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Genetic Diversity, Virulence, and Antibiotic Resistance Determinants of *Campylobacter jejuni* Isolates in Romania

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Abstract: The emergence of antibiotic-resistant *Campylobacter jejuni*, a leading cause of gastroenteritis worldwide, presents a significant public health challenge requiring vigilant surveillance and disease control. This study aimed to characterize *C. jejuni* strains isolated in Romania from 2017-2020, focusing on genetic diversity, virulence, and antibiotic resistance determinants. The isolates underwent phenotypical testing, PCR, and antibiotic resistance assessment using Disc Diffusion Kirby–Bauer for ciprofloxacin, nalidixic acid, erythromycin, and tetracycline. Genetic analysis identified resistance and virulence genes, point mutations, and performed sequence typing (7-gene MLST) to determine genetic relatedness. Results indicated substitutions at position 86 in the amino acid sequence or position 257 in the nucleotide sequence of the gyrA gene in 47 fluoroquinolone-resistant isolates. Additionally, mutations in the rRNA23S gene at positions 2074 and 2075, associated with macrolide resistance, were found in 12 of the 66 isolates. Allelic profiles generated 38 sequence types (STs), including three new STs not present in the reference database. The sequence data analysis revealed a genetically diverse C. jejuni population with a weak clonal structure. This study provides crucial insights into the genetic diversity and antibiotic resistance of *C. jejuni* strains in Romania, highlighting the need for ongoing surveillance and control measures.

Keywords: campylobacter; *C. jejuni*; virulence; antibiotic resistance

1. Introduction

Campylobacteriosis was the most reported zoonosis and foodborne illness of 2022 in EU. A total of 140.241 cases were reported, compared to 129.960 the previous year, with a notification rate of 46.9. The countries with the highest rates are Czechia and Luxembourg while the lowest rates were reported in Bulgaria, Greece, Poland and Romania. The case fatality rate are low, accounting for 0.04% of the reported cases, compared to Listeriosis and West Nile virus infection with a percentage of 18.1% and 8.3% respectively [1,2].

One of the main causing pathogens of gastroenteritis worldwide is *Campylobacter jejuni* which is transmitted to humans by consumption of raw or undercooked meat, milk and dairy products or cross contamination events [3–5] . Another important risk factor is international travel, especially in the Southeast parts of Asia[3]. Moreover, pets and contaminated water were also reported as being a source of infection [6]. Although the illness lasts for only a few days and it's an acute and self-limiting infection, it may cause severe and prolonged symptoms in young and elderly patients. This can lead to complications such as the post infection immune disorder Guillain-Barre syndrome, Miller-Fisher syndrome, meningitis, brain abscess, sepsis, endocarditis, myocarditis, reactive arthritis,

inflammatory bowel disease, celiac disease, cholecystitis, and colon cancer [7] thus requiring antibiotic treatment [8]. Most commonly used antibiotics are macrolides and fluoroquinolones. Tetracycline and gentamycin are used as alternative treatment when the conventional treatment is not efficient [7].

Multidrug resistant *C. jejuni* exhibits resistance against erythromycin, fluoroquinolones and tetracycline. The use of antibiotics as prophylaxis in livestock seems to be the main cause of antibiotic resistance acquisition, either through horizontal gene transfer from other bacteria or mutations [6].

A single-point mutation within the quinolone resistance region – QRDR – gyrA is sufficient to confer resistance to fluroquionolones. One mutation frequently associated with AR is C-257 to T (Thr86Ala). Other single-point mutations, such as Tr86Ala, Ala70Thr, and Asp90Asn were described [9]. (Furthermore, mutations in gyrA gene are not only responsible for the resistance to fluroquionolones, but they also increase the virulence of strains [9]. Another mechanism involved in the decreased susceptibility to these antibiotics is the activity of the CmeABC efflux pump [7].

Most commonly, resistance to macrolides is acquired due to a chromosomal mutation in the V domain of the 23S rRNA gene. Other mechanisms of resistance to macrolide include the presence of the gene ermB (rRNA methyltransferase gene) and point mutations in L4/L22 encoding ribosomal proteins [7].

