



Investigation of Antimicrobial Susceptibilities and Resistance Genes of *Campylobacter* Isolates from Patients in Edirne, Turkey

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Abstract

Background: We aimed to determine the susceptibility of *Campylobacter* isolates obtained from patients to various antimicrobial agents and to investigate some related antimicrobial resistance genes.

Methods: Fifty-six *Campylobacter* isolates obtained from fecal specimens by conventional methods at the Trakya University Health Center for Medical Research and Practice, Department of Medical Microbiology in Edirne, Turkey, from 2017-2017 were included. Antimicrobial susceptibilities were investigated by the gradient strip test method, and species determination was made by multiplex polymerase chain reaction (mPCR). The presence of the *erm(B)* gene and *tet(O)* gene was investigated in all isolates by PCR. DNA sequence analysis was performed to detect the presence of mutations in the 23S rRNA positions 2074 and 2075 in five isolates, including two erythromycin resistant isolates. The *gyrA* gene mutation was investigated by the mismatch amplification mutation assay (MAMA)-PCR.

Results: In 54 *C. jejuni* isolates, resistance to erythromycin was 3.7%; to tetracycline, 59.3%; and to ciprofloxacin, 74.1%. Phenotypically, the *tet(O)* gene was detected in 33 tetracycline-resistant isolates, but no *erm(B)* gene was found in any of the *Campylobacter* isolates. As a result of the DNA sequencing, it was found no mutations in the 23S rRNA gene at the 2074 and 2075 positions. The *gyrA* mutation was observed in all 41 ciprofloxacin resistant *Campylobacter* isolates.

Conclusion: Among the antimicrobial agents tested, ciprofloxacin had the highest resistance rate, and erythromycin had the lowest. Antimicrobial resistance in *Campylobacter* increased significantly compared with previously studies in our region as well as in the entire world. Monitoring the resistance to antimicrobial agents used to treat *Campylobacter* infections is important in determining empiric antimicrobial treatment.

Keywords: *Campylobacter*; Antimicrobial resistance; Resistance genes

Introduction

Campylobacter species are small (0.2–0.8 X 0.5–5 µm), mostly spirally curved and motile Gram-negative bacteria that commonly exist in the intestinal

tracts of domestic and wild animals (1, 2). *Campylobacter* is among the main causes of gastroenteritis all over the world. Although many *Campylobacter* species are pathogenic for humans, *C. jejuni* and *C.*



coli are responsible for the majority of infections (3). *Campylobacter* enteritis is usually self-limited and does not require antimicrobial therapy. However, antimicrobial drugs can be used in severe and prolonged cases of enteritis, bacteremia, or other extraintestinal infections. Macrolides, fluoroquinolones, tetracycline, doxycycline, aminoglycosides, and some β -lactams are among the drugs used in the treatment. Currently, significant increases are being observed in resistance to the antimicrobial drugs used in treatment (4, 5).

High-level tetracycline resistance in *Campylobacter* species is usually associated with the *tet(O)* gene carried by plasmids. Tet(O) protein, encoded by the *tet(O)* gene, is among the ribosomal protection proteins (RPPs), and it causes the release of tetracycline from its binding site on the ribosome (6, 7). Fluoroquinolone resistance in *Campylobacter* species is mainly due to mutations in the *gyrA* gene, which encodes the GyrA subunit of DNA gyrase. The Thr86Ile point mutation in the *gyrA* gene confers high-level resistance to quinolones. It has been reported that a Thr86Ala mutation in the *gyrA* gene in *C. jejuni* causes high-level nalidixic acid and low-level ciprofloxacin resistance (2). In *Campylobacter*, mutations at positions 2074 and 2075 of 23S rRNA cause resistance by reducing the binding of macrolide antibiotics to 23S rRNA (8). The horizontally transferrable *erm(B)* gene encoding rRNA methylase is also associated with high-level resistance to macrolides (9, 10). Because chromosomal mutations are responsible for most resistance to macrolides and fluoroquinolones in *Campylobacter*, DNA sequencing of these target genes is used to detect mutations. To detect these gene mutations, polymerase chain reaction (PCR)-based techniques, including “mismatch amplification mutation assay” (MAMA-PCR), have been developed (11).

