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Türk Hijyen ve Deneysel Biyoloji Dergisi

Investigation of the frequency of *Campylobacter jejuni* and *Campylobacter coli* in acute gastroenteritis cases admitted to a tertiary hospital in Türkiye and determination of the antibiotic susceptibility of isolated strains and various virulence factors

Türkiye'de üçüncü basamak bir hastaneye başvuran akut gastroenterit olgularında *Campylobacter jejuni* ve *Campylobacter coli* sıklığının araştırılması ve izole edilen şuşların antibiyotik duyarlılıkları ve çeşitli virülans faktörlerinin belirlenmesi

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ABSTRACT

Objective: Campylobacteriosis is one of the most common types of gastroenteritis worldwide. In recent years, it has been reported that *Campylobacter* species isolated from humans and animals have increased antibiotic resistance against macrolides and especially fluoroquinolones. This study aimed to determine the frequency of *Campylobacter* species in patients with acute gastroenteritis and to investigate the antibiotic susceptibility profile and various virulence factors in the isolated strains.

Methods: Stool samples from 401 patients at Mersin University Hospital were included in the study between October 2018 and April 2019. Antimicrobial susceptibility testing of *Campylobacter* species was performed by using the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing

ÖZET

Amaç: Campylobacteriosis dünya çapında en sık görülen gastroenterit türlerinden biridir. Son yıllarda insanlardan ve hayvanlardan izole edilen *Campylobacter* türlerinin makrolidlere ve özellikle de florokinolonlara karşı antibiyotik direncini arttırdığı bildirilmektedir. Çalışmamızın amacı, akut gastroenteritli hastalarda *Campylobacter* türlerinin sıklığını belirlemek ve izole edilen örneklerin antibiyotik duyarlılık profilini ve çeşitli virülans faktörlerini araştırmaktı.

Yöntem: Çalışmaya Ekim 2018 ile Nisan 2019 tarihleri arasında Mersin Üniversite Hastanesi'ndeki 401 hastanın dışkı örnekleri dahil edildi. *Campylobacter* türlerinin antimikrobiyal duyarlılık testleri, EUCAST kılavuzuna göre disk difüzyon yöntemi ile yapıldı. *Campylobacter* türlerinin ve çeşitli virülans

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(EUCAST) guidelines. Identification of *Campylobacter* species and various virulence factors were studied with conventional Polimeraz Chain Reaction(PCR).

Results: *Campylobacter* spp. was isolated in 44 (10.9%) of the 401 stool samples included in the study. The PCR method revealed that 36 isolates (81.8%) corresponded to *C. jejuni*, while six isolates (13.6%) were identified as *C. coli*. Two isolates could not be identified to the species level by the PCR method and were reported as *Campylobacter* spp. All 44 *Campylobacter* isolates were resistant to ciprofloxacin. Tetracycline resistance was found to be 97.7% (n=43) and erythromycin resistance was 9.1% (n=4) in the isolated *Campylobacter* strains. *cadF* and *cdtABC* positivity were detected in 90.9% (n=40) and 54.5% (n=24) of the *Campylobacter* isolates, respectively.

Conclusion: Our results have yielded valuable data on the epidemiology of *Campylobacter* in our geographical area, highlighting the importance of including *Campylobacter* culture as a standard component of routine stool culture tests. Macrolide antibiotics have been reaffirmed as the primary treatment option for Campylobacter species. Furthermore, it was suggested that rational antibiotic usage and preventive measures against antibiotic resistance should be implemented.

Key Words: *Campylobacter*, gastroenteritis, PCR, antibiotic, virulence

faktörlerinin tanımlanması konvansiyonel Polimeraz Zincir Reaksiyonu (PCR) ile çalışıldı.

Bulgular: Çalışmaya alınan 401 dışkı örneğinin 44'ünde (%10.9) *Campylobacter* spp. izole edildi. PCR yöntemiyle 36 izolat (%81.8) *C. jejuni*, 6 izolat(%13.6) ise *C. coli* olarak tanımlandı. 2 izolat ise PCR yöntemiyle tür düzeyinde tanımlanması yapılamamış olup *Campylobacter* spp. olarak raporlandı. 44 *Campylobacter* izolatının tamamı siprofloksasine dirençliydi. İzole edilen *Campylobacter* suşlarında tetrasiklin direnci %97.7 (n=43), eritromisin direnci ise %9.1 (n=4) olarak belirlendi. *Campylobacter* izolatlarının sırasıyla %90.9'unda (n=40) ve %54.5'inde (n=24) *cadF* ve *cdtABC* pozitifliği tespit edildi.