To date, more than 60 tetracycline resistance genes have been described, most of them located on plasmids [10]. Mosaic tet genes are common, a vast majority of them being derived from tet(O), tet(W), tet(S), tet(M) and tet32. The resistance levels of these genes is often similar to the non-mosaic genes, however, tet(O/W/32/o/w/o) confers a higher level of resistance than the original genes [10]. There are similarities between tet(O/32/O) and tet(O/M/O), namely the length and insertion position of tet32 and tet(M) within tet(O), thus distinguishing between them is rather difficult [10].

2. Materials and Methods

Bacterial isolates

A total of 66 *C. jejuni* strains isolated between 2017 and 2020 from stool samples were included in the study. The isolates were provided by The Department of Public Health located in Prahova and Maramures regions of Romania. *C. jejuni* strains were recovered on Columbia enriched blood agar media (7%) and incubated for 48h at 42°C in a micro aerobic atmosphere (85% N2, 10% CO2, and 5% O2) using Campygen Compact sachets (Thermo Fisher Scientific Oxoid Ltd, Basingstoke UK). The species were confirmed using the MALDI TOF Mass Spectrometry System - Bruker Daltonik GmbH Biotyper Identification.

Antimicrobial susceptibility

The antimicrobial susceptibility testing to tetracycline (TE - 30μg), nalidixic acid (NA - 30μg), ciprofloxacin (CIP - 5μg), erythromycin (E - 15μg) was performed by Disc Diffusion Kirby–Bauer method according to the protocol of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for fastidious organisms, using the ATCC 33560 C. jejuni as quality control.

Genetic support of resistance and virulence

In order to investigate the genetic support of the antibiotic resistance and virulence, bacterial DNA was extracted with the Invitrogen Pure Link kit, following the producer's instruction and quantified by Quibit 3.0 (Invitrogen). The primers (DNA Technology Biozyme) used for amplifying virulence and resistance genes and their annealing temperatures are presented in Table 1. (Probably sup). The reactions were carried out in a final volume of 50μl, using 2μl of DNA and 1.5U Taq polymerase (Promega).

Table 1. List of primes and reaction conditions used in this study.

	Target gene	primer sequence 5ᶦ- 3ᶦ amplicon length		Reference
Virulece	*CadF*	F2B:5ᶦTGGAGGGTAATTTAGATATG3ᶦ R1B:5ᶦCTAATACCTAAAGTTGAAAC3ᶦ - 400pb	- 94°C 1 min -45°C 1min 30 -72°C 3min	x Konkel et al., 1999 [11]

