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# ISOLATION RATE OF *CAMPYLOBACTER* SPP. AND DETECTION OF VIRULENCE GENES OF *CAMPYLOBACTER JEJUNI* ACROSS THE BROILER CHAIN

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## ABSTRACT

The aim of the study was to identify the isolation rate of thermotolerant campylobacters in a small-scale broiler-meat production farm over a one-year period. The second deliverable of the study was to determine the potential virulence markers. The laboratory investigation was performed on 283 samples (cloacal swabs, caeca, carcass swabs) collected on three sampling points (farm, slaughter line, and cold storage). The isolates obtained with the conventional microbiological method were confirmed with multiplex PCR for identification of campylobacters. The presence of 10 virulence genes was analyzed in the *C. jejuni* isolates (*flaA, racR, virB11, dnaJ, wlaN, cadF, ciaB, cdtA, cdtB, cdtC*). Out of 283 samples, 169 (59.7%) were confirmed as *Campylobacter* spp., 111 (39.2%) *C. jejuni*, and 43 (15.2%) *C. coli*. *C. jejuni* was the most prevalent in all sampling points. *Campylobacter* spp. showed a characteristically seasonal prevalence with the highest isolation rate during the warmer period of the year. We detected the *cadF* and *ciaB* genes in all *C. jejuni* isolates. The *flaA* gene was present in 50% of the examined strains. The *cdt* genes (*cdtA, cdtB,* and *cdtC*) were confirmed in 52.8%, 52.8%, and 47.2% of the *C. jejuni* strains, respectively. *C. jejuni* showed 15 profiles of virulence patterns with four predominant profiles.

**Key words:** isolation rate, *Campylobacter* spp., virulence, *Campylobacter jejuni*, broiler chain

## INTRODUCTION

The genus *Campylobacter* contains 31 species, 11 subspecies, and 3 biovars. Some of them are of high significance for the human and animal health (1). In 90% of the human infections, *C. jejuni* and *C. coli* were isolated in the stool samples (2, 3). It is estimated that  80–90% of poultry flocks are mainly colonized with *C. jejuni* and *C. coli*, and infrequently with *C. lari* and *C. upsaliensis* (4).

*Campylobacter* is the most commonly reported causative agent for human bacterial diarrhoeal disease in the European Union since 2005 (5). The main source of infection is by consumption of food with animal origin, especially undercooked poultry meat, unpasteurized milk, or cross-contaminated meat products during preparation and storage (6). Besides these risk factors there are several more that are worth considering: abroad traveling, exposure to animals, working in slaughterhouses, animal farms visiting, consumption of pork meat, contact with dogs and cats, and swimming in open waters (7, 8, 9, 10).

The infection is usually brief, mild, self-limiting, and generally does not require any treatment. In some cases, the infection is associated with serious clinical manifestations such as bacteremia, reactive arthritis, hemolytic uremic syndrome, meningitis, septicemia, and Guillain-Barré syndrome (1, 11). Macrolides and fluoroquinolones are frequently used as the first and second-choice drugs for high-risk patients (young, elderly, immunocompromised individuals) (12).

Many authors have confirmed the seasonal pattern variation in the occurrence rate of *Campylobacter*. The usual prevalence spikes take

place during the warmer months of the year and are generally associated with the higher survival of *Campylobacter* in the habitat (13).

The molecular pathogenic mechanism in *Campylobacter* infection is still not perfectly understood (14). The extensive research on campylobacters has revealed numerous genes relevant for their virulence and pathogenicity (15): *flaA*, *flhA*, *cadF*, *dnaJ*, and *racR* (genes that are engaged in the stage of adherence and colonization); *virB11*, *ciaB*, and *iam* (genes important for the invasiveness); *cdtA*, *cdtB*, and *cdtC* (genes bound to the cytotoxic effect on the epithelial cells), and *wlaN* (linked with the synthesis of the lipooligosaccharides, resulting in Guillain-Barré Syndrome) (16).

The objectives of this study were to 1) estimate the isolation rate of thermotolerant *Campylobacter* in broilers sampled in farms and slaughterhouse; 2) identify (if present) the seasonal variation in *Campylobacter* prevalence; 3) detect the presence of virulence-related genes in *Campylobacter jejuni* isolates, and 4) evaluate the detected virulence patterns in *Campylobacter jejuni* isolates.

