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Host-associated variability of the *cdtABC* operon, coding for the cytolethal distending toxin, in *Campylobacter jejuni*

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Abstract

Campylobacter, a major cause of food-borne gastroenteritis worldwide, colonize the gastrointestinal tract of a wide range of animals, being birds the main reservoir. The mechanisms involved in the interaction of *Campylobacter* with the different hosts are poorly understood. The cytolethal distending toxin, encoded in the *cdtABC* operon, is considered a pivotal virulence factor during human infection. Differences in the prevalence of *cdtABC* genes in *Campylobacter* isolates from three distinct origins (wild birds, broiler chickens and humans) prompted us to further characterize their allelic variability. The sequence of *cdtABC* is highly conserved among broiler and human isolates. A high diversity of *cdtABC* alleles was found among wild bird isolates, including several alleles that do not produce any functional CDT. These results suggest that specific variants of the *cdtABC* operon might define the host range of specific *Campylobacter jejuni* isolates. Moreover, our data indicate that PCR methodology is inaccurate to characterize the prevalence of the *cdt* genes, since negative PCR detection can be the result of divergences in the sequence used for primer design rather than indicating the absence of a specific gene.

KEYWORDS
allelic variability, *Campylobacter*, cytolethal distending toxin, PCR detection, WGS

1 | INTRODUCTION

Campylobacter is a major enteropathogen worldwide, causing the most common zoonoses reported in humans in the European Union, the United States and Australia (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021; Kaakoush et al., 2015). In most cases, campylobacteriosis is self-limiting gastroenteritis. Severe cases, although rare, occur mostly among children younger than 5 years, elderly and immunocompromised patients (Kaakoush et al., 2015). Moreover, infection by *Campylobacter* can trigger the autoimmune polyneuropathic disorder Guillain–Barré syndrome (Koga et al., 2006). *Campylobacter jejuni* is the species reported to cause most of the campylobacteriosis cases in humans (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021).

Campylobacter jejuni pathogenicity remains poorly understood. Among the array of bacterial products that contribute to its virulence, the cytolethal distending toxin (CDT) seems to play a pivotal role in the interaction of *Campylobacter* with the host. CDT blocks cell division, causing cell cycle arrest at the G_2 stage prior

to mitosis (Whitehouse et al., 1998). CDT is a heteromeric AB-type genotoxin produced by several Gram-negative bacterial pathogens, including *Campylobacter*, enteropathogenic *Escherichia coli*, *Salmonella enterica* serovar Typhi, *Haemophilus duceryi* and *Shigella dysenteriae* (Jinadasa et al., 2011). The toxin consists of three subunits CdtA, CdtB and CdtC. CdtB is the active subunit whereas CdtA and CdtC are required for binding and delivery of CdtB within the target cell by a process that is still poorly understood (Lara-Tejero & Galán, 2001). CdtA/CdtC binds to the lipid rafts, cholesterol-rich microdomains within the host cell membranes (Lai et al., 2016). CdtB has DNaseI-like activity producing DNA double-strand breaks in the host cell that causes arrest of the cell cycle, cellular distension and cell death (Smith & Bayles, 2006). Although CDT is considered a crucial virulence factor during human infection, it remains unclear its role during the colonization of natural hosts, such as birds.

Campylobacter CDT toxin is encoded in the polycistronic operon *cdtABC*. Most *Campylobacter* epidemiological studies reporting prevalence of genes coding for potential virulence factors, including CDT, are based in the use of PCR for gene detection. Very disparate data on *cdtABC* prevalence among *C. jejuni* isolates from human patients suffering of gastroenteritis has been reported using this methodology. Some studies indicate a high prevalence of the *cdtA*, *cdtB* and *cdtC* genes, whereas other reports much lower prevalence, ranging from 100% (50/50) to 15% (3/20; Bang et al., 2003; de Melo et al., 2021; Iglesias-Torrens et al., 2018; Koolman et al., 2015; Pickett et al., 1996; Weis et al., 2014).

The allelic variability of the *cdtABC* genes in *Campylobacter* isolates from three distinct origins (wild birds, broiler chickens and humans) has been characterized. The present study aimed to evaluate the diversity of *cdt* genes among the isolates from different origins, and the possible role that this variability could play in *Campylobacter* host range specificity.

Impacts

- The sequence of *cdtABC* operon, encoding the cytolethal distending toxin in *Campylobacter jejuni*, is highly conserved among broiler and human isolates. Instead, a high diversity is found among wild bird isolates, including several alleles that do not produce any functional CDT.
- Sequence analysis reveals that the *cdtABC* operon seems to accumulate DNA modifications, particularly deletions, especially detected among wild bird isolates.
- The data obtained indicate that PCR methodology is inaccurate to characterize the prevalence of the *cdt* genes, since negative PCR detection can be the result of divergences in the sequence used for primer design rather than indicating the absence of the specific nucleotide sequences of a specific gene.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

The *C. jejuni* strains used in this study are part of a strain collection, previously described (Iglesias-Torrens et al., 2018), composed of isolates obtained from faeces of three different sources: human suffering symptomatic gastroenteritis (50), broiler chicken (50) and wild birds (50). Wild bird isolates were obtained from different species: yellow-legged gulls (*Larus michahellis*) from Barcelona (YLGB) and from Medes Islands (YLGM); Audouin's gulls (*Larus audouinii*) from Ebro Delta (AGD) and Alboran Islands (AGA); feral pigeons (*Columba livia*) from Barcelona (FP); common ravens (*Corvus corax*) from Barcelona (CR); white storks (*Ciconia ciconia*) from Lleida (WS); and norther shoveler (*Spatula clypeata*) from Ebro Delta (NS). Isolates were cultured onto Columbia blood agar (CBA) plates (Sharlau) and incubated at 42°C for 48 hr in microaerophilic conditions (CampyGen, Oxoid).