Gene	Primers / Product	PCR conditions	x	Reference
CeuE	JEJ1:5ˈCCTGCTCGGTGAAAGTTTTG3ˈ JEJ2:5ˈGATCTTTTTGTTTTGTGCTGC3ˈ - 794 bp	-93°C 3 min - 95°C 30 sec -57°C 30 sec 30 -72°C 1min	x	Gonzalez et al., 1997 [12]
virB11	VirBF:5ˈGAACAGGAAGTGGAAAAACTAGC-3ˈ VirBR:5ˈTTCCGCATTGGGCTATATG-3ˈ - 708 bp	-95°C 2min - 95°C 30 sec -52°C 30 sec 35 -72°C 1min	x	Bacon et al., 2000 [13]
flaA	flaAF:5ˈGGATTTCGTATTAACACAAATGGTGC3ˈ flaAR:5ˈCTGTAGTAATCTTAAAACATTTTG3ˈ - 1728 bp	- 94°C 1min - 45°C 1 min 30 - 72°C3min	x	Nachamkim et al., 1993a [14]
cdtA *cdtB* *cdtC*	GNW:5ˈGGAAATTGGATTTGGGGCTATACT-3ˈ IVH:5ˈATCACAAGGATAATGGACAAT-3ˈ - 165bp VAT2: 5ˈ GTTAAAATCCCCTGCTATCAACCA 3ˈ WMI-R:5ˈGTTGGCACTTGGAATTTGCAAGGC3ˈ - 495bp WMI-F:5ˈTGGATGATAGCAGGGGATTTTAAC3ˈ LPF-X:5ˈTTGCACATAACCAAAAGGAAG-3ˈ - 555 bp	- 94°C 1min - 42°C 1 min 30 -72°C3min	x	Pickett et al., 1996 [15]
GZgyrA5 GZgyrA6	F: ATTTTTAGCAAAGATTCTGAT R: CCATAAATTATTCCACCTGT - 673 pb	- 94°C 3min - 94°C 1 min - 50°C 1 min x 30 -72°C 3min -72°C 5min		Gerald Zirnstein et al.,1999. [16]
used for sequencing GZgyrA7 GZgyrA8	F:TTATTATAGGTCGTGCTTTG R:TAGAAGGTAAAACATCAGGTT	- 96°C 2 min - 96°C 20 sec -50°C 20 sec x 40 -60°C 4 min		
23S ARN-F *ERY2074-R*	F: TTAGCTAATGTTGCCCGTACCG R: AGTAAAGGTCCACGGGGTCTGG - 485 pb	- 95°C 5min - 94°C 30 sec - 59°C 30 sec 30 -72°C 45 sec -72°C 5min	x	Alonso et al., 2005 [17]
23S ARN-F *ERY2075-R* **used for sequencing** 23S ARN-F 23S ARN-R	F: TTAGCTAATGTTGCCCGTACCG R: TAGTAAAGGTCCACGGGGTCGC - 485 pb F:TTAGCTAATGTTGCCCGTACCG R: AGCCAACCTTTGTAAGCCTCCG	- 95°C 5min - 94°C 30 sec - 59°C 30 sec 30 -72°C 45 sec -72°C 5min	x	Alonso et al., 2005 [17]
tetO	DMT1 DMT2 559pb	- 95°C 5min - 94°C 1 min - 57°C 1 min 30 -72°C 1 min -72°C 5min	x	Gibreel A et al, 2004 [18]

Resistance (row label spanning the lower section)

The amplicons were purified with the NucleoSpin Gen kit and PCR Clean-up- Macherey Nagel, as recommended by the producers and quantified with Qubit 3.0 (Invitrigen). DNA sequences were determined using primer pairs gyrA7/gyrA8 and BigDye Terminator v3.1 (Applied Biosystems),

according to the manufacturer's instructions, and purified using DyeEx 2.0 Spin kit colonies (Qiagen). Separation and detection of sequences was performed using a SeqStudio genetic analyzer (Applied Biosystems), the obtained sequences were edited and analyzed with the BioEdit Sequence Alignment Editor program, version 7.0.5.3.

C. jejuni strains both sensitive and resistant to fluoroquinolones were compared and analyzed, the detection of mutations in the amino acid sequence at position 86 in the gyrA gene were compared with the sequence in the database with the accession number L04566 DNA sequence in GenBank [19].

3. Results

The analysis of *Campylobacter jejuni* strains isolated from Romania between 2017 and 2020 yielded significant insights into the genetic diversity, virulence, and antibiotic resistance profiles of these pathogens. A combination of phenotypical testing, PCR, and antibiotic susceptibility testing provided a comprehensive overview of the resistance mechanisms and genetic relatedness of the isolates.

Virulence genes

The results obtained for the seven genes encoding virulence factors and toxins specific to *Campylobacter jejuni* strains isolated from co-cultures originating from human faeces are presented in Table 2.

