Molecular and Phenotypical Investigation of Ciprofloxacin Resistance Among

Campylobacter jejuni strains of Human Isolates: High Prevalence of Resistance in Turkey

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ABSTRACT

Introduction: Intestinal infections associated with *Campylobacter* species are one of the most frequently reported zoonosis worldwide. There has been a well-documented increase in the ciprofloxacin resistance in strains, which has increased the importance of rapid detection of the resistance.

Aim: To investigate, using real-time PCR, the incidence of ciprofloxacin resistance among *C. jejuni* strains isolated from humans in Turkey.

Methodology: One hundred and fifty eight *C. jejuni* strains isolated from stool samples of patients were included in the study. The genus and species level identification of the strains were performed by PCR. Minimum inhibitory concentration of ciprofloxacin was determined by Epsilometer test. A cytosine to thymine point mutation at codon 86 was detected by allelic discrimination using Aria-Mx real time PCR system.

Results: Of the 158 strains, 114 (72.2%) were determined to be resistant to ciprofloxacin. The MIC_{50} and the MIC_{90} of ciprofloxacin was found to be 8 and ≥ 32 mg l^{-1}, respectively. By real time PCR, the presence of the mutation was confirmed in all, but one, resistant strain and the absence of the mutation was demonstrated in all, but one, susceptible strain. Allelic discrimimnation could not be determined for two strains.

Conclusion: The rate of ciprofloxacin resistance is high among *C. jejuni* strains and ciprofloxacin should not be used in the treatment of such infections in Turkey. A cytosine to tyhmine mutation at 86th codon is the most frequently detected mechanism for the resistance among *C. jejuni* strains in Turkey. Real time PCR can be used accurately for the quick screening of the resistance.

INTRODUCTION

Campylobacteriosis has been the most frequently reported zoonosis in the European Union (EU) since 2005. In 2017, the European Food Safety Administration reported 246,158 total confirmed cases and the incidence rate of 65.8 cases per 100,000 people [1], although this is likely to be a large underestimation. In the same year, campylobacteriosis surpassed salmonellosis among foodborne infections in the United States of America with the incidence of 19.2 per 100,000 [2]. Although the data related with the incidence of the infection in Africa and Asia are limited, campylobacteriosis is also thought to be endemic in these two continents [3]. *Campylobacter jejuni* is responsible for the majority of infections followed by *C. coli* and these two species together are responsible for more than 90% of human infections [1, 4]. *C. jejuni* infections in humans are generally foodborne and undercooked meat of animals, especially chicken, contaminated with *Campylobacter* is the main source of the infection [4].

Intestinal infections of *C. jejuni* in people are generally self-limiting and resolve in a few days without the need for antibiotic treatment. However, treatment may be required for patients who are at their age extremes, immunosupressed, have prolonged or severe enteritis or sytemic infections [5, 6]. Erythromycin is the first choice of antibiotic for the treatment of confirmed cases of *Campylobacter*. However, since the aetiology of acute gastroenteritis is not generally investigated, ciprofloxacin, a fluoroquinolone, may be prescribed as part of the empirical treatment of undifferentiated diarrhea [6, 7]. However, the rate of ciproflocaxin resistance among *Campylobacter* strains of human origin has been increasing worldwide since the late 1990s in parallel with the increase in the use of other fluoroquinolones in animal husbandry since the mid1990s [3, 7, 8]. Fluoroquinolone resistant *Campylobacter* strains have been classified recently among the "high priority" pathogens related to the discovery, research and development of new antibiotics for drug resistant bacterial infections [9].

Ciproflocaxin resistance mechanisms among *C. jejuni* isolates have been demonstrated to be due to chromosomal mutations in the quinolone resistance determining region (QRDR) of the *gyrA* gene. Although several other mutations have also been reported, a single point mutation C257T in *gyrA,* that results in threonine to isoleucine substitution at the amino acid position 86, has been recognized to be the most frequent mechanism [5, 10]. Detection of this mutation has been the focus of the methods used for the investigation of the molecular mechanisms of

ciprofloxacin resistance. Many of the molecular methods used for the detection of the mutation necessitate the use of agarose gel electrophoresis, which makes the process time-consuming and also liable to contamination. Real-time PCR based allelic discrimation depends on the competition between two probes, one specific for wild type and the other for mutant DNA, that are labelled with different reporter fluors. The technique was reported to discriminate the wild type and mutant strains accurately based on the relative fluorescence emission of the reporters [11].

