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Original Article

Prevalence and distribution of pathogenic genes in *Campylobacter jejuni* isolated from poultry and human sources

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Abstract

Introduction: *Campylobacter jejuni* is one of the most common bacterial causes of human gastroenteritis. Despite its public health importance, the virulence factors and mechanisms underlying *C. jejuni* pathogenesis remain poorly understood and the relationships between these genes and the sources of the strains are not clear. We aimed to determine the virulence profiles of *C. jejuni* isolated from poultry and human cases of Campylobacteriosis.

Methodology: A total of 50 strains of *C. jejuni* isolated from poultry and human cases of Campylobacteriosis were screened by polymerase chain reaction (PCR) for the presence of six pathogenic genes (*flaA*, *iam*, *wlaN*, *cdtA*, *cdtB*, *cdtC*).

Results: A total of 40% (10/25) of the human isolates presented only one virulence marker. In contrast, 64% (16/25) of the poultry-derived strains showed four or five virulence markers. *cdtA* and *flaA* occurred more frequently in poultry-derived strains than in human strains. Ten different virulence profiles were observed among the human isolates and 11 among the poultry strains. Only four profiles were common to both sources: profiles 3 (*flaA*, *cdtA*, *cdtB*, and *cdtC*), 5 (*cdtA* and *cdtB*), 7 (*flaA* and *cdtB*), and 10 (*iam*, *flaA*, *cdtA*, *cdtB*, and *cdtC*). The human-derived strains had a higher Shannon diversity index (1.9396) and Simpson index (0.8367), indicating a more diversified population than found in poultry (1.7742 and 0.7333, respectively).

Conclusions: We found variations in the genetic profiles of the circulating strains based on the isolation source and genes that are potentially pathogenic to humans were detected in poultry-derived strains.

Key words: Broiler; *Campylobacter*iosis; *Campylobacter jejuni*; human; virulence marker.

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Introduction

Campylobacteriosis is a foodborne disease caused by thermophilic *Campylobacter* species, most frequently *C. jejuni* (subspecies *jejuni*) and *C. coli* [1]. It is one of the most common bacteria-caused human gastroenteritis in the world [1-4]. *Campylobacter* infection usually causes abdominal and muscle pain, nausea, headache, fever, and diarrhea. Complications such as Guillain-Barré Syndrome (GBS), an acute neuromuscular paralysis, can occur [4,5].

Campylobacter species are usually present in the intestine of wild and domesticated animals. Poultry is the major reservoir and the primary source of transmission to humans [6,7]. Human cases of Campylobacteriosis are usually associated with the handling, preparation, and consumption of raw or undercooked chicken meat or cross-contamination with these products [8].

Despite its public health importance, the virulence factors and mechanisms underlying *C. jejuni* pathogenesis remain poorly studied in Latin-American countries [9]. Moreover, the relationships between these genes and the sources are unclear [10]. *Campylobacter* virulence factors are mostly associated with motility, chemotaxis, the adhesion to and colonization of intestinal epithelial cells, invasion and translocation capabilities, production of toxins and secreted proteins, and other mechanisms essential for bacterial survival [11].

The cytolethal distending toxin (CDT) consists of three subunits encoded by the *cdtA*, *cdtB*, and *cdtC* genes, which are genetically arranged as an operon [12]. CdtB is the active toxic unit. CdtA and CdtC are subunits required for CDT to bind on target cells and for the delivery of CdtB into the cell's interior [13]. Flagella motility is an important virulence marker for many bacterial pathogens, including *C. jejuni*, as it is necessary to establish infections [14]. *Campylobacter* strains carry one flagellum at each pole. Flagella are composed of several different proteins. The

extracellular filament structural components produce two flagellins, FlaA and FlaB (encoded by *flaA* and *flaB*, respectively) that form the flagellar filament [11,14]. The *Campylobacter* strains responsible for GBS also carry *wlaN*, which encodes a β-1,3-galactosyltransferase enzyme required for the production of sialylated lipooligosaccharide (LOSSIAL). *Campylobacter* LOSSIAL structures cross-react with gangliosides in peripheral nerves leading to clinical symptoms characteristic of this syndrome [15,16]. Invasion and host adaptation are influenced by several factors, for example, the expression of the invasion-associated marker (*iam*) is associated with more invasive *Campylobacter* strains [17].

Considering that *Campylobacter* is transferred from animals to humans, and that poultry serves as its main reservoir, knowing the genetic profile of circulating strains in broiler and human populations is important. Therefore, this study aimed to determine the virulence potential of *C. jejuni* isolated from poultry and humans.