Table 2. Detection of virulence genes by PCR for Campylobacter jejuni isolates from human co-cultures.

species	source	no. of positive PCRs						
Campylobacter jejuni	co-culture	*cad*F	*ceu*E	*vir*B11	*fla*A	*cdt*A	*cdt*B	*cdt*C
		66+/66	**66+**/66	**14+**/66	**66+**/66	**62+**/66	**65+**/66	**65+**/66
%		100%	100%	**21%**	100%	93%	98%	98%

A single specific amplicon for the *flaA* gene corresponding to the expected size of 1728bp was generated for all 66 *C. jejuni* strains (100%) Figure 1a), similar results were also obtained for the cadF (100%) and ceuE genes respectively (100%) Figure 1b,c. For the *virB11* gene, 14 isolates (21%) from the 66 strains *of C. jejuni* were positive (Figure 1d).
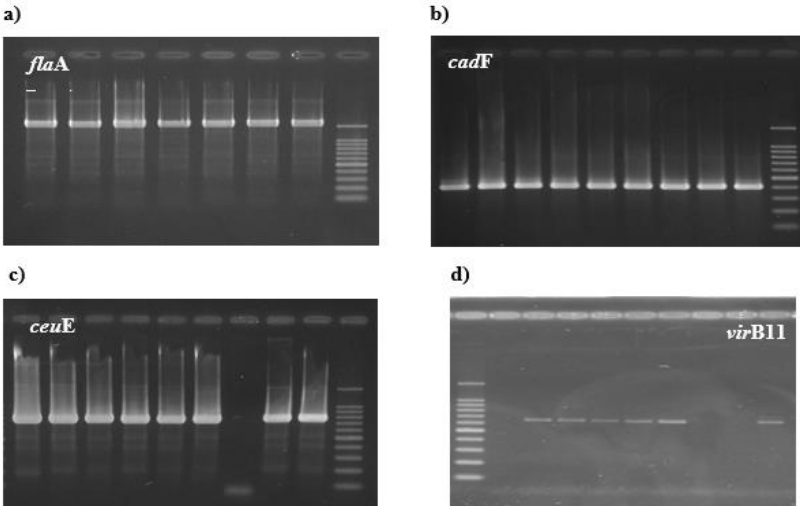

Figure 1. Agarose gel electrophoresis for the detection of virulence genes: a) flaA-1728pb; b) cadF - 400pb; c) ceuE-794pb and d) virB11-708pb by PCR technique (size comparison-molecular weight marker 100 bp Promega).

Primers used for the detection of cdt (cytolethal distending toxin) genes cdtA, cdtB and cdtC, were obtained in all *C. jejuni* isolates analyzed (Figure 2).The cdtA gene PCR product with an expected size of 165bp was observed in 62/66 *C. jejuni* isolates (93%), cdtB gene sequences were

detected in 98% and cdtC gene amplicons with an expected size of 555 bp were also observed in a percentage of 98% (65/66).
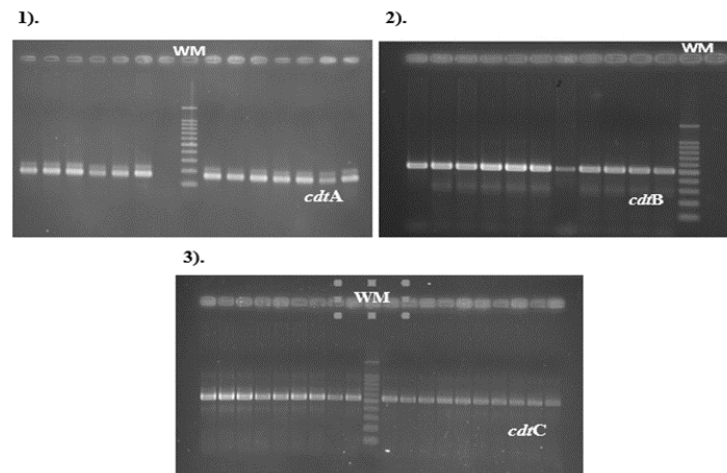
Figure 2. The presence of amplicons obtained with specific primers for *cdt* genes: 1). *cdt*A-165bp; 2). *cdt*B-495pb and 3). *cdt*C- 555pb. (WM- molecular weight marker 100 bp Promega).

