T.R.
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY

DETERMINATION OF *Campylobacter* ssp. ISOLATES FROM STOOL SAMPLES AND THEIR ANTIBIOTIC SUSCEPTIBILITY

Prepared by
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M.Sc. Thesis

March, 2023
KAYSERİ

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COMPLIANCE WITH SCIENTIFIC ETHICS

I hereby declare that all information in this study has been obtained in accordance with academic rules and ethical conduct. At the same time, I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Hussein Ali Kamil

This master's thesis write-up on the topic "**Determination of *Campylobacter* ssp. isolates from stool samples and their antibiotic susceptibility**" has been prepared in accordance with the Thesis Proposal and with the Guidelines for Writing Thesis of Erciyes University.

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيم

" ٱلَّذِينَ قَالَ لَهُمُ ٱلنَّاسُ إِنَّ ٱلنَّاسَ قَدْ جَمَعُواْ لَكُمْ فَٱخْشَوْهُمْ فَزَادَهُمْ إِيمَٰنًا وَقَالُواْ حَسْبُنَا ٱللَّهُ وَنِعْمَ ٱلْوَكِيلُ", صدق الله العظيم", ال عمران (173-147).

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Hussein Ali Kamil,

March 2023, **Kayseri**

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Erciyes University, Department of Biology
M.Sc. Thesis, March 2023
Supervisor: Prof. Dr. Abdurrahman AYVAZ
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ABSTRACT

This study was carried out to determine the prevalence of *Campylobacter* isolates, to reveal antibiotic resistance and to genetically detect quinolone resistance in patients who applied to Erciyes University hospitals with the complaint of diarrhea. Stool samples (150) from individuals with diarrhea who applied to different units at Erciyes University hospitals sent to the microbiology laboratory. Gram stain was used for the initial isolation of the stool samples, and selective medium was used for the final isolation. Species-level diagnosis of *Campylobacter* isolates grown in culture was determined by MALDI-TOF MS. *C. jejune* and *C. coli* strains were determined at the rates of 82.4% (42/51) and 17.6% (9/51), respectively. Antibiotic susceptibility tests (AST) of the isolates were assigned on Mueller Hinton Fastidious (MHF) agar according to the EUCAST recommendations by disc diffusion method. Respectively 94% (47/50), 58% (29/50) and 2% (1/50) resistance rates determined for ciprofloxacin, tetracycline, and erythromycin. *C. jejuni* strains showed higher resistance to all three antibiotics more than *C. coli.* To identify mutations in isolates resistant to ciprofloxacin, PCR products of the *gryA* gene were obtained by mismatch amplification mutation test (MAMA PCR) using a specific primer and sequenced. Thr-86 to-I mutation, which is a marker of quinolone resistance, was observed in all 7 isolates sequenced. Again, in the current study, while 7 silent mutations were identified, 2 novel mutations were detected. *Campylobacter* spp. was isolated at a rate of 35.33% (53/150) from the stool samples examined, and most of the positive isolates (64.15%, 34/25) were obtained from 0-10 age group, especially male (75.5%, 40/53) individuals.

Key words: *Campylobacter* spp., Quinolone resistance, MALDI-TOF MS, AST, MAMA PCR

DIŞKI ÖRNEKLERİNDEN *CAMPYLOBACTER* SPP. İZOLATLARININ ELDE EDİLMESİ VE BU İZOLATLARIN ANTİBİYOTİK DUYARLILIKLARININ BELİRLENMESİ

Hussein Ali Kamil

Erciyes Üniversitesi, Biyoloji Bölümü
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ÖZET

Bu çalışma Erciyes Üniversitesi hastanelerine ishal şikâyeti ile başvuran hastalarda *Campylobacter* izolatlarının prevalansını belirlemek, antibiyotik direncini ortaya çıkarmak ve kinolon direncini genetik olarak saptamak amacıyla yapılmıştır. Erciyes Üniversitesi hastanelerine ishal şikâyeti ile başvuran bireylerden alınan dışkı örnekleri (150) mikrobiyoloji laboratuvarına gönderildi. Gaita örneklerinin ilk izolasyonunda gram boyama, son izolasyonunda ise selektif besiyeri kullanıldı. Kültürde üreyen *Campylobacter* izolatlarının tür düzeyinde teşhisi MALDI-TOF MS ile yapıldı, izolatların sırasıyla %82,4 (42/51) ve %17,6 (9/51) oranlarında C. *jejuni* ve *C. coli* oldukları belirlendi. İzolatların antibiyotik duyarlılık testleri (AST) Mueller Hinton Fastidious (MHF) agarda EUCAST tavsiyelerine göre disk difüzyon yöntemi ile yapıldı. Siprofloksasin, tetrasiklin ve eritromisin için sırasıyla %94 (47/50), %58 (29/50) ve %2 (1/50) direnç oranları belirlendi. *C. jejuni* suşlarının her üç antibiyotiğe karşı *C. coli*'den daha dirençli olduğu görüldü. Siprofloksasine dirençli izolatlardaki mutasyonları belirlemek için *gryA* geninin PCR ürünleri, spesifik bir primer kullanılarak mismatch amplifikasyon mutasyon testi (MAMA PCR) ile elde edildi ve dizi analizi yapıldı. Dizilenen 7 izolatın tamamında kinolon direncinin bir belirteci olan Thr-86 to-I mutasyonu gözlendi. Yine bu çalışmada 7 sessiz mutasyon 2 tane de novel mutasyon saptandı. incelenen dışkı örneklerinden %35,33 (53/150) oranında *Campylobacter* spp. izole edilmiş olup, pozitif izolatların çoğu (%64,15, 34/25) 0-10 yaş grubundan, özellikle erkeklerden elde edilmiştir (%75,5, 40/ 53).

Anahter kelimeler: *Campylobacter* spp., kinolan direnci, MALDI-TOF MS, ADT, MAMA PCR

CONTENTS

DETERMINATION OF *CAMPYLOBACTER* SSP. ISOLATES FROM STOOL SAMPLES AND THEIR ANTIBIOTIC SUSCEPTIBILITY

LIST OF ABBREVIATIONS

CFU	colony forming unit
CDC	Centers for Disease Control and Prevention
QRDR	Quinolone resistance-determining regions
EFSA	The European Food Safety Authority
WHO	The World Health Organization
WGS	Whole genome sequence
MAMA	Mismatch amplification mutation
PCR	Polymerase chain reaction
AST	Antibiotic Sensitivity Test
MIC	Minimal Inhibitory Concentration
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CI	Ciprofloxacin
E	Erythromycin
TE	Tetracycline
CPC	Capsular polysaccharide
LOS	Lipoligosaccharide
IFAT	Immunofluorescence
CsPA	Cold-shock protein

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CHAPTER 1

INTRODUCTION

Campylobacter is a gram-negative rod that moves and has a corkscrew shape with 0.5 to 5 μm in length and 0.2 to 0.9 μm in breadth; it is one of the most common types of bacteria that cause diarrheal diseases worldwide. There are more than 30 species of *Campylobacter* known in the world today, and the species that causes the most of the gastroenteritis in humans is *Campylobacter jejuni*, and *Campylobacter coli* is a less common cause of this disease [1, 2]. Despite the fact that the most widespread species, *C. jejuni*, is known to cause symptoms including abdominal pain, fever, and diarrhea, and it has also been linked to reactive arthritis in Miller-Fisher patients. In addition, this bacterium causes neurological diseases such as Guillain-Barre syndrome [3, 4]. Symptoms can be caused by infection at concentrations as low as 800 CFU/ml. This virulence emphasizes the importance of sanitation for people, who come into contact with infection reservoirs like poultry, polluted water, and raw dairy. Around 1.3 million cases of *Campylobacter* infection are reported annually in the United States alone, according to the Centers for Disease Control. In the US, this has an annual economic cost of between $1.3 and $6.8 billion [2]. The consumption of uncooked milk, uncooked chicken, and unclean water has all been connected to *Campylobacter* species-related illnesses. Patients frequently suffer from a self-limited, 5- to 7-day diarrhea. The elderly and patients with compromised immune systems are those most at risk for morbidity, mortality, and chronic disease. Even though there are treatment and eradication techniques for animal reservoirs, there has been a significant rise in recent cases in both developed and developing nations around the world [2]. The Centers for Disease Control and Prevention (CDC) reported a 14% increase in *C. jejuni* infections in the United States in March 2013. In Europe, 1% of the population is affected by Campylobacteriosis each year. Among foodborne diseases, *Campylobacter* isolation

affected by this type of infection. It has been determined that 90% of the cases in developed countries occur during the summer months and infections are mostly caused by poorly cooked meat sold in open facilities. The disease most commonly affects young children under the age of four and young people between the ages of 15 and 44 [5].

The increasing global prevalence of the disease demonstrates the capacity of *Campylobacter* to survive in a variety of conditions. Bacteria are transmitted to humans through contaminated food and water, as well as contact with animals and other humans. It is getting harder to control *Campylobacter* because of the increase in people's travel opportunities around the world and the transportation of animals to the continents. Health centers around the world are working hard to educate people on signs, symptoms, and places where contact is likely. In this regard, information is provided on issues such as hand hygiene, keeping raw meat separate from other foods while cooking, cooking the meals thoroughly, avoiding raw milk products and untreated water [6].

Campylobacter infection often presents as enteritis with profuse diarrhea and patients suffer from a prodromal symptom period of 1-3 days. The prodromal phase is characterized by high fever, abdominal hardening, disorientation and body aches, and this stage causes the disease to progress more severely. Although symptoms normally appear 24 to 72 hours after consuming the bacteria, they may last longer in people infected with smaller amounts of the microbe. The acute diarrhea phase of *Campylobacter* enteritis can last an average of 7 days, while the illness usually lasts 24 to 48 hours. It is very rare for the feeling of stomach pain to persist for several days to weeks after the diarrhea subsides [7]. While patients may continue to excrete organisms in their stool for several weeks after clinical improvement, antibiotic therapy reduces the chances of prolonged elimination [8].

Abdominal pain and several episodes of diarrhea are common in the acute phase of the disease, with more than ten stools per day. Blood and mucus are common in the stool, caused by bacteria irritating the intestinal epithelium. As inflammatory lesions and mucosal damage develop eventually, appendicitis-like abdominal pains are often

observed [9]. Although the pathophysiology of the disease is unknown, the pVir plasmid is thought to be associated with more invasive diseases and a higher risk of bloody diarrhea [10]. Today, all over the world, it is seen that pathogenic bacteria have developed resistance to antibiotics, and this situation causes increasing problems, especially in the hospital environment, making the treatment more complex.

Diagnosis of *Campylobacter* species and determination of antibiotic resistance are critical for the treatment of infected individuals. Distinguishing between the two most common human species, *Campylobacter coli* and *Campylobacter jejuni*, is critical for epidemiological monitoring, and the detection of resistance genes carried by the same species is becoming increasingly important for treatment processes. Gastroenteritis caused by *Campylobacter* bacteria is a self-limiting disease that often does not require antibiotic therapy. However, antibiotic therapy is used in cases of fever, bloody diarrhea, long-term symptoms, and immunocompromised individuals [11]. While Macrolides and fluoroquinolones are widely used for treatment, antibiotics such as tetracycline, doxycycline and chloramphenicol are also used to treat infections [12]. The rates of resistance to antimicrobials used in treatment have increased significantly and this resistance is an increasingly important public health problem. Continuous use of antibiotics mostly in animal agriculture increases the development of resistance and creates significant concerns worldwide. In addition, inadequate wastewater treatment, contamination of water resources with human and animal waste, and improper preparation of animal-derived food are other important factors contributing to the increase in antibiotic resistance in *Campylobacter* species [13]. Therefore, the use of antibiotics should be used more carefully and in accordance with sensitivity tests, otherwise irregular and unconscious use of antibiotics will worsen the situation. It would be wiser to utilize antimicrobial susceptibility testing, manage treatment well, and monitor epidemiological data [12]. Because of all these concerns, rapid characterization of bacterial pathogen variations is critical for monitoring disease progression and developing epidemic research and prevention techniques [14].