Methodology

Bacterial strains

A total of 50 *C. jejuni* strains isolated from poultry (n = 25) and from human cases of Campylobacteriosis (n = 25) were used for this study. The poultry-derived strains were previously obtained from broiler carcass samples (cooled and frozen) from Brazilian poultry slaughterhouses according to the criteria described by the International Organization for Standardization (ISO 10272-1:2017). The human-derived strains were kindly provided by the Oswaldo Cruz Institute Foundation (Fiocruz, Brazil). All strains were isolated in 2012. The bacterial isolates were stored at −80 °C in brain-heart infusion broth (Oxoid, Hampshire, UK) supplemented with 15% glycerol (Synth, Diadema, Brazil). The bacterial strains were cultured on blood base agar (Oxoid) supplemented with 5% defibrinated sheep blood (Laborclin, Curitiba, Brazil) to reactivate them. The plates were incubated under microaerobic conditions using a gas tank [10% carbon dioxide (CO_2), 5% oxygen (O_2), and 85% nitrogen (N_2)] for 48 h at 42 °C.

DNA extraction

The DNA was extracted using an adapted protocol described by Borsoi *et al*. [18]. Briefly, an aliquot (1 mL each) of the bacterial cultures were boiled at 95 °C for 10 min. After centrifugation at 8,000 × g for 2 min, the supernatants were stored at -20 °C and used as template DNA. The genetic content of the isolates was confirmed by the polymerase chain reaction (PCR)-based detection of 16S rRNA and *mapA* gene [19].

PCR primer design and amplification

C. jejuni isolates were screened for the presence of six pathogenic genes: *flaA*, *iam*, *wlaN*, *cdtA*, *cdtB*, and *cdtC*. The primers, PCR conditions, amplicon lengths, and references are listed in Table 1.

All PCR amplifications consisted of 10 × PCR Buffer [3 µL; 200 mM Tris–HCl (pH 8.4), 500 mM KCl], *Taq* thermostable DNA polymerase (2 U/µL), dNTPs (2.5 µL, 2.5 mM), and extracted template DNA

Table 1. List of primers and PCR conditions used in this study.

Target gene	Primers	Sequence (5'→3')	PCR conditions	Product (bp)	Reference
16S - rRNA	MD16S1 MD16S2	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTATT	95 °C/10 min, 35 cycles: 95 °C/30 s, 59 °C/90 s, 72 °C/1 min, and 72 °C/10 min	857	*Campylobacter* genus identification [19,45]
*map*A	MDmapA1 MDmapA2	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA		589	*C. jejuni* species identification [19]
*fla*A	flaAF flaAR	GGATTTCGTATTAACACAAATGGTGC CTGTAGTAATCTTA AACATTTTG	94 °C/5 min, 30 cycles: 94 °C/1 min, 48 °C/1 min 30 s, 72 °C/1 min, and 72 °C/5 min	1700	[46]
iam	IAMF IAMR	GCGCAAAATATTATCACCC TTCACGACTACTATGCGG	94 °C/5 min, 30 cycles: 94 °C/1 min, 55 °C/1 min, 72 °C/1 min, and 72 °C/5 min	518	[38]
*wla*N	wlaN-DL39 Cj1139cF	TTAAGAGCAAGATATGAAGGTG TGCTGGGTATACAAAGGTTGTG	95 °C/10 min, 25 cycles: 95 °C/30 s, 60 °C/30 s, 72 °C/1 min, and 72 °C/5 min	434	[47]
*cdt*A	cdtAF cdtAR	CCTTGTGATGCAAGCAATC ACACTCCATTTGCTTTCTG	94 °C/5 min, 30 cycles: 94 °C/1 min, 49 °C/1 min, 72 °C/1 min, and 72 °C/5 min	370	[48]
*cdt*B	cdtBF cdtBR	CAGAAAGCAAATGGAGTGTT AGCTAAAAGCGGTGGAGTAT	94 °C/5 min, 30 cycles: 94 °C/1 min, 51 °C/1 min, 72 °C/1 min, and 72 °C/5 min	620	[15]
*cdt*C	cdtCF cdtCR	CGATGAGTTAAAACAAAAAGATA TTGGCATTATAGAAAATACAGTT	94 °C/5 min, 30 cycles: 94 °C/1 min, 49 °C/1 min, 72 °C/1 min, and 72 °C/5 min	182	[15]