Resistance. In this study, the data analysis of the obtained sequences determined the substitution in position 86 in the amino acid sequence or in position 257 in the nucleotide sequence of the gyrA gene for the 47 *C. jejuni* isolates selected with increased resistance to fluoroquinolones with inhibition diameter <26mm, due to a DNA codon mutation from ACA (Thr-threonine) to ATA (Ile-isoleucine) (see Supplementary Figures S1 and S2)).

Out of 12 strains (18%) that were phenotypically resistant to Erythromycin (see Figure 3), 5 isolates harbour a double mutation (2074 A-C, 2075 A-G), while in other 5 only the 2075 single point mutation was detected. (Supplementary Figure S3).
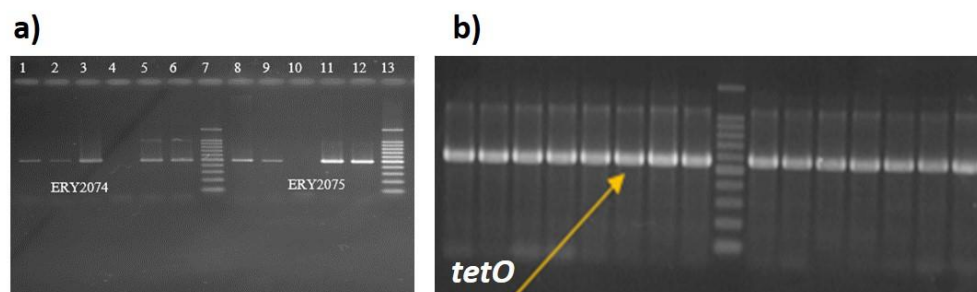
Figure 3. Detection of antibiotic resistance genes. Erythromycin susceptibility testing for 66 C. jejuni isolates and presence of amplicons of 485 bp obtained by PCR with specific primers for the 23S rRNA gene: the presence of amplicons in position 2074 wells 1-3 and 5-6; in position 2075 wells 8-9 and 11-12; wells 7 and 13 BenchTop 100 bp DNA Ladder molecular weight marker (Promega). b) Presence of amplicons of 559 bp obtained by the PCR technique with specific primers for the tetO gene; MK- molecular weight marker BenchTop 100 bp DNA Ladder – (Promega).

Results of tetracycline susceptibility testing determined that 55% of clinical isolates (36 out of 66) showed high-level resistance to tetracycline, whereas 45% of isolates showed sensitivity to this class of antibiotics. Primers DMT1 and DMT2 (Invitrogen) produced a 559 bp amplicon for *36 C. jejuni* strains specific for the tetO gene responsible for tetracycline resistance, phenotypically sensitive strains did not generate amplification for this gene (Figure 3b).

In this study, virulence and resistance profiles to antibiotics such as fluoroquinolones, macrolides, and tetracyclines, classes of antibiotics that are often used first-line in the treatment of infections with *C. jejuni* strains, were observed.

Sequence typing. Sequence typing (7-gene MLST) was performed in order to determine the genetic relatedness between the isolates, by analysing the following genes: aspA, gln, gltA, glyA, pgm, tkt and uncA [20].

The 66 Campylobacter jejuni strains from sporadic cases of gastroenteritis from children aged between 3 months and 9 years provided by the Public Health in our country, were analyzed and compared. PCR products were amplified with oligonucleotide primer pairs, designated after the published sequences for C.jejuni   [21].

Primers used and optimized for amplification and sequencing reactions are shown in Table 3, providing reliable amplification for *C.jejuni* isolates(primers are described at https://pubmlst.org/campylobacter/info/primers.shtml).