Sonuç: Sonuç olarak çalışmamız bölgemizdeki *Campylobacter* epidemiyolojisi hakkında veriler sunmuş ve *Campylobacter* kültürünün rutin dışkı kültürü testlerinin standart bir bileşeni olarak dahil edilmesinin önemini vurgulamıştır. *Campylobacter* türlerinde makrolid grubu antibiyotiklerin birincil tedavi seçeneği olduğu yeniden ortaya konmuştur. Ayrıca akılcı antibiyotik kullanımı ve antibiyotik direncine karşı önleyici tedbirlerin uygulanması gerektiği sonucuna ulaşılmıştır.

Anahtar Kelimeler: *Campylobacter*, Gastroenterit, PCR, Antibiyotik, Virülans

INTRODUCTION

Campylobacter spp. is a zoonotic pathogen that is found commensally in cattle, sheep and especially avian species. When it infects humans, it frequently leads to acute gastroenteritis (1). Foodborne *Campylobacter* infections are a major public health problem in many European countries and worldwide (1-3). Although there are 17 species and 6 subspecies of the genus *Campylobacter*, the most frequently reported species as causative agents in humans are *Campylobacter jejuni* and *Campylobacter coli* (4).

Campylobacteriosis is a self-limiting disease that typically requires only hydration as treatment. However, antibiotic treatment is required in severe or prolonged infections and in immunocompromised patients (5). In recent years, it has been reported that *Campylobacter* species isolated from humans and animals have increased antibiotic resistance (6-8).

Campylobacter species exhibit distinct virulence and survival attributes, including motility, chemotaxis, adhesion, invasion, toxin production(9). Multiple gene regions associated with these virulence factors have been identified. Some of those; the *cadF* gene, which encodes the fibronectin-binding outer membrane protein that plays a role in bacterial adhesion, the *iam* gene, which is an invasion-related marker, and the *cdtA*, *cdtB* and *cdtC* genes, which encode the production of three subunit cytolethal toxin(9) .

This study aimed to determine the isolation frequency of *Campylobacter* species in stool samples obtained from patients admitted to Mersin University Hospital with a preliminary diagnosis of acute gastroenteritis and to investigate antibiotic susceptibility profiles and virulence factors in isolated samples.

MATERIAL and METHOD

Study design

In this study, we examined stool samples from 401 patients who were followed up in various outpatient clinics and wards of Mersin University Hospital between October 2018 and April 2019 and whose anamnesis and clinical findings were compatible with diarrhea caused by *Campylobacter* spp. Stool samples from the same patient were excluded. The stool samples which the erythrocytes and leukocytes were detected in their microscopic examination, were inoculated into the modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid, England) medium. In cases where it was not feasible to process the samples within a 2-hour window, they were instead placed in Cary Blair transport medium and stored at a temperature of +4°C for a maximum duration of 24 hours.

Isolation of *Campylobacter* species from stool samples

Stool samples were inoculated directly or from Cary Blair transport medium onto mCCDA medium. The medium plates were then placed in a 2.5-L jar with Campy-Gen (Oxoid, UK) gas packs (5% O_2, 10% CO_2 and 85% N_2) to provide a microaerophilic environment and incubated at 42°C for 48-72 hours. Colonies displaying a smooth, rounded surface and a gray color on the selective medium, which raised suspicions of *Campylobacter* presence, underwent Gram staining as well as catalase and oxidase tests for confirmation (10). Following the antibiotic susceptibility tests conducted on isolates that were phenotypically identified as *Campylobacter* spp., according to the criteria described in the literature (positive in catalase and oxidase tests; gram negative; and a characteristic gull-wing, curled, or S-shaped appearance in Gram staining), these isolates were transferred into a 10% glycerol broth (Sigma, USA) and stored at a temperature of −20°C for subsequent molecular testing.