2.2 | PCR amplification

Genomic DNA was extracted from cultures grown on CBA plates using the InstaGene Matrix Kit (Bio-Rad Laboratories). PCR reactions (PCR Master Mix x2, ThermoScientific) were performed using 35 ng of DNA as a template and the specific primers for amplification of *cdtA*, *cdtB* and *cdtC* (Table 1) previously used in epidemiological studies (Martínez et al., 2006) and based in the genomic sequence of the reference *C. jejuni* strain 81–176 Amplified PCR products from *cdtB* gene of representative isolates showing different electrophoretic migration were purified using the E.Z.N.A Cycle Pure Kit (OMEGA Bio-tek) and sequenced using the same primers as those used to generate the fragment.

2.3 | Genome sequencing

Whole genome sequencing was performed for a selection of 47 *C. jejuni* isolates, including 12 isolates from broiler, 16 from human and 19 from wild birds. The selection was performed in order to sequence isolates from different origins, sequence types virulence and antibiotic resistance profiles (Iglesias-Torrens et al., 2018). The

TABLE 1 Primers used in this work

Primer		Sequence 5′–3′	PCR product (bp)
cdtA	Fw	CTATTACTCCTATTACCCCACC	422
	Rv	AATTTGAACCGCTGTATTGCTC	
cdtB	Fw	AGGAACTTTACCAAGAACAGCC	531
	Rv	GGTGGAGTATAGGTTTGTTGTC	
cdtC	Fw	ACTCCTACTGGAGATTTGAAAG	339
	Rv	CACAGCTGAAGTTGTTGTTGGC	

DNA extraction was carried out with the Wizard DNA-Purification kit (Promega). The DNA extracted was send to LifeSequencing (Parc Científic, Universitat de València, Spain) to proceed with the DNA library preparation using the Nextera XT DNA Preparation kit and the NexSeq 500 platform. The BioProject has been deposited at NCBI GenBank, accession number PRJNA850915. To find the *cdtABC* nucleotide sequences in each genome we looked for the primers used in previous amplifications by doing the following. We used the "DNAStringSet" function from the Biostrings package (Pagès et al., 2022) in the R (R Core Team, 2020) programming environment (v.3.0.2) to read the primers and genomes in R. The "vmatchPattern" function was used to find all occurrences of the primers with at most 1 mismatch. The start and end positions and the corresponding strand were recorded.

2.4 | Phylogenetic analysis

Evolutionary analyses were conducted with MEGA X (MEGA v10.1.8) (Kumar et al., 2018). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The evolutionary divergence between *cdtABC* sequences was inferred by estimating the distance matrix via the Maximum Composite Likelihood method (Tamura et al., 2004).

2.5 | Ethics approval

No ethical approval was obtained because this study did not involve human subjects, animals or tissue samples and only involved non-invasive procedures.

3 | RESULTS

3.1 | The prevalence of the *cdtA*, *cdtB* and *cdtC* genes is highly variable among *Campylobacter jejuni* isolates from wild birds

The prevalence of genes coding for *cdtA*, *cdtB* and *cdtC* in a collection of 150 isolates of *C. jejuni* from human suffering symptomatic grastroenteritis, broiler chiken and wild birds (see Materials and methods for details) was determined by PCR. The results indicated that the distribution of the *cdt* genes was very variable. The three *cdt* genes were found in all broiler chicken and human isolates. In contrast to what it was observed among human and broiler isolates, 22 out of 50 isolates from wild birds were negative for the detection of one or two of the *cdt* genes (Figure 1), and prevalence varies for each gene, being 58%, 94% and 90% for *cdtA*, *cdtB* and *cdtC*, respectively (Figure 1). The highest variability was detected for the *cdtA* gene. Hence, 14 isolates were *cdtA* negative, three isolates were *cdtAB* negative, four isolates were *cdtAC* negative and one was negative for *cdtC*.

Thirteen out of the fourteen *cdtA*-negative isolates belonged to the clonal complex ST-1,275 which has been described to be highly predominant among wild birds (Hughes et al., 2009; Sheppard et al., 2009). These isolates were collected from Audouin's gulls and yellow-legged gull from three distant location in the Spanish Mediterranean coast (Medes Islands, Ebro Delta, and Alboran Island). The remaining *cdtA*-negative isolate belonged to the singleton ST-8514 and it was collected from a common raven. Three out of four *cdtAC*-negative isolates from Audouin's gulls from Ebro Delta and Alboran Island also belonged to the CC ST-1275. The last *cdtAC*-negative isolate belonged to the singleton ST-996 and was collected from a northern shoveler in Ebro Delta. The three *cdtAB*-negative belonged to the CC ST-952 and were collected from common ravens. The *cdtC*-negative isolate belonged to the CC ST-1034 and was collected from an Audouin's gull in Ebro Delta. Although all *cdt* gene negative isolates were collected from wild birds, it should be point out that *C. jejuni* isolates positive for the three *cdt* genes were also collected from wild birds at the same geographical locations. Our data show that a high diversity in the prevalence of the *cdt* genes was observed among *C. jejuni* isolates from wild birds when compared with isolates from broilers and humans.