Table 3. Sequence of primers for amplification and sequencing of *Campylobacter jejuni* strains.

locus	forward primer	reverse primer	amplicon size
*asp*A	amplification: A9 AGTACTAATGATGCTTATCC	A10 ATTTCATCAATTTGTTCTTTGC	899 pb
	sequencing: S3 CCAACTGCAAGATGCTGTACC	S6 TTCATTTGCGGTAATACCATC	
*gln*A	amplification: A1 TAGGAACTTGGCATCATATTACC	A2 TTGGACGAGCTTCTACTGGC	1262 pb
	sequencing: S3 CATGCAATCAATGAAGAAAC	S6 TTCCATAAGCTCATATGAAC	
*glt*A	amplification: A1 GGGCTTGACTTCTACAGCTACTTG	A2 CCAAATAAAGTTGTCTTGGACGG	1012 pb
	sequencing: S3 CTTATATTGATGGAGAAAATGG	S6 CCAAAGCGCACCAATACCTG	
*gly*A	amplification: A1 GAGTTAGAGCGTCAATGTGAAGG	A2 AAACCTCTGGCAGTAAGGGC	816 pb
	sequencing: S3 AGCTAATCAAGGTGTTTATGCGG	S4 AGGTGATTATCCGTTCCATCGC	
tkt	amplification: A3 GCAAACTCAGGACACCCAGG	A6 AAAGCATTGTTAATGGCTGC	1102 pb
	sequencing: S5 GCTTAGCAGATATTTTAAGTG	S6 AAGCCTGCTTGTTCTTTGGC	
pgm	amplification: A3 TCAGGGCTTACTTCTATAGG	A4 AGCTTAATATCTCTGGCTTC	1150 pb
	sequencing: S5 GGTTTTAGATGTGGCTCATG	S2 TCCAGAATAGCGAAATAAGG	
*unc*A	amplification: A7ATGGACTTAAGAATATTATGGC	A4 ATTCTTTGTCCACGTTCAAG	1120 pb

	sequencing: S3	S4
	AAAGTACAGTGGCACAAGTGG	TGCCTCATCTAAATCACTAGC

Sequences were determined using internal primer pairs of approximately 400–500 bp and BigDye Terminator v3.1 (Applied Biosystems) according to the manufacturer's instructions and purified using the DyEx 2.0 Spin kit colonies (Qiagen).

Sequence separation and detection was performed using a 3130 ABI Prism Genetic Analyzer (Applied Biosystems).

Assignment of alleles and ST type (sequence type)

For each locus distinct allele sequences were assigned, these being internal fragments of the gene containing an exact number of codons, each isolate was designated with 7 numbers constituting an allelic profile or ST (sequence type). (https://pubmlst.org/bigsdb?db=pubmlst_campylobacter_seqdef&page=sequenceQuery)

ST types were identified by numbers assigned to alleles and compared in the database (https://pubmlst.org/bigsdb?db=pubmlst_campylobacter_isolates&page=profiles), which were grouped into clonal complexes (see Supplementary Figure S4).

The length and frequency of the alleles defined by the MLST scheme are shown in Table 4, namely: aspA 477bp, glnA 477bp, gltA 402bp, glyA 507bp, pgm 498bp, tkt 459bp and uncA 489bp. From the 66 C.jejuni isolates, 10 strains were included in CC (clonal complex) 257 (9 strains ST824, 1 strain ST2254), 10 strains in CC353 (4 strains ST400, 4 strains ST353 and 2 strains in ST356), 5 strains in CC21 included in ST50, 4 strains in CC 443 (ST51) and the rest of the strains were included in other types of clonal complexes. The allelic profiles obtained generated 38 types of ST sequences (sequence type) of which 3 new STs of Campylobacter jejuni (10589, 10595, 10686) absent in the database used to establish the ST type (Table 5) and entered in the database international (pubmlst.org) having accession number 106266, 106267 and 106791.

