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Molecular Identification of *Campylobacter* Species Isolated from Patients with Gastroenteritis in Edirne, Turkey

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Abstract

BACKGROUND/AIMS: *Campylobacter* is a major cause of foodborne diarrheal disease, and the incidence of campylobacteriosis has significantly increased in both developed and developing countries. The purpose of the present study was to identify the species of *Campylobacter* isolates and to evaluate the distribution of *Campylobacter* infections according to various characteristics in our region.

MATERIALS AND METHODS: *Campylobacter* isolates obtained from patients at a tertiary hospital in Edirne, Turkey were included in this study. The distribution of *Campylobacter* infections was evaluated according to age, season, and gender. Species identification was performed by multiplex polymerase chain reaction (PCR). The RNA polymerase beta-subunit gene (*rpoB*) of selected samples was amplified, and DNA sequencing was performed.

RESULTS: *Campylobacter* species were isolated from 226 (4.3%) of the 5,241 samples. One hundred and seventy-six (89.3%) of 197 samples were identified as *C. jejuni* and 19 (9.6%) as *C. coli* by multiplex PCR. Two isolates showed a band profile compatible with both *C. jejuni* and *C. coli*. DNA sequencing was performed for 21 isolates. Sixteen isolates were compatible with *C. jejuni* and 5 isolates were consistent with *C. coli*. There was no statistically significant difference in *Campylobacter* isolation rates according to gender and season ($p>0.05$). *Campylobacter* species were most frequently isolated from children in the age group of 0-14 years ($p<0.01$).

CONCLUSION: *Campylobacter* is one of the main causes of diarrhea in Turkey, and this infection is more common in children. This study contributes to information about the situation of *Campylobacter* infection and the genetic features of isolates in Turkey.

Keywords: *Campylobacter*, multiplex PCR, DNA sequencing, Turkey

INTRODUCTION

Campylobacter species are Gram-negative bacteria that are a major cause of gastroenteritis around the world.[1] Although the primary reservoirs of *Campylobacter* species are poultry, cattle, and pigs, these bacteria can be colonized in the gastrointestinal tract of many warm-blooded animals.[2] Transmission to humans is often caused by ingestion of contaminated food or water or by contact with fecal material from infected animals or people.[3] To date, 32 *Campylobacter* species and 9 subspecies have been isolated from various sources. However, the majority of human infections are caused by *C. jejuni* and *C. coli*.[4] In recent years, infections caused by *Campylobacter* species have increased in both developed and developing countries.[1] The infection occurs especially in children younger than 5. However, high incidence can also be seen in other age

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groups.[3,5] According to "The European Union One Health 2019 Zoonoses Report", there was a seasonality in campylobacteriosis cases, with peaks in the summer months.[6]

The aim of this study was to determine the distribution of *Campylobacter* species and to evaluate the effect of age, gender and seasonality on *Campylobacter* infections.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Ethical Committee of Trakya University Faculty of Medicine (approval number: TUTF-BAEK 2016/196).

Bacterial Isolates

Campylobacter isolates obtained from 5,241 fecal samples by conventional methods at the Trakya University Health Center for Medical Research and Practice, Microbiology Laboratory between October, 2013 and May, 2016 were included in this study. Fecal samples were streaked on *Campylobacter* agar (Becton Dickinson, USA) containing 7% horse blood and incubated at 42 °C in a microaerophilic atmosphere for 48 hours. Gram staining, and catalase and oxidase tests were performed on suspected colonies to identify the *Campylobacter* species. Gram-negative, curved rods with positive catalase and oxidase tests were considered to be *Campylobacter*. Laboratory records were examined retrospectively, and the data were evaluated in terms of age, gender, and seasonal distribution.

DNA Extraction

DNA isolation of *Campylobacter* strains that were subcultured and stored at -20 °C was performed using the Invitrogen PureLink Genomic DNA Mini Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Identification of *Campylobacter* Species

A multiplex polymerase chain reaction (PCR) was performed according to the protocol established by Wang et al.[7] with minor modifications. Each multiplex PCR tube contained 2.5 µL of 10X Taq buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, 0.8% (v/v) Nonidet P40], 0.2 mM dNTP, 2 mM $MgCl_2$, 0.5 µM *C. jejuni* and *C. lari* primers, 1 µM *C. coli* primers, 2 µM *C. upsaliensis* primers, 0.2 µM 23S rRNA primers, 1.25 U of Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA), and 2.5 µL of whole-cell template DNA in total 25 µL reaction volume. The primers used for the identification of *Campylobacter* species are shown in Table 1. In all PCR reactions, *C. jejuni* NCTC 13367 and *C. coli* NCTC 11350 were used as positive controls, and *E. coli* ATCC 25922 was used as a negative control. PCR products were subjected to electrophoresis on 1.5% agarose gel, and the bands were evaluated under ultraviolet light.