The aim of this study was to determine the susceptibility of *Campylobacter* isolates obtained from patients to several antimicrobial agents used in the treatment of *Campylobacter* infections and to investigate the presence of related resistance genes and mutations.

Materials and Methods

Ethics statement

This study was approved by the Ethical Committee of Trakya University School of Medicine (TUTF-BAEK 2019/288).

Bacterial isolates

Campylobacter isolates obtained from the fecal samples of 56 patients at the Trakya University Health Center for Medical Research and Practice, Department of Medical Microbiology in Edirne, Turkey, between January 2017 and June 2017 were included in this study. In case of growth in more than one sample of a patient, the first isolate was included in the 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 h. Gram staining and catalase and oxidase tests were performed on suspected colonies of *Campylobacter*. The isolates identified as *Campylobacter* were stored at –80 °C in Mueller–Hinton broth containing 20% glycerol.

Antimicrobial susceptibility testing

Campylobacter isolates were subcultured on *Campylobacter* agar with 5% sheep blood (Liofilchem, Italy) and incubated for 48 h at 42 °C in a microaerophilic atmosphere. The suspension for each bacterial isolate at 0.5 McFarland turbidity was inoculated onto Mueller–Hinton agar with 5% sheep blood. Erythromycin (0.016–256 mg/L), tetracycline (0.016–256 mg/L), and ciprofloxacin (0.002–32 mg/L) strips (Liofilchem, Italy) were placed on these agar media, and the plates were incubated at 42 °C in a microaerophilic atmosphere for 24 h (12). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guideline (13).

DNA extraction

DNA extraction was performed from *Campylobacter* isolates subcultured on *Campylobacter* agar with 5% sheep blood using a commercial kit (Invi-

trogen PureLink Genomic DNA Mini Kit, ThermoFisher Scientific, USA) according to the manufacturer's instructions and stored at -20°C until used.

Species identification

Species identification of *Campylobacter* isolates was performed using the previously described primers for *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* and PCR conditions with minor modifications (14). *C. jejuni* NCTC 13367 and *C. coli* NCTC 11350 were used as the positive control and *E. coli* ACTC 25922 as the negative control in all PCR procedures. PCR products were subjected to electrophoresis on 1.5% agarose gel, and the bands were evaluated under ultraviolet light.

Investigation of antibiotic resistance mechanisms

The presence of *erm*(B) and *tet*(O) genes was investigated with previously described primers and PCR conditions with slight modifications (15, 16). The

MAMA-PCR method was used to detect the mutation [Thr-86 to Ile mutation (ACA-to-ATA in *C. jejuni* and ACT-to-ATT in *C. coli*)] in the *gyrA* gene associated with ciprofloxacin resistance (17, 18). The presence of mutations at positions 2074 and 2075 of 23S rRNA, which is associated with erythromycin resistance, was investigated in two phenotypically erythromycin resistant isolates and three susceptible isolates (two *C. jejuni* isolates and one *C. coli* isolate). The 23S rRNA gene was amplified by PCR using primers defined earlier (19) (Table 1). The amplified PCR products were purified with the Invitrogen PureLink Quick PCR Purification Kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions for the sequencing of the 23S rRNA gene. DNA sequencing was performed (Medsantek, Istanbul) on the ABI 3730XL Sanger sequencing instrument (Applied Biosystems, USA) using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA). Chromatogram files were analyzed with ProSeq v2 and BioEdit programs.

Table 1: Primers used for detection of antibiotic resistance genes

Target gene	Primer name	Oligonucleotide sequence (5' - 3')	Amplicon size (bp)	Reference
<i>ermB</i>	<i>ermB</i> -F	GAAGGAGTGAT-	760	15
	<i>ermB</i> -R	TACATGAACAA		
<i>tet</i> (O)	DMT-1	TCATAGAATTATTTCCCTCCCGT	559	16
	DMT2	GGCGTTTGTGTTATGTGCG		
<i>gyrA</i>	CampyMAMAg _{yr} -A1	ATGGACAACCCGACAGAAGC	265	17
	CampyMAMAg _{yr} -A5	TTTTTAGCAAAGATTCTGAT		
	GZ _{gyrA} C _{coli} 3F	CAAAGCATCATAAACTGCAA	192	18
	CampyMAMAg _{yr} -A8	TATGAGCGTTATTATCGGTC	697	19
23S rRNA	23SRNA-F	TAAGGCATCGTAAACAGCCA	697	19
	23SRNA-R	TTAGCTAATGTTGCCCG-TACCG AGCCAACCTTTGTAA-GCCTCCG		