Antibiotic susceptibility test

Susceptibility testing of isolates against erythromycin 15 µg, tetracycline 30 µg, and ciprofloxacin 5 µg (Oxoid, UK) antibiotics was performed using the disk diffusion method according to the EUCAST guidelines (11). Accordingly, the bacterial suspension of *Campylobacter* spp. Isolates prepared at a density equivalent to 0.5 McFarland turbidity standard, was inoculated on the surface of Mueller Hinton Fastidious agar medium (BD, Germany) containing 20 mg/L ß-NAD, and 5% horse blood and antibiotic disks were placed. And they were incubated in a microaerophilic environment at 42°C for 24 hours, and inhibition zones were evaluated according to EUCAST guidelines (11).

Molecular tests

The rapid DNA extraction method was used for DNA isolation (12). For this purpose, a loopful of bacteria from *Campylobacter* colonies growing on selective medium was suspended in 1 ml of sterile distilled water and kept at 80°C for 20 minutes for the cell lysis. It was then centrifuged at 12,000 xg for 10 minutes, and the supernatant was discarded. The mixture obtained by adding 200 µl of chloroform

and 200 µl of sterile distilled water to the pellet was centrifuged again at 12,000 xg for 10 minutes, and the upper liquid was used as a template for the PCR reaction.

In the identification of *Campylobacter* species using the PCR method, we followed the procedure outlined in the literature (13, 14); primers targeting the *mapA* and *glyA* gene regions were employed for the identification of *Campylobacter jejuni* and *Campylobacter coli*, respectively. Furthermore, virulence factors were examined using the PCR technique, following the procedures outlined in the literature. This included the utilization of primers designed to target the *cadF* gene region and the *cdtABC* gene region (Table 1) (15).

Each sample underwent amplification within a reaction volume of 50 µl. The reaction mixture consisted of 5 µl of 10× PCR buffer (Promega, USA), 1.5 µmol/µl of $MgCl_2$ (Promega, USA), 0.2 µmol/µl of dNTP mix (Sigma, USA), 0.25 pmol/µl of each primer, 2.5 U of Taq DNA polymerase, and 5 µl of sample DNA. The amplification conditions of the samples included initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, binding for 1 minute at the temperatures specified for each gene region in Table 1, elongation at 72°C for 1 minute, and a final elongation step at 72°C for 7 minutes. PCR products were visualized under ultraviolet light after 1% agarose gel electrophoresis containing 0.5 µg/ml of ethidium bromide.

Statistical analysis

STATISTICA version 13.5.0.17 was used for statistical analysis of the data. We employed a chi-square independence test to assess whether the distribution of reproduction was consistent among different categories of categorical variables, including sex, age, ward, and microscopic examination. The statistical significance level was considered $p < 0.05$.

The study was approved by the Mersin University Clinical Research Ethics Committee (Date: 27.04.2018 and Number: 2018/195) and supported by the Mersin University Scientific Research Projects Unit as a project coded (2018-2-TP3-2930).

RESULTS

Of the 401 patients included in the study, 230 (57.3%) were male and 171 (42.7%) were female. The age of the patients ranged from 0 to 95 years, and the analysis was categorized by age groups as follows: stool samples from 123 (30.6%) patients were in the <5-year-old group, 138 (34.4%) patients in the 5-18-year-old group, and 140 (35%) patients in the 18-95-year-old group.

Campylobacter spp. were isolated in 44 (10.9%) of 401 stool samples. Using the PCR method, 36 (81.8%) of the isolates were identified as *C. jejuni* and 6 (13.6%) as *C. coli*. Two strains initially identified as

Table 1. Primer sequences used in the study and expected amplicon sizes

Gene region	Primer sequence	Target amplicon (bp)	Binding temperature (°C)	Source
glyA ***(C. coli)***	F-5'-GTAAAACCAAAGCTTATCGTG-3' R-5'- TCCAGCAATGTGTGCAATG-3'	126	55	[14]
mapA ***(C. jejuni)***	F-5'-CTATTTTTTTTTTTGAGTGCTTGCTTGTG-3' R-5'-GCTTTATTTGCCATTTGTTTTATTA -3'	589	55	[13]
cadF	F-5'-TTGAAGGTAATTTAGATATATG-3' R-5'-CTAATACCTAAAGTTGAAAC-3'	400	45	[15]
cdtABC	F-5'-GGAAATTGGATTTGGGGCTATACT-3' R-5'-TTGCACATAACCAAAAGGAAG-3'	1215	55	[15]

Campylobacter spp. using conventional techniques could not be specified as per the species level via the PCR method. Gel images of the *glyA* and *mapA* genes for the identification of *Campylobacter* species are displayed in Figures 1 and 2.