DNA Sequencing

DNA sequencing was performed for 21 isolates. According to the multiplex PCR results, 13 *C. jejuni* and 6 *C. coli* isolates were randomly selected for DNA sequencing. Sequence analysis was also performed on two samples exhibiting double bands. The RNA polymerase beta-subunit gene (*rpoB*) was amplified using the previously described primers.[8] PCR products were purified using the Invitrogen PureLink Quick PCR Purification Kit (ThermoFisher Scientific, Waltham, MA, USA). DNA sequencing was performed by the Trakya University Technology Research Development Application and Research Center. The *rpoB* gene sequence analysis was carried out on the Applied Biosystems 3500 Series Genetic Analyzer (ThermoFisher Scientific, Waltham, MA, USA) using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA, USA). The sequencing chromatograms were analyzed using the ProSeq v2 and BioEdit programs. The nucleotide sequences of the isolates were compared with sequences in GenBank using the Basic Local Alignment Search Tool.

Statistical Analysis

NCSS (Number Cruncher Statistical System) 2007 software (Kaysville, Utah, USA) was used for statistical analysis. Data were analyzed by descriptive statistical methods. Qualitative data were compared by Pearson chi-square and the Fisher-freeman-halton exact test with a significance level of $p<0.05$.

RESULTS

Bacterial Isolates

A total of 5,241 stool samples were sent to the microbiology laboratory for *Campylobacter* culture between October, 2013 and May, 2016. *Campylobacter* spp. were detected in 226 (4.3%) of these samples, which were obtained from 215 individual patients. Of the patients with *Campylobacter* infection, 44.2% were female and 55.8% were male. There was no statistically significant difference in *Campylobacter* isolation rate by gender ($p=0.584$), and the difference in seasonal rates of *Campylobacter* isolation was not statistically significant ($p=0.141$) (Table 2). The highest culture positivity rate was detected in May ($p=0.001$), while the lowest rate was seen in September ($p=0.014$) (Figure 1). When the 0-5 and 6-14 age groups were evaluated together, *Campylobacter* spp. were isolated more frequently in the 0-14 age group compared to the other age groups ($p<0.01$) (Figure 2).

Identification of *Campylobacter* Species

Bacterial DNA was obtained from 197 isolates of 187 patients. By multiplex PCR, 176 (89.3%) isolates were identified as *C. jejuni* and 19 (9.6%) isolates as *C. coli*. Two samples showed a band profile compatible with both *C. jejuni* and *C. coli*.

DNA Sequencing

The results of the DNA sequencing of 21 isolates were evaluated. The DNA sequences of 13 isolates were found to be compatible with the *C. jejuni* sequences in the GenBank. The DNA sequences of the five isolates

Table 1. Primers used for the identification of *Campylobacter* species (Wang et al.[7])

Oligonucleotide primers	Amplicon size (bp)
23SF: 5'- TATACCGGTAAGGAGTGCTGGAG-3' 23SR: 5'- ATCAATTAACCTTCGAGCACCG-3'	650
CJF: 5'-ACTTCTTTATTGCTTGCTGC-3' CJR: 5'-GCCACAACAAGTAAAGAAGC-3'	323
CCF: 5'-GTAAAACCAAAGCTTATCGTG-3' CCR: 5'-TCCAGCAATGTGTGCAATG-3'	126
CLF: 5'-TAGAGAGATAGCAAAAGAGA-3' CLR: 5'-TACACATAATAATCCCACCC-3'	251
CUF: 5'-AATTGAAACTCTTGCTATCC-3' CUR: 5'-TCATACATTTTACCCGAGCT-3'	204

matched *C. coli*. One isolate was identified as *C. coli* by multiplex PCR and *C. jejuni* by sequence analysis. Additionally, both of the isolates showing double bands in multiplex PCR were found to be compatible with *C. jejuni*. One of the *C. coli* isolates was submitted to the DNA Data Bank of Japan database (accession no: LC511784). The results of multiplex PCR and sequencing analysis of the isolates included in DNA sequencing are shown the Table 3.