## MATERIAL AND METHODS

### Collection of samples

This study was conducted on one broiler farm and slaughterhouse in the Skopje region, N. Macedonia, in the period between March and December 2017. All procedures involving animals were in accordance with the ethical standards and international guidelines for the care and use of animals.

A total of 283 samples were collected. The farm had a capacity of 10.000 broilers with a conventional system. The usual rate of slaughtering was by several thinning cycles of 1.500 broilers per day.

Cloacal swabs were taken from randomly chosen broilers at the farm one week prior to slaughter (n=64). The samples were placed in plastic tubes containing 5 mL of Preston broth.

Broiler caeca were collected at the slaughter line (n=166) during the evisceration phase, and were packed in sterile plastic bags. Samples were collected randomly from at least 40 birds in each sample-collection cycle during the slaughtering process.

Swab samples from broiler carcasses (n=53) were taken in the storage area before shipping to the consumers (cold chamber of the slaughterhouse) using sterile cotton swabs, which were placed in plastic tubes containing 5 mL of Preston broth. The samples were collected the following day after slaughtering, including at least 10 carcasses in each sample-collection cycle.

Samples were stored and transported to the laboratory at 4-8 °C. The laboratory analysis was initiated 3-4 hours after sampling.

### Isolation and confirmation of Campylobacter spp.

The isolation and identification of thermotolerant *Campylobacter* was performed according to the standard ISO 10272-1:2017 (17). 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 DNA preparation for the *Campylobacter* isolates was performed by the 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 15.000 rpm for 5 minutes. Acquired supernatants were kept at -20 °C and used for both PCR methods.

The multiplex PCR for identification and differentiation of *Campylobacter* spp. was performed with adaptations according to the previously published study (18). This multiplex PCR could identify several *Campylobacter* species (*C. jejuni subsp. jejuni; C. coli, C. lari, C. upsaliensis*, and *C. fetus subsp. fetus*). The list of the used primers is shown in Table 1.

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. DNA amplification was carried out in a thermocycler (Techne, UK) using an initial denaturation step at 95 °C for 2 min followed by 35 cycles of amplification (denaturation at 95 °C for 30 sec., annealing at 59 °C for 45 sec., and extension at 72 °C for 45 sec.), ending with a final extension at 72 °C for 10 min.

The multiplex PCR for detection of the virulence genes in the *C. jejuni* isolates was performed as proposed by Datta (19). The list of the used primers is shown in Table 2.

**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 | | |

**Table 2.** Primer sequences used in the multiplex PCR assay and the expected sizes of the products (19)

| Target gene | Primer | Sequence (5'–3') | Annealing temperature (°C) | Product (bp) |
|---|---|---|---|---|
| *flaA* | flaA664 | AATAAAAATGCTGATAAAACAGGTG | 53 | 855 |
| | flaA1494 | TACCGAACCAATGTCTGCTCTGATT | | |
| *cadF* | cadF-F2B | TTGAAGGTAATTTAGATATG | 45 | 400 |
| | cadF-R1B | CTAATACCTAAAGTTGAAAC | | |
| *racR* | racR-25 | GATGATCCTGACTTTG | 45 | 584 |
| | racR-593 | TCTCCTATTTTTACCC | | |
| *dnaJ* | dnaJ-299 | AAGGCTTTGGCTCATC | 46 | 720 |
| | dnaJ-1003 | CTTTTTGTTCATCGTT | | |
| *virB11* | virB-232 | TCTTGTGAGTTGCCTTACCCCTTTT | 53 | 494 |
| | virB-701 | CCTGCGTGTCCTGTGTTATTTACCC | | |
| *ciaB* | ciaB-403 | TTTTTATCAGTCCTTA | 42 | 986 |
| | ciaB-1373 | TTTCGGTATCATTAGC | | |
| *cdtA* | DS-18 | CCTTGTGATGCAAGCAATC | 49 | 370 |
| | DS-15 | ACACTCCATTTGCTTTCTG | | |
| *cdtB* | cdtB-113 | CAGAAAGCAAATGGAGTGTT | 51 | 620 |
| | cdtB-713 | AGCTAAAAGCGGTGGAGTAT | | |
| *cdtC* | cdtC-192 | CGATGAGTTAAAACAAAAGATA | 47 | 182 |
| | cdtC-351 | TTGGCATTATAGAAAATACAGTT | | |
| *wlaN* | wlaN-DL39 | TTAAGAGCAAGATATGAAGGTG | 46 | 672 |
| | wlaN-DL41 | CCATTTGAATTGATATTTTTG | | |

