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Original Article

Distribution of virulence and antibiotic resistance genes in *Campylobacter jejuni* and *Campylobacter coli* isolated from broiler chickens in Tunisia

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Abstract *Background:* Thermo-tolerant *Campylobacter* species are the major cause of food-borne diseases worldwide. This study aimed to evaluate the prevalence of virulence genes and antibiotic resistance determinants in *Campylobacter jejuni* and *Campylobacter coli* isolates, and to investigate the relationship between these two traits.

Methods: A total of 132 *Campylobacter* isolates from poultry were tested for the presence of 13 virulence genes; *flaA*, *cadF*, *racR*, *virB11*, *pldA*, *dnaJ*, *cdtA*, *cdtB*, *cdtC*, *ciaB*, *wlaN*, *cgtB* and *ceuE*. The mechanisms underlying antibiotic resistance phenotypes were also studied by PCR and MAMA-PCR.

Results: PCR results revealed the presence of antimicrobial resistance genes in *C. jejuni* and *C. coli* as follows: *cme*B (80% and 100%), *tet*(O) (100% and 80%), and the bla_{OXA-61} (81% and 93%), respectively. None of these strains harbored the *aph*A-3 gene.

The Thr-86-Ile mutation associated with resistance to quinolones was found in 90% of *C. jejuni* and 80% of *C. coli* isolates. While the A2075G and A2074C mutations linked to the erythromycin resistance were detected in 100% of both species.

Virulence genes were prevalent and ranged from 40 to 100%. A positive relationship was revealed between *cadF, racR*, and *ciaB* genes and resistance to ampicillin, amoxicillin/clavulanic acid, chloramphenicol, and nalidixic acid, in *C. jejuni*. However, no association was observed for *C. coli* isolated strains.

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Conclusion: This study provides for the first time an overview of antibiotic resistance mechanisms and pathogenic profiles of *Campylobacter* isolates, which emphasizes the potential risk for consumer health.

Introduction

Campylobacter is a zoonotic pathogen, which is recognized as one of the major causes of foodborne bacterial diseases, worldwide.[1] *Campylobacter jejuni* and *Campylobacter coli* are the main species responsible for acute gastroenteritis in humans.[2] Indeed, both species are responsible for about 98% of all human *Campylobacter* gastroenteritis cases.[3] However, other species such as *Campylobacter lari* and *Campylobacter upsaliensis* have also been implicated in sporadic cases of gastroenteritis.[4] Campylobacters are capable of causing health complications such as urinary tract infections, sepsis, or certain neuropathies and in particular, reactive arthritis, Guillain-Barré syndrome (GBS), irritable bowel, and Miller Fisher syndrome (MFS).[5] The poultry reservoir is responsible for about 80% of human cases.[6] Recent molecular studies have lighted the main virulence factors involved in the pathogenesis of *Campylobacter* strains.[1] Indeed, the ability of *Campylobacter* to adhere, through *cadF*, *racR*, *virB11*, *pldA*, and *dnaJ* genes, invade intestinal epithelial cells, through *ciaB* and *ceuE* genes, produce toxin, through *cdtA, cdtB, cdtC genes* expression and survive in host cells, are the main virulence factors identified until now.[7] Even though *Campylobacter* infections are usually self-limiting and do not require antibiotic treatment, therapy is required in some prolonged enteritis and septicemia cases. Macrolides (erythromycin and azithromycin), fluoroquinolones (ciprofloxacin), and tetracyclines are the drugs of choice for the treatment of human campylobacteriosis.[8] However, the overuse of antibiotics in human infections and the misuse of antimicrobial drugs in husbandry to control animal diseases or improve growth by antimicrobials addition in food, are the main causes leading to increased resistance rates among *Campylobacter* species.[9]

Several molecular mechanisms involved in antibiotic resistance were widely described. Fluoroquinolone resistance is mainly due to point mutation(s) in the quinolone resistance-determining region (QRDR) of the DNA gyrase gene, *gyrA*. High resistance to tetracycline is generally associated with the acquired *tet*(O)gene, encoding a protective ribosomal protein.[10] Resistance to macrolides is frequently related to point mutations at positions A2074C or A2075G in the V domain of the *23S rRNA* gene, and activation of the CmeABC multidrug efflux pump leading to resistance to several antimicrobials.[11,12] Other genes encoding antimicrobial resistance, such as *erm*(B) (erythromycin resistance), *aadE* or *sat4* (streptomycin/streptothricin resistance), bla_{OXA-61} (β-lactams resistance) and *aph*A-3 (Aminoglycosides resistance), have also been associated with multidrug resistance in *Campylobacter* strains.[13]

Several studies showed a significant association between virulence genes and antimicrobial resistance (AMR) in bacterial pathogens, suggesting a link between antibiotic resistance and potential colonization or invasion capacities of these bacteria.[9,14] Such association has been studied and some reports have shown that infection in humans with antimicrobial resistant strains of *Campylobacter* is associated with a longer period of diarrhea.[9,15] In Tunisia, to the best of our knowledge, little is known about the epidemiology of *Campylobacter* in poultry industries and the molecular basis of antibiotic resistance and virulence patterns. In our previous work, we have reported the prevalence, the geographic distribution of *Campylobacter* infection in various avian farms, in North of Tunisia, and have reported the occurrence of antibiotic resistance in isolated strains.[16] Thus, this study aimed to investigate the genetic basis of AMR and to identify the virulence markers in collected *C. jejuni* and *C. coli* isolates from broilers chickens.

