

The evidence for clonal spreading of quinolone resistance with a particular clonal complex of *Campylobacter jejuni*

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SUMMARY

Campylobacter is the most prevalent cause of bacterial gastroenteritis worldwide and it represents a significant public health risk of increasing severity due to its escalating resistance to clinically important quinolone and macrolide antibiotics. As a zoonotic pathogen *Campylobacter* is transmitted along the food chain and naturally cycles from environmental waters, feedstuff, animals and food to humans. We determined antibiotic resistance profiles, as well as multilocus sequence types and *flaA*-SVR types for 52 *C. jejuni* isolated in Slovenia from human, animal, raw and cured chicken meat and water samples. Twenty-eight different sequence types, arranged in ten clonal complexes, three new allele types and five new sequence types were identified, indicating the relatively high diversity in a small group of strains. The assignment of strains from different sources to the same clonal complexes indicates their transmission along the food supply chain. The most prevalent clonal complex was CC21, which was also the genetic group with 95% of quinolone-resistant strains. Based on the genetic relatedness of these quinolone-resistant strains identified by polymerase chain reaction with a mismatch amplification mutation assay and sequencing of the quinolone resistance-determining region of the *gyrA* gene, we conclude that the high resistance prevalence observed indicates the local clonal spread of quinolone resistance with CC21.

Key words: Antibiotic resistance, *Campylobacter jejuni*, epidemiology, *flaA*, food supply chain, genotyping, MLST, transmittance.

INTRODUCTION

Campylobacteriosis is a leading zoonotic foodborne disease in Europe and around the world. Its latest yearly incidence in Europe and Slovenia is 50.28 and

48.7/100 000, respectively [1, 2]. These numbers are still believed to be strongly underreported, since most campylobacteriosis cases remain unrecognized by surveillance systems due to the mild self-limiting symptoms or undetermined cases of gastroenteritis. In Slovenia, as well as in other countries, there is a trend of increasing campylobacteriosis incidence [2, 3].

Of the thermotolerant campylobacters, *Campylobacter jejuni* is the most infectious, causing around 90% of reported human campylobacteriosis cases in

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Slovenia [2]. Besides the high prevalence of infections, antibiotic resistance of *C. jejuni* against quinolones (ciprofloxacin and enrofloxacin) and to a lesser extent, macrolides (erythromycin and azithromycin) is problematic [4]. In Slovenia in 2010, 78% ($n=60$) of *C. jejuni* strains isolated from meat were resistant against ciprofloxacin, while all of them were susceptible to erythromycin. Of the human *C. jejuni* clinical isolates from the same year, 67.2% and 1.0% ($n=882$) were resistant against ciprofloxacin and erythromycin, respectively [3]. This degree of resistance and the emergence of multidrug-resistant strains can seriously hinder clinical treatment of campylobacteriosis [5].

Campylobacter colonizes the gut of many production animals, especially broilers, in which it rarely causes disease symptoms. The meat of these animals is therefore often contaminated during slaughtering and serves as a vehicle of transmission of this pathogen via the food production chain [6]. *Campylobacter* is widely known for its rapid adaptive ability and genomic instability [7–9]. When different reservoirs harbour strains with specific genotypes, it is possible to determine the source of the infection by pulsed-field gel electrophoresis, multilocus sequence typing (MLST) and/or *fla* gene typing [10–12]. Nevertheless, MLST has become the method of choice for *Campylobacter* genotyping. Moreover, in combination with sequencing of the short variable region within the flagella-encoding *flaB* gene MLST allows further strain differentiation within the same sequence type (ST) [13]. This approach has allowed the correlation of specific genotypes with specific antimicrobial resistance profiles [13, 14]. It was shown that resistance to quinolones and macrolides are mostly associated with point mutations in *gyrA* and 23S rRNA, respectively, acting together with efflux mechanisms [15, 16]. The RND-type efflux pumps of some Gram-negative bacteria are able to extrude different types of antibiotics and can also be induced by their substrates [17]. This indicates that environmental selective pressures play an important role in acquiring non-specific (via active efflux), or specific (via point mutation), resistance against antimicrobial drugs. Specific STs have already been associated with quinolone resistance in strains with point mutations, but it is not yet known whether this is because these genotypes are more prone to mutations conferring resistance or because they are clonal [13].