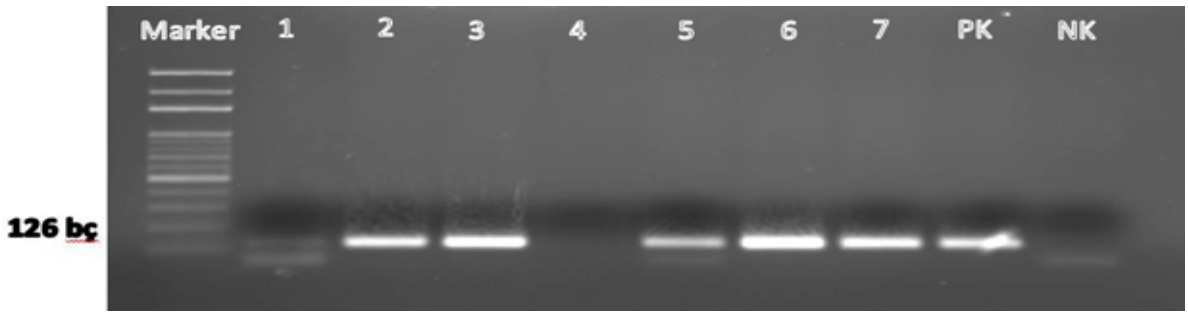

Figure 1. 1% agarose gel electrophoresis image of PCR amplification products at ~126 bp of the glyA region. Column Marker; molecular weight standard (GeneRuler 100 bc DNA ladder, #SM0241, Thermo Fisher Scientific), columns 1 and 4; negative sample, columns 2, 3, 5–7; positive sample, PC; *C. coli* ATCC 33559 as a positive control, NC; distilled water as a negative control

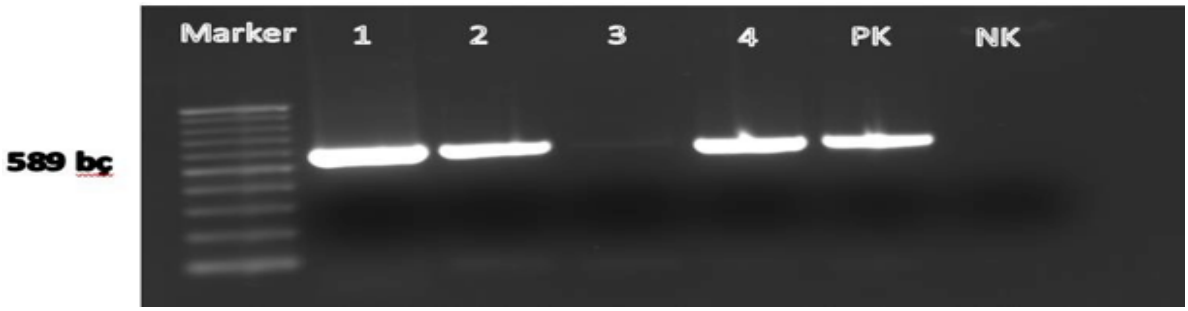

Figure 2. 1% agarose gel electrophoresis image of PCR amplification products at ~589 bp of the mapA gene region. Column Marker; molecular weight standard (GeneRuler 100 bc DNA ladder, #SM0241, Thermo Fisher Scientific), columns 1, 2, and 4; positive sample, column 3; negative sample, PC; *C. jejuni* ATCC 33560 as a positive control, NC; distilled water as a negative control

Of the 44 patients from whom *Campylobacter* spp. were isolated, 28 (63.6%) were male and 16 (36.4%) were female. There was no statistically significant difference in *Campylobacter* positivity between genders (p = 0.097). The age of positive patients ranged from 6 months to 62 years. When categorizing positive patients by age groups, 29.6% (n = 13) in the <5-year-old group, 56.8% (n = 25) in the 5–18-year-old group, and 13.6% (n = 6) in the ≥18-year-old group were positive for *Campylobacter* spp. *Campylobacter* spp. positivity was found to be statistically higher in the 5–18-year-old group compared to other age groups (p = 0.0018).