Materials and methods

Campylobacter strain collection

Herein, we tested the total number of 132 *Campylobacter* strains, including 91 *C. jejuni* and 41 *C. coli*. These isolates were previously recovered from broiler chickens and tested against erythromycin (Ery), ciprofloxacin (Cip), nalidixic acid (Nal), tetracycline (Tet), ampicillin (Amp), amoxicillin/clavulanic acid (Amc), gentamicin (Gen), and chloramphenicol (Chl). Their antimicrobial resistance patterns were shown in our previous study[16] (Data available in supplementary file, Fig. S1).

Culture and growth conditions

Campylobacter culture was performed as described previously.[16] Briefly, all isolates were inoculated into Bolton broth (Oxoid, United Kingdom) with a ratio of 1:10 (v/v). The inoculated broths were incubated at 42 °C for 24–36 h under micro-aerobic conditions (5% O_2, 10% CO_2, and 85% N_2), using a gas pack jar system. One loop full of enrichment broth was streaked onto Karmali agar (SIGMA-Aldrich, Germany) and incubated as above.

DNA extraction

Template DNAs for the PCR test were extracted using the boiling method. *Campylobacter* isolates were grown in 2 ml Bolton broth and plated on Karmali agar. *Campylobacter* colonies were harvested and then suspended in 100 μl TE

buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Cell suspensions were heated at 100 °C for 10 min and then cooled to room temperature. Thereafter, cell suspensions were pelleted by centrifugation at 7871 rcf for 5 min. The supernatant was collected, transferred into a new tube, and then stored at −20 °C before use.

Detection of mutation(s) in the QRDR of *gyrA* and *23S rRNA* genes by PCR

Mismatch amplification mutation assay (MAMA-PCR)

A single point mutation Thr-86-Ile in the quinolone resistance-determining region (QRDR) of the *gyrA* gene was defined as the main mechanism of high-level resistance to fluoroquinolones. *Campylobacter* isolates were subjected to analysis by MAMA-PCR, as previously described for *C. jejuni* and *C. coli* isolates by Zirnstein et al. (1999, 2000).[17,18]

The resistance to macrolide (particularly to erythromycin) is mainly conferred by a single mutation in the *23S rRNA* gene, at positions A2074C or A2075G in the V domain of the *23S rRNA* gene. Detection of these mutations was also carried out by MAMA-PCR, as described previously by Alonso et al. (2005).[19] Primer sets and amplification conditions are listed in Table 1.

Other resistance determinants

All *Campylobacter* isolates were screened for the presence of genetic determinants conferring antimicrobial resistance, by PCR to detect the *tet*(O) (tetracyclines resistance), *aph*A-3 (aminoglycosides resistance), *cme*B (multidrug efflux pump), and bla_{OXA-61} (β-lactams resistance) genes. The PCR mixture consisted of 2.5 μl of DNA template, 0.2 μM of each primer (Carthagenomics Advanced Technologies, Tunisia), 0.2 mM dNTP (Promega, France), 1X Dream DNA polymerase buffer, and 1U Dream Taq DNA polymerase (Thermo Scientific, France), in a final reaction volume of 25 μl. The amplification program consisted of 35 cycles with denaturation at 95 °C for 1min, annealing for 1min, and elongation for 1 min at 72 °C. The annealing temperature for each gene and used primer sets are listed in Table 1.

All reactions were performed in a T100 thermal cycler (BIO-RAD). For the visualization of PCR products, 10 μl were subjected to 1–2% agarose gel electrophoresis containing ethidium bromide (0.5 μg/ml). Lengths of the amplicons were determined in comparison with a 100-bp ladder.

Identification of virulence genes

Campylobacter isolates were tested by PCR for the presence of the following virulence genes: *flaA* (motility), *cadF*, *racR*, *virB11*, *pldA* and *dnaJ* (adherence and colonization), *cdtA*, *cdtB*, *cdtC* (cytotoxin production), *cgtB*, and *wlaN* (Guillain-Barré syndrome), *ciaB* (invasiveness) and *ceuE* encoding a lipoprotein in *C. jejuni* strains. The amplification reaction conditions were as described above. The annealing temperatures and the sequences of primers are shown in Table 2.