We investigated the potential correlation between MLST/*flaA* genotypes, source of the isolates and resistance against seven antibiotics of 52 Slovenian

C. jejuni isolates, in order to elucidate the spread of antibiotic resistance. This is the first report on MLST/*flaA* characterization of Slovenian *C. jejuni* isolates from humans, animals, water, and food.

MATERIALS AND METHODS

Campylobacter strains and growth conditions

Fifty-two *C. jejuni* strains isolated from different sources (human, raw and cured chicken meat from retail, animal, water) and regions in Slovenia were used in our study. Control reference strains included *C. jejuni* ATCC 33560 and *C. coli* strains 158 and 01378 as negative controls. The majority of meat and animal isolates, and all water isolates from Slovenian rivers were obtained through national monitoring in 2008/2009. Five chicken meat isolates were obtained from a laboratory collection and human isolates were from reported campylobacteriosis cases in 2009. *C. jejuni* was confirmed by *hipO* gene species-specific polymerase chain reaction (PCR) [18]. Isolates were grown on Columbia blood agar (Oxoid, UK) under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 42 °C for 24 h and maintained at –80 °C in brain heart infusion broth (Oxoid) with 5% horse blood (Oxoid) and 20% glycerol.

MLST and *flaA* short variable region sequence typing

DNA was extracted from 24-h cultures with PrepMan[®] (Applied Biosystems, USA) according to the manufacturer's instructions and MLST was performed as described by Dingle *et al.* [19]. Seven house-keeping genes, *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, *uncA* were amplified using *Taq* polymerase (Promega, USA) with primers detailed in Korczak *et al.* [20]. The short variable region of the *flaA* gene (*flaA*-SVR) was amplified by primers *flaA*_Cjc-L and *flaA*_Cjc-R [20] according to the protocol of Josefsen *et al.* [21]. The purified PCR products were sequenced with the BigDye terminator v. 3.1 ready reaction cycle sequencing kit (Applied Biosystems) on an ABI3130XL genetic analyser (Applied Biosystems).

The sequences of the seven MLST loci and *flaA* were compared with sequences in the MLST database (www.pubmlst.org/campylobacter) to determine the allele number. The STs, clonal complexes (CCs) and *flaA* type of each strain were assigned from the profiles of the seven alleles in the MLST database with an integrated automated link in the computer program

Table 1. Prevalence of *Campylobacter jejuni* isolates from different sources in identified clonal complexes in this study

CC	No. of STs	Source (no. of isolates)				Total
		Human	Animal	Meat	Water	
21	4	4	10 (53%)	5		19
45	5		1	6 (75%)	1	8
48	3	1	1	1		3
206	1		1			1
353	2	3		3		6
354	3	1	3	1		5
403	1		1			1
464	2		1	1		2
607	1	1				1
658	2				2	2
Not defined	4		1		3	4
Total	28	10	9	11	6	52

CC, Clonal complex; STs, sequence types.

BioNumerics v. 6.6 (Applied Maths NV, Belgium). Relationships between strains were determined by the MLST-based network creation method, minimum spanning tree (MST) and by the unweighted pair-group method with arithmetic mean cluster analysis based on *flaA*-SVR in BioNumerics. New alleles and STs were submitted to the PubMLST database.

Antibiotic susceptibility

The susceptibility of isolates to seven antibiotics (chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, streptomycin, tetracycline) was determined by a broth microdilution method using a commercial diagnostic test for *Campylobacter* minimum inhibitory concentrations (MICs) (Sensititre *Campylobacter* plate–EUCAMP; Trek Diagnostic Systems, USA) according to the manufacturer's instructions. Resistance against antibiotics was expressed as MICs based on cut-off values recommended by European Food Safety Authority (EFSA) [22]. Multidrug resistance was defined as resistance to at least three non-related antibiotics.