Campylobacteriosis is one of the most important travel-associated infections. Although the majority of cases reported in EU are domestic, Turkey is the second most important non-EU country that is responsible for travel-associated *Campylobacter* infections in EU [1]. *Campylobacter* infections with ciprofloxacin resistant strains have been demonstrated to be travel-associated [6, 12]. More than 50% of travel-related *Campylobacter* infections in the United States of America, Denmark, United Kingdom and Norway were shown to be due to ciprofloxacin resistant strains [13-16]. Studies related with the rate of ciprofloxacin resistance and its molecular mechanisms among *Campylobacter* isolates of humans in Turkey, a country that is highly attractive to tourists, are limited [17, 18]. It is evident that studies investigating the rate of ciprofloxacin resistance and its mechanism among *Campylobacter* strains in Turkey will provide valuable data about the treatment of the infection not only for Turkey but also for other countries where travel to Turkey is frequent.

In the present study, the aim was to determine the ciprofloxacin resistance and its molecular mechanism among *C. jejuni* strains isolated from human clinical samples in Turkey and to investigate the use of real time PCR for the quick detection of the resistance.

MATERIALS AND METHODS

One hundred and fifty eight *C. jejuni* strains isolated from stool samples of patients between 2004 and 2010 were included in the study. Strains were identified to genus and species level by PCR [19, 20]. *C. jejuni* ATCC 33560 was used as positive control for the genus and species level PCR.

Phenotypical Ciprofloxacin Resistance

The minimum inhibitory concentration (MIC) of ciprofloxacin was determined by the Epsilometer test on Mueller Hinton agar (Oxoid, Basingstoke, UK) supplemented with 5% horse blood. The resistance of the strains was assessed according to the breakpoint (MIC>0.5 mg l^{-1}) suggested by European Committee on Antimicrobial Susceptibility Testing (EUCAST) [21]. *C. jejuni* NCTC 11168 and *Staphylococcus aureus* ATCC 29213 was used as quality control strains for the growth control of the media and the control of the susceptibility testing, respectively. Statistical analysis of the difference in the rate of the resistance in between 2004-2008 and 2009-2010 was carried out by chi-squared rest and p≤0.05 was regarded as significant.

Real-time PCR

DNA extraction was carried out using a commercially available kit (High Pure Template Preparation Kit, Roche, Germany) according to manufacturer`s recommendations. Singleplex real-time PCR was performed using the primers and probes as described previously [11]. Briefly, the forward (5' TGG GTG CTG TTA TAG GTC GT 3') and reverse (5' GCT CAT GAG AAA GTT TAC TC 3') primers together with the wild-type probe TAQ2 (5' FAM-CCA CAT GGA GAT ATA GCA GTT TAT GAT G-TAM 3') or mutant probe TAQ3 (5' JOE-CCA CAT GGA GAT ATA GCA GTT TAT GAT GC-BHQ1 3') was carried out using AriaMx Real Time PCR Sytem (Agilent Technologies, Cheshire, UK). A 25 µl final reaction mixture contained 10 ng template DNA, 1X Master Mix (Agilent Technologies, Cheshire, UK), 0.8 µM forward and reverse primers and 160 nM TAQ2 or TAQ3. PCR parameters were adjusted as follows: 95°C 10 min, 40 cycles of 95°C 1 min and 55°C 1 min. For each run, nuclease free water, DNA of *C. jejuni* NCTC11168 and that of a strain which was determined to have C257T mutation by sequencing was used as non-template, mutation-negative and mutation-positive control, respectively. Efficiencies of the singleplex real-time PCR reactions were determined by serially diluting 10 ng DNAs of mutation-negative and mutation-positive control strains.

Molecular Detection of Ciprofloxacin Resistance: Allelic Discrimination

Detection of cytosine to thymine point mutation at 257th nucleotide of QRDR of *gyrA* was carried out by allelic discrimination using AriaMx real-time PCR system (Agilent Technologies, Cheshire, UK). The reaction mixture was the same as used in real-time PCR described above

except that the target DNA was 1 ng. Both probes were included in the reaction at 160nM and 30 nM ROX was used as passive reference dye for the normalization of the reporter fluor signal. PCR parameters for allelic discrimination were the same as used in real-time PCR [11]. Non-template, mutation-negative and mutation positive control were used for each run as described in real time PCR.