Statistical analysis

AMR was considered as a binary dependent variable (0 = non resistant; 1 = resistant). The association between the resistance profile of each antimicrobial and the presence/absence of virulence genes was assessed using binary logistic regression models. Every model included the presence/absence of each gene as a binary explanatory variable (0 = present; 1 = absent) and associations were considered significant when $p \leq 0.05$. To determine significant differences in the number of virulence-related genes between bacterial species (*C. jejuni* vs *C. coli*), factorial analysis of variance (ANOVA) was carried out using the number of genes as a dependent variable and the

Table 1 Primers of antibiotic resistance genes, annealing temperatures and size of amplicons.

Gene	Primer Sequences (5′-3′)	Annealing Temperatures (°C)	Product size (bp)	References
***tet*(O)**	F: GCGTTTTGTTTATGTGCG R: ATGGACAACCCGACAGAAG	53	559	Pratt and Korolik (2005)
cmeB	F: AGGCGGTTTTGAAATGTATGTT R: TGTGCCGCTGGGAAAAG	50	444	Olah *et al.*, (2006)
***bla*$_{OXA-61}$**	F: AGAGTATAATACAAGCG R: TAGTGAGTTGTCAAGCC	49	372	Obeng *et al.*, (2012)
aphA-3	F: TGCGTAAAAGATACGGAAG R: CAATCAGGCTTGATCCCC	54	701	Obeng *et al.*, (2012)
23s rRNA	23S rRNA: TTAGCTAATGTTGCCCGTACCG ERY2075: TAGTAAAGGTCCACGGGGTCGC ERY2074: AGTAAAGGTCCACGGGGTCTGG	46	485	Alonso *et al.*, (2005)
Cj-gyrA	gryA1: TTTTTAGCAAAGATTCTGAT gyrA5: AAAGCATCATAAACTGCAA gyrA4: CAAAGCATCATAAACTGCAG	52	265 368	Zimstein *et al.*, (1999)
Cc-gyrA	gyrACc3: TATGAGCGTTATTATCGGTC gyrACc8: TAAGGCATCGTAAACAGCCA gyrACc4: GTCCATCTACAAGCTCGTTA	55	192 505	Zimstein *et al.*, (2000)

Table 2 Primers of virulence genes, annealing temperatures and size of PCR products.

Genes	Primer Sequences (5′-3′)	Annealing Temperatures (°C)	Products size (bp)	References
flaA	F:AATAAAAATGCTCATAAAAACAGGTG R:TACCGAACCAATGTCTGCTCTGATT	55	855	Datta *et al.*, 2003
cadF	F: TTGAAGGTAATTTAGATATG R: CTAATACCTAAAGTTGAAAC	45	400	Konkel *et al.*, 1999
cdtA	F:CCTTGTGATGCAAGCAATC R:ACACTCCATTTGCTTTCTG	55	370	Hickey *et al.*, 2000
cdtB	F:CAGAAAGCAAATGGAGTGTT R:AGCTAAAAGCGGTGGAGTAT	51	620	Hickey *et al.*, 2000
cdtC	F:CGATGAGTTAAAACAAAAAGATA R:TTGGCATTATAGAAAATACAGTT	47	182	Hickey *et al.*, 2000
racR	F:GATGATCCTGACTTTG R:TCTCCTATTTTTACCC	49	584	Hermans *et al.* 2011
dnaJ	F:AAGGCTTTGGCTCATC R:CTTTTTGTTCATCGTT	53	720	Ziprin *et al.*, 2001
virB11	F:TCTTGTGAGTTGCCTTACCCCTTTT R:CCTGCGTGTCCTGTGTTATTTACCC	48	494	Datta *et al.*, 2003
ciaB	F:TTTTTATCAGTCCTTA R:TTTCGGTATCATTAGC	42	986	Ziprin *et al.*, 2014
pldA	F:AAGCTTATGCGTTTTT R:TATAAGGCTTTCTCCA	45	913	Ziprin *et al.*, 2001
wlaN	F:TTAAGAGCAAGATATGAAGGTG R:CCATTTGAATTGATATTTTTG	46	672	Linton *et al.*, 2000
ceuE	F:CCTGCTACGGTGAAAGTTTTGC R:GATCTTTTTGTTTTGTGCTGC	48.9	793	Gonzalez *et al.*, 1997
cgtB	F:TAAGAGCAAGATATGAAGGTG R:GCACATAGAGAACGCTACAA	49.9	561	Gilbert *et al.*, 2000

