Prevalence and Antimicrobial Resistance of Thermophilic Campylobacter Isolates from Raw Chicken Meats

Ghassan ISSA 1  Beren BASARAN KAHRAMAN 2  Mehmet Cemal ADIGUZEL 3  Funda YILMAZ EKER 4  Esra AKKYAYA 4  Gülay Merve BAYRAKAL 4  Ahmet KOLUMAN 5  Tolga KAHRAMAN 4

1 Avrupa Vocational School, TR-34020 Kazlıçeşme, Istanbul - TURKEY
2 Department of Microbiology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcilar, Istanbul - TURKEY
3 Department of Microbiology, Faculty of Veterinary Medicine, Atatürk University, TR-25240 Yavuzlu, Erzurum - TURKEY
4 Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcilar, Istanbul - TURKEY
5 Faculty of Biomedical Engineering, Pamukkale University, TR-20070 Denizli - TURKEY

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Abstract

Globally, the spread of antibiotic resistance via chicken meat consumption cause serious public health concerns. With this respect, the current study aimed to investigate the prevalence of thermophilic Campylobacter species isolated from raw meat samples and their genetic determinants of resistance to various classes of antibiotics. A total of 540 chicken raw meat samples collected from various supermarkets and slaughterhouses in Istanbul, Turkey were analyzed according to EN ISO 10272-1:2006 standard procedure. For identification of the genus and species of the isolates, multiplex PCR assay was held. Minimum inhibitory concentrations of the antimicrobial agents (nalidixic acid, ciprofloxacin, tetracycline, gentamicin, kanamycin, and erythromycin) were initially determined using the broth microdilution method. In addition, the genetic determinants of antimicrobial resistance were investigated by PCR assays. In total, 357 (66.1%) Campylobacter isolates were obtained including 268 Campylobacter jejuni and 89 Campylobacter coli. Resistance to quinolones (nalidixic acid and ciprofloxacin) was the most common in all strains (80.1%), followed by resistance to tetracyclines' (70.3%). The lowest resistance was determined as resistance to kanamycin (4.2%). Gentamicin and erythromycin resistance was not observed in this study. Only five C. coli isolate (1.4%) was classified as multidrug resistant. On the basis of these data, execute widely presence of antimicrobial resistance to quinolones and tetracyclines' in C. jejuni and C. coli isolates from chicken raw meat samples and emphasizes that further multidisciplinary studies and novel strategies in the concept of ‘One Health’ are needed.

Keywords: Campylobacter, Raw chicken meat, Prevalance, Antimicrobial resistance, PCR

Çiğ Tavuk Etlerinden İzole Edilen Termofilik Campylobacter İzolatlarının Prevalansı ve Antimikrobiyal Direnci

Öz


Anahtar sözcükler: Campylobacter, Çiğ tavuk eti, Prevalans, Antimikrobiyal direnç, PCR

İletişim (Correspondence)

+90 212 4737070/17360
beren@istanbul.edu.tr
INTRODUCTION

Poultry is an extremely high nutritive versatile meat, which is of a great importance for human nutrition, so the safety protection measures of poultry meat are very important subject [1]. Thermophilic Campylobacter, including Campylobacter jejuni and Campylobacter coli, is a main bacterial cause of acute gastroenteritis in humans. Raw poultry products are the main reservoir of thermophilic Campylobacter infection in particular via consumption of undercooked products or cross-contamination of ready-to-eat products [2,3]. Also, Campylobacter infection is associated with the development of Guillain-Barre’ syndrome, a neurological disorder affecting the peripheral nervous system. Particularly, the chicken is a natural host of C. jejuni and serves as a major reservoir for this pathogenic organism. Contamination of chicken carcasses often occurs during the slaughtering process and consumption of chicken meat is a significant source of human Campylobacter infections [2-4].

