Research Article

Prevalence, Detection of Resistance Genes and Antimicrobial Resistance of *Campylobacter jejuni* in Broilers in North Macedonia

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Abstract | *Campylobacter jejuni* is one of the most important food borne pathogens. Since the start of the 21st century *C. jejuni* is the leading cause for food borne enteritis. Another point of attention is the change in the antimicrobial resistance of this microorganism towards some critical antimicrobials used in the human and veterinary medicine. In this study samples were taken from three points in the broiler meat production (farm, slaughter line and cold storage of the meat before shipping to the market). A total of 283 samples (cloacal swabs, caeca and carcass swabs) were analyzed for the presence of *C. jejuni*. The isolates of *C. jejuni* were confirmed with the conventional microbiological method and with the use of multiplex PCR method. Both methods confirmed the overall prevalence of *C. jejuni* of 39.2%. In the second part of the study 108 confirmed isolates of *C. jejuni* were analyzed for the presence of resistance genes (*CmeB*, Bla_{oxa-61}, *tet(O)*, *aph-3-1* and *aadE*). The analysis in the third part of the study was concentrated on the antimicrobial resistance of the *C. jejuni* isolates towards three important antimicrobials (ciprofloxacin, erythromycin and tetracycline). The PCR method used revealed highest prevalence for Bla_{oxa-61} (25%), followed by *CmeB* and *tet(O)* genes (19.4%) and *aadE* with 13.9%. The *aph-3-1* gene was not detected in none of the *C. jejuni* isolates. *C. jejuni* isolates in this study showed the highest resistance towards ciprofloxacin (63%) and tetracycline (50%) while the resistance towards erythromycin was very low (5.6%).

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Introduction

The natural habitat for *Campylobacter* spp. is the intestinal tract, specifically the mucosal cells of mammals and birds. Confirmed sources for infections in humans are poultry meat (cross contamination, undercooked meat), contaminated water, contact with animals and raw milk. The highest frequency of isolation in poultry meat is detected for *C. jejuni*, followed by *C. coli*, and *C. lari* (Hansson *et al.*, 2018). *Campylobacter*iosis in humans is usually characterized by mild symptoms and in rare cases neurological problems can occur (Silva *et al.*, 2011). Antimicrobial therapy for the infection is usually not needed because of the self-limiting character but in prolonged cases that may be necessary. Drugs of choice in this case are fluoroquinolones (ciprofloxacin) and macrolides (erythromycin) (Blaser and Engberg, 2008; Zhang

et al., 2020). Tetracycline have been suggested as an alternative choice in the treatment of clinical *Campylobacter*iosis, but in practice they are not often used.

Regarding the classes of antibiotics there are several mechanisms of resistance noticed in *Campylobacter*s for each of them. *Campylobacter* quinolones resistance is mediated by the CmeABC efflux pump and also through a single point mutation in the *gyrA* gene determining area of quinolone resistance (Iovine, 2013; Shen *et al.*, 2018; Whitehouse *et al.*, 2018). *Campylobacter* resistances to tetracycline have been mediated by the protein of ribosomal protection *TetO* that is encoded by the *tet(O)* gene. The *tetO* gene is common in *C. coli* and *C. jejuni*. *C. jejuni* aminoglycoside resistance is achieved through aminoglycoside changing enzymes (Sat, aacA AadE, AphD and AphA) that are encoded by plasmid genes. *Campylobacter* has four main mechanisms for macrolides resistances including: efflux by CmeABC efflux pump, methylation of the ribosome encoded by ermB gene, ribosomal proteins target mutations and mutation in the 23S rRNA gene (Bolinger and Kathariou, 2017). β-lactams resistance in *Campylobacter* spp. is usually mediated by β-lactamases enzymes, which break down the β-lactam ring structure (Iovine, 2013).

Aims of this study were: to detect the prevalence of *C. jejuni* in the broiler production chain in North Macedonia, to analyze the antimicrobial resistance towards three important antimicrobials (ciprofloxacin, erythromycin and tetracycline) and to check for presence of resistance genes in the confirmed isolates of *C. jejuni*.

Materials and Methods

Collection of samples

This study was conducted in North Macedonia during 2017. A total of 283 samples were collected from one farm and one slaughterhouse in the Skopje region of North Macedonia

One week before slaughter cloacal swabs were taken at farm level (n= 64). Cloacal swab samples were collected using sterile cotton swabs and placed in tubes containing 5 ml of Preston broth.