Results

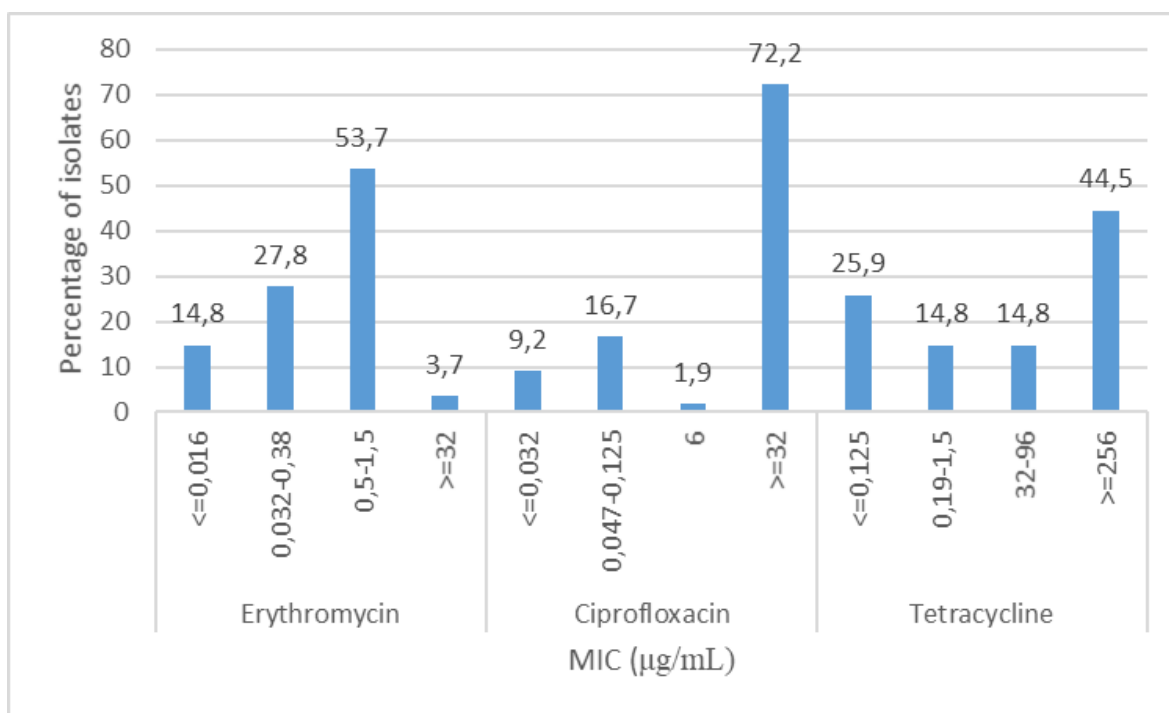
As a result of multiplex PCR using genus and species-specific primers, 54 (96.4%) isolates were identified as *C. jejuni* and two (3.6%) isolates as *C.*

coli. The resistance rates of 54 *C. jejuni* isolates to tetracycline, erythromycin, and ciprofloxacin were determined as 59.3%, 3.7%, and 74.1%, respectively (Table 2). Distribution of MIC values of *C. jejuni* isolates is shown in Fig. 1.