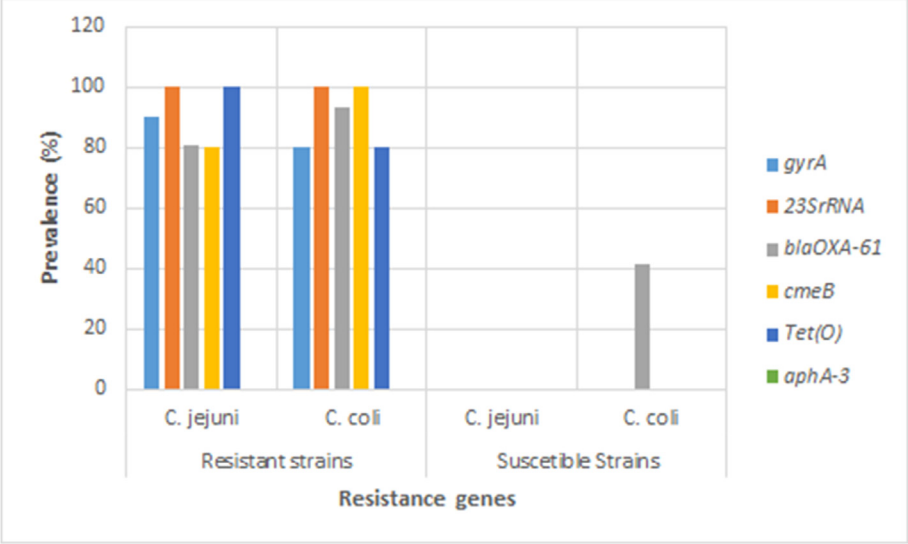

Figure 1. Percentage and distribution of antimicrobial resistance determinants among *C. jejuni* and *C. coli* isolates. Antibiotics and resistance determinants: *Quinolones (*GyrA*), Erythromycin (*23SrRNA*), **β-lactams (bla^{OXA-61}), Tetracycline (*tet*(O)), Gentamicin (*aph*A-3), ***Multidrug resistance *(cmeB)*. *Resistance to Ciprofloxacin or Nalidixic acid or both. **Resistance to Ampicillin or Amoxicillin/clavulanic acid or both. ***Unspecific resistance to multiple antimicrobials acquired by the cmeABC pump efflux activity.

Table 3 Percentage of resistance determinants detected in resistant *C. jejuni* and *C. coli* isolates.

Resistance determinants	*C. jejuni* % (n/N)	*C. coli* % (n/N)	Total % (n/N)
gyrA*	**90** (81/90)	**80** (33/41)	**87** (114/131)
23s rRNA*	**100** (91/91)	**100** (41/41)	**100** (132/132)
A2074C	**14** (13/91)	**27** (11/41)	**18** (24/132)
A2075G	**86**(78/91)	**61** (25/41)	**78** (103/132)
A2075G + A2074C	**0**(0/91)	**12**(5/41)	**4**(5/41)
tet (O)	**100** (91/91)	**80** (33/41)	**94** (124/132)
cmeB	**80** (73/91)	**100** (41/41)	**86** (114/132)
blaOXA-61	**81**(54/67)	**93** (13/14)	**81** (66/81)
aphA-3	**0** (0/13)	**0** (0/4)	**0** (**0**/17)

N: number of antimicrobial resistant isolates.
n: number of isolates harboring resistance determinant.
*gyrA**: *gyrA* Thr-86-Ile mutation.
*23S rRNA**: including *23S rRNA* A2075G and A2074C mutations.

Campylobacter species as a factor. Means were considered significant when $p \leq 0.05$. All analyses were performed using the Infostat R software (www.infostat.com.ar/).

Results

Prevalence of resistance genes and virulence factors

The rates of antimicrobial resistance genes among resistant isolates of *C. jejuni* and *C. coli* were as follows for *tet*(O) (100% and 80%), *cme*B (80% and 100%), and bla_{OXA-61} (81% and 93%), respectively. The *aph*A-3 gene was not detected in any isolate (Fig. 1). Interestingly, when testing these genes in susceptible isolates, the bla_{OXA-61} gene was detected in 41% (11/27) of β-lactams-susceptible *C. coli* isolates (Fig. 1). None of the other genes was detected in the sensitive isolates.

The analysis of the *gyrA* gene showed the presence of the Thr-86-Ile amino acid substitution in 90% of *C. jejuni* and 80% of *C. coli* isolates that were resistant to Cip and/or Nal. Meanwhile, for the only *C. jejuni* isolate exhibiting susceptibility to both quinolones (Cip and Nal), the Thr-86-Ile substitution was not detected.

Macrolide-resistant isolates were tested for the presence of the mutations A2074C and A2075G in the *23S rRNA* gene. The mutation at position A2075G was found in 86% of *C. jejuni* and 61% of *C. coli* isolates. While the A2074C mutation occurred in 14% of *C. jejuni* and 27% of *C. coli* isolates. Interestingly 12% of *C. coli* isolates harbored mutations at both positions (A2074/2075) of the V region (Table 3).

Regarding the virulence genes, all *Campylobacter* isolates contained the *cdtA*, *cdtB* and *cdtC* genes. Among the remaining genes, the *flaA* (100%–96%) was the most prevalent gene in *C. jejuni* and *C. coli*, respectively, followed by *cadF* (95%–89%), *racR* (87%–93%), *virB11* (94%–89%), *pldA* (79%–89%) and *dnaJ* (50%–71%) (Fig. 2). The *ceuE* gene was carried by 53% of *C. jejuni* isolates, while a lower rate was found for the *cgtB*; only 40% of *C. jejuni* and 49% of *C. coli* harbored this gene. The *wlaN* gene was not detected in any tested isolate.

