54                   |                   |
|                       |                                                  |                                 | 18              | 40.90                  |                   |
|                       |                                                  |                                 | 2               | 20.0                   |                   |
|                       |                                                  |                                 | 5               | 50.0                   |                   |
| C. coli               | 4                                                | Cro, Amc, Tet, Gen              | 1               | 10.0                   | 1 (10.0)          |
|                       |                                                  |                                 | 2               | 20.0                   | 2 (20.0)          |
| C. coli               | 5                                                | Cro, Amc, Cip, Tet, Gen        | 2               | 20.0                   | 2 (20.0)          |

Cro - Ceftriaxone, Cip - Ciprofloxacin, Tet - Tetracycline, Amc - Amoxicillin-clavulanic acid, Gen – Gentamicin
18/44 (40.90%) of \textit{C. jejuni} isolates were resistant to beta-lactams, fluoroquinolones and tetracyclines, simultaneously. 9/44 (20.45%), 7/44 (15.90%) and 5/44 (11.36%), were resistant to beta-lactams; beta-lactams with fluoroquinolones and beta-lactams with tetracyclines, respectively. 3/44 (6.81%) of \textit{C. jejuni} isolates were resistant to beta-lactams and aminoglycosides. 1/44 (2.27%) of \textit{C. jejuni} isolates revealed resistance to aminoglycosides and tetracyclines, simultaneously and another isolate was resistant to aminoglycosides only (table 3).

\textbf{Table 3. Number and \% of resistant \textit{C. jejuni} isolates to different groups of antibiotics}

| Antibiotics                          | Number | \%    |
|--------------------------------------|--------|-------|
| Beta-lactams                         | 9/44   | 20.45 |
| Beta-lactams and Fluoroquinolones    | 7/44   | 15.90 |
| Beta-lactams, Fluoroquinolones and Tetracyclines | 18/44 | 40.90 |
| Beta-lactams and Tetracyclines       | 5/44   | 11.36 |
| Aminoglycosides and Tetracyclines    | 3/44   | 6.81  |
| Aminoglycosides                      | 1/44   | 2.27  |

2/10 (20.0%) \textit{C. coli} isolates were resistant to beta-lactams and fluoroquinolones, simultaneously. The same number of isolates revealed simultaneous resistance to beta-lactams, fluoroquinolones, tetracyclines and aminoglycosides. 5/10 (50%) \textit{C. coli} isolates were resistant to beta-lactams, fluoroquinolones and tetracyclines and one \textit{C. coli} isolate only, was simultaneously resistant to beta-lactams, tetracyclines and aminoglycosides (table 4).

\textbf{Table 4. Number and \% of resistant \textit{C. coli} isolates to different groups of antibiotics}

| Antibiotics                          | Number | \%    |
|--------------------------------------|--------|-------|
| Beta-lactams and Fluoroquinolones    | 2/10   | 20    |
| Beta-lactams, Fluoroquinolones and Tetracyclines | 5/10 | 50    |
| Beta-lactams, Fluoroquinolones, Tetracyclines and Aminoglycosides | 2/10 | 20    |
| Beta-lactams, Tetracyclines and Aminoglycosides | 1/10 | 10    |

42 (95.42%), 25 (56.81%), 24 (54.54%) and 5/44 (11.36%) out of 44 \textit{C. jejuni} isolates revealed resistance to beta-lactams, fluoroquinolones, tetracyclines and aminoglycosides respectively. 100%, 50%, 90%, 80% and 30% of \textit{C. coli} isolates revealed resistance to beta-lactams, fluoroquinolones, tetracyclines and aminoglycosides respectively (table 5).

\textbf{Table 5. Number (\%) of resistant \textit{C. jejuni} and \textit{C. coli} isolates to clinically important antibiotics}

| Groups of antibiotics | \textit{C. jejuni} | \textit{C. coli} |
|-----------------------|-------------------|-----------------|
| Beta-lactams          | 42/44 (95.42%)    | 10/10 (100%)    |
| Fluoroquinolones      | 25 (56.80%)       | 9/10 (90%)      |
| Tetracyclines         | 24/44 (54.53%)    | 8/10 (80%)      |
| Aminoglycosides       | 5/44 (11.36%)     | 3/10 (30%)      |