RESULTS

Phenotypical Ciprofloxacin Resistance

Of the 158 strains, 80 and 78 were isolated in between 2004-2008 and 2009-2010, respectively. One hundred and fourteen (72.2%) strains were determined to be resistant to ciprofloxacin. Ciprofloxacin resistance rate was 67.5% in 2004-2008 and 76.9% in 2009-2010. The increase in the resistance rate between two periods was not statistically significant. Thirty eight percent of strains were determined to have high level of resistance with MICs of 32 mg l^{-1} and above. In both periods, MIC_{50} and MIC_{90} of ciprofloxacin was found to be 8 and ≥ 32 mg l^{-1}, respectively. The distribution of ciprofloxacin MICs of the strains is shown in the Table-1.

Table-1. Ciprofloxacin MICs of 158 *C. jejuni* strains [n (%)].

| MIC (mg l^{-1})	2004	2005	2006	2007	2008	2009	2010	Total
0.015	0	0	0	0	1	2	1	4
0.03	4	1	1	3	6	9	3	27
0.06	0	2	2	3	3	3	0	13
2	0	0	0	1	0	3	1	**5 (3.2)**
4	0	2	0	5	2	5	2	**16 (10.1)**
8	2	4	0	8	4	9	5	**32 (20.3)**
16	0	0	0	0	0	0	1	**1 (0.6)**
≥32	1	1	1	12	11	20	14	**60 (38)**
Total	7	10	4	32	27	51	27	158

* The number of resistant strains is underlined.

Real-time PCR and Allelic Discrimination

The efficiencies of real-time PCR reactions for mutation-negative and mutation-positive control strains were found to be 93% and 105% using TAQ2 probe and 93% and 96% using TAQ3 probe, respectively. One strain which was susceptible to ciprofloxacin (MIC:0.03 mg l^{-1}) did not yield any amplicon with either of the probes and no amplicon was detected for another strain that was resistant to ciprofloxacin (MIC:32 mg l^{-1}) with TAQ2 probe. All other strains gave positive result in the singleplex PCRs with both probes.

Upon allelic discrimination, all strains, but one, that were susceptible to ciprofloxacin by E-test (MIC>0.5 mg l^{-1}) gave a FAM signal (ΔRn:FAM) greater than 0.076 and a JOE signal (ΔRn:JOE) less than 0.066. All strains, but one, that were were resistant to ciprofloxacin had the signal of ΔRnFAM<0.073 and ΔRnJOE>0.066 (Figure-1). One strain that was susceptible and another one that was resistant to ciprofloxacin did not yield any amplicon with either of the probes and TAQ2, respectively. Allelic discrimination could not be determined for these two strains.

Figure-1: An illustration of allelic discrimination.
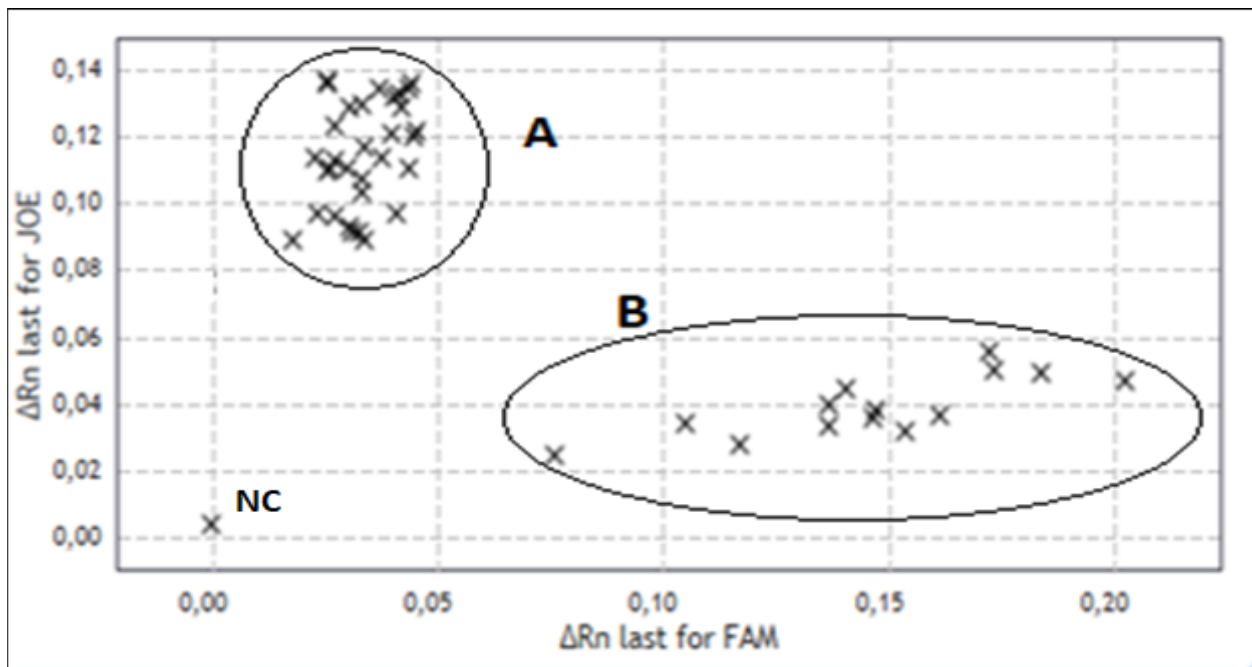
* **A:** Resistant strains with ΔRnFAM<0.073 and ΔRnJOE>0.066 ; **B:** Wild-type strains with ΔRn:FAM>0.076 and ΔRn:JOE <0.066 ; **NC:** Non-template control