Mutation assay PCR and *gyrA* sequencing

Mismatch amplification mutation assay (MAMA) PCR for detection of the Thr⁸⁶-Ile mutation, which confers resistance against ciprofloxacin was performed on all phenotypically ciprofloxacin-resistant isolates. Additionally, the quinolone resistance-determining

region (QRDR) of *gyrA* was sequenced (Macrogen, South Korea) to study the genetic relatedness of ciprofloxacin-resistant isolates from different CCs. Both procedures were as described previously [23]. Thirty-two sequences of *gyrA* QRDR were deposited in GeneBank (accession numbers KF831198–KF831229).

Statistical analysis

The discriminatory index of the typing methods was calculated by Simpson's index of diversity according to Hunter & Gaston [24]. Statistical significance ($P < 0.05$) of association of specific genotypes with quinolone resistance and source of the strains was tested using IBM SPSS Statistics v. 20 (IBM, USA), with Fisher's exact two-tailed test and Pearson's χ^2 test.

RESULTS AND DISCUSSION

The 52 *C. jejuni* isolates were recovered from animals (chicken, bovine, turkey skin and faeces), raw and cured chicken meat from retail, human patients and environmental water samples during 2008–2009 in different regions of Slovenia. The isolates were grouped by MLST into 28 STs, four of which were novel additions to the PubMLST database; one of these new STs (ST5207 – *C. jejuni* 1603) was a novel combination of previously known alleles but three, ST5205, ST5206, ST6309, included novel allele sequences in *tkt*, *aspA* and *glyA* loci, respectively. Each of the isolates with