Association between antibiotic resistance and occurrence of virulence genes

Regarding the relationship between the presence of virulence genes and the antimicrobial susceptibility/resistance patterns among isolates, the number of virulence-associated genes detected in resistant *Campylobacter* isolates was higher than in susceptible ones.

Resistant isolates to Ery-Cip-Chl-Tet showed more virulence genes than Amp-Amc-Nal-Gen resistant ones (Fig. 3). Strains with susceptibility to Cip-Tet-Ery showed fewer virulence genes than those susceptible to Gen-Nal-Amc (Fig. 4).

Binary logistic regression models were performed for every isolate, using the antimicrobial resistance profiles as a dependent variable and the presence/absence of the virulence genes as an independent variable. The *flaA, cdtA, cdtB, cdtC, wlaN* genes, and the antibiotics erythromycin

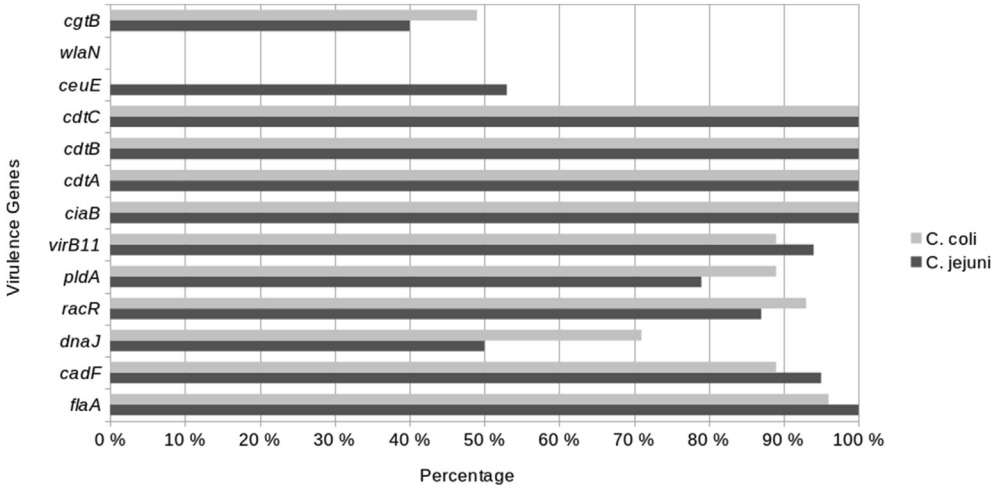

Figure 2. Prevalence of virulence genes in *C. jejuni* and *C. coli*.

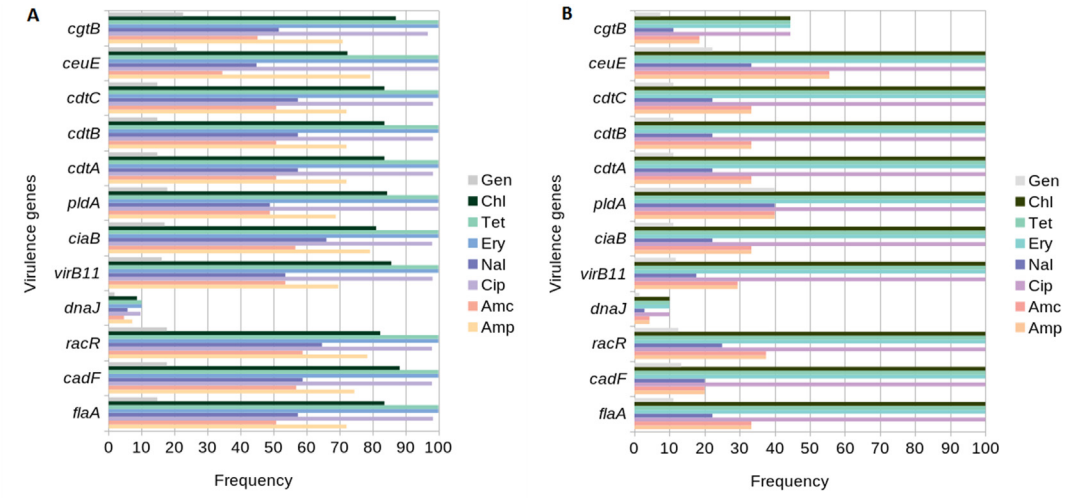

Figure 3. Frequency and distribution of virulence genes in resistant strains of *C. jejuni* (A) and *C. coli* (B). Cip, ciprofloxacin; Nal, nalidixic acid; Ery, erythromycin; Tet, tetracycline; Amp, ampicillin; Amc, amoxicillin/clavulanic acid; Gen, gentamicin and Chl, chloramphenicol.