The emergence of antimicrobial resistance is not a new phenomenon, nor an unexpected one. Several reports have been published about antibiotic resistance problem and the reasons behind the increasing rates. These reports have highlighted that poultry meat may play a major role in transmission [3,9]. The uncontrolled and excessive use of antibiotics in the treatment of infections in humans and veterinary medicine may be the reason for high rates of resistance, in poultry [1,10]. In Turkey, antibiotics feed additives were widely used for control of the growth in poultry, but in 2006 the usage of antibiotics in broiler flocks were forbidden by the European Union (EU) Council Directive 90/167/EEC [11,12]. All of the countries in EU have flocks were forbidden by the European Union (EU) Council Directive 90/167/EEC [11,12]. All of the countries in EU have been started to investigate the prevalence of Campylobacter spp. in broiler carcasses and the antimicrobial resistance in broiler flocks [10]. However, the number of the studies on antibiotic-resistant Campylobacter isolated from poultry meat in Turkey, is rather limited.

This study was aimed to carry out to determine the prevalence and antimicrobial resistance of thermophilic Campylobacter species isolated from chicken raw meat samples available in retail trade in Istanbul, Turkey.

MATERIAL and METHODS

Sample Collection

A total of 540 chicken raw meat samples including chicken thigh, breast and wings were collected from various supermarkets and slaughterhouses in Istanbul, Turkey, between January 2015 and March 2016. With this aim each month, 6 thigh, 6 breast, and 6 wings were obtained from slaughterhouse and same amounts were collected from different markets. A sum of 540 samples were analysed for Campylobacter contamination.

Campylobacter species detection and isolation were performed according to EN ISO 10272-1:2006 standard procedures [13]. A 25 g portion of each sample was homogenized in a stomacher and were enriched in Bolton broth (Oxoid, USA) for 4 h at 37°C and then incubated for up to 44 h at 42°C under microaerophilic conditions created by using a CampyGen gas pack (Oxoid, USA). The enriched samples were subsequently subcultured by spreading 10 µL aliquots on modified Charcoal Cefoperazone Deoxycholate agar (CCDA, Oxoid, USA) and incubated for up to 48 h at 42°C under microaerophilic conditions. Suspected colonies were cultured onto plates of Columbia Blood agar (Oxoid, USA) containing 5% horse blood, and were confirmed by microscopic analysis, oxidase testing (Oxoid, USA), microaerophilic growth at 25°C and aerobic growth at 42°C. The remainder of each plate was harvested and stored in 1 mL of nutrient broth plus 10% glycerol at 80°C. Conventional culture method was verified using ISO 16140 method. According to this method 30 positive and 30 negative samples were analysed using the method. The results obtained showed a specificity and sensitivity of 95%.

For identification of the genus and species of the isolates, multiplex PCR was carried out following the PCR assay method described by Linton et al.[14] and Denis et al.[15]. Simultaneous amplification of 16SrRNA gene fragment (genus-specific), mapA gene (for C. jejuni) and ceeE gene (for C. coli) was carried using primers and protocol. The details of primers and cycling conditions are given in Table 1. Amplified PCR products were visualized by electrophoresis in 1.5% agarose gel stained with ethidium bromide. For quality control, C. jejuni ATCC 33291, C. jejuni ATCC 33560 and C. coli ATCC 33559 strains were used.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MIC) of antimicrobial agents (ciprofloxacin, erythromycin, gentamicin, kanamycin, nalidixic acid and tetracycline) was determined with a microbroth dilution method [16]. The clinical breakpoints were interpreted according to the EUCAST [16] guidelines for Campylobacter as regards erythromycin, nalidixic acid, gentamicin, ciprofloxacin and tetracycline, and to CLSI guidelines for Enterobacteriaceae [17] as regards kanamycin (MIC≤16 susceptibility, MIC=32 intermediate, MIC≥64 resistant), because there was no ECOFFS for Campylobacter.

Campylobacter jejuni ATCC 33560 was used as reference strains for quality control assurance in each batch of broth microdilution plates [16].