When considering the ST-complex determined for each isolate, in most cases, the isolates belonging to a specific ST-complex shared the same pattern of *cdtABC* prevalence (Figure 1). The only exception was within the ST-1275 complex which was the most represented ST among wild birds (16 out of 50 isolates). All the ST-1,275 complex isolates were *cdtA* negative whereas only three of them were *cdtC* negative by PCR.

3.2 | Different *cdtB* alleles detected among the wild bird isolates

A puzzling observation was done during the PCR genotyping analysis of the different *cdt* genes. Most isolates PCR-positive for *cdtB* depicted a band that migrated as expected for the canonical *cdtB* sequence of the reference strain 81–176 (531 bp). However, some isolates showed a band that migrated more than expected, indicating that the PCR amplified band carried a shorter sequence (Figure 2a). These results suggested the presence of different *cdtB* alleles among distinct isolates. Hence, from the 46 *cdtB*-positive wild bird isolates, 17 apparently carried the shorter *cdtB* allele, representing 36.9% of the *cdtB*-positive isolates. Sixteen isolates belonged to the CC ST-1275 and the remaining isolate to the singleton ST-8514 (Figure 2b). Remarkably, all the isolates that carried a shorter *cdtB* allele were PCR-negative for *cdtA*. It should be noticed that 100% of the broiler and human isolates carry a *cdtB* allele that by PCR amplification resembled the canonical allele of 81–176. The amplicons from the reference strain 81–176, the isolates W02, W54, carrying an apparently canonical allele; and W20, W25, W53, showing a shorter *cdtB* allele, were sequenced (Figure 2c).The amplicons from W20, W25 and W53 showed the same feature when compared to 81–176 amplicon, a deletion of 51 nucleotides, resulting in an amplicon of 480

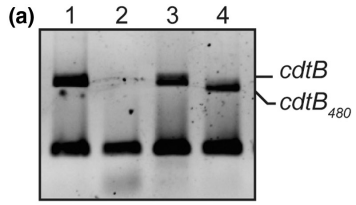
Strain	*cdtABC*	ST-CC (ST)	Strain	*cdtABC*	ST-CC (ST)	Strain	*cdtA*	*cdtB*	*cdtC*	ST-CC (ST)	Species	D4	D154	I6	D9	D668	D25	D51	D51 (PCR)	D17	D28
H02		21 (4664)	B06		21 (883)	W30				45 (45)	YLGB										
H06		21 (21)	B07		21 (50)	W32				45 (45)	FP										
H08		21 (3769)	B14		21 (50)	W36				45 (45)	FP										
H11		21 (21)	B17		21 (883)	W37				45 (45)	FP										
H12		21 (50)	B20		21 (21)	W40				45 (45)	FP										
H32		21 (21)	B36		21 (21)	W43				45 (45)	FP										
H34		21 (50)	B46		21 (21)	W44				45 (45)	FP										
H35		21 (50)	B50		21 (50)	W47				45 (8512)	FP										
H37		21 (883)	B22		45 (652)	W29				48 (48)	YLGB										
H52		21 (21)	B27		45 (45)	W33				179 (179)	FP										
H54		21 (1214)	B31		45 (45)	W34				179 (2209)	FP										
H59		21 (21)	B42		45 (137)	W39				179 (2209)	FP										
H60		21 (883)	B09		48 (48)	W41				179 (220)	FP										
H66		21 (50)	B49		206 (46)	W46				179 (2209)	FP										
H68		21 (19)	B13		257 (2254)	W48				179 (2209)	FP										
H73		21 (50)	B52		257 (367)	W49				179 (2209)	FP										
H58		42 (4016)	B53		257 (367)	W28				354 (354)	YLGM										
H61		42 (459)	B54		257 (367)	W54				354 (354)	CR										
H48		45 (45)	B55		257 (367)	W55				354 (354)	WS										
H38		48 (48)	B56		257 (367)	W56				354 (354)	WS										
H71		49 (49)	B57		257 (367)	W12				446 (3552)	AGA										
H51		52 (52)	B33		283 (267)	W50				952 (8513)	CR										
H05		61 (61)	B05		353 (400)	W51				952 (8513)	CR										
H40		61 (61)	B18		353 (400)	W52				952 (8513)	CR										
H57		61 (61)	B28		353 (5)	W05				1034 (4001)	AGD										
H65		61 (61)	B29		353 (5)	W03				1275 (1223)	AGD										
H70		61 (61)	B30		353 (400)	W04				1275 (1275)	AGD										
H09		206 (572)	B40		353 (400)	W09				1275 (1275)	AGD										
H13		206 (572)	B48		353 (356)	W10				1275 (1223)	AGD										
H14		206 (572)	B23		354 (354)	W14				1275 (1292)	AGA										
H62		206 (572)	B37		354 (8498)	W15				1275 (1223)	AGD										
H64		206 (227)	B45		354 (354)	W16				1275 (1292)	AGA										
H03		257 (257)	B08		464 (464)	W18				1275 (1223)	AGD										
H50		257 (257)	B15		464 (464)	W19				1275 (3629)	AGD										
H56		257 (257)	B21		574 (305)	W20				1275 (1275)	AGD										
H67		257 (2254)	B25		574 (305)	W21				1275 (1223)	AGD										
H69		257 (2254)	B04		607 (607)	W22				1275 (1268)	AGD										
H36		353 (400)	B19		607 (7110)	W24				1275 (637)	YLGM										
H63		353 (353)	B24		607 (1707)	W25				1275 (8511)	YLGM										
H49		354 (354)	B38		607 (904)	W26				1275 (3049)	YLGM										
H74		354 (354)	B41		607 (607)	W27				1275 (637)	YLGM										
H19		443 (5799)	B47		607 (904)	W02				S996	NS										
H46		443 (51)	B51		607 (7110)	W06				S4355	AGA										
H18		464 (464)	B02		S2334	W07				S4355	AGA										
H04		607 (904)	B10		S1710	W08				S4355	AGA										
H01		S441	B16		S531	W11				S1261	AGD										
H07		S1710	B26		S1710	W13				S1343	AGA										
H33		S8479	B35		S1710	W17				S2351	AGD										
H53		S531	B39		S7114	W23				S1343	AGA										
H72		S441	B44		S441	W53				S8514	CR										