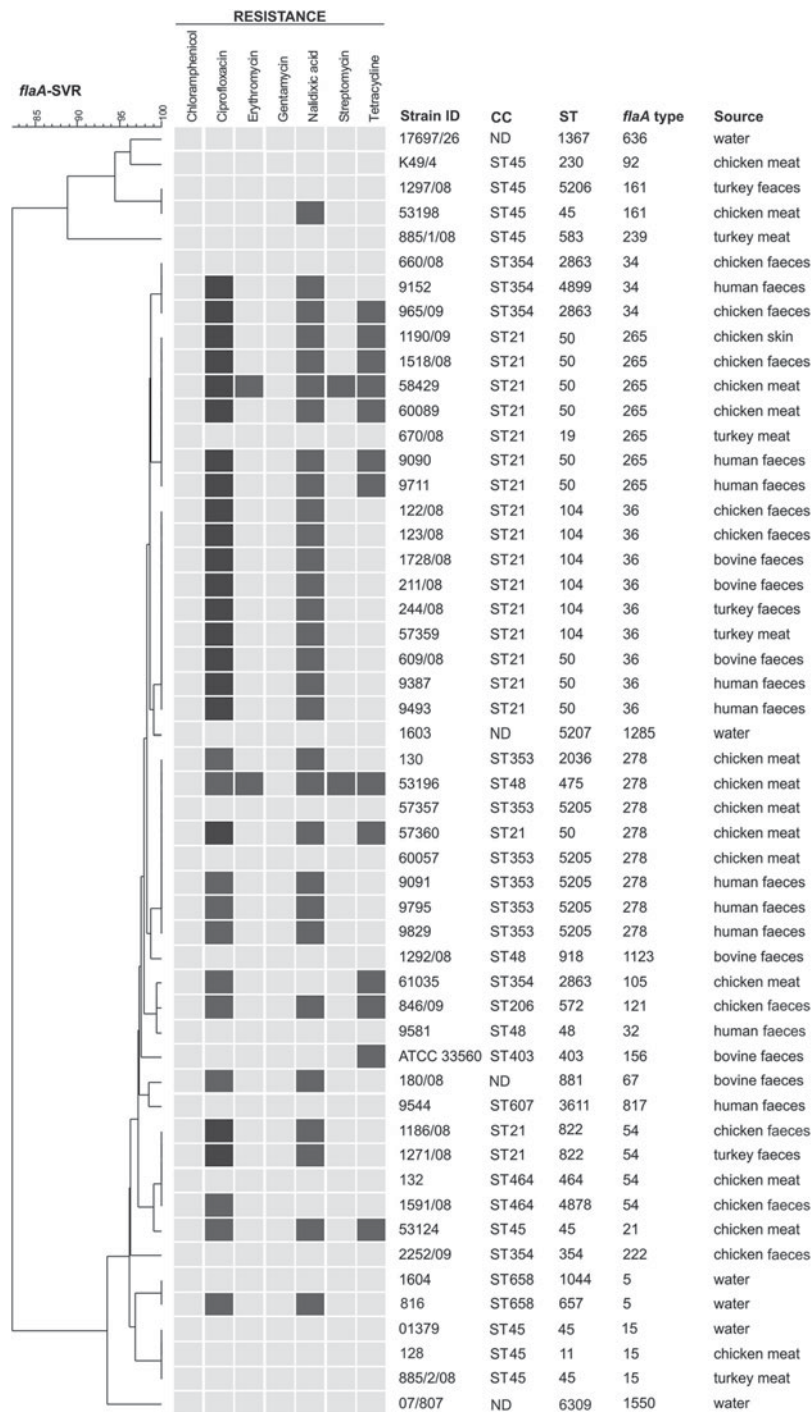


Fig. 1. Phylogenetic tree constructed on the basis of the short variable repeat of the *flaA* region showing correlation between antibiotic resistance, origin of the strains and MLST type. Light grey squares represent antibiotic-sensitive phenotypes; dark grey squares represent antibiotic-resistant phenotypes; black squares represent ciprofloxacin-resistant phenotypes of clonal complex ST21. CC, Clonal complex; ST, sequence type; ND, not determined.

the novel *tkt* allele type originated from the same region in Slovenia (data not shown) and were isolated from raw and cured chicken meat and human faeces, which is suggestive of chicken being the most probable source of infection.

As shown in Table 1, 48 *C. jejuni* isolates were assigned to ten pre-defined CCs and 24 STs by MLST. Four isolates could not be assigned to a CC. CC21 and CC45 predominated and accounted for 19 and eight, respectively, of the total 52 isolates typed.