Caeca were collected (n= 166) at the slaughter line during the evisceration phase broiler and packed in sterile bags.

Swab samples from broiler carcasses (n=53) were taken from the storage area before shipping to the consumers (cold chamber of the slaughterhouse) using sterile cotton swabs and placed in tubes containing 5 ml of Preston broth.

After the collection, the samples were transferred to the laboratory at an appropriate temperature (4-8°C) and were analyzed 3-4 hours after sampling.

Isolation and confirmation of Campylobacter spp.

The isolation and identification of thermotolerant *Campylobacter* was done according to the ISO 10272-1 (ISO, 2017). The positive isolates were sub-cultured on mCCDA agar plates and stored in glycerol broth at -80°C.

Extraction of DNA and PCR analysis

The confirmed *Campylobacter* isolates were used for extracting the DNA with the conventional boiling method. The procedure included suspension of the cultures in 0.5 ml of TE buffer, boiling at 95°C for 10 min and centrifugation at 15000 rpm for 5 minutes. The supernatants were kept at -20°C and used for both PCR methods.

The multiplex PCR for identification of *Campylobacter* spp. was done according to the previously published study by Wang *et al.* (2002). The list of the used primers is shown in Table 1.

The multiplex PCR for detection of the resistance genes in the *C. jejuni* isolates was performed as proposed in the published study by Obeng *et al.* (2012). The list of the used primers is shown in Table 2.

For the antimicrobial testing of the isolates disk diffusion method (Kirby Bauer method) was used (EUCAST, 2020). The inoculums were prepared with density adjusted to 0.5 McFarland turbidity standard. The inoculum was delivered with sterile swabs on Columbia blood agar and the following antimicrobial agents were used: erythromycin, ciprofloxacin and tetracycline. The EUCAST breakpoints (EUCAST, 2020) were used for the classification of the isolates (Table 3).

Table 1: *Primer sequences used in the multiplex PCR assay and the expected sizes of the products (18).*

Target gene	Primer name	Sequence (5'–3')	Annealing temperature (°C)	Product (bp)
C. jejuni hipO	CJF	ACTTCTTTATTGCTTGCTGC	59	323
	CJR	GCCACAACAAGTAAAGAAGC		
C. coli glyA	CCF	GTAAAACCAAAGCTTATCGTG	59	126
	CCR	TCCAGCAATGTGTGCAATG		
C. lari glyA	CLF	TAGAGAGATAGCAAAAGAGA	59	251
	CLR	TACACATAATAATCCCACCC		
C. upsaliensis glyA	CUF	AATTGAAACTCTTGCTATCC	59	204
	CUR	TCATACATTTTACCCGAGCT		
C. fetus sapB2	CFF	GCAAATATAAATGTAAGCGGAGAG	59	435
	CFR	TGCAGCGGCCCCACCTAT		
C. jejuni 23S rRNA	23SF	TATACCGGTAAGGAGTGCTGGAG	59	650
	23SR	ATCAATTAACCTTCGAGCACCG		

**PCR amplifications were performed in a mixture (25 µL) consisting of 12.5 µL of 2×Platinum Multiplex PCR Master Mix (Applied Biosystems, UK), 2.5 µL of template DNA, and 5 µL of primer mix (0.5 µM C. jejuni and C. lari primers; 1 µM C. coli and C. fetus primers, 2 µM C. upsaliensis primers; 0.25 µM 23S rRNA primer). Distilled water was added to make 25 µL.*

Table 2: *Primer sequences used in the multiplex PCR assay and the expected sizes of the products.*

Target gene	Primer name	Sequence (5'–3')	Annealing temperature	Product bp
tet(O)	tet(O)-F	GCGTTTTGTTTATGTGCG	54	559
	tet(O)-R	ATGGACAACCCGACAGAAG		
cmeB	cmeB-F	TCCTAGCAGCACAATATG	54	241
	cmeB-R	AGCTTCGATAGCTGCATC		
bla_{OXA-61}	BlaOXA-61-F	AGAGTATAATACAAGCG	54	372
	BlaOXA-61-R	TAGTGAGTTGTCAAGCC		
aph-3-1	aphA-3-1-F	TGCGTAAAAGATACGGAAG	54	701
	aphA-3-1-R	CAATCAGGCTTGATCCCC		
aadE	aadE1-F	GAACAGGATGAACGTATTCG	54	837
	aadE1-R	GCATATGTGCTATCCAGG		