Recently, matrix-assisted laser desorption ionization (MALDI) and time-of-flight (TOF) MS (MALDI-TOF MS) has been used as an alternate approach for the fast identification and categorization of *Campylobacter spp.* [15, 16]. MALDI-TOF MS, like other DNA detection technologies rely on DNA fingerprints, employs unique protein or lipid

patterns from the entire bacterial cell. MALDI-TOF MS data are repeatable and may be utilized to differentiate bacteria at the genus and species levels [17].

As in all other bacteria, it is important to investigate the resistance genes and determine the mutations that occur in *Campylobacter* species. Fluoroquinolones affect DNA gyrase, a type II topoisomerase that catalyzes the negative supercoiling of relaxed or positively supercoiled double-strand, covalently closed circular DNA, in *Campylobacter* spp, *E. coli*, and other gram-negative bacteria [18]. Fluoroquinolone resistance in gram-negative bacteria is caused by mutations in the *gyrA* gene that change the amino acid sequence close to the protein's putative active site [19]. The main site for mutations causing quinolone resistance in *E. coli* is the quinolone resistance determining region (QRDR) of the *gyrA* gene, which includes codons 67 to 106, close to the Tyr-122 catalytic site of DNA gyrase [20]. The *gyrA* gene of *C. jejuni* has a similar QRDR, but it appears that the transient, covalent DNA-protein bridge that forms during the DNA strand passage process of DNA top isomerization occurs at the gyrATyr-125 residue as the catalytic site [19]. By changing the amino acid sequence close to the putative active region of the GyrA protein, mutations in the *gyrA* gene of gram-negative bacteria result in resistance to fluoroquinolones [19]. The main site for mutations causing quinolone resistance in *E. coli* is the quinolone resistance determining region (QRDR) of the *gyrA* gene, which includes codons 67 to 106, close to the Tyr-122 catalytic site of DNA gyrase [20]. The *C. jejuni gyrA* gene has a comparable QRDR, but it appears that the gyrA Tyr-125 residue is the catalytic location for the transitory, covalent DNA-protein bridge that forms during the DNA strand passing process of DNA topoisomerization [19].

In this study, stool samples were taken from patients who applied to different units of Erciyes University hospitals to characterize *Campylobacter* isolates in people living in the Kayseri region, and after *Campylobacter* isolates were obtained by conventional methods, they were diagnosed at the species level by MALDI_TOF MS method. Antibiotic susceptibility test was applied to the isolates and the susceptibility of each was determined and the presence of resistance genes was screened by PCR method using the relevant primers. In addition, sequence analysis was performed to determine whether there was a mutation in the *gyrase A* gene.

CHAPTER 2

LITERATURE REVIEW

2.1. Classification of the genus *Campylobacter*

Campylobacter is found mostly in the gastrointestinal tracts of vertebrates (e.g., birds, cattle, swine, sheep, and dogs), although it may also be detected in mussels these organisms. The majority is usually seen in bird species due to their relatively high body temperatures. There are other non-zoonotic *Campylobacter* species prevalent in the human oral microbial population (*C. concisus, C. showae, C. gracilis, C. ureolyticus, C. curvus, and C. rectus*) that can cause periodontitis [21, 22, 23 and 24]. Human gastroenteritis is mostly caused by *C. jejuni*, with minor contributions from *C. coli, C. fetus, C. lari, and C. upsaliensis* [25].

2.2. Epidemiology

In 2018, approximately 67,000 *Campylobacter* enteritis were detected in Germany, resulting in 80-90 infections per 100 000 people [26]. Infection is seasonal with a spike throughout the summer.

The European Food Safety Agency (EFSA) reported an incidence of around 64 cases per 100 000 populations in Europe for the same year [27]. Furthermore, the European Centre for Disease Prevention and Control reports the incidence as 246,000 cases in Europe in 2018, of whom approximately 21,000 were hospitalized, and 60 recorded fatalities (0.03%) [28]. In the United States, around 20 cases per 100 000 people are recorded each year [28]. The World Health Organization (WHO) predicts 44 to 93 incidences per 10,000 people worldwide [29]. However, it should be noted that in low- and middle-income countries, the cases are much higher than stated due to the lack of definitive diagnostic methods and data collection difficulties. Such statistics are rarely

collected in countries such as Afghanistan, Central Africa, Haiti, Nepal, Bangladesh, and Egypt. Therefore, the prevalence of this pathogen is much higher worldwide and ranks second after infant losses due to malnutrition [30]. Child mortality from diarrhea is over 25% in African and Southeast Asian countries and the most common cause of diarrhea in children with reported cases is *Campylobacter* species [31].

2.3. Transmission

Campylobacter is found in a variety of sources including meats, contaminated waters, poultry animals and other animals. Bacteria can survive in a variety of agricultural environments, including air, dust, soil, and even abiotic surfaces. Organic fertilizer transmission is considered [32].

Campylobacteriosis is a highly contagious zoonotic disease, and the natural reservoirs of the disease agent are *C. coli* and *C. jejuni* living in the gastrointestinal tract of birds and other animals [33]. Newborn chicks are mostly *Campylobacter* free until 7 days after hatching [34], so the cause of the infection is not clearly understood and is thought to be spread perhaps by infected flies [35]. Tools, boots, birds, and rodents are thought other potential vectors [36]. However, *C. jejuni* isolated from wild birds is typically different from human *campylobacteriosis* and *C. jejuni* isolated from food and is therefore of much less public health importance [37]. Due to insufficient cooking of poultry meat and cross-contamination (through knives, plates, etc.) especially in private homes during meal preparation, the disease agent can easily spread. The peak of infection cases in the summer and early autumn seasons when barbecue gatherings are common in Western countries explains this situation [38]. *C. jejuni* commonly causes *campylobacteriosis* in humans because it has a low minimum infection dose (MID) value [39]. To the best of our knowledge, no new experimental-based data for this MID, as well as MID for other *Campylobacter* species, are available in recent literature.

2.4. Symptoms

As it is known, *C. jejuni* (75%) and *C. coli* (10%) are the species that cause campylobacteriosis at the highest rate among *Campylobacter* species, and the rate of these two species creating campylobacteriosis together is 14%. Other species, such as *C. lari*, *C. upsaliensis*, and *C. fetus*, have a symptom incidence rate of only 1% [39].

Campylobacter spp. cause infection after an incubation period of 2-5 days and symptoms begin with a prodromal stage characterized by fever, headache, and muscle discomfort. This is followed by acute enterocolitis with watery and sometimes bloody diarrhea, causing cramp-like stomach pains [40]. In most cases, the condition resolves spontaneously within 5-7 days without any problems. However, persistent and severe cases are more likely to occur in children and the elderly over 65 years of age. Sepsis has been observed in immunocompromised individuals, such as HIV-positive patients, and the risks are reduced when active antiretroviral drugs are used [41].

2.5. Colonization of hosts

The colonization of the bacterium has not been fully clarified [42]. Strain-dependent pathogenicity and specific colonization ability were reported by some other researchers [43, 44]. *C. jejuni* is a bacterium that contains a capsule and there are glycans in this layer, especially the polysaccharides in this layer are very important in the host-bacterial interaction and ensure the emergence of virulence and antigenicity. These bacteria form a capsular polysaccharide (CPS) within the capsule structure, these CPSs exhibit various structures and expressions due to the phase change in bacteria [43].

Campylobacter flagella are very effective in colonization and pathogenicity because these structures facilitate the passage of the viscous mucin layer of the host's gut [45]. Wassenaar et al. demonstrated the importance of flagella in chicken colonization using *flaA* and *flaB* mutants of the bacterium [46]. Type VI secretion system T6SS was described for some *Campylobacter* strains, and this also contribute to the colonization of the bacteria. *C. jejuni* T6SS is required for adherence to and invasion into T84 human colonic epithelial and murine RAW264.7 macrophages and is also required for persistent colonization in IL- 10-deficient mice [47].

2.6. Genome and phase variation

The genome of C. jejuni NCTC strain 11168 was published in 2000 and many genes' functions are now known [48]. Whole Genome Sequencing (WGS) of various pathogenic and pathogenic mutants is used to identify virulence gene clusters responsible for pathogenicity and develop drugs against Campylobacter [49]. The reversible exchange of phenotypes caused by random errors during DNA replication is

known as phase variation, which allows the bacteria to form a variety of phenotypes and subpopulations, allowing them to adapt to and survive in a variety of environments [50]. Many of these phase-variable loci are involved in cell surface modification, such as capsular polysaccharides (CPS) or lipooligosaccharides (LOS) and transferees for LOS sialylation and CPS biosynthesis are highly phase-variable. The flagellum and its associated motility exhibit high phase variability, allowing the bacterium to avoid detection by the host's immune system or bacteriophages [51].

2.7. Diagnosis of Campylobacter infection

New molecular methods, phenotypic isolation, determination of the causative agent, biochemical characterization, PCR, RFLP, RAPD, multiplex PCR, and real-time PCR are used in the diagnosis of campylobacteriosis [52]. In addition to biochemical tests to confirm the genus and species of isolates, molecular methods such as MALDI-TOF MS have been used recently for diagnosis and typing [53].

2.7.1. Conventional methods

2.7.1.1. Direct demonstration

After moist preparation and staining, it is possible to identify the organism by observing the motility pattern and shape in fresh semen, vaginal mucosa and stool samples with dark field/phase contrast microscopy. Microscopic examination is done after a small amount of sample is mixed with physiological saline and dispersed, followed by staining with 1% carbol fuchsin [54]. It is also argued that direct microscopic examination of stools is more practical than culturing on a selective medium [55].

2.7.1.2. Culture and identification

Because *Campylobacter* is fastidious and microaerophilic, it requires selective media and optimum environmental conditions before plating on agar. Corry et al. (1995) outlined the isolation of *Campylobacter* from feces by plating on selective media or filtration on non-selective agar [56]. However, enrichment to improve the culture sensitivity of possibly environmentally stressed bacteria or low concentrations of bacteria in household and companion animal sperm or feces is preferable.

However, the enrichment method is preferred to improve culture sensitivity of environmentally stressed bacteria or low bacterial concentrations in household and companion animal sperm or feces [57].

Bolton water, Preston water, Park and Sanders water, CCDB, Campy-BAP, Balser Wang medium, modified charcoal cefoperazone deoxycholate agar (mCCDA), CAT medium, Karmali agar, Campycefex agar and some other media are preferred for *Campylobacter* separation and are used for *Campylobacter* separation in samples. It is recommended to make two or more media as well as several supplements to increase the likelihood of detection [58].

2.7.2. Species differentiation of *Campylobacter*

Campylobacter jejuni and *Campylobacter coli* are phylogenetically and genetically linked, making differentiation challenging. When growing at 42°C, the odds of finding *C. jejuni* and *C. coli* are greater than with other *Campylobacters* [59]. The immunofluorescence antibody test (IFAT) can be used to identify *C. fetus* directly from materials or validate a strain's identity after separation, but cannot distinguish between distinct types [60].

Since *Campylobacter jejuni* and *Campylobacter coli* are closely related phylogenetically and genetically, they are difficult to distinguish. At a growth temperature of 42°C, it is more likely to acquire *C. jejuni* and *C. coli* than other *Campylobacters* [Morris et al., 1985]. Immunofluorescent antibody testing (IFAT) is suitable for directly identifying *C. fetus* from materials but cannot differentiate between different types [60].

2.7.2.1. MALDI-TOF MS analysis

Since it does not require culture and DNA extraction for the Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS) method, it gives results in a very short time and accurately [61]. Instead, it can be used to directly identify microorganisms from positive blood culture broths with good diagnostic precision [90]. MALDITOF MS employs the unique protein or lipid patterns from the intact bacterial cell. Data generated by MALDI-TOF MS are reproducible and can be used to

discriminate bacteria both at the genus and species levels [62]. The VITEK® MS (bioM'erieux, Inc., Durham, NC) and the Biotyper are the two main, publicly accessible MALDI-TOF mass spectrometers that are extensively used in the detection of bacteria of public health significance (Bruker Daltonics, Inc., Billerica, MA). These devices provide distinct datasets for the detection of microorganisms retrieved from human specimens, such as Gram-negative and -positive bacteria, anaerobic bacteria, and yeast.