For this protocol 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. Amplification was performed with initial denaturation step at 95 °C for 2 min followed by 35 cycles of amplification (denaturation at 94 °C for 1 min, annealing at a temperature specific to the primer pair for 1 min, and extension at 72 °C for 1 min) and final extension at 72 °C for 10 min.
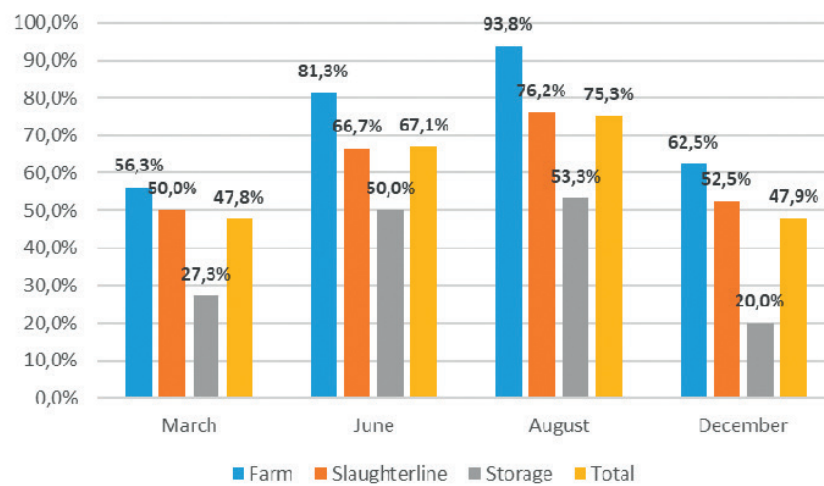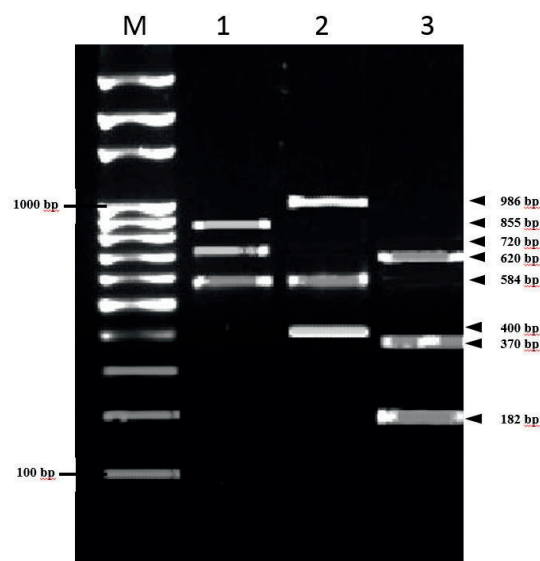
## RESULTS

*Isolation rate of thermotolerant Campylobacter, C. jejuni, and C. coli at broiler farm and at the slaughterhouse*

The results of the study indicated that *Campylobacter* spp. was present in all phases of the production. The distribution of campylobacters was highest in the cloacal swabs (73.4%). The results of the laboratory examinations showed that *C. jejuni* was the most prevalent species on the farm and in the slaughterhouse (Table 3).

**Table 3.** Isolation rate of *Campylobacter* spp. at the farm, at the slaughter line and at storage

| Sampling point | Number of samples | Type of sample | Campylobacter spp. | C. jejuni | C. coli | C. lari | C. upsaliensis |
|---|---|---|---|---|---|---|---|
| **Farm** | 64 | Cloacal swab | 47 (73.4%; CI 62.6-84.2) | 31 (48.4%) | 13 (20.3%) | 2 (3.1%) | 1 (1.5%) |
| **Slaughter line** | 166 | Cecum | 102 (61.4%; CI 54.4-68.4) | 68 (40.9%) | 25 (15.1%) | 6 (3.6%) | 3 (1.8%) |
| **Storage** | 53 | Carcass swab | 20 (37.7%; CI 24.9-50.6) | 12 (22.6%) | 5 (9.4%) | 1 (1.9%) | 2 (3.8%) |
| **TOTAL** | 283 | / | 169 (59.7%; CI 54.1-65.3) | 111 (39.2%) | 43 (15.2%) | 9 (3.2 %) | 6 (2.1%) |

CI, confidence intervals with 95% confidence level



**Figure 1.** Seasonal prevalence of *Campylobacter* spp. along the broiler chain



**Figure 2.** Visualization of PCR amplicons of virulence genes. Lane marked with M: 100-bp ladder; Lane 1: *racR*, *dnaJ* and *flaA* genes; Lane 2: *cadF*, *racR* and *ciaB* genes; Lane 3: *cdtC*, *cdtA* and *cdtB* genes

This study identified high prevalence of *Campylobacter* in carcasses (37.7%), consisting mainly of isolates of *C. jejuni* (22.6%), followed by *C. coli* (9.4%).

