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Phenotypic and genotypic examination of antimicrobial resistance in thermophilic *Campylobacter* species isolated from poultry in Turkey

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Abstract

Introduction: The study aimed to isolate thermophilic *Campylobacter* from chickens raised three rearing methods, determine its antimicrobial susceptibilities, and examine resistance-related genes by PCR. **Material and Methods:** Cloacal swabs or intestinal contents were taken in Istanbul, Sakarya, and Izmir provinces. Chickens were from small village-based family-run businesses (n = 70), organically raised (n = 71), and conventionally raised broilers (n = 79). The samples were cultured on modified charcoal cefoperazone desoxycholate (mCCD) agar. Suspect isolates were identified with multiplex PCR (mPCR). As per EUCAST standards, MIC values were derived by broth microdilution for tetracycline, ciprofloxacin, nalidixic acid, kanamycin, gentamicin, and erythromycin in isolates of *C. jejuni* (n = 98) and *C. coli* (n = 83). **Results:** In *C. jejuni*, 78.6% tetracycline, 87.8% ciprofloxacin, and 81.6% nalidixic acid resistance was detected, but none was to kanamycin, gentamicin, or erythromycin. In *C. coli*, 98.8% ciprofloxacin and 63.9% nalidixic acid resistance was detected, whereas resistance to non-quinolones was not observed. C257T (Thr-86-Ile) mutation in the *gyrA* gene of all phenotypically quinolone-resistant isolates was detected through a mismatch amplification mutation assay PCR (MAMA-PCR). It emerged that all isolates bore the *tet* (O) resistance gene. **Conclusion:** Common tetracycline, nalidixic acid, and ciprofloxacin resistance exists in *Campylobacter* isolated from chickens raised three rearing methods.

Keywords: chicken, antimicrobial resistance, PCR, stools, thermophilic *Campylobacter*.

Introduction

Thermophilic *Campylobacter* species, including *Campylobacter jejuni* and *Campylobacter coli*, are among the most common bacterial gastroenteritis agents in both developed and developing countries (29). Thermophilic *Campylobacter* species are flora bacteria in chicken and poultry intestines, these being the main reservoir for this pathogen. Infection in humans occurs as a result of intake of water and food contaminated with *Campylobacter* by the digestive system. Poultry-based foods play a particularly important role in the spread of the disease (29). Thermophilic *Campylobacter* infections are also closely related to Guillain-Barré syndrome, a neurological disorder (10, 25, 29).

Macrolide, aminoglycoside, tetracycline, and especially fluoroquinolone antibiotics are frequently used for campylobacteriosis treatment in human medicine. These groups of antibiotics are also widely used in veterinary medicine. Unconscious and unnecessary use of them causes resistant strains infecting people through contaminated nutrients (5, 9, 29).

In Turkey and in other countries, there have been many studies with the purpose of determining the prevalence of antimicrobial-resistant *Campylobacter* species (1, 4, 5, 6, 11, 16, 17). In Turkey, where poultry

production represents an important sector of animal husbandry and significant supplier to poultry consumption, but also outside the country, it is important to reveal the current state of antimicrobial resistance of *Campylobacter* isolated from poultry. In this study, we aimed to examine thermophilic *Campylobacter* isolated from chickens raised in different ways and specifically to examine possible phenotypic and genotypic resistance of the isolates to tetracycline, ciprofloxacin, nalidixic acid, kanamycin, gentamicin, and erythromycin.

Material and Methods

Sample collection. Between October 2014 and May 2015, a total of 220 cloacal swabs or intestinal contents were taken from chickens in Istanbul, Sakarya, and Izmir provinces. These were chickens typical of those raised in villages by small family-run businesses (n = 70), organically reared animals (n = 71), and conventionally reared broilers (n = 79) (Table 1). The samples were delivered to the laboratory as soon as possible in cold containers and examined bacteriologically without any delay.