FIGURE 1 Collection of *Campylobacter jejuni* isolates from human patients (H), broiler chicken (B) and wild birds (W). The strains are organized attending to the host and ST clonal complexes (ST-CC). Sequence types are also indicated (ST). The species of the wild bird strains are specified (YLGB: yellow-legged gulls from Barcelona; YLGM: yellow-legged gulls from Medes Islands; AGD: Audouin's gulls from Ebro Delta: AGA: Audouin's gulls from Alboran Islands; FP: feral pigeons; CR: common ravens; WS: white storks; NS: northern shoveler). *cdtA*, *cdtB* and *cdtC* positive strains by PCR amplification are indicated in colour background (green for human isolates, red for broiler chicken isolates and blue for wild bird isolates). The different nucleotide deletions and insertions determined by WGS or PCR analysis are indicated in yellow. The shadowed strain names (clear green, pink and clear blue) indicate the strains that the complete *cdtABC* operon sequence have been determined.

nucleotides (as compared with 531 bp amplicon from 81–176), that explained the shorter band detected by PCR. From now, we will refer to this *cdtB* allele as $cdtB_{480}$. Interestingly, in the genome of the strain 81–176, the 51 bp deleted sequence from $cdtB_{480}$ allele was flanked by two 10 bp direct repeats (Figure 2c), suggesting that the $cdtB_{480}$ allele was most probably generated by a site-specific recombination event.

3.3 | Whole-genome sequencing reveals a high diversity in the *cdtABC* locus among wild bird isolates

To further characterize the diversity within the *cdtABC* locus, whole-genome sequencing (WGS) was performed for 47 *C. jejuni* isolates, including 12 isolates from broiler, 16 from human and 19 from wild birds. The sequence of the *cdtABC* locus was characterized



(b)

$cdtB_{480}$ (17)

CC ST-1275 (W03,W04,W09,W10,W14,W15,W16,W18,W19, W20,W21,W22,W24,W25,W26,W27)

Singleton 8514 (W53)

(c)

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81-176  ATCGTGTAAATTTAGCGCTATAGTTTCAAGAATGCAAGCTGAAGAAGTGATT-GTTTTACCTCCACCTACTACAGTTTCAAGACCCATTATA
W20     ATCGTGTAAATTTAGCGCTAT----------------------------------------------------AGTTTCAAGACCCATTATA
W25     ATCGTGTAAATTTAGCGCTAT----------------------------------------------------AGTTTCAAGACCCATTATA
W53     ATCGTGTAAATTTAGCGCTAT----------------------------------------------------AGTTTCAAGGCCCATTATA
W54     ATCGTGTAAATTTAGCGCTATAGTTTCAAGAATGCAAGCTGAAGAAGTGATT-GTTTTACCTCCACCTACTACAGTTTCAAGACCCATTATA
W02     ATCGTGTAAATTTAGCGCTATAGTTTCAAGAATGCAGGCTGAAGAAGTGATTTGTTTTACCTCCGCCTACTACAGTTTCAAGACCCATTATA
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FIGURE 2 Identification of the $cdtB_{480}$ allele. (a) PCR amplification of *cdtB* gene from strains 81–176 (lane 1), W50 (lane 2), W54 (lane 3) and W54 (lane 4). The amplification of the housekeeping *gltA* gene was used as internal control. (b) Isolates that carry the $cdtB_{480}$ allele. (c) Sequences obtained from the PCR amplification of strains carrying the $cdtB_{81\text{-}176}$ or the $cdtB_{480}$ allele. Shadow square indicates the 10 bp direct repeats flanking the 51 bp deletion.

(Figure S1, Appendix S1). Surprisingly, sequences of the three ORFs of the *cdtABC* operon were found in all 19 isolates from wild birds, including isolates that were negative by PCR for *cdtA* (5 isolates), *cdtAB* (2 isolates), *cdtAC* (2 isolates) and *cdtC* (1 isolate; Figure 1). Sequence alignment of the *cdtABC* sequences from all 47 sequenced isolates and the reference strain 81–176, revealed that 38 of the isolates carried a *cdtABC* operon of similar length to the $cdtABC_{81\text{-}176}$, whereas 9 isolates, all of them from wild birds, had suffer drastic DNA modifications, resulting in shorter *cdtABC* operons (Figure S1, Appendix S1).

The optimal tree of the evolutionary analyses of the *cdtABC* operons (Figure 3a) showed that most of the operons from broiler and human isolates formed a very compact cluster (cluster I in Figure 3a). Only four wild bird isolates were found scattered within this cluster I, whereas most of the *cdtABC* operons from wild bird isolates were distant from the broiler/human cluster. The four wild bird isolates founded within the broiler/human cluster I were W12, a ST-446 isolated from AGA; W29 a ST-48 isolated from YLGB and W28 and W56, two ST-354 isolated from YLGM and WS, respectively. The four isolates were determined as *cdtABC* positive by PCR detection.