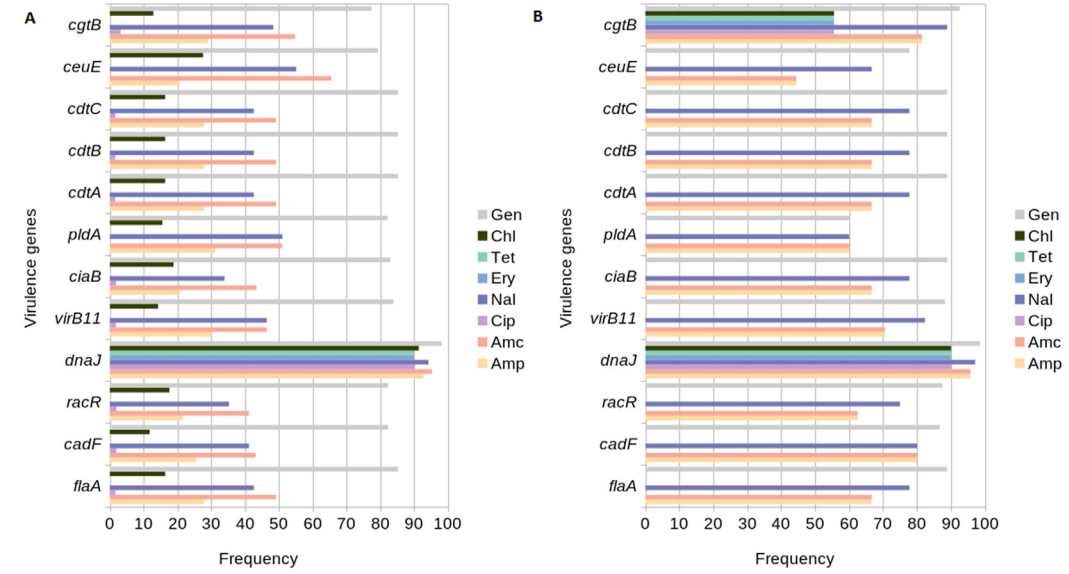

Figure 4. Distribution of virulence genes in susceptible strains of *C. jejuni* (A) and *C. coli* (B). Cip, ciprofloxacin; Nal, nalidixic acid; Ery, erythromycin; Tet, tetracycline; Amp, ampicillin; Amc, amoxicillin/clavulanic acid; Gen, gentamicin and Chl, chloramphenicol.

and tetracycline, which show no variability (100% resistant), were excluded from the analysis. When analyzing the association between resistance to antimicrobials and the presence/absence of virulence genes, it was observed that *C. jejuni* strains show a statistically significant association ($p < 0.05$ and an OR >1) between the antimicrobial resistance and the presence of virulence genes. The *cadF* gene presented a positive association with resistant strains to amoxicillin/clavulanic acid and chloramphenicol. The *racR* was associated with resistant strains to β-lactams drugs (both ampicillin and amoxicillin/clavulanic acid) and nalidixic acid, while the *ciaB* was associated with ampicillin-resistant isolates (Table 4). Regarding *C. coli* isolates, no association between resistant strains and virulence genes was observed (Table 5). As a regard to the *ciaB* gene, the binary logistic regression model failed to generate results, probably because variables are non-linear or highly correlated.

Discussion

Campylobacter species are involved in foodborne infections, and the emergence of MDR strains is becoming a serious health concern. Due to the limited studies on *Campylobacter* in Tunisia, there are no available data on molecular pathogenic traits of local *Campylobacter* strains. Hence, this study evaluated the occurrence of virulence

and antimicrobial resistance-associated determinants in *Campylobacter* species isolated from broiler chickens.

Several studies, worldwide, have shown that the expression of genes involved in motility, colonization, invasion of epithelial cells, and production of toxins plays an important role in the development of *Campylobacter*-associated diseases.[20] In the current study, most of the isolates were shown to contain associated-virulence genes related to the pathogen adhesion, colonization, and invasion traits. This was consistent with the results of previous studies, where genes such as *flaA, cadF, racR, virB11, ciaB, and pldA* were frequently present.[1,21–24] Moreover, the *cdtA, cdtB, and cdtC* genes, necessary for the expression of the CDT toxin, were detected in all *Campylobacter* isolates, as reported by several studies.[25] Regarding, *C. jejuni*, the *ceuE* gene conferring the capacity to chelate iron, is harbored by 53% of the tested isolates. The *wlaN* gene was not detected in any *Campylobacter* isolate; in contrast with the study carried out in Iran, where high prevalence among *Campylobacter* isolates (82.22%) was shown.[26] However, the prevalence of the *cgtB* gene was 49% in *C. coli* and 40% in *C. jejuni*. Therefore, we can suggest that the high rates of these genes among the studied isolates may indicate their important pathogenic potential and the high risk for human health.