Isolation. Stool swabs taken directly and in a sufficient amount to represent the intestinal contents comprehensively were planted onto the Campylobacter selective supplement modified charcoal cefoperazone desoxycholate (mCCD) agar surface. This microaerobic medium was incubated for 24–48 h at 42°C and the colonies were first evaluated in terms of colony morphology and colour. The Gram characteristics of the colonies were determined, and suspicious colonies were purified by passage through blood agar. Catalase positive/negative, oxidase-positive isolates were examined for their motile ability, and those which were mobile were considered suspicious (7).

Table 1. Samples and their origin

Source		Aim to raise	Rearing method	Use of antibiotics
Province/District	Number of samples			
Izmir	1–71	Broiler	Organic	Unused
Sakarya	72–150	Broiler	Conventional	Unknown
Istanbul				
Catalca-1	151–166	Layer hen	Village	Unknown
Catalca-2	167–175	Layer hen	Village	Unknown
Arnavutkoy-1	176–193	Layer hen	Village	Unknown
Arnavutkoy-2	194–206	Layer hen	Village	Unknown
Avcilar	207–220	Layer hen	Village	Unknown

Table 2. Primers and amplicon lengths used in the study

Target gene		Amplified gene	Primer sequence (5'-3')	Size (bp)	References
mPCR	*Campylobacter* spp. (16S rRNA)	MD16S1 MD16S2	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTATT	857	(26)
	C. jejuni (*mapA*)	MDmapA1 MDmapA2	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA	589	
	C. coli (*ceuE*)	COL3 MDCOL2	AATTGAAAATTGCTCCAACTATG TGATTTTATTATTTGTAGCAGCG	462	
MAMA-PCR	Quinolone resistance gene	CampyMAMAgryA-F CampyMAMAgyrA-R	TTTTTAGCAAAGATTCTGAT CAAAGCATCATAAACTGCAA	265	(18, 35)
		CampyMAMAgryA1-F GZgyrA4	TTTTTAGCAAAGATTCTGAT CAGTATAACGCATCGCAGCG	368	
		GZgyrACcoli3F-F CampyMAMAgyrA8-R	TATGAGCGTTATTATCGGTC TAAGGCATCGTAAACAGCCA	192	(18, 34)
		GZgyrACcoli3F-F GZgyrACcoli4R-R	TATGAGCGTTATTATCGGTC GTCCATCTACAAGCTCGTTA	505	
Erythromycin resistance gene		23S rRNA-F 23S rRNA-R	TTAGCTAATGTTGCCCGTACCG AGCCAACCTTTGTAAGCCTCCG	697	(3)
		ERY2075-R	TAGTAAAGGTCCACGGGGTCGC	485	
		ERY2074-R	AGTAAAGGTCCACGGGGTCTGG	485	
Kanamycin resistance gene		*aphA*-3 F *aphA*-3 R	GGGACCACCTATGATGTGGAACG CAGGCTTGATCCCCAGTAAGTC	600	(15)
Tetracycline resistance gene		*tetO* F *tetO* R	GGCGTTTTGTTTATGTGCG ATGGACAACCCGACAGAAGC	559	(22)
CmeABC efflux system		*cmeA*- F *cmeA*- R	TAGCGGCGTAATAGTAAATAAAC ATAAAGAAATCTGCGTAAATAGGA	435	(27)
		cmeB- F *cmeB*- R	AGGCGGTTTTGAAATGTATGTT TGTGCCGCTGGGAAAAG	444	
		cmeC- F *cmeC*- R	CAAGTTGGCGCTGTAGGTGAA CCCCAATGAAAAATAGGCAGAGTA	431	