The rest of *cdtABC* operons from wild birds were grouped in three different clusters. Cluster II was characterized by carrying no deletions or 1 bp deletion, cluster III by having deletions shorter than 50 bp in total and cluster IV by carrying several deletions causing an overall deletion of more than 700 bp.

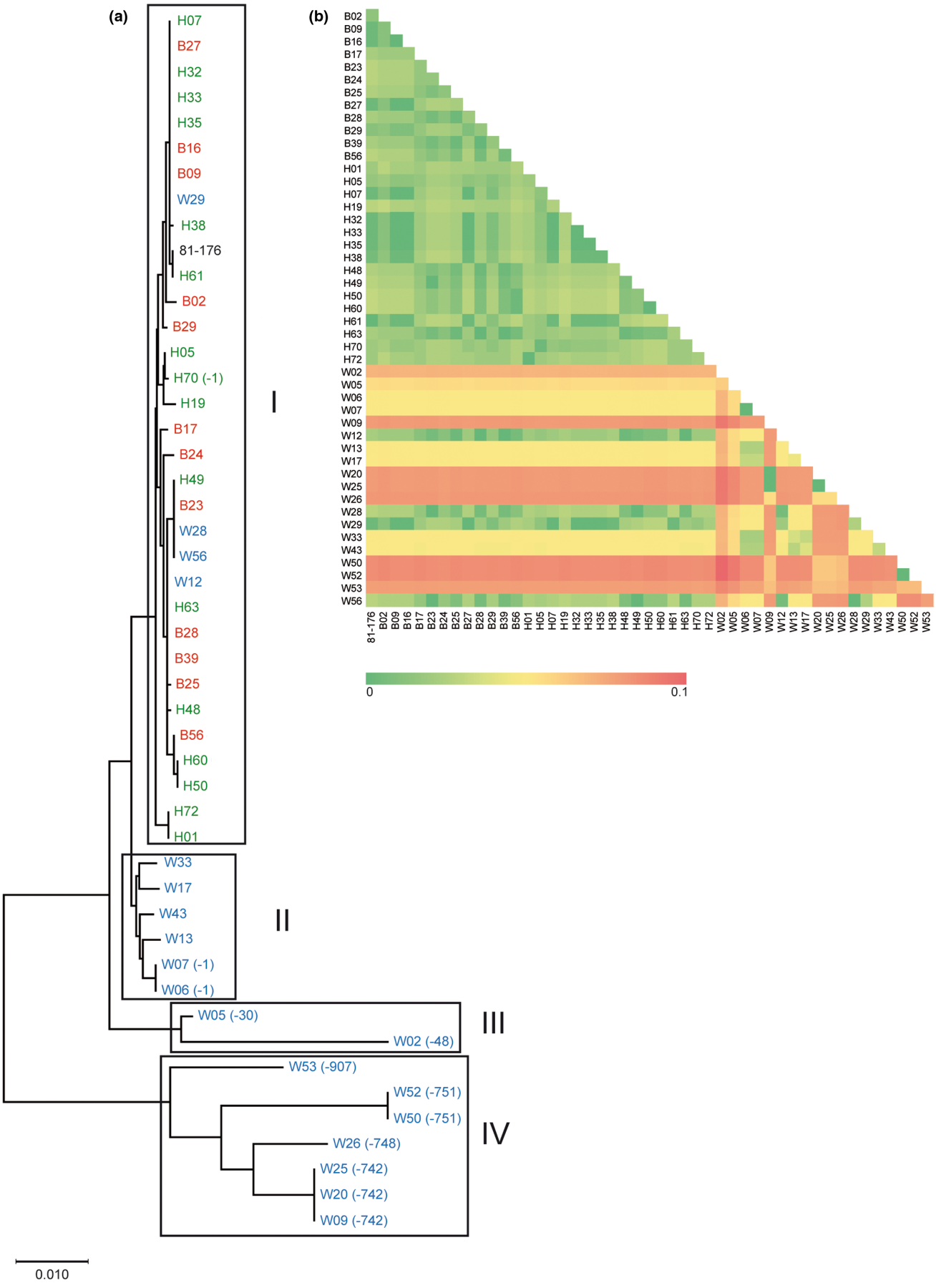
The evolutionary divergence between *cdtABC* sequences was also estimated and the resulting heat map showed the differences between most of the *cdtABC* operons from wild bird isolates respect those isolated from broiler and human (Figure 3b). As expected, similar clustering of the *cdtABC* operons as observed in the optimal tree (Figure 3a) was found.