DISCUSSION

Campylobacteriosis is a zoonosis that transmits to humans mainly via consumption of chicken meat and is the leading cause of foodborne infections in Europe. There was a substantial increasing trend in the number of reported confirmed cases between 2008 and 2017 from 40.4 to 64.8 per 100,000 population in EU [1, 22]. Similar to many EU countries, the isolation rate of *Campylobacter* spp. from patients with acute gastroenteritis was reported to be high, at 5.4%, in Turkey [23].

Although antibiotic treatment is not neccessary for the majority of cases, ciprofloxacin was the drug of choice when treatment was required until the mid-2000s. Due to the emergence of ciprofloxacin-resistant strains of human origin worldwide coinciding with the use of fluoroquinolones in food producing animals, erythromycin replaced ciprofloxacin for treatment. However, because the infection can be clinically indistinguishable from acute enteritis caused by other pathogenic bacteria that are generally susceptible to the antibiotic, ciprofloxacin may still be used for the treatment of campylobacteriosis especially in regions where *Campylobacter* is not investigated routinely. This would eventually lead to the failure of the treatment [4]. Therefore, identifying the infectious agent responsible for gastroenteritis and rapid detection of the ciprofloxacin resistance have clinical importance in terms of the treatment of the infections.

Various antibiotic susceptibility testing methods, including disk diffusion, agar dilution, broth dilution and E-test have been used for the detection of ciprofloxacin resistance among *Campylobacter* isolates. Although EUCAST [21] and Clinical Laboratory Standard Institute (CLSI) [25] recommend broth microdilution method for the determination of the MIC, E-test was reported to be a reliable quantitative method for the investigation of ciprofloxacin resistance showing excellent concordance with microdilution [26, 27].

A high rate (73.4%) of ciprofloxacin resistance among *C. jejuni* human isolates detected in the present study shows similarity to the limited number of previous studies carried out in this country [17, 18]. The high rate of fluoroquinolone resistance among *C. jejuni* chicken isolates reported in a recent study in Turkey may reflect the over-use of the antibiotic in animal husbandry, which may be responsible for the high rate of resistance among human strains detected in the present study [28]. Likewise, high rate of resistance was reported from various European countries

such as Portugal, Estonia, Lithuania, Italy and Spain [29] and from countries in Asia including Korea [30], China [31] and India [32]. On the other hand, the rate of resistance was reported to be low in Australia [33] and Sweeden [34]. The geographical difference in the prevalence of the resistance may be due to the difference in the rate of the use of ciprofloxcacin in human infections and livestock production. The correlation between the over-use of the antibiotic among animals and the increasing rate of resistance among *Campylobacter* of human origin has been well described [3, 35, 36].

Ciprofloxacin resistance among *C. jejuni* strains is predominantly as a result of chromosomal mutations related with the *gyrA* gene that is the target of the antibiotic. Although numerous different mutations have been reported, a cytosine to thymine transition at the 257th nucleotide (86th codon) is the most frequently detected mutation that is responsible for ciprofloxacin resistance [5, 10]. In contrast to many other bacteria, mutations that result in the development of resistance have been reported to increase the fitness of *Campylobacter* spp. leading to the persistence of the resistant strains even after cessation of the antibiotic pressure [12, 36-39]. Such a phenomenon increases the importance of the emergence of resistant strains due to the over- and mis-use of fluoroquinolones in animal husbandry and humans resulting in the treatment failure for over a long period of time.