(2 µL). For protocol 1 (*cdtA* and *cdtC*) the mixture (30 µL) consisted of $MgCl_2$ (1 mM) and primers (0.5 µL each, 20 pmol). Protocol 2 (*cdtB*) was composed of a mixture (30 µL) with $MgCl_2$ (1.5 mM) and primers (0.5 µL each, 20 pmol). For protocol 3 (*iam*), the mixture (25 µL) was composed of $MgCl_2$ (1.5 mM) and primers (0.5 µL each, 20 pmol). Finally, for protocols 4 (*flaA*) and 5 (*wlaN*), the mixture consisted of $MgCl_2$ (1.2 mM) and primers (0.5 µL each, 10 pmol). Sterile Milli-Q water was used to achieve the final reaction volume (25 µL or 30 µL). All amplification reactions were performed in a thermal cycler according to the cycling conditions described in Table 1. The DNA bands were stained with ethidium bromide to visualize the PCR products. Aliquots of 10 µL were subjected to electrophoresis in a 2% agarose gel in Tris–acetylated EDTA (TAE) buffer for 2 h at 100 V, viewed under an ultraviolet transilluminator, and photographed. The size of the PCR amplicons was determined through comparison with a 100-bp DNA ladder (Invitrogen, Carlsbad, CA, USA). *C. jejuni* ATCC 33560 and two strains of *C. jejuni* from our laboratory stock collection were used as positive controls. In all PCRs, a mixture of all constituents of the PCR mixed without the addition of extracted DNA was used as a PCR control.

Statistical analysis

The data were subjected to statistical analysis using the PASW Statistics software (IBM, Hong Kong). Descriptive statistics (frequency distribution) were used to determine the presence of the virulence genes according to the isolation source (poultry and human). Chi square ($\chi 2$) and Fisher's exact tests were used to compare the frequencies of virulence-associated genes within and between the isolation sources. Statistical significance was defined as $p < 0.05$, and the Bonferroni correction was applied to adjust the confidence intervals for multiple hypothesis testing; these adjusted p values are indicated in the tables. Shannon and Simpson diversity index values were determined based on virulence profiles. This index is calculated as the natural logarithm of the proportion of individuals in one particular group divided by the total number of individuals. Populations with higher indexes are considered more diverse [20]. Simpson diversity index values (D) were calculated using the following formula:

$$D = 1 - \left(\frac{n(n-1)}{N(N-1)}\right)$$

where n represents the total number of bacterial strains with a particular virulence profile and N is the total number of bacterial strains from the respective source (human or poultry). The value of this index ranges from 0 to 1 with higher values indicating greater sample diversity [21].

Results

The occurrence of virulence genes according to the isolation source is shown in Table 2. Only one strain (poultry origin) did not present any virulence markers. However, no strain presented all of the virulence markers. Among the human isolates, 40% (10/25) presented only one virulence marker. In contrast, 64% (16/25) of the poultry-derived strains presented four or five virulence markers. Statistical analysis indicated that the isolation sources differed significantly ($p < 0.05$) regarding *cdtA* and *flaA*. For both genes, *C. jejuni* isolated from poultry presented higher frequencies of these pathogenicity-associated genes than the strains isolated from humans. Among the poultry-derived strains, *wlaN* and *iam* were significantly less frequent ($p < 0.05$) than the other genes. Among the human isolates, the frequency of *cdtB* was significantly higher ($p < 0.05$) than the other genes.

The distribution of virulence profiles according to isolation source is described in Table 3. The distribution of *C. jejuni* strains indicated 10 virulence profiles among the human-derived strains and 11 profiles in the samples of poultry origin strains. Only four profiles were shared by both isolation sources: profiles 3 (*flaA*, *cdtA*, *cdtB*, and *cdtC*), 5 (*cdtA* and *cdtB*), 7 (*flaA* and *cdtB*), and 10 (*iam*, *flaA*, *cdtA*, *cdtB*, and *cdtC*). Among the human isolates, profiles 1 (*cdtB*) and 2 (*cdtA*, *cdtB*,

Table 2. Detection of virulence genes among *Campylobacter jejuni* strains.

Gene	Distribution		
	Poultry % (n = 25)	Human % (n = 25)	Total % (n = 50)
*cdt*A	84 (21)^{a,A}	44 (11)^{b,A}	64 (32)^{A}
*cdt*B	88 (22)^{a,A}	96 (24)^{a,B}	92 (46)^{B}
*cdt*C	76 (19)^{a,A}	48 (12)^{a,A}	62 (31)^{A}
*fla*A	80 (20)^{a,A}	20 (5)^{b,A}	50 (25)^{A}
*wla*N	16 (4)^{B}	0	8 (4)^{C}
iam	4 (1)^{a,B}	12 (3)^{a,A}	8 (4)^{C}

Different lowercase letters in the same line indicate significant differences ($p < 0.05$) in detection of virulence-associated genes between sources of isolation (Fisher's exact test). Different uppercase letters in the same column indicate significant differences ($p < 0.05$) in frequency detection of virulence-associated genes within the same source of isolation (Fisher's exact test; adjusted p value = 0.0085).

and *cdtC*) were the most common, representing 36% (9/25) and 20% (5/25) of these strains, respectively. Among the poultry strains, profile 3 was predominant, representing 52% (13/25) of these strains.