Antibiotic resistance, which represents a global problem for animal and public health, is widely reported. The spread of antimicrobial resistance is becoming of high concern due to the widespread use of antibiotics in the poultry industry.[1,27,28] Our previous results showed high rates of resistance to several antibiotics among *Campylobacter*.[16] Investigation of the molecular basis of drug resistance in tested isolates showed that antibiotic resistance phenotypes correlate well with both the occurrence of genes and the mutations encoding antibiotic resistance. Indeed, tetracycline resistance in *Campylobacter* is conferred by the presence of the *tet*(O) gene. However, tetracycline-resistant *C. jejuni* isolates with negative PCR for *tet*(O) gene, might harbor other genetic determinants conferring antibiotic resistance. These results are comparable to reports from Italy, showing high resistance rates (67.87%) to tetracycline in broilers.[29] Based on the high occurrence of resistance to tetracycline and the presence of *tet*(O) gene in the majority of these isolates, the results might suggest that tetracycline would not be a good alternative for the treatment of campylobacteriosis.[30,31] The point mutations A2075G and/or A2074C in the *23S rRNA* gene, which are associated with the resistance to erythromycin, were found in all erythromycin-resistant *Campylobacter* isolates. Other studies have also reported high prevalences of such mechanism of resistance in *Campylobacter* isolates from various origins.[32,33]

Resistance to ciprofloxacin has been linked to the presence of two different mechanisms. The first is the presence of the operon *cmeABC*, which is the most common multidrug efflux pump found in *Campylobacter*, contributing to the resistance to fluoroquinolones by decreasing the amount of the drug in the cells.[34,35] The second resistance mechanism is related to point mutation(s) in the QRDR of *gyrA* gene, in particular, the Thr-86-Ile mutation, frequently observed in fluoroquinolone-resistant isolates.[27,36] Such mutation was detected in 90% and 80% of quinolones-resistant *C. jejuni*

Table 4 Relationship between susceptibility/resistance patterns and associated-virulence genes in *C. jejuni* isolates.

	Ampicillin		Amoxicillin/ Clavulanic acid		Ciprofloxacin		Naldixic acid		Chloramphinicol		Gentamicin	
	OR	*p-values*	*OR*	*p-values*	*OR*	*p-values*	*OR*	*p-values*	*OR*	*p-values*	*OR*	*p-values*
cadF	2.111111e+00	0.303026832	6.3437500[b]	0.03172378[b]	5.851132e-08	0.9976300	1.500000e+00	0.55974728	5.000000e+00[b]	0.03861846[b]	2.478188e+07	0.99341410
racR	6.000000e+00[b]	0.014911479[b]	16.0000000[b]	0.01224128[b]	5.851132e-08	0.9976300	6.416667e+00[b]	0.02943628[b]	5.185185e-01	0.55625084	2.478188e+07	0.99341410
dnaJ	8.333333e-01	0.755104697	0.4642857	0.17162191	7.258963e+07	0.9973858	9.122807e-01	0.86112147	1.333333e+00	0.68273000	1.576087e+00	0.53123423
virB11	5.729253e-08	0.992480121	6.3157895	0.11081109	1.749553e-07	0.9974188	2.820555e-08	0.99216055	4.000000e+00	0.16122466	8.146879e+06	0.99282372
ciaB	1.260000e+01[b]	0.004375017[b]	13.1250000[b]	0.02071075[b]	1.654123e-07	0.9967232	8.272602e+07	0.99027222	3.718154e-08	0.99408107	2.365543e+07	0.99412548
pldA	7.153846e-01	0.649302359	0.8593750	0.81784195	1.935723e+08	0.9965063	3.000000e-01	0.09634416	4.523810e-01	0.47846275	2.594595e+00	0.39099606
ceuE	1.642857e+00	0.413710706	0.3296703	0.05544940	7.742894e+07	0.9973300	3.545455e-01	0.05840358	2.010345e-01[a]	0.04210814[a]	2.434783e+00	0.24226672
cgtB	7.777778e-01	0.668692996	0.6334842	0.41011224	1.291507e-08	0.9972844	5.614035e-01	0.27679960	1.687500e+00	0.45721810	4.083333e+00	0.09735914

[a] Indicates an association between antimicrobial susceptibility and the associated-virulence gene.
[b] Indicates an association between AMR and the associated-virulence gene.
Antimicrobial susceptibility is defined as an OR <1 and $p \leq 0.05$, and antimicrobial resistance (AMR) is defined as an OR > 1 and $p \leq 0.5$.

Table 5 Relationship between susceptibility/resistance patterns and associated-virulence genes in *C. coli* isolates.