Table 4. Genetic diversity of MLST loci for *C. jejuni* strains.

locus	fragment size	no alleles/ frequency
aspA	477 pb	12 alleles(allele 8,9,2 and 7)
glnA	477 pb	14 alleles(allele 17 and 2)
gltA	402 pb	11 alleles(allele 2 and 5)
glyA	507 pb	17 alleles(allele 2,10, and 838 -new allele)
pgm	498 pb	16 alleles(allele 11 and 10)
tkt	459 pb	16 alleles(allele 5,3 and 1)
uncA	489 pb	13 alleles(allele 6,5 and 632-new allele)

The most common type sequences found among *C. jejuni* isolates in this study were ST824 (9 strains), ST400 (4 strains), ST50 (5 strains), ST51 (4 strains), ST2079 and ST2066 (3 strains) (Figure 4).
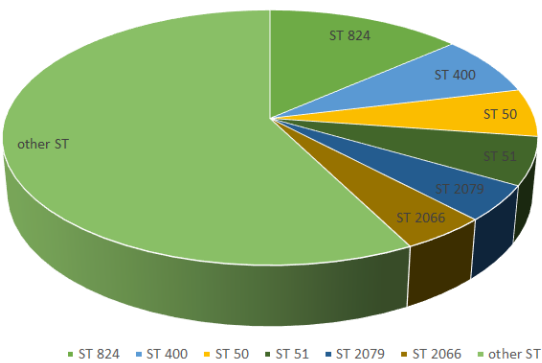
Figure 4. ST (sequence type) of *C. jejuni*.

The strains of *C. jejuni* in this study were typed by this method, the allelic profiles obtained generated various types of ST sequences (sequence type) already known, these data indicated a genetically diverse population, having a weak clonal structure.

The advantages of MLST include high discrimination among isolates, reproducibility, simplicity of interpretation, and generate data that are comparable to other laboratories by accessing them from international databases.

4. Discussion

In this study, we analysed the sequence data of antibiotic resistance and virulence determinants collected in Romania. Furthermore, the relatedness of the strains was assessed.

To our knowledge, data available on the genetic analysis of antibiotic resistant *C. jejuni* strains recovered from human campylobacteriosis cases in Romania is very scarce. Annually, up to 10% of the population is affected by foodborne diseases according to WHO.

The prevalence for the seven virulence genes and toxins of the analyzed *C. jejuni* strains was determined by PCR. The sequences for the three virulence genes (cadF, flaA, ceuE) as well as the cdtC toxin were detected in 98% - 100% of the isolates analyzed in this study.

A high prevalence of 100% of cadF, ceuE and flaA gene was consistent with similar results of previous studies using identical PCR detection assays [11] (, this high prevalence may be explained by that the cadF gene represents an important virulence factor as well as the flaA gene that plays an important role in *Campylobacter* pathogenesis, because Campylobacter motility requires flagellum production.

The low prevalence of 21% of the virB11 gene among the isolates analyzed compares with studies reported by Bacon et al., 2000, the low virulence being due to isogenic mutations. VirB-D – plasmid-mediated – associated with plasmid pVir [22]. The detection of mutations in the gyrA gene in the QRDR (quinolone resistance determining region) region associated with fluoroquinolone resistance (for resistance to nalidixic acid and ciprofloxacin - widely used antibiotics) was determined in 47 strains of the 66 C.jejuni isolates that have shown resistance to nalidixic acid and ciprofloxacin, widely used antibiotics. The data analysis of the obtained sequences determined the substitution in position 86 in the amino acid sequence or in position 257 in the nucleotide sequence of the gyrA gene for the 47 C. jejuni isolates selected with increased resistance to fluoroquinolones with inhibition diameter < 26mm, due to a DNA codon mutation from ACA (Thr-threonine) to ATA (Ile-isoleucine). Also tested were 3 sensitive strains having inhibition diameter ≥50mm around antibiotic discs and did not show mutation at position 86 in the amino acid sequences for *C. jejuni* strains. The quinolone resistance mutation at amino acid position 86 is due to environmental events, these unique DNA sequences in the QRDR region may be useful during the investigation of an epidemiological outbreak.

An alarmingly increasing rate of resistance to quinolone resistance was reported in a neighbouring country, Turkey, registering up to 74.3% (2013) resistant isolates [7,24].