Discussion

C. jejuni is one of the most reported causes of gastroenteritis worldwide [1]. However, most studies analyzing *Campylobacter* occurrence and pathogenicity focus on European and North American isolates [22]. The absence of an internal monitoring program adopted by poultry companies and national governments, especially in developing countries, is likely responsible for the lack of more precise data in these regions, including South America [23,24]. The pathogenic potential of *C. jejuni* strains isolated from Brazilian poultry has increased over the last decade [25]. Recently, *C. jejuni* strains isolated from poultry in Brazil were characterized according to their zoonotic risk [26]. However, studies comparing virulence factors in human and poultry sources in South American countries remain scarce. Therefore, studies that identify and monitor pathogenic markers in *C. jejuni* isolated from this region are extremely important for the development of effective management strategies.

Campylobacter spp. pathogenicity depends on the presence of virulence factors that differ among isolates of different origin [27]. In the present study, *Campylobacter* strains isolated from poultry presented more virulence markers than those obtained from humans. We expected that human-derived strains would present more virulence marks, as previously reported [28], because these isolates were obtained from people presenting Campylobacteriosis, rather than presumably healthy chickens.

The presence of the *wlaN* gene is associated with the occurrence of GBS in patients with Campylobacteriosis [16]. This gene was only detected in the poultry-derived strains in the present study, which was unexpected. Although previous studies have detected this gene in human isolates in other countries [28,29], studies analyzing Brazilian samples also did not detect *wlaN* [30]; neither did these studies find a higher occurrence in *C. coli* strains than in *C. jejuni* [31]. The *wlaN* gene is associated with GBS. However, it is not essential, and other genes (e.g., *cgtB*) have also been associated with the occurrence of this syndrome [16,28]. The presence of *wlaN* in poultry isolates poses challenges to public health because the consumption of undercooked chicken meat is the most common transmission route of *C. jejuni* to humans [16].

The genes associated with bacterial motility are usually essential for the development of *Campylobacter* infection [28]. The *flaA* gene was significantly more common in the poultry-derived strains (80%) than in the human ones (20%). This result was not expected, since human-derived strains were isolated from persons suffering from Campylobacteriosis, and the poultry-derived strains were collected from carcasses of presumably healthy broilers. This differs from previous studies, which found this gene to be highly conserved among *C. jejuni*, independent of the strain's origin [26,28,32]. Similarly, other Brazilian studies found a high frequency (> 80%) of *flaA* among human and poultry isolates [25,33], which was not confirmed in the present study.

Table 3. Distribution of virulence profiles among *Campylobacter jejuni* strains, according to the source of isolation.

Profile number	Virulence profile	Distribution		
		Human % (n = 25)	Poultry % (n = 25)	Total % (n = 50)
1	*cdtB*	36 (9)	0	18 (9)
2	*cdtA, cdtB, cdtC*	20 (5)	0	10 (5)
3	*flaA, cdtA, cdtB, cdtC*	8 (2)	52 (13)	30 (15)
4	*cdtB, cdtC*	8 (2)	0	4 (2)
5	*cdtA, cdtB*	8 (2)	4 (1)	6 (3)
6	*iam*	4 (1)	0	2 (1)
7	*flaA, cdtB*	4 (1)	4 (1)	4 (2)
8	*iam, cdtA, cdtB, cdtC*	4 (1)	0	2 (1)
9	*flaA, cdtB, cdtC*	4 (1)	0	2 (1)
10	*iam, flaA, cdtA, cdtB, cdtC*	4 (1)	4 (1)	4 (2)
11	*wlaN, cdtA, cdtB*	0	8 (2)	4 (2)
12	*flA, cdtB, cdtC*	0	8 (2)	4 (2)
13	*wlaN, flaA, cdtA, cdtB, cdtC*	0	4 (1)	2 (1)
14	*cdtA, cdtC*	0	4 (1)	2 (1)
15	*flaA, cdtA, cdtB*	0	4 (1)	2 (1)
16	*wlaN, flaA, cdtA, cdtC*	0	4 (1)	2 (1)
17	No virulence genes detected.	0	4 (1)	2 (1)

The expression of all three *cdt* genes is required to maximize the effect of CDT toxin [10,34]. In this study, one strain from each source did not present any of the *cdt* genes. Van Deun *et al.* [35] reported that not all *Campylobacter* strains produce toxins. In contrast, 75% (15/20) of poultry and 45% (9/20) of the human-derived strains possessed all three genes. Our results differ from previous reports which indicate that these genes occur with similar [10] or even a higher frequency in human-derived strains [36]. Furthermore, more pronounced CDT production is associated with human-derived strains [10,35]. Differences in cytotoxicity levels between isolates obtained from humans and poultry have also been previously described [10,35].