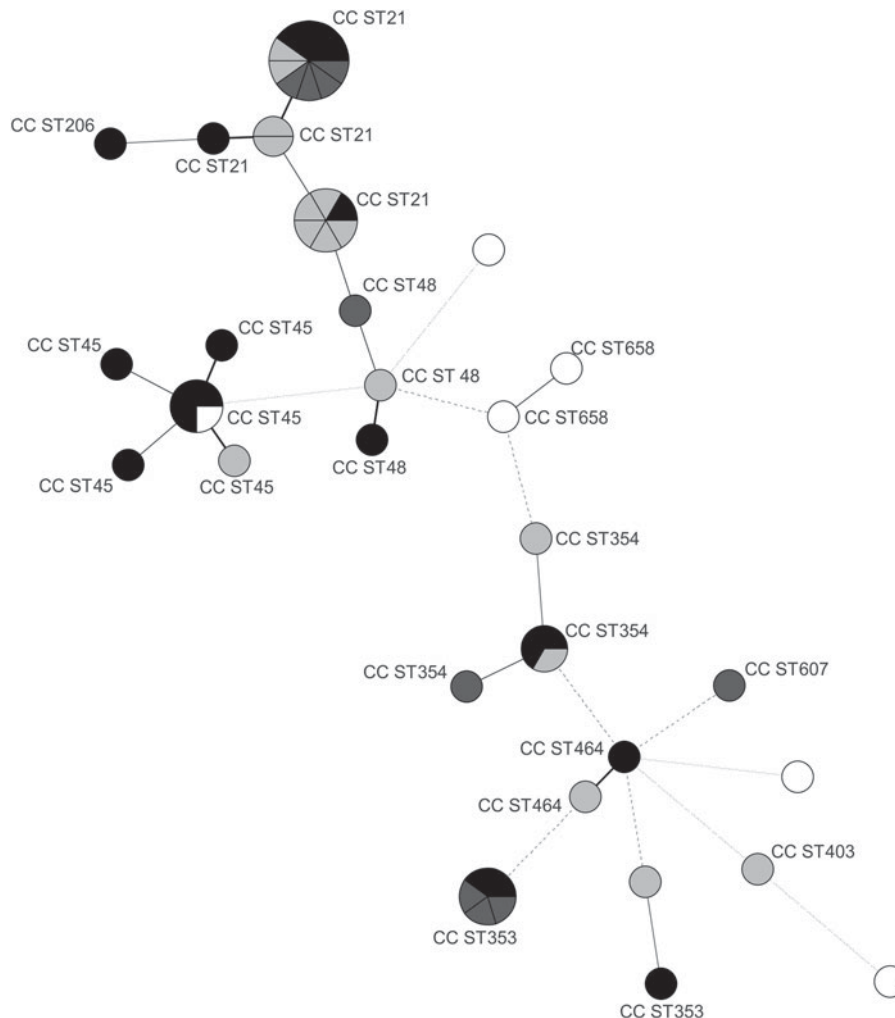


Fig. 2. Minimum spanning tree generated from MLST comparisons of *Campylobacter jejuni* strains isolated from animals, humans, meat, and water in Slovenia. Each circle represents one sequence type (ST), the clonal complexes (CC STs) are indicated by numbers. Node sizes represent higher strain numbers within one ST. Node colour indicates the origin of the strains: black (meat), dark grey (human), light grey (animal), white (water). The connecting lines between STs depict the number of allelic differences between them: one allele difference (black bold lines), two alleles difference (grey bold lines), three alleles difference (grey dashed lines), more than three alleles difference (grey dotted lines).

ST50 and ST104 were the most frequent STs accounting for 10 and six, respectively, of the total (Fig. 1, Table 1). Twenty-three (79%) STs appeared only once, indicating high genetic diversity. STs belonging to CC21 and CC45 were previously shown to be predominant in Europe and typical for poultry [13, 20, 25, 26]. In our study, 12/19 isolates assigned to CC21 and 6/8 assigned to CC45 were isolated from poultry meat or faeces.

The distribution of isolates in the MST analysis (Fig. 2) indicates some correlation between specific genotypes and their main source of isolation. The majority (71%) of human isolates was distributed among CC21 and CC353 and most (67%) of the meat isolates

among CC21 and CC45, while animal isolates were mostly (67%) assigned to CC21 and CC354. Water isolates were genetically the most diverse group and 2/5 could not be assigned to any existing CC. Their distinct STs present an interesting group with little in common with isolates from human and animal sources. Water as an abiotic source, represents a different environment to which bacteria need to adapt phenotypically and genetically. Similar genetic differences between *C. jejuni* strains originating from biotic and abiotic sources have been found in the past and resulted in the identification of specific genetic markers able to distinguish between isolates from biotic and abiotic sources [27].

Table 2. *Distribution of antibiotic resistant strains in clonal complexes*

CC	No. of isolates	STR	CIP	TET	ERY	NAL	MDR
21	19	1	18 (95%)	7 (37%)	1	18 (95%)	1
45	8		1	1		2	
353	6		4 (67%)			4 (67%)	
354	5		3 (60%)	2		2 (40%)	
206	1		1	1		1	
403	1			1			
464	2		1				
48	3	1	1	1	1	1	1
607	1						
658	2		1			1	
Other	4		2			1	
Total	52	2	7	6	2	6	2

CC, Clonal complex; STR, streptomycin; CIP, ciprofloxacin; TET, tetracycline; ERY, erythromycin; NAL, nalidixic acid; MDR, multidrug-resistant strain (resistant against ≥ 3 unrelated antibiotics).

flaA-SVR typing revealed 22 different genotypes in the *C. jejuni* isolates (Fig. 1), and was thus a less discriminatory method than MLST (28 STs). Indeed, *flaA*-SVR typing additionally discriminated isolates within only one ST (ST50), whereas MLST distinguished between isolates in more *flaA* types. This was reflected by the higher discriminatory power of MLST (0.942) over *flaA*-SVR (0.928). The combination of both typing methods increased the discriminatory power to 0.967.