Detection of Antimicrobial Resistance Genes

All of the phenotypically resistant isolates were analyzed for the presence of ery, tet(O), aphA-3, gyrA (Thr-86-Ile
mutation), \textit{cme}A, \textit{cme}B and \textit{cme}C genes, representing resistance to erythromycin, tetracycline, aminoglycoside, and quinolones, and CmeABC efflux system components, respectively.

Mismatch Amplification Mutation Assay (MAMA-PCR) for the detection of point mutations at position 2075 and 2074, which present high-level erythromycin resistance, were performed [18]. Genes \textit{tet}(O) and \textit{aph}A-3 were detected by PCR assay as described [19]. Thr-86-Ile mutations in the quinolones resistance determining region (QRDR) of gene \textit{gyr}A were detected by MAMA-PCR [20,21]. The presence of the \textit{cme}A, \textit{cme}B and \textit{cme}C genes were determined by PCR assays [22]. The primers sequences, product sizes and cycling conditions are listed in Table 2.

Multi-drug resistance (MDR) was defined as resistance to three or more antimicrobial agents with different mechanisms of action, as previously described [23].

### RESULTS

The prevalence rate of \textit{Campylobacter} spp. in chicken raw meat samples were found in 66.1%. Monthly distribution is summarized in Fig. 1. Totally 357 \textit{Campylobacter} isolates, whereas \textit{C. jejuni} was identified in the remaining 268 (75.07%) and \textit{C. coli} 89 (24.93%).

Distribution of \textit{C. jejuni} according to tight, breast and wing samples were 73 (27.23%), 106 (39.55%) and 89 (33.22%). The presence of \textit{C. jejuni} from different parts at slaughterhouse level was not significant with months and no seasonal change was observed. On the contrary, seasonal distribution of \textit{C. jejuni} was observed in market samples. \textit{C. jejuni} was mostly isolated during summer months with a rate of 88.88% (48 of 54 samples) and was lowest during January with a rate of 5.56% (1 of 18 samples).

### Table 1. Primer sequences, product sizes and cycling conditions

<table>
<thead>
<tr>
<th>Primer Specific For</th>
<th>Target(s)</th>
<th>Primers (5' to 3', as synthesized)</th>
<th>Size (bp)</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter</td>
<td>16S rRNA</td>
<td>ATCTATGTCATTACATTTAATACCCAGACGCTAAGGCAG</td>
<td>857</td>
<td>95°C 60 s; 95°C 15 s; 59°C 60 s; 72°C 90 s (35 cycles); 72°C 3 min</td>
</tr>
<tr>
<td>\textit{C. jejuni}</td>
<td>mapA</td>
<td>CTATTTATTTTTTTGAGTTCGTCGTCGGTTATTGCAATTGTTATTATTATTATA</td>
<td>589</td>
<td></td>
</tr>
<tr>
<td>\textit{C. coli}</td>
<td>cceE</td>
<td>AATGGAATGTTGCACTCAACTTATGTAATTTTATTGACAGGGCG</td>
<td>462</td>
<td></td>
</tr>
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</table>