2.8. Antimicrobial therapy

Macrolides (azithromycin and erythromycin), fluoroquinolones (ciprofloxacin) and tetracycline are the most commonly used and recommended antibiotics for treatment [63].

2.8.1. Ciprofloxacin

Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic and has been approved for the treatment of large numbers of infections. It is an appropriate treatment option, especially in patients with mixed infections or prone to gram-negative infections [64]. Members of the interprofessional team must review its indications, coverage, contraindications, and adverse event profile to manage patients' infectious diseases optimally. Lawrence patented it in 1983 and the US Food and Drug Administration approved it in 1987 [65]. It has been suggested for the treatment of Campylobacteriosis as well as for many other bacterial diseases [66], by inhibiting bacterial DNA topoisomerase and DNA gyrase, it prevents DNA replication [67].

2.8.2. Erythromycin

A macrolide antibiotic called erythromycin was first discovered in 1952. It has a sign for a non-infectious pathology and is helpful for treating a number of infections. Neutral conjunctivitis is a traditional treatment for neonatal infections, *chlamydia*, and various respiratory infections, such as pneumonia and Legionnaires' disease [68]. It has FDA approval for the prophylaxis of syphilis, pelvic inflammatory disease (PID), intestinal amebiasis, rheumatic fever, and skin infections [69]. It works well to treat acne when combined with retinoid cream or benzoyl peroxide, and Clinicians can use it during

pregnancy to protect the newborn from contracting Group B streptococcal infection [70].

Many gram-positive and gram-negative bacteria, including Campylobacter, as well as several other microorganisms are susceptible to erythromycin. Since erythromycin is a bacteriostatic antibiotic and inhibits bacterial growth by preventing protein synthesis rather than eradicating it entirely [71]. Erythromycin blocks the synthesis of peptide chains by binding to the 23S ribosomal RNA molecule in the 50S subunit of the bacterial ribosome. Since ribosomes in human cells have only 40S and 60S subunits, they do not act on human tissues and are therefore a good antibiotic for human use [71].

2.8.3. Tetracycline

Derivatives such as methacycline, doxycycline, and minocycline were discovered later, tetracycline was first found in the 1940s [72]. It is a group of broad-spectrum antibiotics that are effective against a variety of gram-positive and gram-negative bacteria as well as unusual microorganisms like chlamydiae, mycoplasmas, rickettsia, and protozoan parasites [73]. Despite their success, researchers continued to look for analogs with better water solubility to enable parenteral administration or improve oral absorption [74].

Tetracycline inhibits protein synthesis by preventing aminoacyl-tRNA from binding to the bacterial ribosome, and therefore, it must reach its target in the cytoplasm to be able to act in all kinds of bacteria, regardless of the cell wall structure [75].

2.9. Antimicrobial Resistance Mechanisms in *Campylobacter*

Members of the *Campylobacter* genus typically do not tolerate high oxygen concentrations because they are microaerophilic organisms. Nonetheless, coping with environmental and oxidative stress is beneficial for survival in the environment outside of the host. Individual factors are discussed in depth in a review by Murphy et al. (2006) [76].

C. jejuni can grow optimally at temperatures between 37 and 42 °C and cannot reproduce at temperatures below 30 °C. In addition to being mobile, features such as metabolism and chemotaxis allow it to remain active at low temperatures, so that *C.*

jejuni can survive longer at temperatures ranging from 0 to 42 °C. Therefore, bacteria contaminated during the processing of food can survive in this wide temperature range. [77]. Cold-shock proteins (CspA) are proteins that are expressed under cold conditions and act as chaperones to facilitate protein translation. So far, no cold-shock proteins have been found in *Campylobacter*. Heat shock response [78] occurs in response to heat, in which some genes are expressed in a modified form; these genes code for periplasmic genes or genes involved in regulatory or metabolic systems. The RacR/RacS system plays an important role in the formation of heat shock proteins. The dnaJ gene, which is transcriptionally controlled by RacR and codes for a heat shock protein, is one such example [76]. Because *C. jejuni* is extremely sensitive to drought, the bacteria can only be found on wet surfaces [79].

2.9.1 Resistance to Quinolones

Quinolones are compounds that inhibit DNA replication by targeting DNA gyrase and topoisomerase IV enzymes, thus preventing cell proliferation [67]. Other mechanisms of this resistance exist as well, such as an efflux system and decreased outer membrane permeability [80]. GyrA and GyrB, or DNA gyrase, and ParC and ParE, or topoisomerase IV, respectively, are two pairs of subunits that make up the large enzymatic quaternary structures known as gyrase and topoisomerase gene products [80]. Resistance to fluoroquinolones is primarily brought on by amino acid substitutions in the topoisomerase's quinolone resistance-determining region (QRDR). On the surface of these enzymes, the DNA-binding domain contains QRDR. The single GyrA modifications Thr86Ile, Asp90Asn, Thr86Lys, Thr86Ala, Thr86Val, and Asp90Tyr have all been linked to fluoroquinolone resistance in Campylobacter species. However, the C257T change in the *gyrA* gene, which results in the Thr86Ile substitution in the gyrase and confers high-level resistance to this class of antibiotics, is the mutation that is most frequently seen in quinolone-resistant *Campylobacter* [81]. T86 K, A70 T, and D90 N are additional reported mutations associated with resistance, but they are less frequent and do not contribute significantly to the level of quinolone resistance seen with the Thr86Ile mutation [82]. As was previously mentioned, mutations in the *gyrA* gene, which codes for a portion of the GyrA subunit of DNA gyrase, appear to be the primary cause of fluoroquinolone resistance mechanisms in *Campylobacter* [83]. It was discovered that the point mutation Thr86Ile in the *gyrA* gene, which is homologous to

Ser83Leu in *E. coli*, conferred a high-level resistance to ciprofloxacin [84]. The Thr86Ala mutation, which results in high levels of nalidixic acid resistance and low levels of ciprofloxacin resistance in *C. jejuni*, is one of the other reported mutations of the GyrA region [85]. Furthermore, Asp85Tyr, Asp90Asn, and Pro104Ser double point mutations of the *gyrA* gene have been identified [84]. The lack of a secondary target for fluoroquinolones in *C. jejuni* and *E. coli* suggests that a specific alteration to the GyrA subunit is all that is required to confer a fluoroquinolone-resistant phenotype [81].

Antimicrobial resistance to a number of antimicrobials, including fluoroquinolones and macrolides, has been linked to the CMEABC multidrug efflux pump [79]. CmeABC is encoded by an operon made up of the three genes *cmeA*, *cmeB*, and *cmeC*, which, respectively, code for an outer membrane protein, an inner membrane drug transporter, and a periplasmic fusion protein [86]. The most prevalent efflux system in *Campylobacter*, the CmeABC multidrug efflux pump, cooperates with GyrA mutations to produce fluoroquinolone resistance [87]. Eflux pump inactivation via insertional inactivation of *cmeB* or with efflux pump inhibitors demonstrates that CmeABC plays a significant role in both intrinsic and acquired resistance of *Campylobacter*. Furthermore, even with GyrA mutations, the ciprofloxacin minimum inhibitory concentration (MIC) values are reduced to susceptible strain levels when the efflux pump is blocked [87]

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Instruments

The equipment used during the studies are indicated in the Table 3.1. Most of the equipment used is in the laboratories of ERU, Faculty of Medicine, and Department of Medical Microbiology. Studies on PCR and electrophoresis were carried out in Microbial Biotechnology laboratory at Faculty of Agriculture.

Table 3.1. List of instruments used in the research

Equipment	Company	Origin
Automated Gram stain	Biomeriux	France
MALDI-TOF-MS	BD	USA
VORTEX	BD	USA
Densitometer	BD	USA
Autoclave	Memer	Germany
Thermocycler	AB	UK
Incubator	BD	USA
Electrophoresis	AB	UK

3.1.2. Culture Media

Necessary materials and media for cultivation and biochemical tests used in the diagnosis of *Campylobacter* species are given in Table 3.2.

Table 3.2. Substances used in cultivation and biochemical tests

Materials	**Company**	**Origin**
Campy selective media	THERMO-AKLAB	TURKEY
Blood agar media	THERMO-AKLAB	TURKEY
Muller Hinton Fastidious Agar	OXOID	USA
Skim milk	BD	USA
Glycerol	BD	USA
Campy gas pack	BD	USA
KWIK STIK	OXOID	USA
Gram stain test	Biomeriux	France

3.1.3. MALDI-TOF MS Analysis

As it is known, matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionized diagnostic processes due to its features such as easy identification of microorganisms, fast and accurate results, and even direct analysis of the sample. Therefore, in this study, MALDI-TOF MS Biotyper Sirius (Bruker Daltonics) device was used for the species-level diagnosis of bacterial samples taken from different units of Erciyes University hospitals. The materials used during MALDI-TOF MS analysis are given in Table 3.3.

Table 3.3. Materials used in MALDI-TOF MS analysis

Materials	Company	Origin
Acetonitrile (ACN)	BD	USA
Deionized water	BD	USA
Trifluoro Acetic acid (TFA)	BD	USA
HCCA (a-Cyano-4-hydroxycinnamic acid) Matrix	BD	USA

For analysis with Biotyper Sirius MALDI-TOF MS systems, one to two colonies of each isolate were directly spotted on the respective manufacturer's proprietary sample plates, essentially following the manufacturer's protocols. One microliter of CHCA matrix solution (a-cyano-4-hydroxycinnamic acid,) was applied to the samples and then air-dried at room temperature for crystallization. For the species identification of each isolate and two spots were examined for the Biotyper system.

The VITEK MS MALDI-TOF MS system used in the study was equipped with a 337 nm fixed-focus nitrogen laser with a 50 Hz frequency. The mass range between 1 and

500 kDa produced a single composite mass spectrum. American Type Culture Collection (ATCC) 8739 isolates of *Escherichia coli*-A was used as the positive control. The sample spectra were initially analyzed by VITEK MS in Vitro Diagnostic (IVD) Analysis Software (version 3.2). It is a comprehensive, proprietary clinical database for the identification of bacteria and fungi. Briefly, scores between 60 and 100% probability indicate an excellent discrimination value and a reliable identification, and a score below 60% represents low discrimination identification and includes a list of choices for possible identification. In all cases, no identification was determined when no match was found for the composite spectra or not enough spectral peaks were generated during the analysis. In addition, this MALDI-TOF MS system is equipped with the VITEK MS Research Use Only (RUO) system software, including the SARAMIS database for the rapid detection of bacterial isolates. In this study, VITEK MS IVD analysis, VITEK MS RUO system and SARAMIS softwares were used for species identification. At the same time, The Biotyper MALDI-TOF MS system was used for the identification of these bacterial isolates by comparing the protein mass patterns with the available Bruker reference databases. The Biotyper has a built-in microflex TM LT/SH MALDI-TOF mass spectrometer with an N2 laser providing a 60 Hz repetition rate. The samples were examined by the RUO reference library with 2380 species for bacterial identification. During analysis, the generated mass peaks were matched with reference spectra in the database; the comparison of spectra also generated a numerical value for precise and rapid identification of the bacteria. A value >2.0 was considered species level, and a value between 2.0 and 1.7 was considered a reliable genus-level identifications.

3.1.4. Antibiotics Susceptibility Test (AST)

Antibiotics used in the Susceptibility Test (AST) with their concentration, Disk diffusion zone breakpoints MIC value and manufacturer illustrated in Table 3.4.