The identification of isolates for *Campylobacter* spp. (16S rRNA; 23), *C. jejuni* (*mapA* gene), and *C. coli* (*ceuE* gene) was performed by multiplex mPCR (Table 2, 26). To extract genomic DNA, a loopful of bacterial colonies harvested from agar plates was suspended in 0.5 mL of sterile water, heated at 95°C for 10 min, and centrifuged at 5,000 rpm for 5 min at 4°C (20). Amplification of the chromosomal region was performed with a PCR mixture which contained 5 µL of 10× PCR buffer, 1.5 mmol of $MgCl_2$, 2 µL of dNTP mix (2.5 mM each of deoxynucleoside triphosphate), 1 µL of forward and reverse primers (2.5 pmol/µL MD16S1/S2, 10 pmol/µL MDmapA1/A2, and 10 pmol/µL COL3/MDCOL2), 0.2 µL of Taq polymerase (5 U/µL, Takara Bio Inc, Japan), 5 µL of target DNA, and up to 50 µL of distilled water. Amplification was performed in a Maxygene thermal cycler (Axygen, USA) with 35 cycles of 95°C for 60 s as initial denaturation, 95°C for 15 s as denaturation, 59°C for 60 s as the annealing step, 72°C for 90 s as extension, and 3 min as the final extension step at 72°C. The products obtained after PCR were subjected to electrophoresis at 200 V in 1% agarose gel for 30 min and were stained with ethidium bromide (0.5 µg/mL) (26). *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used as positive control strains.

Antimicrobial susceptibility test. Phenotypic antimicrobial resistance evaluation of the isolates to ciprofloxacin, erythromycin, gentamycin, kanamycin, nalidixic acid, and tetracycline was performed with the broth microdilution method (12). Cation-adjusted Mueller-Hinton broth (CAMHB, Oxoid, UK) enriched with 5% haemolysed defibrinated horse blood and containing 20 mg/L of β-nicotinamide adenine dinucleotide (β-NAD) was used in order to determine the minimum inhibitory concentration (MIC). Antibiotics diluted in appropriate concentrations were distributed to 50 µL microplates. A bacterial suspension was prepared at a density of 0.5 McFarland with 24 h bacterial culture in tryptic soy broth (TSB), diluted to 1:100 with TSB, and was distributed to all wells in 50 µL volumes. In this way, the liquid in each well totalled 100 µL. The last well containing the suspension of media and bacteria was evaluated as a negative control. The microplate was capped and allowed to incubate for 24 h at 42°C. At the end of the incubation, the lowest antimicrobial concentration without bacterial growth was recorded as the MIC value. To check the accuracy of the assay, 10 µL of negative control suspension was spread over the blood agar surface and was incubated. At the end of the incubation, 20–80 colonies demonstrated the accuracy of the test. The *C. jejuni* ATCC 33560 strain was tested as a quality control. Microplates were incubated at 42°C for 24 h in microaerobic (5% CO_2) conditions. Thermophilic *Campylobacter* isolates resistant to three or more antimicrobial classes were defined as multidrug resistance isolates (12, 19).

Determination of antimicrobial resistance genes. The isolates phenotypically determined as resistant were examined by PCR in the broad sense of antimicrobial resistance (3, 15, 22, 27, 34, 35). A total of 25 µL of PCR mixture contained 2.5 µL of 10× PCR buffer, 1.5 µL of $MgCl_2$ (25 mM), 1.25 µL of dNTP (2 mM), 0.25 µL of each primer (1.0 mg/mL), 0.2 µL of Taq polymerase (5 U/µL, Takara Bio Inc, Japan), and 1 µL of target DNA. The amplified PCR products were viewed on 1.5% agarose gel. The primer sequences and amplification conditions used in the study are demonstrated in Table 2.

Statistical analysis. The SPSS package programme (IBM, USA) was used for the statistical analysis. Pearson's Chi-squared (x^2) test was used for comparisons, and P values <0.05 were considered significant (28).

Results

In total 181 (82.3%) *Campylobacter* spp. were isolated from cloacal swabs and intestinal contents. The distribution of isolates according to breeding types is shown in Table 3. *C. coli* was isolated in all samples from conventional breeding, while *C. jejuni* was isolated only in village-reared chickens and organically reared broilers.

While the isolates from organic broilers and village chickens were resistant to ciprofloxacin, nalidixic acid, and tetracycline, the isolates of conventional broilers were found to be resistant to ciprofloxacin and nalidixic acid (Table 4). No isolate was determined as multiple antibiotic resistant.