### Table 2. Primer sequences, product sizes and cycling conditions

<table>
<thead>
<tr>
<th>Primer Specific For</th>
<th>Target(s)</th>
<th>Primers (5' to 3', as synthesized)</th>
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<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin resistance</td>
<td>23S rRNA-F</td>
<td>TTAAGCTAATGTTGCCCGTACCGAGCCAACCTTTGTAAGCCTCCG</td>
<td>697</td>
<td>94°C 5 min; 94°C 30 s; 59°C 30 s; 72°C 45 s; (30 cycles); 72°C 5 min</td>
</tr>
<tr>
<td>\textit{C. jejuni} mapA</td>
<td>GZgyrA5-F</td>
<td>ATTTTATAGCAGATGTTGTGAGTTAGTATT</td>
<td>857</td>
<td></td>
</tr>
<tr>
<td>\textit{C. coli} ceuE</td>
<td>GZgyrA4</td>
<td>ATTATTTTCAAAAAATTCTCAGTATCAAACTTTAGCAGGCA</td>
<td>265</td>
<td>94°C 3 min; 94°C 30 s; 50°C 30 s; 72°C 20 s; (30 cycles); 72°C 5 min</td>
</tr>
<tr>
<td>Quinolones resistance</td>
<td>GZgyrAcoll3F-F</td>
<td>GZgyrAcoll4R-R</td>
<td>192</td>
<td>95°C 7 min; 95°C 1 min; 72°C 2 min (30 cycles); 72°C 5 min</td>
</tr>
<tr>
<td>Aminoglycoside resistance</td>
<td>apha-3 F</td>
<td>apha-3 R</td>
<td>600</td>
<td>95°C 30 s; 55°C 1 min; 72°C 1 min (30 cycles); 72°C 5 min</td>
</tr>
<tr>
<td>Tetracycline resistance</td>
<td>tetO F</td>
<td>tetO R</td>
<td>559</td>
<td>95°C 7 min; 95°C 15 s; 58°C 15 s; 72°C 30 s; (30 cycles); 72°C 5 min</td>
</tr>
<tr>
<td>cmeABC</td>
<td>cmeF-F</td>
<td>cmeF-R</td>
<td>435</td>
<td>94°C 7 min; 94°C 1 min; for cmeA 49.8°C, for cmeB 50.8°C, for cmeC 52.3°C 90 s; 72°C 2.5 min (31 cycles); 72°C 5 min</td>
</tr>
<tr>
<td>\textit{C. jejuni} mapA</td>
<td>GZgyrA5-F</td>
<td>GZgyrA6-R</td>
<td>673</td>
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<td></td>
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</table>
Prevalence and Antimicrobial Resistance...

Resistance to quinolones (nalidixic acid and ciprofloxacin) was the most common finding (80.1%), followed by resistance to tetracyclines (70.3%). Conversely, the lowest resistance was recorded against to kanamycin (4.2%). Furthermore, all isolates were detected susceptible to gentamicin and erythromycin. Only five C. coli isolates (1.4%) were evaluated as multidrug resistant.

The antibacterial susceptibility testing results of 357 Campylobacter isolates against six different antibacterial agents are exhibited in Table 3.

The phenotypic and genotypic results were fully concordant. Comparison of phenotypic and genotypic resistance to antimicrobial agents was shown in Table 4.
In this study, all isolates were resistant to at least one antibacterial agent, while most of the isolates were resistant to tetracycline, nalidixic acid, and ciprofloxacin. 20% of the isolates were resistant to two antibacterial agents and 1.4% of the isolates to more than two antibiotics.

DISCUSSION

Poultry products are the most important single source of human Campylobacteriosis. The European Food Safety Authority (EFSA) reported 246,307 laboratory confirmed cases in the EU [24]. Turkey was one of the most often reported as the probable country of infection outside EU (5.5%). Han et al. [31] in Korea, Guyard-Nicodème et al. [27] in France, and Maesaar et al. [26] in Estonia were reported Campylobacter spp. prevalence from broiler chicken meat 68.3%, 76%, and 88.8%, respectively.

Regarding previous studies in Turkey, Hızlısoy et al. [33] found 100% of the chicken meat samples positive for Campylobacter species. Abay et al. [34] reported that among 100 carcass samples examined, 96% C. jejuni strains were isolated. In this study, it has been demonstrated that Campylobacter spp. are frequently present (66.1%). Withal, when comparing the reported prevalence of Campylobacter spp. among our country during recent years, the results of the present study are considerably lower. Seasonal distribution of samples were showing similarity with the results of Koluman [26] and Pamuk [27].