Table 3.4. The antibiotics used in this study

Antibiotics	Conc of Discs (mg/L)	MIC		Diameter of Inhibition zone (mm)		Company
		R> NOTE[A]	S≤ NOTE[A]	R< NOTE[A]	S≥ NOTE[A]	
Ciprofloxacin (CIP)	5	0.5	10^{-2}	26	50	Oxoid –UK
Erythromycin (E)	15	4^{1}	4^{1}	20^{A}	20^{A}	Oxoid –UK
Tetracycline (TE)	30	2^{1}	2^{1}	30^{A}	30^{A}	Oxoid –UK

R: Resistant, S: Sensitive, MIC: Minimal Inhibition Concentration,

In this study, we used the antibiotic disks Ciprofloxacin, Erythromycin, and Tetracycline, which are considered the last resort for infection treatment. Disk diffusion tests were typically performed to *Campylobacter spp* isolates on Muller Hinton Fastidious agar which contains %5 lysed horse blood and 20mg/L β-NAD. Antibiotic discs were put in media already inoculated with the *Campylobacter spp* strain, where the colony grew intensively. The antibiotics in the discs began to disperse among the bacterial colonies, and inhibition zones around the antibiotic discs were observed after 41± 1 °C one day of incubation in microaerobic conditions. The zones for inhibition are measured and contrasted with EUCAST guidelines.

MIC stands for minimum inhibitory concentrations, meaning the low antibiotic concentration that inhibits the observable growth of a microorganism after overnight incubation and is used to determine the antibiotic concentration that prevents bacterial development.

3.1.5. Molecular studies

Mutation detection primers and other reagents used for PCR in detecting *gryA* gene are given in the Table 3.5.

Table 3.5. Mutation detection primers and other reagents in detecting *gryA* gene

No	Material		Company	Original
1	F: ACTGGTTCTAGCCTTTTGGAA		Nucleo Gene	Turkey
	R: TTGGTACACAACCAGCGAAA		Nucleo Gene	Turkey
2	F: ATTTTTAGCAAAGATTCTGAT	673 bp	Nucleo Gene	Turkey
	R: CCATAAATTATTCCACCTGT		Nucleo Gene	Turkey
	GZgryA			
3	R: TTT TTAGCAAAGATTCTG AT	265 bp	Prisma	Turkey
	F: CAAAGC ATCATAAACTGCAA *CampyMAMAgyrA1*		Prisma	Turkey
4	Hotstart PCR master mix		Nucleo Gene	Turkey
5	Genomic DNA extraction kit		Nucleo Gene	Turkey
7	Agarose 100Gr		Nucleo Gene	Turkey
8	DNA ladder		Nucleo Gene	Turkey

PCR for detection of *gryA* gene (normal) was conducted using the primers and PCR conditions with a few minor modifications [88]. After performing standard PCR products was sequenced. A conserved, forward primer, CampyMAMA*gyrA*1, and a

reverse, mutation detection primer CampyMAMA*gyrA*5 (Table 1), were used together in a PCR to generate a 265-bp PCR product that was a positive indication of the presence of the Thr-86-to-Ile (ACA→ATA) mutation in the *C. jejuni gyrA* gene [89].

3.2. Method

Most of the methods applied in this study were carried out in the clinical microbiology laboratory of Erciyes University hospitals. One hundred and fifty duplicate rectal swab specimens were obtained at random from patients with different age groups hospitalized in different units (Intensive Care, Neurosurgery, Pediatric Intensive Care, anesthesia, hematology, general surgery, neonatal intensive care, bone marrow, nephrology, and medical oncology) at Erciyes University's Medical Faculty Hospitals in Kayseri, Turkey. These specimens were kept at -20 °C in the deep freezer.

All clinical specimens were checked by the gram stain and cultured on Blood agar and Campylobacter selective media to get pure colony. Bacteria transferred from plates to skim milk and stored in deep freezer (-20 °C) until subculturing on blood agar. Analysis by MALDI-TOF-MS was performed to identify the clinical specimens at the species level. Antibiotic susceptibility testing was performed using Kirby-Bauer disc diffusion method on Mueller-Hinton Fastidious agar blood agar to determine whether the samples were resistant to antibiotics such as fluoroquinolones, macrolides and tetracyclines using EUCAST (The European Committee on Antimicrobial Susceptibility Testing, (2020-2022) guidelines. The antimicrobial discs used were ciprofloxacin (5 mg, Oxoid, UK), erythromycin (15 mg, Oxoid, UK), and tetracycline (30 mg, Oxoid, UK). Then, using the relevant primers, the GZgryA gene was detected by normal PCR and sequence analysis of this gene was performed.

3.2.1. Cultivation

All media used for cultivation and biochemical tests were sterile and ready to use. In the laboratory, only the Skim milk (BD, USA) is prepared to store samples. For this purpose, 51.5 g Skim milk was dissolved in 1000 ml of distilled water and autoclaved for 15 minutes (15 lbs pressure and 121 ° C temperature). The sterilized media were poured into tubes and the collected bacterial samples were inoculated into this media and stored in the deep freezer for further studies. The following culture media were used for the growth of bacterial isolates.

Mueller-Hinton Fastidious Agar

This medium was used in the AST processes. Since this medium is a non-selective and non-differential medium that promotes the growth of organisms, it is the recommended medium for both antibiotic susceptibility testing (AST) for fastidious microorganisms like Campylobacter spp. (refere EUCAST guidelines)

Blood agar

Blood agar is a general purpose and enriched medium prepared from tryptic soy agar or Columbia agar base containing 5% sheep blood, used for the growth of microorganisms that are difficult to grow and used to differentiate bacteria according to their hemolytic properties [90].

Campy selective media

Campylobacter Selective Agar was used to obtain pure colonies of Campylobacter isolates [91]. The samples, which were previously stored in skim milk medium, were taken from the deep freezer and cultured on Campylobacter Selective Agar to ensure the development of the isolates.

3.2.2. Detection of bacterial species with conventional and molecular methods

3.2.2.1 Gram staining

Gram staining was performed in the laboratory for the 150 rectal swab samples cultivated in a sterilized working area, and the blood agar plates were incubated in the incubator at 48 °C for 48 hours in a microaerophilic atmosphere. A loop of pure colony was taken and placed on the slide and slightly heated to dry and fix. This preparation was then placed on an automatic gram staining system and examined under a microscope and S-shaped or rod- See gull wings shaped- bacteria were identified under the microscope (S-shaped, or in pairs showing, See gull wings) [92].

3.2.2.2. Culturing in selective media

Pure colonies were obtained by culturing gram negative isolates in campy selective media and to get pure colonies of *Campylobacter* species. Isolates from these pure colonies were used for MALDI TOF MS and PCR analyzes.

3.2.2.3. Antibiotic Susceptibility Test (AST)

In the Antibiotic Susceptibility Test (AST), the McFarland Criteria are used to standardize the approximate number of bacteria in the liquid suspension of a bacterial cell. To achieve more accurate results for the antibiotic susceptibility test, the bacterial suspension concentration must be between 0.5 and 0.65 McFarland. Using a sterile cotton swab and mixing with buffer saline (zero McFarland), the bacterial mixture was blended well using a vortex, and the density was read with a McFarland densitometer, the suspension density for inoculum would be 0.5-0.65 McFarland.

AST was performed on Mueller Hinton fastidious agar using the disk diffusion method, according to EUCAST recommendations, by inoculating the plates with previously formulated bacterial broth suspension by swabbing the inoculum in three directions and placing the Ciprofloxacin, Erythromycin, and Tetracycline antibiotic disks on. ATCC 33560 *Campylobacter jejuni* isolate were used as quality control.

3.2.2.4. MALDI-TOF-MS test

Cultured bacteria in blood agar and incubated them at 42 °C in a microaerophilic atmosphere for 48 hours. Then one to two colonies of each isolate were directly spotted on the manufacturer's proprietary sample plate, following the manufacturer's protocol. One to two colonies of each isolate were directly spotted on the manufacturer's proprietary sample plate. Then the samples were crystallized. For crystallization 2 microliters of CHCA matrix solution (-cyano-4- hydroxycinnamic acid) were applied to samples and air dried at room temperature. The obtained samples were placed on the manufacturer's registered sample plate and placed in the MALDI-TOF-MS device, then, the device was operated for the diagnosis process. Within a short period of time (2 or 3 minutes) the device performed a species-level analysis of *Campylobacter* isolates.

3.2.2.5. Detection *gryA* gene by Polymerase Chain Reaction (PCR)

DNA Extraction

In order to isolate DNA 1.5 ml of bacterial samples stored in skim milk medium were taken and centrifuged at 5000x-g for 10 minutes in a 2 ml tube, then the supernatant was discarded and 400 ml of lysis buffer, 20 ml of Proteinase K and 20 ml of Lysozyme

were added to the pellet. It was incubated at 56°C until complete disintegration. After 400 binding buffer was added onto the incubated sample mixture, the mixture was vortexed for 15 s. After transferring 800 ml of the mixture to the spin column, centrifuging at 10,000 x g for 2 minutes, the liquid in the lower tube was discarded and the column was placed in a new collection tube. After adding 500 µl of wash buffer I to the spin column, it was centrifuged at 10 000x-g for 1 minute, and after the liquid was removed, the column was placed in a new collection tube. The same process was repeated after adding 750 ml of Wash Buffer II to the spin column and centrifuged again at 10 000x-g for 1 minute. After the liquid was removed, the column was placed in a new collection tube and optionally the procedure was performed in duplicate. The spin column was then centrifuged again at 10 000x-g for 3 minutes to remove the ethanol in the residue. After these washings, the spin column was transferred to a 1.5 ml micro centrifuge tube and incubated for 1 minute at room temperature after adding 200 µl of elution buffer to the center of the column. After the column was centrifuged again at 10 000x-g for 1 minute, DNA was obtained and used in PCR processes. If not used immediately, stored at -20 °C.

Mismatch amplification mutation assay (MAMA PCR)

The Mismatch amplification test (MAMA PCR) is a modified polymerase chain reaction (PCR) variant developed to provide specific amplification of gene sequences. The mismatch amplification test (MAMA PCR) is a modified polymerase chain reaction (PCR) variant developed to provide specific amplification of gene sequences. This is a PCR method based on a special primer design. Accordingly, a single nucleotide mismatch in the 3' proximity of the reverse oligonucleotide primer inhibits the elongation process of Taq DNA polymerase. Thus, primers can produce a wild-type PCR fragment, whereas mismatch amplification mutation assay (MAMA) from any gene cannot synthesize a mutated product at the location covered by mismatch positions on the primer. Primers designed for this purpose are given in Table 3.5 above.

This method was applied as follows: First, after thawing, the master mix was started by gently vertexing. After a thin-walled PCR tube was taken and placed in ice, a mixture consisting of 10µl Hot start master mix(2x), 1µl CampyMAMAgyrA1-F, 1µl CampyMAMAgyrA8-R, 2µl DNA template and 6µl Nuclease-free water was prepared for 20µl reaction volume.

The following program was applied for this prepared 20µl PCR mix: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 20 s. Following amplification, the 10 µl of PCR products were electrophoresed. For electrophoresis, 80 ml TE 1X mixed with 0.8 g agarose in a beaker and heated in a microwave, then 0.8 µl safety dye was added and the mixture was allowed to cool. For the DNA ladder 1 µl DNA loading dye, 1.5 µl DNA ladder, and 4 µl distilled water mixed within a tube and vortexed. 10 µl aliquots of each PCR product were loaded with 3 µl loading dye onto horizontal 2.0%, 0.5 Tris-borate-EDTA agarose gels and electrophoresed

Detection of gyrA QRDR mutations associated with quinolone resistance.

Mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, have been characterized among the *Campylobacter* isolates. The *gyrA* genes of the isolates were amplified by PCR. Primers GZ*gyrA*5 and GZ*gyrA*6 (Table 3.6) were chosen for PCR amplification of a 673-bp product containing the QRDR of the *gyrA* gene of quinolone-resistant and -susceptible isolates of *C. jejuni* after analysis of the NCTC 11168 *gyrA* gene sequence in GenBank.