Determination of minimal inhibitory concentration (MIC) was performed in all \textit{Campylobacter} isolates for erythromycin, ciprofloxacin and tetracycline. Sixty three (64.95%) out of 97 isolates were susceptible to ciprofloxacin with MIC between 0.032 and 0.25 µg/ml, but 34 (35.05%) out of 97 isolates were resistant to ciprofloxacin with MIC between 0.5 and > 32 µg/ml. Graph 3 presents inhibition zone diameter distributions versus MIC values for ciprofloxacin. Fourteen (41.17%), nine (26.47%) and six (17.64%) out of the total 34 isolates of \textit{Campylobacter} revealed high level of resistance to ciprofloxacin with MIC > 32 µg/ml, 16 µg/ml and 8 µg/ml, respectively. The inhibition zone diameters for that isolates ranged between 6 and 14 mm. The rest of five (14.7%) out of 34 \textit{Campylobacter} isolates revealed low resistance to ciprofloxacin with MICs between 0.5 and 4 µg/ml and their inhibition zone diameters were between 14 and 18 mm, except in one isolate with the inhibition zone of 25 mm. (Graph 3.)
All investigated isolates of *Campylobacter* revealed good susceptibility to Erythromycin with MIC’s between 0.125 and 1 µg/ml and with the inhibition zone diameters between 22 and 36 mm (Graph 4).

An antimicrobial susceptibility of 97 *Campylobacter* isolates to tetracycline is presented on graph 5. Sixty five (67.02%) out of 97 isolates were susceptible to tetracycline with MIC between 0.125 and 1.0 µg/ml, but 32 (36.08%) out of 97 isolates were resistant to tetracycline, with MIC between 2.0 and 256 µg/ml. 15 (46.87%), 8 (25%) and 3 (9.37%) out of the total 32 isolates of *Campylobacter* were highly resistant to tetracycline with MIC 256 µg/ml, 64 µg/ml and 32 µg/ml, respectively. Their inhibition zone diameters were 6 and 9 mm long. The rest of six (18.75%) out of 32 *Campylobacter* isolates revealed low resistance to tetracycline with MICs between 2 and 4 µg/ml and their inhibition zone diameters were between 30 and 36 mm long.

29/34 (85.3%) and 26/32 (81.25%) of *Campylobacter* isolates revealed high level of resistance to ciprofloxacin and tetracycline, respectively.

**DISCUSSION**

The isolation rate of *Campylobacter* spp. in the last two years of the study increased for more than 2 times in comparison with the isolation rate during the previous four years. With the isolation rate of 2.53 our results are comparable with the results revealed in the reports from some Balkan countries [7], but it is much lower than the isolation rates in many European countries [8]. Several factors such as consumer awareness, severity of a disease and its surveillance by the clinicians, affect the degree of isolation rate of *Campylobacter* as a food borne pathogen. Since *C. jejuni* was identified in 87.63% of patients with acute enteritis caused by *Campylobacter* spp., it was detected as the most prevalent *Campylobacter* species. *C. coli* was identified in 12.3% patients and *C. lari*
was not detected at all. This species distribution of *Campylobacter* is similar with that existing in our neighboring countries, as well as in many countries worldwide [7, 9]. This variation might be attributed to demographic, geographic and study period differences between these studies. It was not found any statistically significant difference in patient’s gender (p>0.05). *Campylobacter* isolation rate was highest in children under 15 years of age. In the remaining three groups of patients at the age from 15 to 30, from 31 to 50 and above 50, the isolation rate was very similar and more than twice lower than in children up to 15 years. This difference was statistically significant (p<0.05) and these findings were in agreement with the studies conducted on similar age groups in many developing countries [10, 11].

Most cases of *Campylobacter* enteritis do not require antimicrobial treatment, being clinically mild and self-limiting. However, a substantial proportion of these infections require treatment. Cephalosporines and fluoroquinolones are very often empirically prescribed for diarrheal diseases, because of the effectiveness against a big range of enteropathogenic bacteria [12, 13]. Our results revealed quite high resistance to ceftriaxone and ciprofloxacin, which are in agreement with the results from some other studies [14-17]. The most important reasons for this type of resistance is the treatment of infections, other than gastroenteritis, as well as their consummation in cases with self-medication. Treatment of patients with ceftriaxone might be very unreliable, because of the high resistance to this agent, most often due to the activity of beta lactamase enzyme. One third of *C. jejuni* isolates indicating resistance to ceftriaxone were susceptible to amoxicillin-clavulanic acid. These finding indicates beta lactamase mediated resistance in *C. jejuni* isolates, which should be investigated in the future. 29/44 (65.90%) of *C. jejuni* isolates revealed high level of resistance (8 – 32 µg/ml) to ciprofloxacin. 26/32 (81.25%) resistant *C. jejuni* isolates to tetracycline revealed high level of resistance with MIC between 32 and 256 µg/ml. In many developed countries, resistance of *Campylobacter* isolates to fluoroquinolones and tetracyclines occurs, due to their use in food animals and travel to developing countries [18, 19, 20], but similar data are still not known for our country. The high level of resistance in *C. jejuni* isolates to ceftriaxone, ciprofloxacin and tetracycline, simultaneously indicates the probability for a wide use or overuse of these antimicrobials in human, as well as in veterinary medicine. Those type of data are still not available in our country.