Table 2: Antimicrobial susceptibility test interpretation criteria and resistance percentages of *Campylobacter jejuni* isolates

Antimicrobial agent	MIC ($\mu\text{g}/\text{mL}$) interpretation criteria			Number of <i>C. jejuni</i> isolates		
	S	I	R	S n (%)	I n (%)	R n (%)
Erythromycin	≤ 8	16	≥ 32	52 (96.3)	-	2 (3.7)
Ciprofloxacin	≤ 1	2	≥ 4	14 (25.9)	-	40 (74.1)
Tetracycline	≤ 4	8	≥ 16	22 (40.7)	-	32 (59.3)

MIC: Minimum inhibitory concentration, S: Susceptible, I: Intermediate, R: Resistant

**Fig. 1:** Distribution of minimum inhibitory concentration (MIC) values of *Campylobacter jejuni* isolates

Twelve (22.2%) *C. jejuni* isolates were sensitive to all three tested antimicrobials. No isolates resistant to all three antimicrobials studied were found. The resistance distributions of *C. jejuni* isolates to the tested antimicrobials are shown in Fig. 2. While resistance to tetracycline and ciprofloxacin was observed in one of the *C. coli* isolates, the other isolate was found to be sensitive to all three antimicrobials. The *tet(O)* gene was detected in all 33 isolates phenotypically resistant to tetracycline, and

the *erm(B)* gene was not found in any of the *Campylobacter* isolates. As a result of the sequence analysis of the 23S rRNA gene region, it was determined that the adenine nucleotide was located at positions 2074 and 2075 and there was no mutation. The 23S rRNA gene sequence of the *C. jejuni* isolate was registered in the DNA Data Bank of Japan (Accession no. LC680886).

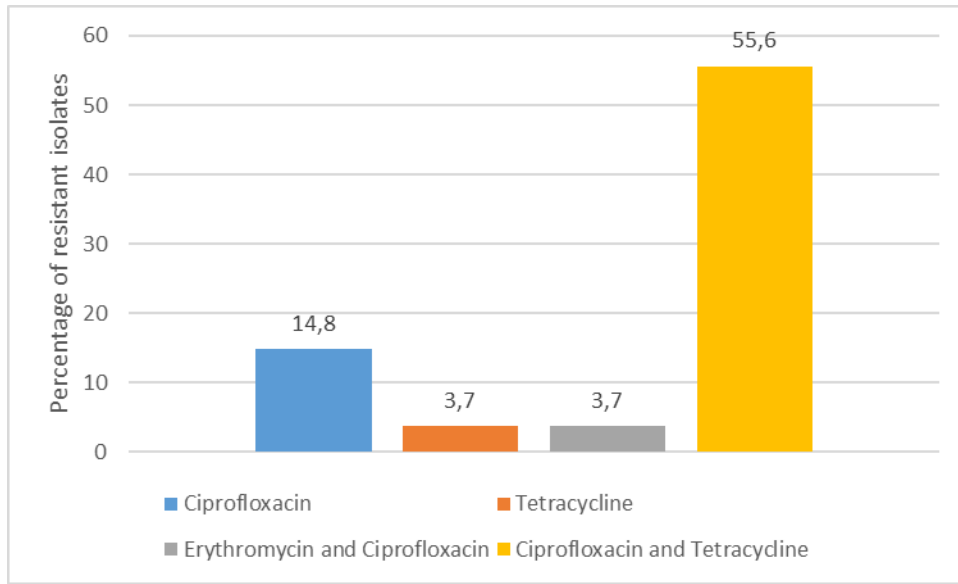


Fig. 2: Distribution of resistance to antimicrobial agents in *Campylobacter jejuni* isolates

While the *gyrA* mutation was observed in all 41 *Campylobacter* isolates that were phenotypically resistant to ciprofloxacin, no mutation was found in any of the susceptible isolates by the MAMA-PCR.

Representative gel of antimicrobial resistance genes investigated from *Campylobacter* isolates is shown Fig. 3.