The following method was used for PCR: A thin-walled PCR tube was taken and placed in ice, a mixture consisting of 20µl Hot start master mix (2x), 2µl GZgyrA5-F, 2µl GZgyrA6-R, 4µl DNA template and 12µl Nuclease-free water was prepared for 40 µl reaction volume. PCR cycling conditions are the same as for MAMA PCR above. Following amplification, the 10 µl of PCR products were electrophoresed. After amplification, 10 µl of PCR product was subjected to electrophoresis as described above and PCR products giving positive bands were sent for sequencing in tubes containing 20 µl sample

CHAPTER 4

RESULTS

4.1. Isolation and Identification of *Campylobacter spp.*

Campylobacter spp. isolates, which are the subject of this study, were obtained from patients who applied to different departments of Erciyes University Hospitals with diarrhea. A total of 150 rectal swab samples were randomly taken from the patients and the incidence of *Campylobacter* isolates according to age groups is given in Table 4.1. As can be seen from the table, it is observed that most of the patients (64.15%) who applied with the complaint of diarrhea are children and young individuals.

Table 4.1. Distribution of the isolates according to age groups

Age Groups	Number of collected samples	Percent frequency of positive isolates (%)	Male (%)	Female (%)
0-10	60	64.15 (34)	73.50 (25)	26.50 (9)
11-30	66	26.42 (14)	78.60 (11)	21.40 (3)
31-50	14	3.77 (2)	100.00 (2)	00.00 (0)
51-64	10	5.66 (3)	66.70 (2)	33.30 (1)
Total	150	100.00	75.5 (40)	24.50 (13)

The number of isolates with positive results is indicated in parentheses.

While the rate of positive males in the 0-10 age group was 2.77 times higher than that of females, this rate was calculated as 3.66 times in the 11-30 age groups. Therefore, it has been observed that males are infected at a much higher rate in almost all age groups. Again, when the samples were examined, no infection was observed in female over 41 years of age, while it was determined that the highest age group with positive results in male patients was 53.

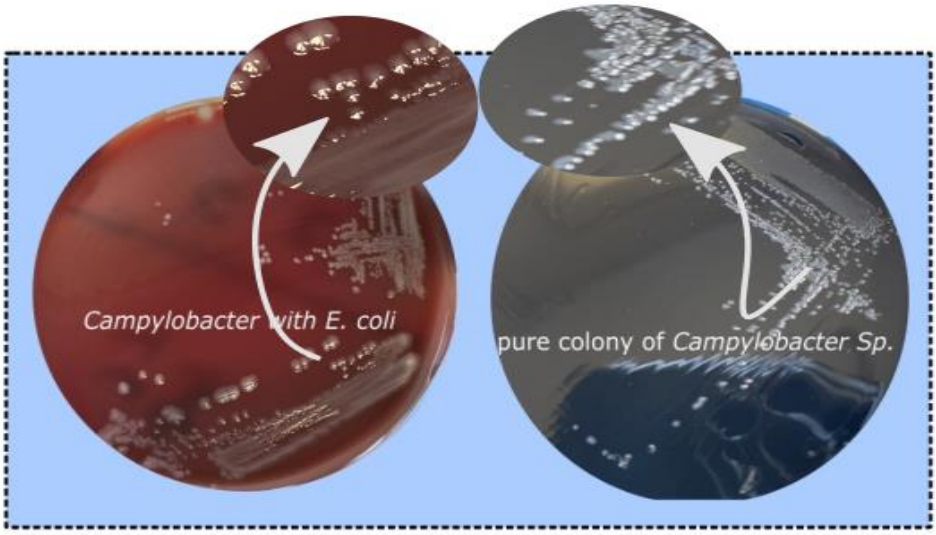


Figure 4.1. Campylobacter spp. growth in blood and selective agar media

Morphological diagnosis of bacterial isolates was made on blood agar and Campy selective medium (Figure 4.1). Totally pure Campylobacter colonies were obtained in 53 of the 150 samples tested in campy selective media. However, when the same procedure was performed on blood agar, while *Campylobacter* colonies were obtained in 52 of them, except for 1, in only 1 plate, *E. coli* grew together with *Campylobacter*. *Campylobacter* colony morphology, like its polar flagella and rod-shaped bacteria, appeared as helical morphology. After growing bacterial isolates on blood agar, *Campylobacter* colonies were non-haemlytic, smooth, glossy, and convex with an entire edge (Figure 4.2).

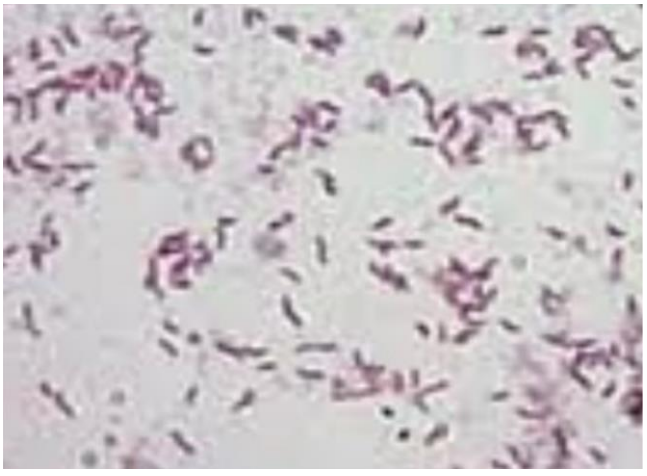


Figure 4.2. Morphology of *Campylobacter* under microscope after Gram stinging

It was confirmed that 53 of the 150 positive isolates were *Campylobacter* after performing Gram stain tests were positive due to the chemical and physical properties of *Campylobacter* cells. In briefly it is a Gram-negative, microaerophilic non-fermenting, spiral rod, and gull wing shaped bacteria (Figure 4.2).

4.2. MALDI-TOF MS analysis

The isolates were cultured on Campy selective agar plating media and incubated at 42ºC for at least 48h in microaerophilic conditions. Samples were subcultured on blood agar medium to sufficiently reproduce these naive colonies from Campy selective medium. Then, the MALDI-TOF MS system was used to identify these colonies at the species level and 53 isolates that gave positive results in Campy selective medium were used for this analysis and the procedure was repeated 2 times for each. Several colonies from two plates of each isolate were spotted on the sample plate for their identification. A score of 2 or more (>2) indicates a very strong diagnosis. This method, which was applied for these 53 samples, was also applied to the reference strain and the score values were compared. As a result of these analyzes, it was determined that 42 samples were *Campylobacter jejuni* and 9 samples were *Campylobacter coli*. Only 1 of the samples could not be identified by the device, perhaps because not enough samples could be loaded to the plate. *Campylobacter* reference strain (ATCC) was also used for comparison, and in the MALDI TOF MS analysis, the second replicate confirmed the first in all samples (Table 4.3). Both species found at a much higher rate in adult intensive care units. While no *C. coli* observed in Hematology, Pediatric intensive care and Anesthesia units, *C. jejuni* isolated from all units. Only one *C. coli* isolate observed at the anesthesia unit (Table 4.2).

Table 4.2. Distribution of *Campylobacter* isolates by hospital units.

Unit	*C. coli*		*C. jejuni*	
	N	*%*	*N*	*%*
Intensive care	6	66.67	15	35.71
Neurosurgery	1	11.11	12	28.57
Hematology	0	0.00	6	14.29
General surgery	2	22.22	5	11.90
Pediatric intensive care	0	0.00	3	7.14
Anesthesia unit	0	0.00	1	2.38
Total	9	17.65	42	82.35

N: Number of isolates

Table 4.3. Detection of *Campylobacter* spp. by MALDI-TOF MS

Sample name	Sample ID	Organisms (best match)	Score* value	Organisms (second best match	Score* value
B1 (+++) (A)	1. standard	*Campylobacter jejuni*	2.28	*Campylobacter jejuni*	2.22
B2 (+++) (A)	2. standard	*Campylobacter jejuni*	2.21	*Campylobacter jejuni*	2.10
B3 (+++) (A)	3. standard	*Campylobacter jejuni*	2.16	*Campylobacter jejuni*	2.09
B4 (-----) (C)	4. standard	No organism identified	1.64	No organism identified	1.56
B5 (+++) (A)	5. standard	*Campylobacter coli*	2.18	*Campylobacter coli*	2.13
B6 (+++) (A)	6. standard	*Campylobacter jejuni*	2.06	*Campylobacter jejuni*	2.05
B7 (+++) (A)	7. standard	*Campylobacter jejuni*	2.30	*Campylobacter jejuni*	2.27
B8 (+++) (A)	8. standard	*Campylobacter jejuni*	2.01	*Campylobacter jejuni*	1.93
B9 (+++) (A)	9. standard	*Campylobacter jejuni*	2.12	*Campylobacter jejuni*	2.12
B10 (+++) (A)	10. standard	*Campylobacter jejuni*	2.13	*Campylobacter jejuni*	2.11
B11 (+++) (A)	11. standard	*Campylobacter jejuni*	2.01	*Campylobacter jejuni*	1.85
B12 (+++) (A)	12. standard	*Campylobacter Coli*	2.01	*Campylobacter Coli*	2.01
C1 (+++) (A)	13. standard	*Campylobacter jejuni*	2.18	*Campylobacter jejuni*	2.14
C3 (+++) (A)	14. standard	*Campylobacter jejuni*	2.24	*Campylobacter jejuni*	2.08
C4 (+++) (A)	15. standard	*Campylobacter jejuni*	2.12	*Campylobacter jejuni*	2.12
C5 (+++) (A)	16. standard	*Campylobacter jejuni*	2.15	*Campylobacter jejuni*	2.07
C6 (+++) (A)	17. standard	*Campylobacter jejuni*	2.10	*Campylobacter jejuni*	2.05
C7 (+++) (A)	18. standard	*Campylobacter jejuni*	2.12	*Campylobacter jejuni*	2.12
C8 (+++) (A)	19. standard	*Campylobacter jejuni*	2.14	*Campylobacter jejuni*	2.08
C9 (+++) (A)	20. standard	*Campylobacter jejuni*	2.21	*Campylobacter jejuni*	2.14
C10 (+++) (A)	21. standard	*Campylobacter jejuni*	2.20	*Campylobacter jejuni*	2.04
C11 (+++) (A)	22. standard	*Campylobacter jejuni*	2.33	*Campylobacter jejuni*	2.23
C12 (+++) (A)	23. standard	*Campylobacter jejuni*	2.00	*Campylobacter jejuni*	2.01
D1 (+++) (A)	24. standard	*Campylobacter jejuni*	2.36	*Campylobacter jejuni*	2.32
D2 (+++) (A)	25. standard	*Campylobacter jejuni*	2.14	*Campylobacter jejuni*	2.11
D3 (+++) (A)	26. standard	*Campylobacter jejuni*	2.25	*Campylobacter jejuni*	2.25
D4 (+++) (A)	27. standard	*Campylobacter jejuni*	2.34	*Campylobacter jejuni*	2.32
D5 (++) (A)	28. standard	*Campylobacter Coli*	2.28	*Campylobacter Coli*	2.24
D6 (+++) (A)	29. standard	*Campylobacter jejuni*	2.28	*Campylobacter jejuni*	2.23
D7 (+++) (A)	30. standard	*Campylobacter Coli*	2.28	*Campylobacter Coli*	2.22
D8 (+++) (A)	31. standard	*Campylobacter jejuni*	2.41	*Campylobacter jejuni*	2.35
D9 (+++) (A)	32. standard	*Campylobacter jejuni*	2.23	*Campylobacter jejuni*	2.16
D10 (+++) (A)	33. standard	*Campylobacter Coli*	2.26	*Campylobacter Coli*	2.25
D11 (+++) (A)	34. standard	*Campylobacter jejuni*	2.14	*Campylobacter jejuni*	2.12
D12 (+++) (A)	35. standard	*Campylobacter jejuni*	2.33	*Campylobacter jejuni*	2.29
E1 (+++) (A)	36. standard	*Campylobacter jejuni*	2.33	*Campylobacter jejuni*	2.26
E3 (+++) (A)	37. standard	*Campylobacter jejuni*	2.31	*Campylobacter jejuni*	2.30
E4 (+++) (A)	38. standard	*Campylobacter jejuni*	2.02	*Campylobacter jejuni*	2,24
E5 (+++) (A)	39. standard	*Campylobacter jejuni*	2.24	*Campylobacter jejuni*	2.14
E6 (+++) (A)	40. standard	*Campylobacter jejuni*	2.22	*Campylobacter jejuni*	2.11
E8 (+++) (A)	41. standard	*Campylobacter jejuni*	2.25	*Campylobacter jejuni*	2.18
E9 (+++) (A)	42. standard	*Campylobacter jejuni*	2.19	*Campylobacter jejuni*	2.26
E10 (+++) (A)	43. standard	*Campylobacter Coli*	2.28	*Campylobacter Coli*	2.16
E11 (+++) (A)	44. standard	*Campylobacter Coli*	2.31	*Campylobacter Coli*	2.18
E12 (+++) (A)	45. standard	*Campylobacter jejuni*	2.12	*Campylobacter jejuni*	2.24
F1 (+++) (A)	46. standard	*Campylobacter jejuni*	2.31	*Campylobacter jejuni*	2.25
F2 (+++) (A)	47. standard	*Campylobacter Coli*	2.12	*Campylobacter Coli*	2.05
F3 (+++) (A)	48. standard	*Campylobacter Coli*	2.17	*Campylobacter Coli*	2.16
F4 (+++) (A)	49. standard	*Campylobacter jejuni*	2.15	*Campylobacter jejuni*	2.00
F5 (+++) (A)	50. standard	*Campylobacter jejuni*	2.03	*Campylobacter jejuni*	2.01
F6 (+++) (A)	51. standard	*Campylobacter jejuni*	2.12	*Campylobacter jejuni*	2.09
F7 (+++) (A)	52. standard	*Campylobacter jejuni*	2.19	*Campylobacter jejuni*	2.14
F9 (+++) (A)	ATCC	*Campylobacter jejuni*	2.23	*Campylobacter jejuni*	2.18