**Each multiplex PCR tube contained 25 µL of mixture: 12.5 µL Platinum Multiplex PCR Master Mix (Applied Biosystems, UK), 2.5 µL of template DNA, 5 µL of primer mix (0.5 µM of the used primers), and 5 µL of distilled water.*

Results and Discussion

Prevalence of C. jejuni at broiler farm and at the slaughterhouse

The results of the study indicated that *C. jejuni* was present in all phases of the production. The distribution of this microorganism was highest in the cloacal swabs (48.4%). The results from this study confirm that *C. jejuni* had the highest prevalence in the broiler farms (Table 4), which is in line with previous studies (Schets *et al.*, 2017; Sibanda *et al.*, 2018). The level of prevalence of *C. jejuni* on the broiler farm was very similar with the level confirmed in two studies in Malaysia and Vietnam (Saleha, 2002; Schwan, 2010).

Table 3: *Zone of inhibition and concentration of the used antibiotic discs.*

Antimicrobial agent	Disc content	Zone of inhibition (mm)	
		resistant	susceptible
Ciprofloxacin	5 µg	≤26	26≥
Erythromycin	15 µg	≤20	20≥
Tetracycline	30 µg	≤30	30≥

** Disk diffusion (EUCAST standardised disk diffusion method). Medium: Mueller-Hinton agar + 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F). Inoculum: McFarland 0.5; Incubation: Microaerobic environment, 41±1°C, 24h; Quality control: C. jejuni ATCC 33560.*

This study also revealed high prevalence of *C. jejuni* (40.9%) in the slaughterhouse (evisceration phase) in

the cecum samples. This phase (with cecum samples) shows a great variation in the prevalence. In some studies, the prevalence was in accordance with our results (Di Giannatale *et al.*, 2010), but the literature also revealed studies that confirmed even higher prevalence (Perez-Arnedo and Gonzalez-Fandos, 2019).

Table 4: *Prevalence of C. jejuni at the farm, at the slaughter line and at storage.*

Sampling point	No. of samples	Type of sample	*C. jejuni*
Farm	64	Cloacal swab	31 (48.4%)
Slaughter line	166	Cecum	68 (40.9%)
Storage	53	Carcass swab	12 (22.6%)
Total	283	/	111(39.2%)

**The isolation and identification of thermotolerant Campylobacter was done according to the ISO 10272-1 Microbiology of the food chain, Horizontal method for detection and enumeration of Campylobacter spp., Part 1: Detection method.*

Table 5: *Prevalence of resistance genes in C. jejuni isolates n positive (%).*

Species	No.	*CmeB*	Bla_{OXA-61}	*tet(O)*	*aph-3-1*	*aadE*
C. jejuni	108	21(19.4)	27(25.0)	21(19.4)	0 (0)	15 (13.9)

Table 6: *Antimicrobial resistance of Campylobacter jejuni.*

Antimicrobial agent	resistant (R)	susceptible (S)
	n positive (%)	
Ciprofloxacin	68 (63)	40 (37)
Erythromycin	6 (5.6)	94 (94.4)
Tetracycline	54 (50)	54 (50)

**Samples were tested according to the EUCAST guidelines for the disk diffusion method for C. jejuni and C. coli.*

The last point of sampling (cold storage) detected 22.6% of positive samples. This is important because from this point the poultry meat is dispatched to the consumers. If we follow the *Campylobacter* prevalence along the production chain we find reduced prevalence but not eliminated (Zhu *et al.*, 2017).

Presence of resistance genes in C. jejuni

In total 111 of the isolstaes were confirmed as *C. jejuni* by both the classical method and the multiplex PCR. Three of the isolates were dismissed because of technical issues with the template DNA purity. Therefore, 108 isolates were subjected to further analysis for the detection of resistance genes.

Resistance genes showed different prevalence in the *C. jejuni* isolates. Highest prevalence was noted for Bla_{oxa-61} gene (25%) which is lower than the prevalence detected in similar studies (Bardon *et al.*, 2017; Obeng *et al.*, 2012). The analysis for the *CmeB* and *tet(O)* genes showed same prevalence (19.4%). The *CmeB* gene presence (part of the CmeABC operon) in this study was much lower that the confirmed presence in a similar study (Olah *et al.*, 2006) where the authors detected this gene in 85,5% of the *C. jejuni* isolates. The *tet(O)* gene has an interesting plasmid location therefore its transfer between the microorganisms like *Campylobacter*s is very possible. In our study the prevalence noted (19.4%) was higher that the prevalence detected in two other studies (Du *et al.*, 2018; Obeng *et al.*, 2012) where the authors confirmed the *tet(O)* gene in 8.3% and 11.2% of the *C. jejuni* isolates.