The genetic marker *iam* has been associated with the adherence and invasion of HEp–2 cells *in vitro*, and is usually positively associated with *in vitro* invasion [10,38]. Furthermore, a correlation between the clinical occurrence of diarrhea and the isolation of *Campylobacter* strains that adhere to and invade HEp–2 cells has been established [32]. In this study, a lower number (4/40) of *C. jejuni* strains presented this gene, regardless of the isolation source. Among the poultry strains, only one (4%) was positive for *iam*, and three human isolates (12%) presented this gene. The poultry strains analyzed in this study had a lower occurrence of *iam* than those evaluated in previous reports [39,40]. These results show that the occurrence of this virulence marker depends not only on the isolated *Campylobacter* species but also on the isolation source [26]. According to Carvalho *et al.* [40], the absence of *iam* in invasive strains supports the existence of function polymorphisms, high heterogeneity in the *iam* locus, and the contribution of additional loci.

In the present study, a variety of virulence profiles were identified, but only four (3, 5, 7, and 10) were shared by the human and poultry sources. Interestingly, profiles 5, 7, and 10 were not common in either of the two isolation sources. Profile 3 was the most common (52%) among the poultry-derived strains, and profiles 1 and 2 among the human isolates (56%).

These virulence profiles were also used to determine the diversity among *C. jejuni* strains. The Shannon diversity index is a statistical indicator that assumes all groups are represented in a sample and that they are randomly sampled. Populations with higher indexes are considered more diverse [20]. In this study, the human-derived strains had a higher Shannon diversity index (1.9396) than those isolated from poultry (1.7742), indicating a more diversified population in the former. Similarly, Simpson index values were greater for the human-derived strains (0.8367) than their poultry counterparts (0.7333). These results differed from previous studies, which found that poultry isolates were more diverse than the human ones [39]. A possible reason for the difference found in the present study is that the poultry isolates were obtained from a single Brazilian state (RS, Brazil), and, therefore, the strain types may be restricted. On the contrary, although the human-derived strains were also obtained from a single institution, they were isolated from human samples received from across the country. However, it is noteworthy that previous studies have found that, despite the evidence that virulence-associated genes in *C. jejuni* are widely dispersed in both species, poultry isolates presented a high occurrence of virulence markers [10,41,42]. A previous study has evaluated the genotypic relationship between human and poultry strains of *C. jejuni* in Brazil and observed that human isolates presented limited virulence capacity when compared to their poultry counterparts, and were different in molecular typing. They also observed that the ability to cause GBS was similar for both strains [41].

Previous studies have demonstrated that *C. jejuni* isolated from Brazilian poultry presented a high level of diversity of circulating genotypes, since no clones were observed in strains isolated in the same period (2012) of the isolates from this study [43]. In addition, a concern regarding this high frequency of virulence genes in poultry isolates is their stability in chicken juice through conditions that mimic the transmission route and their transmission through host models, which demonstrate the potential transmission of *C. jejuni* from food to human [44]. It is possible that the overlap observed in distribution of virulence-associated genes among human and chicken isolates suggests that Campylobacteriosis may be linked with chicken meat [22]. However, their relationship with clinical severity in humans and the expression of virulence factors should be further investigated.

Studies that identify virulence traits are crucial to better understand the risk of Campylobacteriosis associated with different strains and the zoonotic potential of animal strains. However, despite the high prevalence of pathogenic factors found in the present study, it is difficult to predict the *in vivo* virulence of *C. jejuni* strains during human infection [10,28]. Thus, the detection of virulence-associated genes should be complemented by gene expression analyses and by determining the pathogenicity through *in vivo* models [10].

Conclusions

This study has shown that there are variations in the genetic profiles of the circulating strains based on the isolation source (poultry or human) and that genes potentially pathogenic in humans were detected in poultry-derived strains. In this study, poultry strains presented more virulence markers, making these strains potentially capable to adapt to the environment, invade, and cause disease in humans. Our findings support the potential risk of transmitting highly virulent *C. jejuni* from chicken meat to human. Their relationship with clinical severity in humans and the expression of virulence factors warrant further investigation.

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