The detection of mutations in the rRNA23S gene at position 2074 and 2075 associated with macrolide resistance (for erythromycin resistance) was determined in 12 strains (18%) of the 66 *C. jejuni* isolates. The data obtained by sequencing were compared and analyzed with the sequence from the *C. jejuni* NCTC 11168 23Sribosomal database, which confirmed the presence of a point mutation at position 2075 (A-G) in all 12 isolates of C.jejuni with high erythromycin resistance, 5 isolates showed double mutation in both position 2074 (A-C) and position 2075, 5 strains showed mutation in position 2074 and 7 strains did not show this mutation.

The results are consistent with similar results due to the presence of the mutation in position 2075 for *C. jejuni* strains with increased resistance, therefore the validation of these tests for the detection of point mutations in the rRNA23S gene associated with erythromycin resistance was confirmed.

Results of tetracycline susceptibility testing determined that 55% of *C. jejuni* clinical isolates (36 of 66 isolates) showed high level resistance, whereas 45% of isolates showed sensitivity to this class of antibiotics. Due to the long widespread use of tetracyclines, a number of determinants of resistance

to this class of antibiotics have been observed in a variety of bacteria, generally this resistance is mediated by one of four mechanisms: efflux pumps, chemical modification of tetracyclines, ribosomal protection proteins and by mutations in rRNA [23]. Efflux pump and ribosomal protection genes are the most important mechanisms of tetracycline resistance, the acquisition of new tetracycline resistance genes is mostly associated with mobile components such as plasmids or transposons which are often conjugative elements [25].

Increasing antibiotic resistance of Campylobacter spp. strains isolated from both humans and animals is expected to become a significant public health problem in both developed and developing countries [26]. Most Campylobacter infections have been found to be self-limiting, antibiotics may be prescribed in severe cases or in immunocompromised patients [27], virulence and antibiotic resistance profiles such as fluoroquinolones were observed in this study, macrolides and tetracyclines, classes of antibiotics that are often used first-line in the treatment of infections with *C. jejuni* strains.

An ECDC surveillance reported that the highest level of resistant *C. jejuni* isolated from broilers were detected in Romania and Cyprus.

Also, an increased level to resistance was detected in pig isolates, but the resistance to colistin in broilers decreased [27].

Moreover, ECDC reported in disease surveillance that the European countries with the highest percentage of reported *C. jejuni* infections in 2022 are Germany followed by Spain and Czechia, Romanian being located on the 21st place. The mean rate of notification reported by the 30 EU/EEA countries was 46.83 cases per 100.000 population with the highest rate in children under 5 years old. Romania was the only country where all the reported cases were hospitalised [28]. It is therefore necessary to undertake ongoing monitoring of both the prevalence and molecular characteristics of bacterial virulence and resistance regarding effective treatment information for *Campylobacter* infections.

The diversity of house keeping genes for the 66 isolates of *C. jejun*i was analyzed by MLST, the allelic profiles obtained generated 38 types of ST sequences (sequence type) of which 3 new Campylobacter jejuni STs (10589, 10595, 10686) were absent in the database used for ST typing and entered in the international database under accession numbers 106266, 106267 and 106791. Schemes for the characterization and discrimination of various bacterial isolates are essential for epidemiological, genetic and evolutionary studies.

The study provides the first information on the circulation of a polyclonal population of *Campylobacter jejuni* at the local level. The molecular typing technique by MLST discriminates between *C. jejuni* isolates effectively and generates data that can be applied to investigate population structure and its evolutionary mechanisms.

Schemes for the characterization and discrimination of diverse bacterial isolates are essential for epidemiological, genetic and evolutionary studies. Ideally these schemes generate data that are relevant to all these domains, but before the advent of high-throughput nucleotide sequencing technology, this goal proved elusive [29]

The need for appropriate molecular typing schemes for *C. jejuni* isolates is essential because this human pathogen possesses extensive reservoirs from animals and the environment, and the relationships between diseases associated with the animal population or the environment remain completely unclear [30].

This study demonstrates that the molecular typing technique by MLST discriminates between *C. jejuni* isolates effectively and generates data that can be applied to investigate population structure and its evolutionary mechanisms.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest

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