**Score values greater than 2 indicate that the detection is strong.*

Where was the highest percentage of *Campylobacter coli* in stool samples received from the intensive care unit (66.67%), also for *Campybacter jejuni,* the highest percentage (36.4%) was found in intensive care unit. The lowest percentage of *Campylobacter coli* was (11.11%) from the neurosurgery unit, while for *Campylobacter jejuni* the lowest percentage was (2.38%) from the anesthesia unit. When the species identifications of the obtained isolates were compared, the incidence of *C. jejuni* isolate was much higher than that of *C. coli.*

4.3. Determination of the antibiotic resistance of *Campylobacter spp*

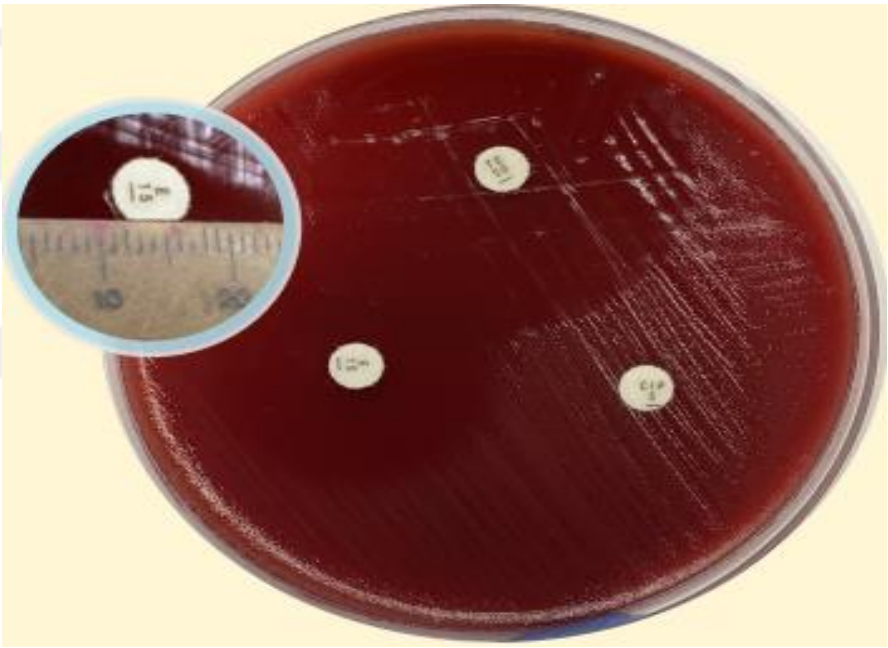


Figure 4.3. Antibiotic susceptibility (AST) of *Campylobacter* spp isolates on Mueller Hinton Fastidious agar medium

AST was applied to determine whether *Campylobacter* isolates showed resistance to 3 types of antibiotics and the results were recorded for *C. coli, C. jejuni* and reference strains and are given in Table 4.4. Antibiotic susceptibility determined by disc diffusion method on Mueller Hinton Fastidious agar media (Figure 4.3.).

Discs impregnated with Tetracycline, Ciprofloxacin and Erythromycin were used for this testing. At the same time, the sensitivity of the ATCC 33560 *Campylobacter jejuni* strain reference was determined by the same method and compared. Inhibition zone diameters (mm) obtained are given in Table 4.4.

Table 4.4. Inhibition zones of isolates after Antibiotic Susceptibility Test (AST)

No of Isolate	Species	Zone of Inhibition (mm)		
		TE	CIP	E
HA1	*C. jejuni*	38	0	27
HA2	*C. jejuni*	0	0	33
HA3	*C. jejuni*	32	0	21
HA4	*C. coli*	38	0	25
HA5	*C. jejuni*	41	38	25
HA6	*C. jejuni*	0	0	26
HA7	*C. jejuni*	35	0	22
HA8	*C. jejuni*	0	0	27
HA9	*C. jejuni*	0	0	30
HA10	*C. jejuni*	0	0	29
HA11	*C. coli*	0	0	25
HA12	*C. jejuni*	37	35	27
HA13	*C. jejuni*	40	0	27
HA14	*C. jejuni*	40	0	27
HA15	*C. jejuni*	40	0	30
HA16	*C. jejuni*	42	0	32
HA17	*C. jejuni*	0	0	27
HA18	*C. jejuni*	12	0	27
HA19	*C. jejuni*	34	0	35
HA20	*C. jejuni*	45	0	30
HA21	*C. jejuni*	37	0	27
HA22	*C. jejuni*	0	0	27
HA23	*C. jejuni*	0	0	25
HA24	*C. jejuni*	40	0	25
HA25	*C. jejuni*	0	0	27
TE: Tetrocyline, CIP: Cyproflaxacine, E: Erytromycine				

No of Isolate	Species	Zone of Inhibition (mm)		
		TE	CIP	E
HA26	*C. jejuni*	0	0	32
HA27	*C. coli*	45	42	0
HA28	*C. jejuni*	0	0	31
HA29	*C. jejuni*	0	0	26
HA30	*C. jejuni*	0	0	25
HA31	*C. coli*	0	0	25
HA32	*C. jejuni*	40	0	25
HA33	*C. jejuni*	0	0	32
HA34	*C. jejuni*	0	0	31
HA35	*C. jejuni*	0	0	35
HA36	*C. jejuni*	0	0	32
HA37	*C. jejuni*	37	0	25
HA38	*C. jejuni*	10	0	30
HA39	*C. jejuni*	16	0	27
HA40	*C. jejuni*	45	0	35
HA41	*C. coli*	40	0	25
HA42	*C. coli*	40	0	25
HA43	*C. jejuni*	0	0	26
HA44	*C. jejuni*	15	0	30
HA45	*C. coli*	0	0	25
HA46	*C. coli*	0	0	25
HA47	*C. jejuni*	0	0	25
HA48	*C. jejuni*	0	0	30
HA49	*C. jejuni*	0	0	35
HA50	*C. jejuni*	0	0	25
51	ATCC	35	38	28

As can be seen from the table above, *Campylobacter* isolates showed different levels of resistance for each antibiotic. While 5 of 8 *C. coli* isolates (HA11, HA25, HA31, HA45 and HA46) are resistant to both Tetracycline and Ciprofloxacin, 3 of them (HA4, HA41 and HA42) are resistant against Ciprofloxacin. Only one *C. coli* species (HA27) showed resistance against erythromycin. For example, isolate HA4 of *C. coli* was sensitive to Tetracycline and erythromycin, but resistant to ciprofloxacin. Interestingly *C. coli* isolate HA11 was resistant to TE and CIP, but sensitive to erythromycin. All *C. jejuni* isolates are susceptible to erythromycin, including the reference strain. As can be seen from the table, all other *C. jejuni* isolates show resistance to Ciprofloxacin except HA5 and HA12 isolates. This seems to be an indication that isolates of this species cannot be treated with ciprofloxacin in the years ahead. 21 isolates of the *C. jejuni* genus appear to be resistant to both Tetracycline and Ciprofloxacin. More than half of the *C. jejuni* isolates (24 isolates, 57.14%) were found to be resistant to both Tetracycline and

Ciprofloxacin. As a result of the AST results, it is understood that more *C. jejuni* isolates are resistant to Ciprofloxacin when compared to Tetracycline, but it is also revealed that it is difficult to treat these bacteria with these two antibiotics. Among all the isolates used in this study, only 1 (HA27) was resistant to Erythromycin, and all other isolates were found to be susceptible to this antibiotic.

The antibiotic susceptibilities of Campylobacter species are summarized in Tables 4.5 and 4.6. Zone diameter reference values for Erythromycin, Ciprofloxacin and Tetracycline for *Campylobacter coli* were determined as 24, 50 and 30 mm, respectively. However, the reference value for *C. jejuni* is 20 mm for Erythromycin, while the reference values for other antibiotics are the same as for *C. coli*. All *C. jejuni* isolates are susceptible to Erythromycin, but 1 *C. coli* isolate is resistant to this antibiotic.

Table 4.5 Antibiotic susceptibility of the *Campylobacter coli* isolates

Antibiotics	Zone diameter (mm)		Prevelance of sensitive isolates (%)	Prevelance of resistant isolates (%)
	S≥	R<		
Erythromycin	24[A]	24[A]	7 (87.5%)	1 (12.5%)
Ciprofloxacin	50	26	1 (12.5%)	7 (87.5%)
Tetracycline	30[A]	30[A]	5 (62.5%)	3 (37.5%)

Table 4.6 Antibiotic susceptibility of the *Campylobacter jejuni* isolates

Antibiotics	Zone diameter (mm)		Prevelance of sensitive isolates (%)	Prevelance of resistant isolates (%)
	S≥	R<		
Erythromycin	20[A]	20[A]	42.00 (100%)	0.00 (0%)
Ciprofloxacin	50	26	2.00 (4.76%)	40.00 (95.23%)
Tetracycline	30[A]	30[A]	16.00 (38.09%)	26.00 (61.90%)