The most common *flaA* alleles were 36, 278 and 256 with prevalences of nine (17%), eight (15%) and seven (13%) out of a total of 52 alleles. All *flaA* allele types 256 and 36 were segregated into CC21, while most of the strains with allele type 278 were assigned to CC353, as well as to CC21 and CC48. Cluster analysis of *flaA*-SVR sequences revealed six major clusters at a similarity level of 95%. Statistical analysis confirmed *flaA* allele type 36 (8/9) as typical for animal isolates and type 278 (5/8) as typical for meat isolates.

The highest frequency of antibiotic resistance was recorded for the quinolones ciprofloxacin and nalidixic acid (61% and 58%), followed by tetracycline (25%), streptomycin and erythromycin (4%) (Table 2). All *C. jejuni* isolates were susceptible to gentamicin and chloramphenicol. These results are in accordance with trends reported by the EFSA [3]. Antibiotic resistance in *C. jejuni* is becoming a significant issue, not only in Slovenia, but also in other European countries [3] with the most problematic being resistance against quinolones, which are the second group of drugs of choice for campylobacteriosis treatment. In Slovenia quinolones are widely used for the non-specific

treatment of bacterial gastroenteritis as well as for enterococcal and *E. coli* infections in poultry flocks. The prevalence of ciprofloxacin-resistant animal *Campylobacter* isolates in Europe is reported to range from 37% to 84%, depending on the country [3] which is consistent with the results reported here, where 72% of animal and 44% of meat isolates were ciprofloxacin resistant and this increased to 80% for human isolates. Almost all (94%) ciprofloxacin-resistant strains from our study were cross-resistant to nalidixic acid. Interestingly, tetracycline resistance occurred almost exclusively (92%) in ciprofloxacin-resistant strains. Although erythromycin resistance was rare, it is of concern that both erythromycin-resistant isolates were from raw chicken meat and exhibited multidrug resistance to ciprofloxacin, nalidixic acid, streptomycin and tetracycline, which seriously compromises the choice of treatment for infections caused by the ingestion of these organisms.

The high incidence of quinolone resistance compared to erythromycin resistance was previously demonstrated to be due to the faster development of ciprofloxacin-resistant mutants during exposure to antibiotics, improved biological fitness of resistant mutants and their ability to persist in the environment after the selective pressure is removed [28–30]. By contrast, macrolide resistance appears to require longer exposure to antibiotics to develop, and resistant strains are less likely to survive in the absence of selective pressure [31–33]. We also observed persistence of ciprofloxacin-resistant strains of the same genotypes (e.g. ST50) which were isolated from animals, meat, and human samples.