Table 3. The proportion of thermophilic *Campylobacter* strains isolated from cloacal and intestinal swab samples

Rearing method	Sample number	*Campylobacter* spp. (%)	*C. jejuni (%)*	*C. coli (%)*
Village	70	36 (51.4)[†]	34 (48.6)[‡]	2 (2.9)[‡]
Organic	71	66 (93.0)[‡]	64 (90.1)[§]	2 (2.8)[‡]
Conventional	79	79 (100.0)[§]	-	79 (100.0)[§]
Total (%)	220	181 (82.3)	98 (44.6)	83 (37.7)

[†, ‡, §] The statistical difference between the ratios with different symbols in the same column is significant (P < 0.05)

Table 4. Resistance rates of thermophilic *Campylobacter* isolates according to the method of raising

Isolate	Antibiotic	Resistance status	Resistant isolates (%)			Chi-squared (P value)
			Village-raised (n = 34)	Organic (n = 64)	Conventional (n = 0)	
C. jejuni	Tetracycline	Resistant	13 (38.2)†	64 (100.0)‡	-	50,310 (<0.001)
		Non-resistant	21 (61.8)	0 (0.0)	-	
	Ciprofloxacin	Resistant	22 (64.7)†	64 (100.0)‡	-	(<0.001)*
		Non-resistant	12 (35.3)	0 (0.0)	-	
	Nalidixic acid	Resistant	17 (50.0)†	63 (98.4)‡	-	34,744 (<0.001)
		Non-resistant	17 (50.0)	1 (1.6)	-	
	Kanamycin	Resistant	-	-	-	-
	Gentamycin	Resistant	-	-	-	-
	Erythromycin	Resistant	-	-	-	-
Isolate	Antibiotics	Resistance status	Village-raised (n = 2)	Organic (n = 2)	Conventional (n = 79)	Chi-Squared (P value)
C. coli	Tetracycline	Resistant	-	-	-	-
	Ciprofloxacin	Resistant	2 (100.0)†	1 (50.0)§	79 (100.0)‡	(0.048)**
		Non-resistant	0 (0.0)	1 (50.0)	0 (0.0)	
	Nalidixic acid	Resistant	2 (100.0)†	-	51 (64.6)†	(0.542)*
		Non-resistant	0 (0.0)		28 (35.4)	
	Kanamycin	Resistant	-	-	-	-
	Gentamycin	Resistant	-	-	-	-
	Erythromycin	Resistant	-	-	-	-
Total			36	66	79	

†, ‡, § The statistical difference between the ratios bearing different symbols on the same line is significant
* Fisher's exact test value was applied because cells have expected count less than 5
** Fisher's exact test for a 2×3 contingency table was applied

It was revealed by the PCR that all isolates that were phenotypically resistant to ciprofloxacin (86 *C. jejuni* and 82 *C. coli*) contained point mutations in the *gyrA* gene of Thr-86-Ile of the DNA gyrase enzyme. The chain reaction also showed that all isolates that were phenotypically tetracycline-resistant (77 *C. jejuni*) contained the *tet* (O) gene involved in the synthesis of the ribosomal protective protein.

Discussion

This study clearly demonstrates that thermophilic *Campylobacter* species are commonly seen in chickens raised by three different methods. The antimicrobial resistance rate differs according to the chicken rearing method and this difference stands out in conventional broiler isolates. Isolates with multiple antimicrobial resistance were not detected in this study.

While both *Campylobacter* species (*C. jejuni* and *C. coli*) were isolated from organic broilers and village chickens, only *C. coli* was isolated from conventionally reared broilers (P = 0.048). *C. jejuni* was the most dominant microorganism isolated from both organic broilers and village chickens. This finding is similar to those of previous studies (5, 33). The isolation of only *C. coli* from the samples from conventionally reared chickens was determined to be derogative finding. There are, however, studies indicating that *C. coli* is isolated as the dominant species in commercial ducks and organic and free-range chickens (24, 29). It was thought that the possibility of isolating *C. coli* from conventionally reared broilers may depend on the hygiene of the poultry and shelter, the type of breeding of the animals, the season, and the drugs used.