4.4. Detection of the T-86 to-I mutations (ACA→ATA) by MAMA PCR

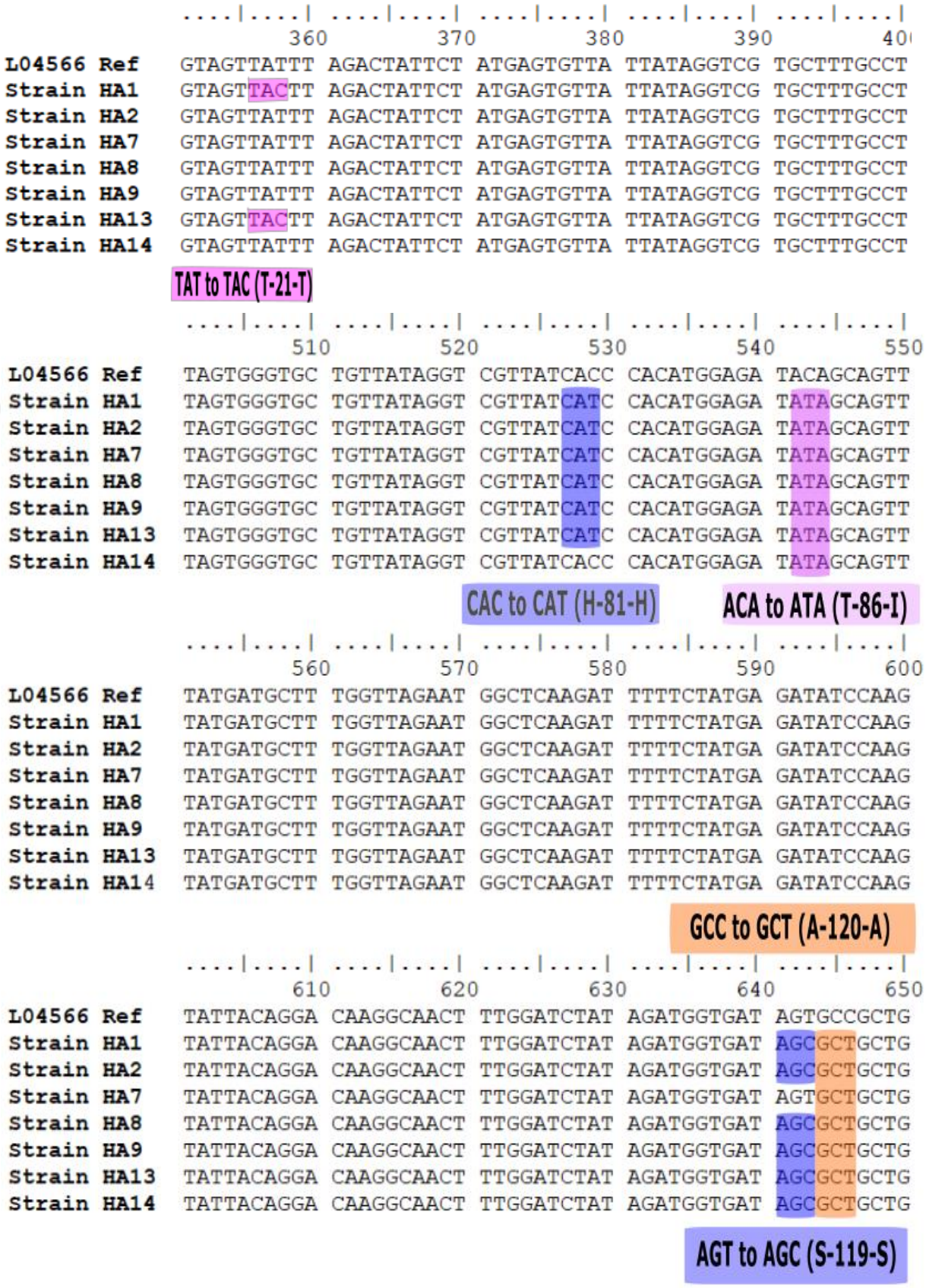
A rapid PCR method was developed for the detection of the T-86 to-I (ACA-to-ATA) mutation at amino acid position 86 in the DNA sequence of *C. jejuni* isolates. Isolates with the wild-type amino acid 86 codon (ACA, ciprofloxacin susceptible) were not amplified with the reverse mutation primer CampyMAMA*gyrA*5, whereas the isolates with the mutated amino acid 86 codon (ATA, ciprofloxacin resistant) generated a 265-bp PCR product with the CampyMAMA*gyrA*5 reverse mutation primer and the

CampyMAMA*gyrA*1 forward conserved primer. As shown in Figure 4.4, some *Campylobacter spp*. samples resistance to ciprofloxacin had a mutation in codon 86 we checked seven samples for Thr-86-to-Ile mutations (ACA→ATA), all show positive result for this mutation because it's most common in *C. jejuni* samples that showed resistance for ciprofloxacin.

Figure 4.4. Agarose gel of *C. jejuni* MAMA PCR products. Lane A, 0.8 mg of a 100-bp DNA ladder. Other lanes contain the isolates.

4.5. Detection of *gyrA* QRDR mutations associated with quinolone resistance

We identified seven nucleotide mutations in the QRDR in their gyrA DNA sequence data, which are depicted in Figure 4.5. Interestingly, there was three novel mutation one silent mutations (CTA→TTA) L208-to-L and the other two changes the amino acid (AAT→AGT) N203-to-S and (GCA→ACA) Thr201-to-Ile. The other mutations, which are also reported in previous studies, comprises an amino acid alteration at position 86 of the GyrA protein was present in 7 ciprofloxacin-resistant strains as a result of a DNA codon change from ACA (threonine) to ATA (isoleucine). Also we got seven silent mutation we had H 81-to- H (CAC→CAT) in five samples, S-to-S (AGT→AGC) in one isolate, A120- to- A (GCC→GCT) was in all seven samples, S145- to-S in one sample and V161-to-V (GTT→GTC) showed just in one sample (Figure 4.5).


360 370 380 390
L04566 Ref GTAGTTATTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
Strain HA1 GTAGTTACTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
Strain HA2 GTAGTTATTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
Strain HA7 GTAGTTATTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
Strain HA8 GTAGTTATTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
Strain HA9 GTAGTTATTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
Strain HA13 GTAGTTACTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
Strain HA14 GTAGTTATTT AGACTATTCT ATGAGTGTTA TTATAGGTCG TGCTTTGCCT
TAT to TAC (T-21-T)
510 520 530 540 550
L04566 Ref TAGTGGGTGC TGTTATAGGT CGTTATCACC CACATGGAGA TACAGCAGTT
Strain HA1 TAGTGGGTGC TGTTATAGGT CGTTATCATC CACATGGAGA TATAGCAGTT
Strain HA2 TAGTGGGTGC TGTTATAGGT CGTTATCATC CACATGGAGA TATAGCAGTT
Strain HA7 TAGTGGGTGC TGTTATAGGT CGTTATCATC CACATGGAGA TATAGCAGTT
Strain HA8 TAGTGGGTGC TGTTATAGGT CGTTATCATC CACATGGAGA TATAGCAGTT
Strain HA9 TAGTGGGTGC TGTTATAGGT CGTTATCATC CACATGGAGA TATAGCAGTT
Strain HA13 TAGTGGGTGC TGTTATAGGT CGTTATCATC CACATGGAGA TATAGCAGTT
Strain HA14 TAGTGGGTGC TGTTATAGGT CGTTATCACC CACATGGAGA TATAGCAGTT
CAC to CAT (H-81-H)
ACA to ATA (T-86-I)
560 570 580 590 600
L04566 Ref TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
Strain HA1 TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
Strain HA2 TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
Strain HA7 TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
Strain HA8 TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
Strain HA9 TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
Strain HA13 TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
Strain HA14 TATGATGCTT TGGTTAGAAT GGCTCAAGAT TTTTCTATGA GATATCCAAG
GCC to GCT (A-120-A)
610 620 630 640 650
L04566 Ref TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGTGCCGCTG
Strain HA1 TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGCGCTGCTG
Strain HA2 TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGCGCTGCTG
Strain HA7 TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGTGCTGCTG
Strain HA8 TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGCGCTGCTG
Strain HA9 TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGCGCTGCTG
Strain HA13 TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGCGCTGCTG
Strain HA14 TATTACAGGA CAAGGCAACT TTGGATCTAT AGATGGTGAT AGCGCTGCTG
AGT to AGC (S-119-S)

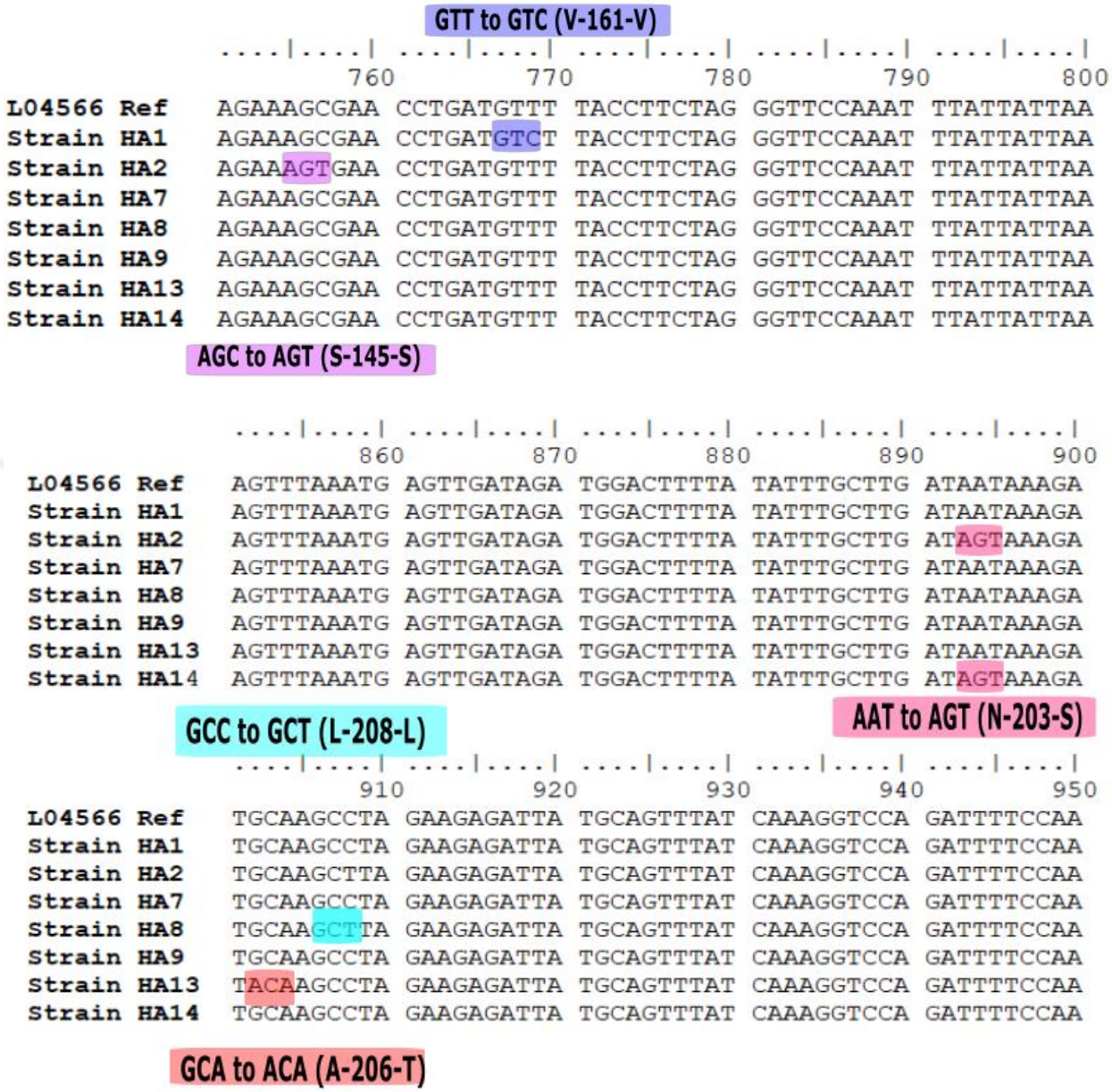


Figure 4.5. Comparison of *C. jejuni gyrA* QRDR DNA sequences. Sequence L04566 is a portion of the *gyrA* QRDR of *C. jejuni* UA580 (GenBank no. L04566). All isolates have a T86-to-I (ACA-to-ATA) *gyrA* mutation frequently associated with fluoroquinolone resistance. Silent mutations were noted at amino acid positions 21, 81, 119, 120, 145, 161 and 208. One of the other 2 novel mutations is located at the 203 positions as N203 to-S (AAT-to-AGT) for HA2 and HA14 and the other at the 206 positions as A206 to-T (GCA to ACA) just for HA13.