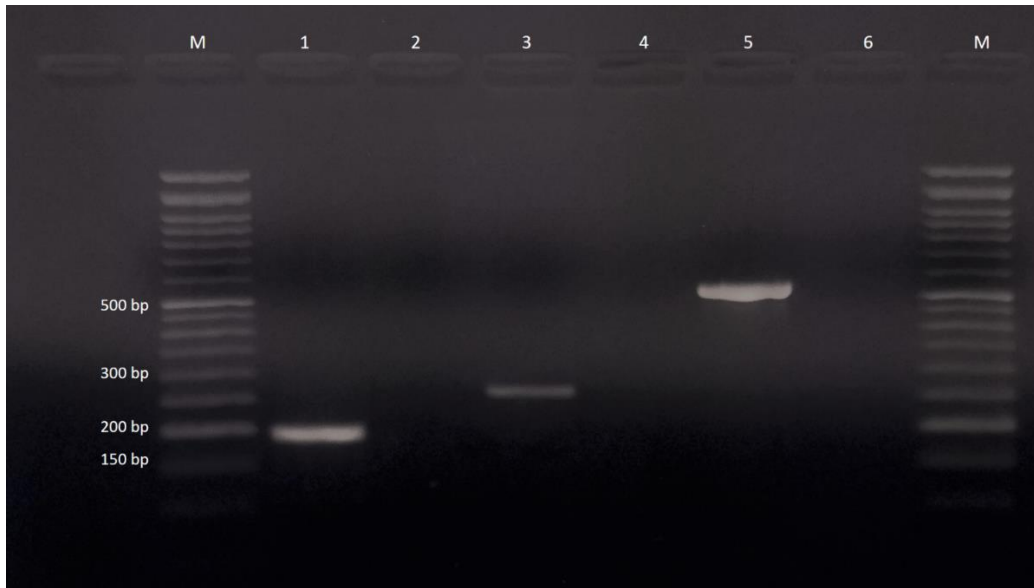


Fig. 3: Representative gel of antimicrobial resistance genes investigated from *Campylobacter jejuni/ coli* isolates. M; DNA Marker (100bp Opti-DNA Marker, Cat. No. G016, Applied Biological Materials, BC, Canada), lane 1-2; MAMA-PCR products of the *gyrA* gene of ciprofloxacin resistant and susceptible *Campylobacter coli* isolates, respectively, lane 3-4; MAMA-PCR products of the *gyrA* gene of ciprofloxacin resistant and susceptible *Campylobacter jejuni* isolates, respectively, lane 5-6; PCR products of the *tet(O)* gene of tetracycline resistant and susceptible *Campylobacter jejuni* isolates, respectively

Discussion

Antimicrobial resistance is an important public health problem in developed and developing countries. There is also a significant increase in resistance to antimicrobial drugs used in the treatment of *Campylobacter* infections, and this may limit the antimicrobial agent options used in the treatment. The widespread use of antibiotics in livestock is facilitating the spread of resistant strains of *Campylobacter*. In addition, the inability to eliminate adequately these antibiotic-resistant bacteria during wastewater treatment, the inappropriate discharge of human and animal wastes into water resources, and the improper preparation of food of animal origins are among the problems that contribute to the spread of resistance in *Campylobacter* (5,20,21).

C. jejuni is responsible for the majority (85-95%) of human *Campylobacter* infections, followed by *C. coli* (5-10%) (22,23). In our study, the most common *Campylobacter* species isolated from fecal samples was *C. jejuni* (96.4%), and *C. coli* was the second most common (3.6%) species. Our results are consistent with those of other studies conducted in Turkey and elsewhere (24-26).

Fluoroquinolones are broad-spectrum antimicrobial agents widely used in the treatment of many infections. They target two bacterial enzymes, DNA gyrase and DNA topoisomerase IV for an antimicrobial effect (27,28). Resistance to fluoroquinolones in *Campylobacter* tends to increase both in clinical isolates and in livestock. The major resistance mechanism to the fluoroquinolones is modification of the quinolone resistance-determining region (QRDR) of the corresponding topoisomerase. Numerous GyrA modifications associated with fluoroquinolone resistance have been described in *Campylobacter* species. The most frequently observed mutation in quinolone-resistant *Campylobacter* is the C257T change in the *gyrA* gene, and this mutation causes amino acid changes with Thr86Ile (2,27).