The quinolone group antibiotics were used as feed additives in previous years (30). El-Adawy *et al.* (9) reported resistance to nalidixic acid and ciprofloxacin in organically grown turkeys. It has been shown that quinolone-resistant *Campylobacter* strains in the environment could be identified on a 30-metre wind-exposed field and that quinolone-resistant *Campylobacter* strains could also contain quinolone-sensitive strains even in the absence of antimicrobial use. This finding is consistent with other studies showing that some quinolone-resistant strains can survive on farms for several rotations (24). The detection of nalidixic acid and ciprofloxacin resistance from organic farming isolates in this study was found to be statistically significant (P < 0.001). It was thought that the detection of this resistance could be a legacy effect of the production by the farms where the samples were collected of reared broilers in previous years. While the use of quinolone antibiotics in village-raised chickens and floor-reared broilers is unknown, high resistance to nalidixic acid and ciprofloxacin is detected. Alfredson and Korolik (2) studied poultry coops and reported that the quinolones used in the treatment of infections led to the development of ciprofloxacin-resistant *Campylobacter* by their entering the food chain as a result of selective effect. These authors stated that a large number of resistant clones had been selectively transferred as a result of quinolone treatment. It has been shown in previous studies that

the use of quinolone antibiotics as feed additive increases the incidence of quinolone resistance in thermophilic *Campylobacter* isolates in Turkey (1, 4, 6, 30, 32). The high quinolone resistance in this study is compatible with the findings of researchers both in Turkey and in other countries.

C257T (Thr-86-Ile) mutation has been nominated as the main resistance mechanism in quinolone resistance (34, 35). While Aslantaş (4) discovered the same mutation on all his isolates, Kurekci and Onen (21) reported Ala40Ser mutation in addition to this mutation. In this study, the statistical significance of the C257T (Thr-86-Ile) mutation in the *gyrA* gene was found to be significant for all isolates resistant to ciprofloxacin ($P < 0.001$). This finding is similar to other studies showing the significance of an increase in resistance (8, 9, 18). In this study, tetracycline resistance was detected in *C. jejuni* strains isolated from organic broiler production ($P < 0.001$), and all these isolates carried the *tet* (O) gene, which was compatible with the results of Hungaro *et al.* (18). High tetracycline resistance in thermophilic *Campylobacter* strains isolated from the organic production type has also been reported in another study (24). Since antibiotics of the tetracycline group have been used as feed additives for both treatment and protection in farms and poultry for a long time (14), resistant strains could have been transmitted over the years and can be found extensively in animal housing structures regardless of the rearing method. This finding revealed that antibiotic use was not the only reason for the development of resistant bacteria.

No isolates from any of the three different production types showed resistance to kanamycin, gentamicin, or erythromycin in the study. It seems that there is low resistance against these antibiotics, as demonstrated in other studies in Turkey (1, 6, 17). According to an erythromycin resistance report concerning with European countries; it was reported to be 0% in Lithuania, Austria, and Denmark, 2% in Germany, 7% in Portugal, and 8% in Belgium (13). It was posited that the low resistance to these antibiotics was due to their rare use in prophylaxis and therapy in the poultry industry.

In this study, isolates carrying multiple antibiotic resistance were not detected. Elsewhere, multiple-antibiotic-resistant isolates have been reported in some studies (1, 4, 31). The difference between reported rates could depend on the antibiotic test sensitivity of methods used by researchers, the number of antimicrobial agents, and the method of sampling.

The literature reveals that the antimicrobial resistant *Campylobacter* isolates can be transmitted and are long-lasting. It also brings to light that antibiotic use is not the only reason for the development of resistant bacteria and reveals the problem of removing resistant isolates from farms. The rate of antimicrobial-resistant thermophilic *Campylobacter* increases every year. Particularly, against the antibiotics such as the quinolone group which are no longer used as feed additives, the resistance is bordering on becoming permanent. Widespread resistance to quinolone and tetracycline, determined even in chickens which had no history of antibiotic use, compels us to apply these antimicrobials nationally only in a controlled and conscious manner.

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