CHAPTER 5

DISCUSSION

When we examine the studies conducted in Turkey on human campylobacteriosis cases, it is observed that various studies have been conducted on the genetic analysis of antibiotic resistance of *C. jejuni* isolates. However, enough studies are needed to explain the situation in terms of genetics. An increasing number of reports are accumulating around the world that *C. jejuni* isolates have developed resistance to therapeutic antibiotics. Concerns have been raised that this strain of bacteria has become more resistant over the years, particularly to fluoroquinolones. Similar concerns have been shared by researchers in Turkey, and studies since 1989 reveal that this bacterium was initially sensitive to fluoroquinolones, but gradually developed resistance over the years. While no resistance was observed in the first known study in Turkey in 1989, subsequent studies show that the development of resistance has increased over the years and reached 74.3% in 2013 [93]. In another study reported from Turkey, it was stated that the *C. jejuni* isolate, isolated from humans and stored at -20 °C for about 20 years, showed quinolone resistance at a rate of 42.5%. All these studies depicted that the development of resistance has increased dramatically over the years and reached 94% in the current study, and that treatment with quinolone group antibiotics will not be possible soon [94]. This study was conducted to determine the quinolone resistance of Campylobacter isolates genetically, as well as to determine the prevalence of Campylobacter isolates in patients who applied to Erciyes University hospitals with diarrhea, to diagnose them at the species level, and to reveal the therapeutic power of two other antibiotics such as tetracycline and erythromycin. Ciprofloxacin resistance in *C. jejuni* was 95.23% in the present research, compared to 70.6% in the Turkish study by Savasan et al (2004) [95]. When many literature studies on the subject are examined, quinolone resistance for *Campylobacter* species worldwide emerged at the end of the 1980s and has increased greatly since then, and these findings indicate that antibiotic

therapy is safe for Campylobacter. In other areas of the globe, the prevalence of Campylobacter infection in humans is quickly rising. Since the increasing use of antibiotics for animal production in Europe, USA, and Asia causes an increase in quinolone resistance in human *Campylobacter* isolates, some countries such as Australia, Denmark and Finland limit the use of quinolones in animal production, and there is low and moderate resistance development in these countries. The rate of resistant strains in Denmark's own production poultry was found to be much lower than in imported products [96, 97, 98, 99,100, 101and102].

All these reports show that while *Campylobacter* species had minimal or no resistance to antimicrobials nearly 30 years ago, especially fluoroquinolones (FQs), they have become nearly 100% resistant to FQs in almost all continents of the world today. This situation necessitates the fact that FQs will be excluded from treatment in the near future and that other groups of antimicrobials currently provide treatment should be used with caution and resistance processes followed more closely[67].

Especially in the summer months, with the increase in diarrhea cases, approximately 500 rectal Enterobacterales samples are sent to the Central Microbiology Laboratory from Erciyes University Hospitals every week, 14% of which are identified as *Campylobacter* spp. Due to the increasing resistance development all over the world, especially in children, the use of ciprofloxacin in Erciyes University Hospitals should be reconsidered and its use should be limited except for patients with suppressed immunity and chronic diseases.

In many studies, the mutation in the *gryA* gene, especially Thr-86 to Ile (ACT to CATT), was determined to be the main cause of ciprofloxacin resistance, and the presence of this mutation was screened with the relevant primers in various studies and its relationship with resistance was clearly determined. It was emphasized that too many mutations in this *gryA* gene would increase ciprofloxacin resistance day by day and the importance of sequencing studies to detect the mutation [103]. In addition to the presence of Thr-86 to Ile mutation in all 7 isolates sequenced in the current study, 3 other novel mutations were detected, indicating mutation in the gene continues, explaining the high resistance rate of 95.23% in the current study. That 7 silent

mutations determined in this study and another 4 reported previously in the same region shows that the mutation process continues increasingly [104].

Current study also focused on the *C. jejuni* and *C. coli* resistance to erythromycin and tetracycline. *C. jejuni* has been studied in more detail in other studies and in the current study as it is isolated at a higher rate and is the main cause of severe bacterial diarrhea [105 and 106]. In the current study, 1 (12.5%) of 8 *C. coli* isolates were resistant to erythromycin, while none of 42 *C. jejuni* isolates were resistant. Previously, the development Macrolide resistance has been investigated in different countries [107]. Previous reports generally depicted the prevalence of erythromycin resistance of *Campylobacter* isolates (including both *C. jejuni* and *C. coli*) from chickens and cattle lover than 10%. However, a report from the USA revealed that more than 40% of *C. coli* isolated from poultry and pigs exhibited erythromycin resistance [108]. While reports from various countries represented susceptibility of *C. jejuni* to macrolides, increasing data are accumulating about *C. coli*'s resistance [109]. The detection of resistant isolates, even among a small number of *C. coli* isolates in the current study, necessitates close monitoring of the situation in the relevant hospital, and other studies also point to this issue [110]. While no resistant isolates were observed in many *C. jejuni* isolates, this situation should be followed carefully for *C. coli.* The reason why *C. coli* has higher macrolide resistance needs to be investigated in detail.

As a result of conventional phenotypic studies such as culturing, blood agar, selective medium and gram staining, the isolation rate of *Campylobacter* was observed to be 35.3% in stool samples used in the current study. However, there are reports that the *Campylobacter* isolation rate is lower in similar studies conducted in previous years. This indicates that the incidence of *Campylobacter* isolates has increased over the years. For example, in a study conducted in the UK in 2002, the isolation rate of *Campylobacter* was found to be approximately 6.7% in the screening of selective media [111]. Again, in Kayseri, with similar phenotypic methods, isolation rates were found to be as 1.43% and 4.5% in 2007 and 2013, respectively, and the current study shows that the isolation rate has increased many times over the years [112 and 93]. Numerous scientific reports indicate that the incidence and prevalence of Campylobacteriosis are increasing at significant rates in developed and developing countries, while the dramatic increase in North America, Europe and Australia is alarming [113].

The results of the present study reveal that campylobacteriosis is seen at a much higher rate in children aged 0 to 10 years. The incidence decreases with increasing age, and bacteria have not been isolated from males and females over the age of 41 and 55, respectively. In addition, men are affected more than women at all age levels. In accordance with our findings, other studies also showed that young children and young men are highly affected [114, 115 and 116].

The higher incidence of infection in children and young men suggests that these people have more contact with poultry and birds and use more polluted water [117 and 117]. Of course, it is wise to consider the prevalence of infection is also significantly affected by population-level immunity. In most developing countries, campylobacteriosis infection is generally limited to children, and infection rates decrease with age, suggesting that early exposure to infection will provide protective immunity [119, 120 and 121].

It is very difficult to identify *Campylobacter*-like microorganisms based on phenotypic features, as they do not use carbohydrates and have little metabolic activity [122]. *Campylobacter* spp. identification by serological, biochemical and molecular methods requires high cost and time, so the differentiation of organisms with MALDI-TOF MS is completed in a shorter time and is more rapid. In this study, 51 Campylobacter strains as *C. jejuni* and *C. coli* could be identified in a few minutes with MALDI TOF MS. The method gives very good results and has been made suitable for routine use [Beamish et al 2001]. *Bacillus spp*. strains isolated from bovine rectal swabs are difficult to identify with these conventional methods as their growth pattern and colonial morphology are very similar to those of Campylobacter, and therefore MALDI-TOF mass spectrometry has been greatly improved in terms of delay, cost, and accuracy [124 and 125].

In most studies, *Campylobacter* isolates were identified at genus level using phenotypic methods, but from 2003 to 2009, identifications at species level could be made by molecular and standard phenotypic methods. The use of MALDI-TOF MS after 2010 enabled the identification of *C. jejuni* and *C. coli* strains with 99.4% and 100% accuracy, respectively. In studies conducted in northern European countries, it was stated that the rate of *C. coli* was much lower than that of *C. jejuni*, which is consistent with ours. Another study showed *C. jejuni* infection had a distinct seasonality, unlike *C.*

coli infection, and therefore its incidence was 2 times higher than *C. coli*. Also, summer eating patterns differ, with more barbecued, undercooked chicken eaten and possible salad cross-contamination [126]. In the current study, there is a 4.5-fold difference between the rates of infection caused by *C. jejuni* and *C. coli* with MALDI-TOF MS analysis, and the rates were determined as 82.23% and 17.64%, respectively. Again, a report from Turkey that the rate of *C. jejuni* is much higher than that of *C. coli* confirms our findings [127]. This indicates that especially poultry-derived foods are more contaminated with *C. jejuni* during the warmer period of the year.

As a result, a generalization can be made about this study. Previous reports from the same region indicate that the prevalence of *Campylobacter* isolates has increased over time. This indicates a more complex situation, especially due to the development of antibiotic resistance. We can conclude that the use of ciprofloxacin should be avoided and directed to erythromycin in the treatment of enteritis caused by *Campylobacter*. Considering all these results, it is certain that campylobacteriosis will be among the most important infectious diseases that may threaten global health in the coming years. In the coming years, it will be of great importance to study the resistance mechanisms developed against various antibiotics, especially genetically, to monitor the development of resistance and to develop more strategic treatment methods.

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Appendix (2)

Comparison of conventional traditional method findings with MALDI-TOF MS for 50 stool samples in this study

No	Gander /Age	Conventional culturing method					**MALDI-TOF MS**
		Gram staining	Selective media	AST / MIC(µ g / ml)			
				TE	CIP	E	
HA1	F/64	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA2	M/5	+	+	R<30	R<26	S≥24	*C. jejuni*
HA3	M/1	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA4	F/41	+	+	S≥30	R<26	S≥20	*C. coli*
HA5	F/21	+	+	S≥30	S≥50	S≥24	*C. jejuni*
HA6	M/4	+	+	R<30	R<26	S≥24	*C. jejuni*
HA7	M/2	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA8	M/11	+	+	R<30	R<26	S≥24	*C. jejuni*
HA9	M/8	+	+	R<30	R<26	S≥24	*C. jejuni*
HA10	M/11	+	+	R<30	R<26	S≥24	*C. jejuni*
HA11	M/11	+	+	R<30	R<26	S≥20	*C. coli*
HA12	M/2	+	+	S≥30	S≥50	S≥24	*C. jejuni*
HA13	F/8	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA14	M/4	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA15	M/10	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA16	M/13	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA17	M/12	+	+	R<30	R<26	S≥24	*C. jejuni*
HA18	M/55	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA19	M/10	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA20	M/15	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA21	F/10	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA22	M/11	+	+	R<30	R<26	S≥24	*C. jejuni*
HA23	M/9	+	+	R<30	R<26	S≥24	*C. jejuni*
HA24	F/1	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA25	M/8	+	+	R<30	R<26	S≥24	*C. jejuni*
HA26	M/2	+	+	R<30	R<26	S≥24	*C. jejuni*
HA27	M/3	+	+	S≥30	S≥50	R<24	*C. coli*
HA28	F/13	+	+	R<30	R<26	S≥24	*C. jejuni*
HA29	F/10	+	+	R<30	R<26	S≥24	*C. jejuni*
HA30	M/3	+	+	R<30	R<26	S≥24	*C. jejuni*
HA31	M/52	+	+	R<30	R<26	S≥20	*C. coli*
HA32	M/9	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA33	F/7	+	+	R<30	R<26	S≥24	*C. jejuni*
HA34	M/19	+	+	R<30	R<26	S≥24	*C. jejuni*
HA35	M/6	+	+	R<30	R<26	S≥24	*C. jejuni*
HA36	F/5	+	+	R<30	R<26	S≥24	*C. jejuni*
HA37	M/3	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA38	M/6	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA39	M/2	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA40	M/39	+	+	S≥30	R<26	S≥24	*C. jejuni*
HA41	M/12	+	+	S≥30	R<26	S≥20	*C. coli*
HA42	M/20	+	+	S≥30	R<26	S≥20	*C. coli*
HA43	M/10	+	+	R<30	R<26	S≥24	*C. jejuni*
HA44	F/3	+	+	R<30	R<26	S≥24	*C. jejuni*
HA45	M/10	+	+	R<30	R<26	S≥20	*C. coli*
HA46	F/14	+	+	R<30	R<26	S≥20	*C. col*
HA47	M/2	+	+	R<30	R<26	S≥24	*C. jejuni*
HA48	M/13	+	+	R<30	R<26	S≥24	*C. jejuni*
HA49	M/14	+	+	R<30	R<26	S≥24	*C. jejuni*
HA50	M/8	+	+	R<30	R<26	S≥24	*C. jejuni*

*AST: Antibiotic Susceptibility Testing, MIC: Minimum Inhibitory Concentration, CIP (Ciprofloxacin), TE (Tetracycline), E (Erythromycin), M: Male, F: Female, (+): Positive, (-): Negative, R: Resistant S: Sensitive.

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