*C. coli* isolates revealed even higher resistance to the six antibiotics tested in our study. MIC values of all *Campylobacter* isolates for ciprofloxacin, erythromycin and tetracycline showed very good correlation with their inhibition zone diameters. There was no any discrepancy between the results obtained with Epsilon test and disk diffusion test, according to EUCAST recommendations. As shown in Graph 4, all isolates revealed MIC and inhibition zone diameters for erythromycin in the sensitive range. Regarding the level of resistance to ciprofloxacin and tetracycline we obtained concerning results, because of the high level of resistance to these two antibiotics. This finding indicates the need for performing the more rational prescribing of these antibiotics in the treatment of *Campylobacter* enteritis as well as other infections.

**CONCLUSION**

The increase of the isolation rate of *Campylobacter* from patients with acute enteritis indicates the need for permanent isolation and identification of *Campylobacter* from every clinically diagnosed patient as acute enteritis. Erythromycin is the most effective antibiotic for treatment of *Campylobacter* enteritis in our patients.

Since high percentage of *Campylobacter* isolates in our study revealed resistance against many of the clinically important groups of antibiotics (cephalosporines, fluoroquinolones, tetracyclines), it is essential to perform susceptibility testing on every single *Campylobacter* isolate. The high level of *Campylobacter* resistance to beta-lactams, fluoroquinolones and tetracyclines require more rational approach in the treatment of *Campylobacter* enteritis.

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Резиме

АНТИМИКРОБНА ЧУВСТВИТЕЛНОСТ НА ИЗОЛАТИТЕ НА CAMPYLOBACTER БО ГЛАВНИОТ ГРАД НА СЕВЕРНА МАКЕДОНИЈА

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Вовед: Инфекциите со Campylobacter вообичаено се само лимитирачки, но во одредени случаи со тешка форма на ентеритис, компромитиран имунолошки систем и бактериемија, потребен е соодветен антимикробен третман. Целта на нашата студија беше да се одреди стапката на изолација на соевите на Campylobacter кај пациенти со акутен ентеритис во главниот град на Северна Македонија и нивната антимикробна осетливост.

Материјал и методи: Во студијата беа вклучени 3820 пациенти кај кои клинички биле дијагностицирани со акутен ентеритис. Изолацијата и идентификацијата на Campylobacter беше направена во примероци од фецес со примена на класичните микробиолошки методи. Антимикробната чувствителност беше тестирана кај сите примероци со помош на диск-дифузиската техника, користејќи ги дисковите за цефтриаксон, амоксицилин-клавулонска киселина, еритромицин, ципрофлоксацин, тетрациклин и гентамицин. Дополнително, кај сите изолати на Campylobacter беа испитувани минималните инхибиторни концентрации на еритромицин, ципрофлоксацин и тетрациклин, користејќи ги епсилон-тестовите на концентрационални градиенти.

Резултати: Campylobacter species беше изолиран кај 97 пациенти. Иако средната стапка на изолација на Campylobacter spp. за време на целниот период на студијата беше 2,53 %, статистички значаен пораст беше забележан во 2016 и 2017 година во споредба со податоците од претходните четири години. Стапката на изолација на Campylobacter spp. не покажа статистички значајна разлика меѓу мажите и жените (p > 0,05). 46,4 % од пациентите со ентеритис предизвикан од Campylobacter беа деца на возраст под 15 години. Четирисепст и три изолати на C. jejuni беа чувствители на сите шест антибиотици, но другите 44 изолати покажаа резистенција кон еден антибиотик. Изолатите на C. coli беа истовремено резистентни на три антибиотици. Единствено два изолати на C. coli покажаа чувствителност против сите шест антибиотици. 40,9 % и 50 % од изолатите на C. jejuni и на C. coli соодветно, покажаа резистенција кон бета-лактами, флуроксениолони и тетрациклини истовремено.

Заклучок: Порастот на стапката на изолација на Campylobacter од пациенти со акутен ентеритис укажува на потребата за перманентна изолација и идентификација на Campylobacter од секој клинички дијагностициран пациент со акутен ентеритис. Еритромицинот е најефективниот антибиотик за третман на ентеритис предизвикан од Campylobacter кај пациентите од нашата студија. Високото ниво на резистенција на Campylobacter кон бета-лактамите, флуроксениолоните и тетрациклилните бара порационален пристап во третманот на акутниот ентеритис предизвикан од Campylobacter.

Ключни зборови: акутен ентеритис, Campylobacter, антимикробна чувствителност.