In this study the presence of *aadE* gene was detected in 13,9% of the *C. jejuni* isolates. The literature review on this gene usually shows a low prevalence and in several published papers we noted low prevalence (Cantero *et al.*, 2018; Obeng *et al.*, 2012; Wysok *et al.*, 2020; Du *et al.*, 2018) with 6.3%, 0%, 0%, 0.8%, respectively.

The *aph-3-1* gene generally has low prevalence in *C. jejuni* isolates and that was also confirmed in our study. The same prevalence (0%) was confirmed in other studies (Devi *et al.*, 2019) but there are cases when this gene had shown higher prevalence confirmed by Qin *et al.*(2012) with 7.3% and the study done by Issa *et al.* (2018) with 3.7%.

Antimicrobial resistance of C. jejuni

The results of the the tests identify that *C. jejuni* isolates expressed highest antimicrobial resistance towards ciprofloxacin (63% of resistant isolates) and tetracycline (50%). Much lower antimicrobial resistance was detected towards erythromycin (5.6%).

Concerning high level of resistance of *Campylobacter jejuni* to ciprofloxacin was also confirmed in several other studies through the world ranging from 71 to 98% (Wieczorek *et al.*, 2018; Nguyen *et al.*, 2016; Chen *et al.*, 2010).

Tetracycline resistance in isolates of *C. jejuni* shows a great variation depending on the study region, climatic conditions and the frequency of usage of this antimicrobial in the feed. We have noted similar rate of prevalence in the study performed in Belgium (Mattheus *et al.*, 2012) with 45.3% prevalence of *tet(O)* gene. Higher prevalence of this gene in *C. jejuni* isolates were detected in other studies with prevalence of 77,4 and 98.2% (Nguyen *et al.*, 2016; Bester and Essack, 2008).

Erythromycin is still a first-choice drug in clinical management of the *Campylobacter*iosis (Rivera-Mendoza *et al.*, 2020). Therefore, it is of vital importance to follow the resistance level of this antimicrobial in *Campylobacter* isolates. In our study we detected 5.6% of resistant isolates of *C. jejuni* that is similar to the results obtained in other studies (Gupta *et al.*, 2004; Mattheus *et al.*, 2012) where the authors confirmed 2% and 12.1% of isolates of *C. jejuni* that were resistant to erythromycin.

Conclusions and Recommendations

This study confirmed high prevalence of *Campylobacter jejuni* in the broiler meat production chain. This statement is valid both at the farm and the slaughterhouse (evisceration phase and cold storage) in different type of analyzed samples.

We must address the level of *C. jejuni* prevalence in the cold chamber which is the last stage before the chicken goes on sale therefore posing a great risk to the consumers.

The findings of this study highlight the need for efficient measures to control *Campylobacter* spp. contamination in the production chain.

The isolates of *C. jejuni* showed high levels of phenotypic resistance to ciprofloxacin and tetracycline. This fact underlines the need for prudent use of antimicrobials in poultry production to minimize the emergence and spread of antibiotic-resistant *Campylobacter* spp. strains.

The resistance gene Bla_{oxa-61} was detected in 25% of the *C. jejuni* isolates, *cmeB* and *tet(O)* genes were present in 19.4%. Lower level of prevalence was confirmed for *aadE* gene (13,9%) and *aph-3-1* gene was not detected in any of the *C. jejuni* isolates.

Finally, we recommend measures that should be implemented to prevent the occurrence of resistant bacteria or resistance genes in the food chain: reduction of the use of antibiotics, encourage narrow-spectrum specific antibiotic therapy instead of broad spectrum antimicrobials, and replacement of antibiotics with improvements in hygiene and flock management.

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Novelty Statement

This study is among the first to focus on the prevalence of *C. jejuni* in the broiler production chain in North Macedonia, subsequently analyzing the antimicrobial resistance towards ciprofloxacin, erythromycin and tetracycline combined with the presence of resistance genes in the confrmed isolates.

Authors's Contribution

LJA conceived and designed the study and wrote the manuscript.
LJA, ZP, KB and MP performed the experiments.
MP contributed to the final version.
SM and DJ gave critical revision.
PS supervised the project and approved it for publishing.

Conflict of interest

The authors have declared no conflict of interest.

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