*Seasonal variation in Campylobacter spp. prevalence*

As previously mentioned, in several studies, *Campylobacter* spp. has shown a seasonal trend of higher prevalence in the warmer period of the year. In the current study (Fig. 1) we confirmed this trend with highest prevalence in the samples taken in August (75.3%), followed by the June sampling period (67.1%).

*Presence of virulence genes in C. jejuni*

Among the isolates, 111 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 virulence genes (Fig. 2; Table 4).

*Evaluation of the detected virulence patterns in C. jejuni*

In total, we detected 15 virulence patterns in the *C. jejuni* isolates. In 19.4% of the confirmed *C. jejuni* isolates, the analysis detected the profile No. 7. The second highest prevalence was detected for the profile No. 12 (in 13.9% of the isolates). The profile No. 1 (all of the virulence genes present except *virB11*) was confirmed only in 3 isolates (2.8%) of *C. jejuni* (Table 5).

**Table 4.** Seasonal prevalence of virulence genes in *C. jejuni*

| Virulence gene | March | June | August | December | Average |
|---|---|---|---|---|---|
| | | | n positive (%) | | |
| *flaA* | 8 (40) | 17 (58.6) | 20 (48.8) | 9 (50.0) | 54 (50.0) |
| *racR* | 5 (25) | 8 (27.6) | 11 (26.8) | 6 (33.3) | 30 (27.8) |
| *virB11* | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *dnaJ* | 8 (40) | 12 (41.4) | 21 (51.2) | 7 (38.9) | 48 (44.4) |
| *wlaN* | 2 (10) | 5 (17.2) | 7 (17.1) | 1 (5.6) | 15 (13.9) |
| *cadF* | 20 (100) | 29 (100) | 41 (100) | 18 (100) | 108 (100) |
| *ciaB* | 20 (100) | 29 (100) | 41 (100) | 18 (100) | 108 (100) |
| *cdtA* | 11 (55) | 15 (51.7) | 22 (53.7) | 9 (50.0) | 57 (52.8) |
| *cdtB* | 11 (55) | 15 (51.7) | 22 (53.7) | 9 (50.0) | 57 (52.8) |
| *cdtC* | 9 (45) | 14 (48.3) | 20 (48.8) | 8 (44.4) | 51 (47.2) |

**Table 5.** Virulence patterns of *C. jejuni* isolates

| No. | Virulence patterns | | | | | | | | | n | % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | *flaA* | *cadF* | *racR* | *dnaJ* | *ciaB* | *wlaN* | *cdtA* | *cdtB* | *cdtC* | 3 | 2.8 |
| 2. | *flaA* | *cadF* | *racR* | *dnaJ* | *ciaB* | *cdtA* | *cdtB* | *cdtC* | | 6 | 5.6 |
| 3. | *flaA* | *cadF* | *dnaJ* | *ciaB* | *cdtA* | *cdtB* | *cdtC* | | | 3 | 2.8 |
| 4. | *flaA* | *cadF* | *ciaB* | *cdtA* | *cdtB* | | | | | 9 | 8.3 |
| 5. | *flaA* | *cadF* | *racR* | *dnaJ* | *ciaB* | *cdtC* | | | | 6 | 5.6 |
| 6. | *flaA* | *cadF* | *dnaJ* | *ciaB* | *wlaN* | *cdtC* | | | | 6 | 5.6 |
| 7. | *flaA* | *cadF* | *ciaB* | | | | | | | 21 | 19.4 |
| 8. | *cadF* | *racR* | *dnaJ* | *ciaB* | *wlaN* | *cdtA* | *cdtB* | *cdtC* | | 3 | 2.8 |
| 9. | *cadF* | *racR* | *dnaJ* | *ciaB* | *cdtA* | *cdtB* | *cdtC* | | | 12 | 11.1 |
| 10. | *cadF* | *dnaJ* | *ciaB* | *wlaN* | *cdtA* | *cdtB* | *cdtC* | | | 3 | 2.8 |
| 11. | *cadF* | *dnaJ* | *ciaB* | *cdtA* | *cdtB* | *cdtC* | | | | 3 | 2.8 |
| 12. | *cadF* | *ciaB* | *cdtA* | *cdtB* | | | | | | 15 | 13.9 |
| 13. | *cadF* | *dnaJ* | *ciaB* | *cdtC* | | | | | | 3 | 2.8 |
| 14. | *cadF* | *ciaB* | *cdtC* | | | | | | | 3 | 2.8 |
| 15. | *cadF* | *ciaB* | | | | | | | | 12 | 11.1 |