Upon analyzing the distribution of *Campylobacter* spp. isolation based on clinical units, it was observed that the rate of positivity in samples from the pediatric emergency clinic (68.2%; n = 30) was greater than in the other units (31.8%; n = 14) (p = 0.0237).

Antibiotic susceptibilities of 44 *Campylobacter* isolates were tested by the disk diffusion method, and all 44 (100%) isolates were resistant to ciprofloxacin. Tetracycline resistance was detected in 43 (97.7%) isolates, while erythromycin resistance was observed in 4 (9.1%) isolates (Table 2).

When the cadF and cdtABC gene regions encoding *Campylobacter* virulence factors were analyzed by PCR, *cadF* was found positive in 40 (90.9%) and *cdtABC* was found positive in 24 (54.5%) of 44 *Campylobacter* isolates (Table3).

Table 2. Antibiotic Susceptibility Results of *Campylobacter* Isolates

Campylobacter species	Ciprofloxacin	Tetracycline	Erythromycin
	Resistant n (%)	Resistant n (%)	Resistant n (%)
C. jejuni (n = 36)	36 (100)	35 (97)	1 (2,8)
C. coli (n = 6)	6 (100)	6 (100)	3 (50)
Campylobacter spp. (n = 2)	2 (100)	2 (100)	0 (0)
Total (n = 44)	44 (100)	43 (97,7)	4 (9,1)

Table 3. Distribution of *cadF and cdtABC* virulence genes of *Campylobacter* isolates

Campylobacter species	*cadF* n (%)	*cdtABC* n (%)
C. jejuni (n = 36)	34 (94,4)	24 (66,6)
C. coli (n = 6)	6 (100)	0 (0)
Campylobacter spp. (n = 2)	0 (0)	0 (0)
Total (n = 44)	40 (90,9)	24 (54,5)

DISCUSSION

Among the factors that cause ~550 million foodborne infectious diseases annually worldwide, *Campylobacter* species have been reported as the second most common agent after Norovirus (16). According to the European Food Safety Authority report, the most frequently reported causative agent in the European Union (EU) in 2019 was *Campylobacter* species, with 220,682 cases (2). Campylobacteriosis was isolated at a rate of 1%-13% in Türkiye (17). According to the 2012 data from the National Enteric Pathogens Laboratory Network (UEPLA) in Türkiye, confirmed cases of enteric pathogens, *Campylobacter* species accounted for ranking second in prevalence after *Salmonella* serotypes (18).

In countries such as the United Kingdom and the United States, ~90% of *Campylobacter* species isolated from gastroenteritis cases are *C. jejuni*, while *C. coli* is responsible for the majority of the remaining ~10% of cases (19). Of the 44 *Campylobacter* isolates in our study, 36 (81.8%) were *C. jejuni*, 6 (13.6%) were *C. coli*, and 2 (4.6%) were *Campylobacter* spp. (other than *C. jejuni* and *C. coli*). The distribution rates of *Campylobacter* species distribution rates in our study are similar to other data worldwide.

Although campylobacteriosis is a disease that affects all age groups, it is estimated that more than half of all *Campylobacter* cases occur in children >5 years of age globally (3). In large-scale studies conducted in Germany, England and Wales, it was reported that the age distribution of *Campylobacter* cases varied geographically. In a study conducted in the United Kingdom, *Campylobacter* cases were more common in men, whereas in a study conducted in Germany, young adult women were more likely to be affected by *Campylobacter* infections (20,21). In our study, although *Campylobacter* infection was detected at a higher rate in the male gender, no statistical difference was found between genders (p = 0.097). Examining the age distribution of the 44 cases in our study, we observed the highest positivity rate (56.8%) in individuals aged 5–18 years (p = 0.0018). In our study, the higher prevalence of *Campylobacter* cases under the age of 18 years is similar in the literature (3,22) .