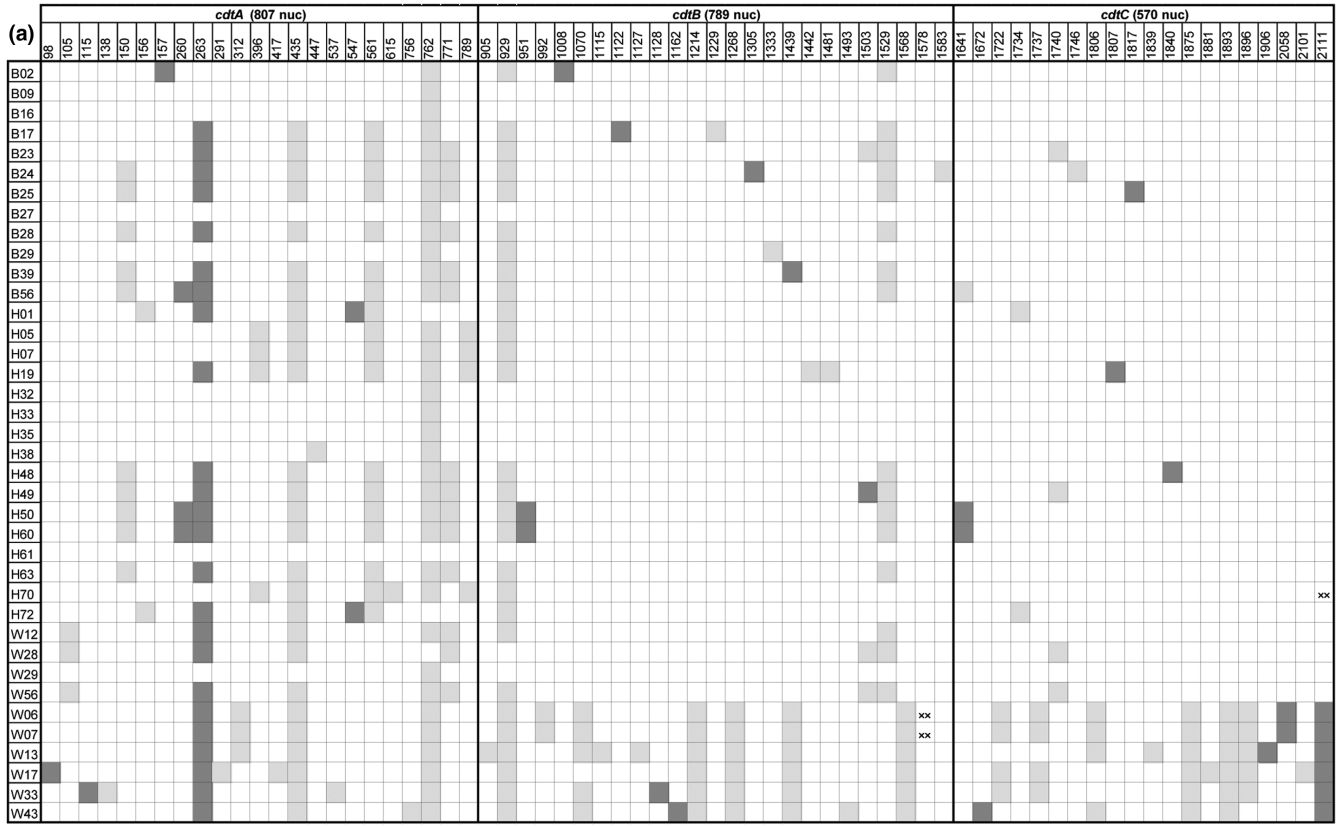
The single nucleotide polymorphism (SNP) distribution respect to the $cdtABC_{81\text{-}176}$ for the 38 isolates with a presumably intact *cdtABC* operon, corresponding to cluster I and II (Figure 3a), is shown in Figure 4a. Overall, it was manifest that *cdtA* accumulated higher number of SNPs as compared with *cdtB* and *cdtC*. The frequency of nucleotide substitution for *C. jejuni* isolates from broilers, humans and wild birds, was 0.48%, 0.53% and 0.53% for *cdtA*, 0.25%, 0.16% and 0.65% for *cdtB*, and 0.06%, 0.09% and 0.77% for *cdtC*, respectively (Figure 4b). Moreover, it was also patent that a group of wild bird isolates (W06, W07, W13, W17, W33, W43), corresponding to cluster II, carried *cdtB* and *cdtC* genes with a higher degree of variation than the isolates from cluster I. The results point out that the *cdtABC* operons from wild birds showed a higher variability (0.64%) as compared with the *cdtABC* operons from broiler and human isolates (0.28% in both groups; Figure 4b). The nucleotide substitution frequency was also calculated for the arbitrarily chosen *ciaB*, *cadF* and *glnA* genes, revealing similar divergence among the three groups of isolates.

3.4 | Several *Campylobacter jejuni* isolates from wild birds carry nonfunctional *cdtABC* operons

The analysis of the nucleotide sequence of the *cdtABC* operons from wild bird isolates showed that this operon is highly variable

FIGURE 3 (a) Evolutionary relationships of 47 *cdtABC* sequences obtained from the WGS, inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.01161943 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 2,181 positions in the final dataset. (b) Evolutionary divergence between sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 2,206 positions in the final dataset.





(b)

	cdtA	cdtB	cdtC	cdtABC
B	0.48	0.25	0.06	0.28
H	0.53	0.16	0.06	0.28
W	0.53	0.65	0.77	0.64

	ciaB	cadF	glnA
B	0.71	1.21	0.62
H	0.82	1.09	0.76
W	0.80	1.34	0.67

FIGURE 4 Single nucleotide polymorphism. (a) Distribution of SNPs in *cdtA*, *cdtB* and *cdtC* as compared to the $cdtABC_{81\text{-}176}$ for the 38 isolates with a presumably intact *cdtABC* operon, corresponding to cluster I and II of the optimal tree (Figure 3a). Squares in light grey indicate silent mutations, dark grey squares indicate missense mutations and asterisks indicates nonsense mutations. (b) Frequency of nucleotide substitution for *cdtA*, *cdtB*, *cdtC* and *cdtABC*. The frequency determined for the indicated arbitrary chosen genes is also shown. In all cases sequences were compared to the sequence of the reference strain 81–176.

and prompted to accumulate DNA modifications. When considering deletion and insertion larger than 3 bp, several modifications were identified within the *cdtABC* operon. Remarkably, the modifications occurred within the *cdtA* and *cdtB* genes. No deletions and insertions larger than 3 bp were found within *cdtC* (Figure 5a, Figure S1, Appendix S1). Among *cdtA* genes, four deletions were detected, D4, D9, D25 and D154 of 4, 9, 25 and 154 bp, respectively. An insertion of 6 bp (I6) was also identified in four *cdtA* alleles. Within the *cdtB* gene three specific deletions were detected D17, D28 and the above described D51. Interestingly, six isolates carried a large deletion of 668 bp (D668) that affected both *cdtA* and *cdtB* genes.