Various methods have been used for investigating the molecular mechanisms of the ciprofloxacin resistance among *C. jejuni* strains and the majority of them rely on the detection of the point mutation at the codon 86 of *gyrA* gene. The two most frequently used methods, mismatch amplification mutation assay (MAMA) and PCR-RFLP, were demonstrated to be accurate but labourious because of the need of agarose gel electrophoresis [11, 40, 41, 42]. Fluorogenic PCR depends on the the use of two probes, one specific for the wild-type and the other one for the mutant strains, which are tagged with different reporter fluors in a competitive manner in a multiplex real time PCR reaction. The method is practical because it obviates the need for post-PCR manipulation. Wilson DL *et al*. [11] reported that the technique is sensitive and rapid for the the detection of cytosin to thymine transition that is related with ciprofloxacin resistance. Mutant strains that were resistant and wild type strains that were susceptible to ciprofloxacin were reported to yield different emission of the reporter fluors leading to the determination of the presence or absence of the mutation [11]. In the present study, the same primers and probes as suggested by

Wilson DL *et al.* [11] were used first time for the allelic discrimination using an AriaMx Real Time PCR Sytem. PCR detected that all of the resistant and susceptible strains, but two, had a higher emmision of reporter fluor tagged to mutant and wild-type probe, respectively. Thus, all of the strains, except two, that were phenotypically resistant or susceptible to ciprofloxacin were determined to have the mutation or not, respectively. Of the two strains that the real time PCR method is inefficient for determining the presence or absence of the mutation, one (ciprofloxacin susceptible) did not give any amplicon with both probes and the other one (ciproflocaxcin resistant) did not give any amplicon with TAQ2. The most reasonable explanation is that these strains carry mutation(s) in the regions of DNA where the probes bind [11].

In many studies, strains with a ciprofloxacin MIC of 4 mg l^{-1} and above were reported to have the related mutation where as those with MIC of 0.5 mg l^{-1} and lower did not [40, 43-49]. In a study carried out by Padungtod *et al.* [50], fluorogenic PCR was used to detect the molecular mechanisms of the resistance and the mutation was found to be present in the *gyrA* of the strain with ciprofloxacin MIC of 2 mg l^{-1}; although the strain was considered as susceptible since the breakpoint for the resistance was regarded as 4 mg l^{-1} and over according the CLSI. In another study in which breakpoint for resistance was accepted as 0.5 mg l^{-1} in accordance with EUCAST, strains with ciprofloxacin MIC of 1 and 2 mg l^{-1} were found to have the mutation [41]. On the other hand, Hormeno *et al.* [42] did not detect any mutation by MAMA-PCR in a strain with ciprofloxacin MIC of 2 mg l^{-1}. Such discrepancies between the MIC and the presence/absence of the mutation may be due to the differences in the methods used for the detection of the mutation.

There are studies reporting the absence of C257T mutation in some high level resistant strains indicating that the mutation in question is not the only mechanism for the resistance [51, 52]. Point mutations other than Thre86Ile at the codon of 86 such as Thre86Ala, Thre86Lys, Thre86Val and mutations outside the codon such as Asp90Asn and Asp90Tyr, that are all related with the resistance, have been reported [53, 54, 55]. Moreover, in addition to the point mutations in *gyrA*, a CmeABC efflux pump has also been reported to contribute to the development of ciprofloxacin resistance [53-57]. In a recent study, a resistance-enhancing variant of the efflux pump that was linked to the enhanced multidrug resistance has been discovered in *C. jejuni* of animal origin [58].

Although numerous *gyrA* mutations related to the emergence of ciprofloxacin resistance have been reported in other countries, in two studies carried out in Turkey, one related with *C. jejuni* of human and the other of chicken isolates, all of the strains that were resistant to ciprofloxacin was determined to have C257T mutation at the codon of 86 [17, 28]. Parallel to these studies, in the present study, strains that were found to be resistant and susceptible to ciprofloxacin were determined to give different emissions of the reporter fluors with wild type and mutant probes in allelic discrimination using Aria-Mx real time PCR system indicating that the system can be used accurately for the quick screening of the resistance. Moreover, the present study highlights the high rate of ciprofloxacin resistance and the fact that ciprofloxacin should not be used in the treatment of *Campylobacter* infections in Turkey, and that the threonine to isoleucine transition at 86th codon is the most frequently detected mechanism of the resistance among *C. jejuni* strains.

Authorship Contributions

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Conflict of interest.

The authors declare that there are no conflict of interests

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