DISCUSSION

Campylobacter is one of the most common causes of diarrheal diseases worldwide.[9] There has been a significant increase in the incidence of campylobacteriosis in North America, Europe, and Australia. Although epidemiological data are insufficient, it is known that campylobacteriosis is endemic in Africa, Asia, and the Middle East.[1] In European Union countries, 220,682 cases (59.7 per 100,000 population) were reported in 2019.[6] In a study performed in Denmark, it was found that the incidence of *Campylobacter* infection was higher in males. For domestic cases, age groups 20-29 and 0-4 years had a higher incidence compared to other age groups. For travel-related cases, the incidence was highest in young adults aged 20-29 years. Also, it was revealed that cases not related to travel increased from May to October and peaked in August.[5] In a study conducted in the United States, the highest incidence of campylobacteriosis was observed in males and children under five years of age in most regions, while in three states, the highest incidence was found in people over 60 years of age. The incidence was highest from June to August and lowest from December to February for all regions and age groups.[10] In Ireland, the highest incidence of *Campylobacter* infection was reported in children aged 0-4 years. Cases reported in males were more than female cases in the surveillance system.[11] In our study, 44.2% of the patients with *Campylobacter* infection were female, and 55.8% of the patients were male; but no statistically significant difference was found by gender (p>0.05). Infection most commonly occurred in May (p=0.001), and least commonly in September (p=0.014); nonetheless, we observed no seasonal patterns. The majority of cases were detected in children, and these data are consistent with other studies in Turkey.[12-14]

C. jejuni is responsible for the majority (85-95%) of human *Campylobacter* infections, and *C. coli* is the second most common (5-10%) species.[3,15] Kayman et al.[13] isolated thermophilic *Campylobacter* spp. from 5.4% of 3,287 stool samples of patients with gastroenteritis; and using multiplex PCR, they identified 85% of the isolates as *C. jejuni* and 15% as *C. coli*. In a study performed in Iran, 420 fecal samples from hospitalized children were analyzed. In 8.8% of these samples, the authors isolated *Campylobacter* spp. In that study, 75.7% of *Campylobacter* isolates were

Table 2. Distribution of *Campylobacter* isolates according to gender and season, Edirne, 2013-2016

Parameters	No. sampled (%)	No. positive (%)	p-value
Gender			
Female	2,412 (46.0)	100 (44.2)	0.584
Male	2,829 (54.0)	126 (55.8)	
Season			
Winter	1,362 (26.0)	56 (24.8)	0.141
Spring	1,635 (31.2)	86 (38.0)	
Summer	1,003 (19.1)	38 (16.8)	
Autumn	1,241 (23.7)	46 (20.4)	
Total	5,241 (100)	226 (100)	

*statistical significance defined as p≤0.05.

Table 3. Results of multiplex PCR and sequencing analysis of the isolates included in DNA sequencing

Sample no.	Multiplex PCR	DNA sequencing	
		Matched sequence (accession no.)	Identity (%)
1-10	*C. jejuni*	*C. jejuni* (CP028910)	100
11	*C. jejuni*	*C. jejuni* (DQ174200)	100
12	*C. jejuni*	*C. jejuni* (HM486854)	100
13	*C. jejuni*	*C. jejuni* (AF372097)	100
14-17	*C. coli*	*C. coli* (AF372098)	100
18	*C. coli*	*C. coli* (HG326877)	98
19	*C. coli*	*C. jejuni* (CP054847)	99
20	*C. jejuni* and *C. coli* (double bands)	*C. jejuni* (CP012217)	100
21	*C. jejuni* and *C. coli* (double bands)	*C. jejuni* (CP053854)	100

PCR: polymerase chain reaction.

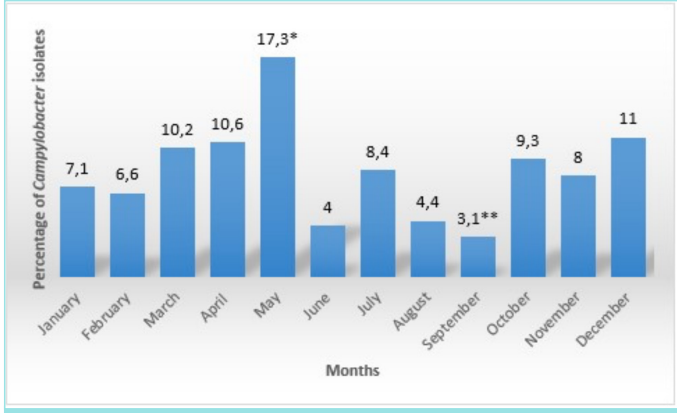

Figure 1. Distribution of *Campylobacter* isolates by month, Edirne, 2013-2016.
*p=0.001, **p=0.014.