Campylobacteriosis cases are mostly self-limiting without a need for antibiotic treatment, but antibiotic treatment is required in severe or prolonged infections and in immunocompromised patients (5). Although the resistance rate to macrolide antibiotics, which are the preferred antibiotics for treatment, remains relatively low and consistent, strains isolated from both humans and animals have exhibited a notably high resistance rate to fluoroquinolones and tetracycline antibiotics (7,8,18,23). In a study conducted by Akan et al. (24) in 1994 in Türkiye, one (0.8%) quinolone and one (0.8%) macrolide resistant strains were detected in 119 *Campylobacter* isolates, all of which were tetracycline-sensitive. In 2011, a study conducted by Kayman et al. (25) found that among 127 *Campylobacter* isolates, 73.9% exhibited resistance to ciprofloxacin, 24% to tetracycline, and 6.3% to erythromycin. Based on the 2015 data from UEPLA, our country had recorded resistance rates of 86.5% to ciprofloxacin, 66.7% to tetracycline, and 7.2% to erythromycin (18). In our study, all *Campylobacter* isolates were resistant to ciprofloxacin, 97.7% to tetracycline. Accordingly, ciprofloxacin and tetracycline resistance in *Campylobacter* isolates in Türkiye have increased over the years. We observed significantly elevated rates of ciprofloxacin and tetracycline resistance. While the limited number of isolates in our study might suggest elevated resistance rates, the prevalence of resistant infections could be attributed to the transmission of resistant strains from poultry to humans (8,23). In a study where resistance rates to ciprofloxacin, tetracycline, and erythromycin among *Campylobacter* isolates from humans and chickens ranged from 81%–93%, 38%–56%, and 6%–7%, respectively, the clonal analysis using Pulsed field gel electrophoresis (PFGE) further confirms this perspective by revealing a close genetic relationship between human and chicken isolates (8).

Infections caused by *Campylobacter* species can have diverse clinical symptoms. In addition to the host response, the pathogenicity of the agent also significantly contributes to clinical variability. Genes associated with pathogenicity in *Campylobacter* species are *flaA*, *cadF*, *virB11*, *ciaB*, *cdtABC* (cytolethal expanding toxin A, B, and C), and *cgtB* (26). In our study, among *Campylobacter* virulence factors, *cadF*-encoding adhesion protein was found to be positive in 90.9% (n = 40) and *cdtABC*-encoding cytoletal toxin production was found to be positive in 54.5% (n = 24). In a study conducted with clinical *C. jejuni* isolates, *cadF* was found to be 100% positive and *cdtA*, *cdtB*, and *cdtC* were found to be positive at a rate of 97.5% each (27). While all *C. jejuni* isolates isolated from slaughterhouse samples were positive for *cdtA*, *cdtB*, and *cdtC*, one sample was found to be negative for *cdtC* (28). In a study by Iglesias-Torrens et al. (23), all human and chicken *C. jejuni* isolates were positive for *cdtA*, *cdtB*, and *cdtC*, while at least one subunit of cdt genes was found negative in 46% of avian bird isolates. *cadF* was positive in all chicken and wild avian isolates, while 10% of human isolates were negative. *cadF* was found to be positive in all *C. jejuni* and *C. coli* isolates of human and chicken origin in a study by Wysok et al. (29). *cdtABC* has been reported in 89.8% and 40% of human *C. jejuni* and

C. coli, respectively, compared to 100% and 33.3% of chicken *C. jejuni* and *C. coli*, respectively. In our study, the presence of the *cadF* gene was detected at a range of 94.4%-100%, while the cdtABC gene group was identified at a range of 66.6%-0% in *C. jejuni* and *C. coli* isolates, respectively (Table 3). In a study with 58.4% *cdtABC* positivity, although the cdt subunit genes were independently positive in 15 samples, the *cdtABC* group PCR test was found to be negative (15), which is consistent with our study. This variability may be due to the primers and PCR techniques employed, as well as the genetic and epidemiological traits of the bacteria (19). In our study and in our country, a high rate of virulence factor-related genes was detected in human and animal isolates. Additional data is required to clarify the relationship between these genes found in human and animal isolates and their association with disease patterns. Further studies on the relationship between virulence factors and patient clinics are needed.

In conclusion; *Campylobacter* cases were detected in 10.9% of patients with acute gastroenteritis in our hospital. Our findings provide important data on the epidemiology of *Campylobacter* in our region and are particularly significant in highlighting the increasing resistance rates of *Campylobacter* isolates, especially against ciprofloxacin and tetracycline. These results suggest that campylobacteriosis should be considered in patients presenting with symptoms of acute gastroenteritis, and that routine stool culture tests should include *Campylobacter* culture. Based on our study, macrolide antibiotics remain the first-line treatment option.

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ETHICS COMMITTEE APPROVAL

* The study was approved by the Mersin University Clinical Research Ethics Committee (Date: 27.04.2018 and Number: 2018/195).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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