High fluoroquinolones resistance levels among Campylobacter poultry meat isolates have been widely stated, in Poland [5,28], Italy [29], Turkey [6], Korea [9], and many other European countries [30]. In the current study, resistance to quinolones (nalidixic acid and ciprofloxacin) was the most common and these results substantiate other authors’ findings. The broad use of this class of antibiotics in poultry may be the reason for this crucial problem.

The tetracycline’s, being the first major group of antimicrobial agents, are among the most frequently used therapeutics in veterinary medicine. In the current study, the resistance rate to tetracycline was determined as 70.3%. The prevalence was higher in comparison to those detected by Abay et al. [34], Guyard-Nicodème et al. [27], Maesaar et al. [26], Wei et al. [31], Wieczorak et al. [19].

The aminoglycosides are a group of antimicrobials used both in human and veterinary medicine. Gentamicin is the most widely used aminoglycoside in poultry. EFSA [31] reported that the gentamicin resistance was comparatively very low (0.3%) in C. jejuni isolates and resistance were not detected in C. coli isolates from broiler meat. Wei et al. [29] call attention to the high prevalence of gentamicin-resistant Campylobacter isolated in food-producing animals in China. Moreover, low to moderate resistance ranging from 0 to 27% was observed in various studies [32-34]. Kanamycin is an aminoglycoside antibiotic which is effective in the treatment of severe infections caused by Gram-negative bacteria [8]. In the current study, all isolates were susceptible to gentamicin. Also, kanamycin resistance was determined in 15 strains (4.2%).

Macrolides are still the most effective antibiotics against Campylobacter infections. Macrolide resistance in Campylobacter spp. has been the result of the point mutation(s) occurring in ribosomal RNA or proteins. The authors reported high resistance to erythromycin in Spain [31]. However, in European countries, low resistance levels were stated from 0 to 8% [32]. According to EU summary report, the variable occurrence of resistance to erythromycin among Campylobacter species were reported, depending on the country of isolation [24]. In this study, all of the isolates were susceptible to erythromycin which is the drug of choice for the treatment of human Campylobacteriosis. This result is in agreement with those reported for chicken meat isolates Wieczorak et al. [19] in Poland and Guyard-Nicodème et al. [27] in France. Because of the low level of resistance might be consequences of the ban of macrolides as a growth promoter in broilers.

Otherwise, except these individual resistance mechanisms, multidrug efflux system CmeABC contributes to Campylobacter resistance to multiple drugs, including fluoroquinolones, β-lactams, erythromycin, and tetracycline [19,36]. The authors indicated that the effect of CmeABC on aminoglycoside resistance (like gentamicin) was less apparent [36]. In this study, only five C. coli isolate (1.4%) was classified as multidrug resistant. Contrary, the authors reported much higher percentages ranged from 44.9 to 86% [34,37]. The use of antimicrobial drugs in food animals has been regulated in European countries, the conflicted results may base on the implementation of legislation. In some developing countries, even where legislation does exist and is enforced, their enforcement may be a problem and virtually non-existent [10,37]. The absence and/or weakness of regulations and implementation particularly about usage of antibiotics in the food animals, also inadequate hygiene and sanitation, may have accelerated the emergence and dissemination of antimicrobial resistance.

Over recent decades, antibiotic resistance undoubtedly represents a global public health problem. The global author’s highlight that poultry meat is an important risk of human exposure to antimicrobial resistance due to residual resistance of high impact antibiotic application of 20th century or illegal applications as growth promoters [34,39]. In the current study provides baseline information on the highlights the widespread presence of this emerging foodborne pathogen and resistance profiles in poultry meat. These data emphasize that further multidisciplinary studies, surveillance programmes and reports in animals, and humans, as well as food, are important in terms of manifesting the current status of resistance against antimicrobial drugs and emerging
health problems. Therewithal, the data acquired here will be useful for risk assessment for public health hazard C. jejuni. It is significant that the population and demographic character of Istanbul is highly variable with widespread chicken consuming behaviour. This picture can be generalized with significant variations of other cities to determine a significant hazard map to prevent C. jejuni borne infections.

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