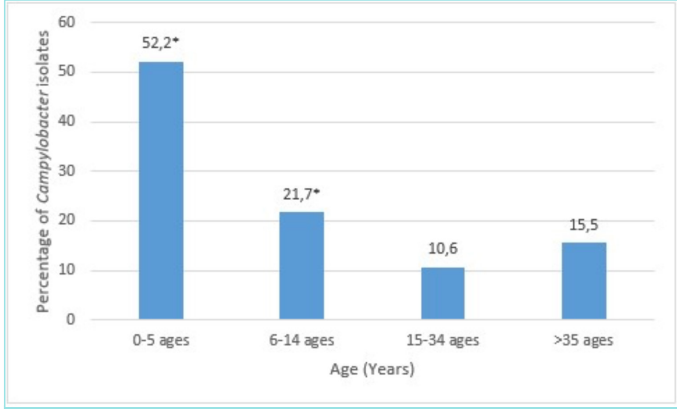

Figure 2. Distribution of *Campylobacter* isolates by age group, Edirne, 2013-2016.
*p<0.01.

identified as *C. jejuni* and the remaining isolates were identified as *C. coli*.[16] In a study carried out in Edirne in 2005, *Campylobacter* was detected in 4% of fecal samples sent to the microbiology laboratory in our hospital, and 81% of these strains were *C. jejuni*. *Campylobacter* was detected mostly in July, August, and September, and there were no differences between age groups and gender distributions.[17] In our study, *Campylobacter* spp. was detected in 4.3% of the samples sent to the laboratory for stool culture. Multiplex PCR identified 89.3% and 9.6% of the isolates to be *C. jejuni* and *C. coli*, respectively. Two isolates showed a band profile compatible with both *C. jejuni* and *C. coli* reference strains. In terms of *Campylobacter* isolation rate and species distribution, our results are consistent with those of other studies conducted in Turkey and elsewhere.[13,15] However, we considered that the inclusion of *Campylobacter* isolates obtained from patients admitted to the single-center to be a limitation of this study. The *rpoB* gene is used in the species identification and phylogenetic analysis of *Campylobacter*. However, *rpoB* gene sequencing may lead to misidentification between the two closely related species, *C. jejuni* and *C. coli*.[8,18] In our study, one isolate showed a band profile consistent with *C. coli* in multiplex PCR, and this isolate was identified as *C. jejuni* by sequence analysis.

CONCLUSION

Campylobacter is a significant bacterial agent in gastroenteritis; therefore, it is important to investigate *Campylobacter* in routine laboratory diagnosis. In the present study, it was determined that there was no statistically significant difference in *Campylobacter* isolation rates according to gender and season in our region. However, it was observed that *Campylobacter* infection is more common in children. This study contributes to information about the epidemiological features of *Campylobacter* in Turkey. To obtain more comprehensive epidemiological data about *Campylobacter* infection and examine the the genetic features of *Campylobacter*, there is a need for further research conducted over an extended period and which uses multiple molecular methods.

MAIN POINTS

- *Campylobacter* species were isolated from 4.3% of the fecal samples.
- The most common *Campylobacter* species isolated from fecal samples was *C. jejuni* (89.3%) followed by *C. coli* (9.6%).
- No statistically significant difference was found in the *Campylobacter* isolation rate according to gender and season ($p>0.05$).
- *Campylobacter* species were most commonly isolated from children aged 0-14 years ($p<0.01$).

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ETHICS

Ethics Committee Approval: This study was approved by the Ethical Committee of Trakya University Faculty of Medicine (approval number: TUTF-BAEK 2016/196).

Informed Consent: Informed consent is not necessary due to the retrospective nature of this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: C.E., N.Ş., K.T., M.C.U., Ş.B., Design: C.E., N.Ş., K.T., M.C.U., Ş.B., Supervision: C.E., N.Ş., K.T., Materials: C.E., N.Ş., Ş.B., Data Collection and/or Processing: C.E., N.Ş., M.C.U., Analysis and/or Interpretation: C.E., N.Ş., K.T., M.C.U., Literature Search: C.E., Ş.B., Writing: C.E., Critical Review: N.Ş., K.T.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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