	Ampicillin		Amoxicillin/Clavulanic acid		Naldixic acid		Gentamicin	
	OR	*p*-values	OR	*p*-values	OR	*p*-values	OR	*p*-values
cadF	2.161717e-09	0.99577264	2.161717e-09	0.99577264	5.000000e-01	0.6166028	17792122	0.9964629
racR	2.552689e+07	0.99513554	2.552689e+07	0.99513554	1.418160e+07	0.9953032	16,521,256	0.9971248
dnaJ	8.673659e+07	0.99552820	8.673659e+07	0.99552820	4.625952e+07	0.9956820	52,394,336	0.9973625
virB11	9.793595e-09	0.99628071	9.793595e-09	0.99628071	5.036706e-09	0.9961466	5,672,642	0.9968636
ciaB	NA	NA	NA	NA	NA	NA	NA	NA
pldA	1.500000e+00	0.71063966	1.500000e+00	0.71063966	3.666667e+00	0.2762823	569,690,286	0.9967289
ceuE	1.000000e+01	0.06653898	1.000000e+01	0.06653898	4.000000e+00	0.2768084	244,152,980	0.9973926
cgtB	3.571429e+00	0.30544117	3.571429e+00	0.30544117	1.666667e+00	0.6903733	62,873,203	0.9967366

Antimicrobial susceptibility is defined as an OR <1 and $p \leq 0.05$, and AMR is defined as an OR > 1 and $p \leq 0.5$.
N/A: Not applicable.
* Indicates an association between antimicrobial susceptibility and the associated-virulence gene whereas.
**Indicates an association between AMR and the associated-virulence gene.

and *C. coli* isolates, respectively. The remaining resistant isolates didn't harbor the Thr-86-Ile substitution, and the observed resistance might be explained by an efflux pump activity since they are carrying the *cme*B gene or by the existence of other mutations.

The bla_{OXA-61} gene encoding resistance to β-lactams was detected in the majority (80—95%) of isolates resistant to one of the tested antibiotics or both of them (ampicillin and amoxicillin/clavulanic acid). However, even though there is a strong correlation between resistance to β-lactam drugs and the presence of the bla_{OXA-61}, our study showed that 41% of β-lactams-sensitive *C. coli* carried this gene. Our results corroborate with other reports, which suggest that the presence of bla_{OXA-61} gene might have other function than mediating β-lactams resistance in *Campylobacter*.[37,38]

Regarding gentamicin resistance, isolates were screened for the presence of *aph*A-3 gene. Indeed, the main mechanism of aminoglycoside resistance in *Campylobacter* spp. Is *via* aminoglycoside-modifying enzymes (AphA, AadE, Sat) and probably *via* the efflux pump systems even though the mechanism is not yet clear. By contrast to the results of Durate and collaborators,[39] which reported the detection of *aph*A-3 gene in gentamicin resistant-strains, none of our *Campylobacter* strains harboured this gene, suggesting the presence of other mechanism, such us the aadE—sat4—aphA-3 resistance cluster or point mutations.

When looking at the relationship between the prevalence of virulence genes and antimicrobial resistance, our results showed that several virulence genes are associated with resistant strains. Indeed, amoxicillin/clavulanic acid-resistant strains were associated with the presence of *cadF*, *racR*, and *ciaB*, ampicillin-resistant ones with the presence of *racR* and *ciaB*, those resistant to nalidixic acid with the presence of *racR* and chloramphenicol-resistant ones with the presence of *cadF* and *ceuE*.

The existence of positive or negative associations between antimicrobial resistance and virulence genes in bacteria has been shown but still controversial in *Campylobacter* genus.[22,40,41] Indeed, some studies showed an *in vitro* increased invasion of resistant strains as compared to susceptible ones,[42] while others described the tendency of susceptible strains to cause more severe infections than resistant strains.[41] In the current study, analysis of the relationship between the presence of virulence genes and the antimicrobial resistance among *C. jejuni* isolates showed some positive associations ($p < 0.05$ and an OR >1). Some of the virulence genes associated with antimicrobial-resistant strains of *C. jejuni* are involved in bacterial adhesion and invasion capacity, which support the suggestion that resistant strains have higher adhesion and invasion capacity than sensitive ones. Nevertheless, further investigations should be conducted to explore more in-depth the relationship between the pathogenic traits and the antimicrobial resistance in *Campylobacter* strains.

Conclusions

The present study is the first to assess the occurrence of virulence and antimicrobial resistance genes among *Campylobacter* strains isolated from broiler chickens, in Tunisia. It was shown that the rate of strains harboring multiple virulence factors is significantly high; this might be considered when analyzing the pathogenic potential of such bacterial agents for public health in our country. The emergence of antimicrobial resistance among *Campylobacter* isolates is becoming of great concern and may present a serious public health threat. Multiple genetic determinants conferring drug resistance were identified and might be considered as a serious source of spreading antibiotic resistance in the environment. A positive association between virulence genes and drug resistance was shown only for *C. jejuni* isolates, however these results require more in-depth investigation, particularly for strains of human origins.

In conclusion, this study provided for the first time an overview of molecular mechanisms of antimicrobial resistance and virulence traits in *Campylobacter* strains in Tunisia and drawn attention to the need to consider this foodborne pathogen as an emerging public health concern.

Ethics statement

The study was approved by the Institute Pasteur of Tunis Biomedical Ethics Committee, under the reference number: 2018/12/I/LR16IPT03.

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Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.07.001.