In a study, ciprofloxacin resistance was found in 55.8% of 199 *C. jejuni* isolates obtained from patients (29). In another study in which ciprofloxacin resistance was found in 96.8% of human isolates, the Thr86Ile mutation in the *gyrA* gene was detected in all resistant isolates, and no mutation was found in susceptible strains (30). In some studies performed in Turkey, ciprofloxacin resistance was found to be 73.9% and 61.9% (24,31). In a study from Turkey in which *C. jejuni* isolates obtained from patients with acute gastroenteritis were included, 74.3% ciprofloxacin resistance was detected, and a mutation causing a Thr-86-Ile change in the *gyrA* gene was detected in all these resistant isolates (32). In the present study, ciprofloxacin resistance was found to be 74.1% in *C. jejuni* isolates, consistent with other studies, and mutation in the *gyrA* gene was found in all resistant isolates.

Macrolides are among the drugs commonly used in the treatment of *Campylobacter* infections. Macrolides inhibit protein synthesis by binding to the 50S subunit of the ribosome (2). Although macrolide resistance is lower than fluoroquinolone resistance in *Campylobacter*, an increase in resistance is observed in certain regions (4). Mutations at positions 2074 and 2075 of 23S rRNA in *Campylobacter* block the binding of macrolides to 23S rRNA. The A2075G mutation is the most common among the mutations identified (8). In addition, the rRNA methylase gene *erm(B)* has been reported to be associated with macrolide resistance in *Campylobacter* (9). In a study by Zhou et al., which included *C. jejuni* strains isolated from stool samples taken from patients with diarrhea between 1994 and 2010, a significant increase was found in resistance to ciprofloxacin, nalidixic acid, doxycycline, tetracycline, florfenicol, and chloramphenicol. Resistance to erythromycin and gentamicin was determined to be relatively low, and no significant change was found in the resistance rates in the specified period (33). In some studies, erythromycin resistance in *Campylobacter* was found to be 2%, 0.7% and 6.3% (33-35). In a study performed in our country in 2019, resistance to erythromycin was found in 4.8% of *Campylobacter* iso-

lates (24). In the current study, the rate of erythromycin resistance was determined to be 3.6% in *Campylobacter* isolates. The *erm(B)* gene was not found in both isolates resistant to erythromycin, and no mutations were detected at positions 2074 and 2075 of 23S rRNA gene in these resistant isolates. These findings suggest that other mechanisms lead to erythromycin resistance in these isolates.

Tetracyclines are broad-spectrum antimicrobials widely used in human and veterinary medicine. They inhibit protein synthesis by binding to the 30S subunit of the ribosomes of susceptible microorganisms. Acquisition of the *tet(O)* gene encoding the ribosomal protection protein Tet(O) in *Campylobacter* is one of the important mechanisms conferring tetracycline resistance (8,36). In some studies including *Campylobacter* isolates obtained from patients, tetracycline resistance was found to be 74.6%, 25%, and 64%, and in these studies, the *tet(O)* gene was detected in all tetracycline-resistant isolates (30, 32, 37). In our study, 58.9% of the *Campylobacter* isolates were found to be resistant to tetracycline, and the *tet(O)* gene was found in all 33 resistant isolates. The *tet(O)* gene was not detected in any of the susceptible isolates. Among the antimicrobials we tested, the highest resistance was to ciprofloxacin and the lowest was to erythromycin. In a study performed in Edirne in 2005, 8% resistance was found in *C. jejuni* strains to erythromycin and ciprofloxacin, while no resistance was found to tetracycline (38). In another study performed in 2020, these rates were found to be 12.1%, 68.2%, and 40.9%, respectively (39). When the data of the present study are evaluated, there is a serious increase in antibiotic resistance in *C. jejuni* in our region, as in other parts of the world. The low number of *C. coli* isolates in our study seems a limiting factor for our interpretation of antibiotic resistance in this species. In our study, 58.9% of *Campylobacter* isolates were found resistant to two antibiotics. Multidrug resistance and high MIC values lead to a decrease in antimicrobial agent options in *Campylobacter* infections (33).

Conclusion

The monitoring of resistance to the antimicrobial drugs used in the treatment of *Campylobacter* is important in terms of choosing antibiotics for empirical treatment. In addition, determining the factors and mechanisms that cause antibiotic resistance will contribute to the planning of appropriate measures to prevent resistance. For this reason, it is thought that studies including *Campylobacter* isolates obtained from humans, animals, and foods and investigating many factors that may be effective in resistance will be useful.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

There are no conflicts of interest.

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