Considering the mentioned modifications, five *cdtABC* operon variants were identified (Figure 5b). The *cdtABC* v1 has only a deletion (D28) within *cdtB*. The *cdtABC* v2 seems to derive from variant 1 since in addition to D28, an additional deletion (D25) was found in *cdtA*. The other three variants underwent drastic modifications, as they accumulate at least five different deletions within *cdtA* and *cdtB*, including the large D668. The variant 3 was detected in two isolates. The variant 4 which is identical to variant 3 but with an insertion of 6 bp, is the most prevalent (detected in four isolates). The variant 5, similar to variant 3 but carrying the deletion (D154) was detected in one isolate.

The production of the CDT subunits was predicted from the nucleotide sequence of the different *cdt* alleles (Table 2, Figures S2, S3 and S4, Appendix S1). All the isolates from broiler and human origin, with the only exception of H70, expressed apparently full CDT subunits with a theoretical size of 268, 265 and 189 amino acids for *cdtA*, *cdtB* and *cdtC*, respectively. The *cdtC* sequence of H70 isolate carried a deletion causing a nonsense mutation, and a predicted protein 18 AA shorter (Figure 4a and Table 2). High diversity was again found among the wild bird isolates (Table 2). The wild bird isolates grouped in clusters I and II seems to carry functional

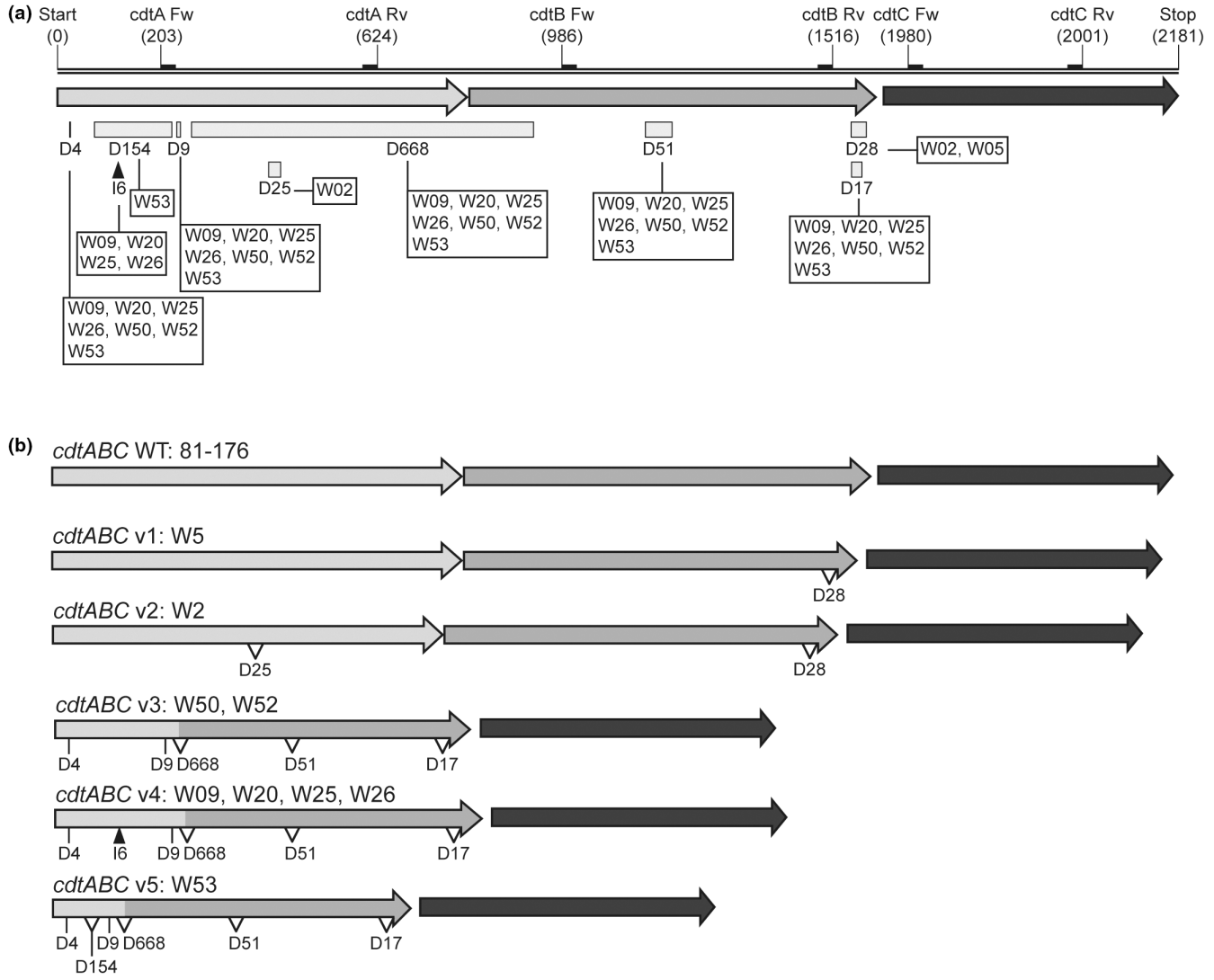


FIGURE 5 Rearrangements detected within the *cdtABC* operons. (a) Schematic representation of the *cdtABC* gene cluster. The arrows indicate the sequences of the three *cdt*A, *cdtB* and *cdtC* genes. The relative position of primers used for PCR amplification of each gene is indicated. The different deletions and insertions found are represented, indicating the number of nucleotides affected and the wild bird isolates that carries each of them. (b) Representation of the five variants of the *cdtABC* operon identified as compared with the *cdtABC* operon of strain 81–176. The deletions and insertions determined in each of the variants are showed, as well as the isolates where have been characterized.