## DISCUSSION

In this study, *Campylobacter* spp. was present in all type of samples collected along the broiler meat production chain (cloacal swabs, caeca, and carcass swabs). The confirmed level of *Campylobacter* spp. on the broiler farm was very similar with the level confirmed in two studies performed on farms with comparable breeding conditions (20, 21).

This study also revealed high isolation rate of *Campylobacter* spp. (61.4%) in the slaughterhouse (evisceration phase) in the cecum samples. Analysis of cecal samples showed a great variation in the isolation rate (region, age of the flock, seasonality, on farm pre-harvest measures, farm management) (22). In some studies, the isolation rate was in accordance with our results (23), but there are studies that report even higher isolation rate (24).

At the last point of sampling (cold storage), 37.7% of the samples were positive. The point of concern is that 22.2% of the samples have shown to be positive for *C. jejuni*. This is important as from this point the poultry meat is dispatched to the consumers. If we follow the *Campylobacter* spp. presence along the production chain, we find reduced level but not eliminated level of contamination (25).

Our results have confirmed a well-defined seasonal pattern of flock colonization with *Campylobacter* spp. As observed by many authors, it is characterized with highest prevalence in summer or autumn (26, 27, 28). The mechanism by which temperature affects *Campylobacter* colonization of broilers is probably linked with microbial survival, higher numbers of wildlife vectors, increased ventilation and fan speeds, insects, and rodents (29).

The pathogenicity of thermotolerant *Campylobacter* found in the broiler meat in addition to its isolation rate gives a clear picture of the public health risk. In this study we detected several of the virulence genes in *C. jejuni* isolates. Our results showed that *cadF* and *ciaB* genes were detected in all of the confirmed strains (Table 5). Similar observations indicated that the *cadF* and *ciaB* genes were present in the *Campylobacter* isolates from chicken carcasses and droppings (19, 30).

The analyses detected an isolation rate of 50% of the *flaA* gene which coordinate the motility and virulence (31, 32). Comparable levels of prevalence of this gene were detected in other studies (33, 34).

The frequency of *cdt* genes (52.8%, 52.8%, and 47.2%) that was observed in our *C. jejuni* isolates was close to the prevalence detected in studies by other authors (35, 36). This holotoxin (cdtABC) produced by campylobacters is a factor which has a great role in the development of the disease. All three toxin subunit genes (*cdtA*, *cdtB*, and *cdtC*) are necessary for the cytotoxin expression (37).

The *wlaN* gene (prevalence of 13.9%) is responsible for the metabolism of specific lipooligosaccharides (LOS) connected with disease sequelae such as Guillain-Barre syndrome (38, 39). Concerning the genes connected with adherence and colonization (40) the study results confirmed 44.4% prevalence of the *dnaJ* gene and lower prevalence of the *racR* gene (27.8%). The *virB11* gene, which is responsible for the expression of invasion (41), was not detected in the *C. jejuni* isolates.

The authors acknowledge that at the time of sample collection for the purpose of the current research, the country had a small-scale broiler production based on few farms and only one operating slaughterhouse imposing limitations of sample number.

## CONCLUSION

This study confirmed high isolation rate of *Campylobacter* spp. in the broiler meat production chain. This statement is supported both at the farm and the slaughterhouse (evisceration phase and cold storage) in different type of analyzed samples. *C. jejuni* was more frequently isolated than *C. coli* at the three sampling points.

The *Campylobacter* isolation rate in the cold chamber samples was notable and relevant for public health consideration. *C. jejuni* isolates had varying virulence patterns (n=15) and diverse pathogenic potential which are not always necessarily expressed *in-vivo*. The detected virulence genes and their patterns surely demonstrate the pathogenic potential of *Campylobacter jejuni* isolated across the broiler chain.

## AUTHORS' CONTRIBUTIONS

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

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