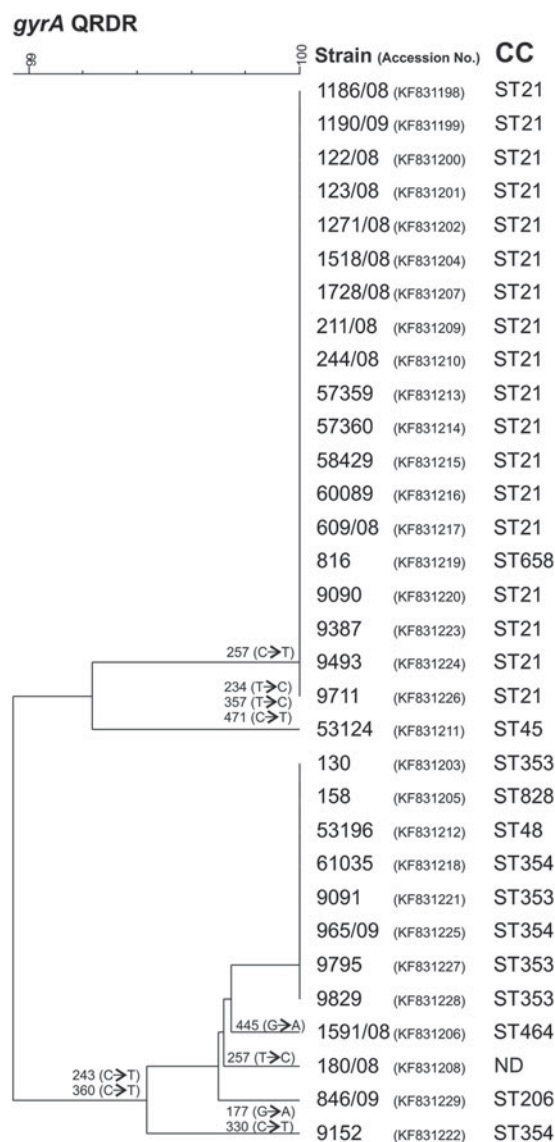


Fig. 3. Relationship between *Campylobacter jejuni* strains based on the sequences of quinolone resistance-determining region (QRDR) of the *gyrA* gene in comparison to clonal complex (CC). The position of silent mutations in relation to the reference strain *C. jejuni* NCTC 11158 are shown on the branches. The accession numbers of the sequences are shown in parentheses after the strain designation. ND, Not determined.

Analysis of antibiotic resistance and MLST genotypes revealed that all but one of the 19 strains of *C. jejuni* belonging to the predominant CC21 were quinolone resistant ($P < 0.05$). Over half of ciprofloxacin-resistant genotypes within CC21 belonged to ST50 and over 30% to ST104 and only 13 (42%) of these resistant strains were identified in all other CCs ($P < 0.05$). A similar pattern was observed with *flaA* types, where 47% of ciprofloxacin-resistant CC21 genotypes were assigned to *flaA* 36 and 32% to *flaA* 265.

All ciprofloxacin-resistant strains in CC21 also showed cross-resistance to nalidixic acid. An association between CC21 and quinolone resistance has already been found in Belgium [34] and Switzerland [13] accounting for resistance rates in these countries of 66% and 30%, respectively.

In order to determine whether the association of quinolone resistance with CC21 was due to individual mutational events or an increased ability to spread clonally [24], we investigated the most common ciprofloxacin resistance conferring mutation Thr⁸⁶-Ile and analysed the QRDR by sequence typing. The results show that all phenotypically ciprofloxacin-resistant isolates, with the exception of strain 180/08, have the mutation ACA to ATA in the 86th codon. Six additional silent mutations in the QRDR were identified, and on the basis of these results we constructed the dendrogram shown in Figure 3, which clearly indicates the high degree of genetic relatedness of ciprofloxacin-resistant isolates from CC21, which together with one strain from CC658 and two strains with unassigned CCs form a separate group compared to the ciprofloxacin-resistant strains from all other CCs. As quinolone resistance is quickly developed under antibiotic selective pressure and can persist long after cessation of treatment, we conclude that the high incidence of such strains within CC21 found here is not due to high genetic plasticity of this particular CC [24], but rather to acquired efficiency of clonal spreading. This hypothesis is additionally supported by the fact that not all investigations from other countries [35] found a correlation between quinolone resistance and CC21.

In conclusion, this study is the first report on the genetic variability of *C. jejuni* isolates from Slovenia based on MLST, which contributes new allele types and STs to the PubMLST database. CC21 was the predominant genetic group with representative isolates from a variety of different sources and this group was highly associated with quinolone resistance, confirming its clonal spreading along the food supply chain. We also identified distinct genotypes of strains from water sources with high antibiotic susceptibility.

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DECLARATION OF INTEREST

None.

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