cdtABC since the predicted proteins are of same length as the one produced by the 81–176 strain. The only exception was the isolates W06 and W07 that carried a *cdtB* gene encoding a slightly shorter protein of 259 amino acids. The *cdtABC* from the two isolates in cluster III encoded a shorter CdtB toxin, since had a theoretical length of 145 and 179 residues for W02 and W05, respectively. Similarly, the CdtC subunit from both isolates was also shorter with 82 and 40 as compared with the 189 residues of $CdtC_{18\text{-}176}$. CdtA was apparently expressed in W05 whereas was non-expressed in W02 by the presence of a codon stop in the third codon.

The sequence of the *cdtABC* operon from the the seven *C. jejuni* isolates from cluster IV indicated that these isolates did not produce any functional CDT toxin since apparently any of the three subunits could be produced. SNPs and the deletions and insertions in the sequences caused early stop codons within *cdtA* and *cdtC*, and the accumulated deletions did not allow generation of a CdtB protein.

4 | DISCUSSION

In this research, the existing variability within the *cdtABC* operon has been studied in a collection of *C. jejuni* isolates from three different origins: broiler, human and wild birds. Our data shows that the *cdtABC* operon seems to be highly conserved among broiler and human isolates whereas a great diversity was found among wild bird isolates as shown by the prevalence of the three *cdt* genes by PCR, the fragment length polymorphism of the *cdtB* amplicon and the *cdtABC* sequence analysis from a selected group

TABLE 2 Summary of the results of PCR amplification, sequence homology with the primers used, deletions and insertions determined, total bp and predicted AA from *cdtA*, *cdtB* and *cdtC* genes of wild bird isolates, grouped in clusters as defined in Figure 3a

			PCR	Primers	Deletions	Insertions	bp	AA
cdtA	WT	81–174	POS	22/22 22/22			807	268
	I	W12	POS	22/22 22/22			807	268
		W28	POS	22/22 22/22			807	268
		W29	POS	22/22 22/22			807	268
		W56	POS	22/22 22/22			807	268
	II	W06	POS	22/22 22/22			807	268
		W07	POS	22/22 22/22			807	268
		W13	POS	22/22 22/22			807	268
		W17	POS	22/22 22/22			807	268
		W33	POS	22/22 22/22			807	268
		W43	POS	22/22 22/22			807	268
	III	W02	NEG	20/22 20/22	D25, 1(−1)	6 (+1)	787	1
		W05	POS	22/22 22/22			807	268
	IV	W09	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	567	11
		W20	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	567	11
		W25	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	567	11
		W26	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	256	11
		W50	NEG	20/22 ND	D4, D9, D668	1 (+1)	250	11
		W52	NEG	20/22 ND	D4, D9, D668	1 (+1)	250	11
		W53	NEG	ND/ND	D4, D154, D9, D668, 1 (−1)		94	11
cdtB	WT	81–174	POS	22/22 22/22			798	265
	I	W12	POS	22/22 22/22			798	265
		W28	POS	22/22 22/22			798	265
		W29	POS	22/22 22/22			798	265
		W56	POS	22/22 22/22			798	265
	II	W06	POS	22/22 22/22	1 (−1)		797	259
		W07	POS	22/22 22/00	1 (−1)		797	259
		W13	POS	22/22 22/22			798	265
		W17	POS	22/22 22/22			798	265
		W33	POS	22/22 22/22			798	265
		W43	POS	22/22 22/22			798	265
	III	W02	POS	22/22 22/22	D28	2 (+1)	772	145
		W05	POS	22/22 21/22	D28	1 (+1)	771	179
	IV	W09	POS	20/22 21/22	D51, D17	1 (+1)	607	NO
		W20	POS	20/22 21/22	D51, D17	1 (+1)	606	NO
		W25	POS	20/22 21/22	D51, D17	1 (+1)	606	NO
		W26	POS	20/22 21/22	D51, D17, 1 (−2), 2 (−1)	1 (+1)	601	NO
		W50	NEG	20/22 20/22	D51, D17, 1 (−1)	2 (+1)	605	NO
		W52	NEG	20/22 20/22	D51, D17, 1 (−1)	2 (+1)	605	NO
		W53	POS	21/22 20/22	2 (−1), D51, D17		602	NO

TABLE 2 (Continued)

			PCR	Primers	Deletions	Insertions	bp	AA
cdtC	WT	81–174	POS	22/22 22/22			570	189
	I	W12	POS	22/22 22/22			570	189
		W28	POS	22/22 22/22			570	189
		W29	POS	22/22 22/22			570	189
		W56	POS	22/22 22/22			570	189
	II	W06	POS	22/22 22/22			570	189
		W07	POS	22/22 22/22			570	189
		W13	POS	22/22 22/22			570	189
		W17	POS	22/22 22/22			570	189
		W33	POS	22/22 22/22			570	189
		W43	POS	21/22 22/22			570	189
	III	W02	NEG	19/22 21/22	1 (−3) 2 (−1)	3 (+1)	568	82
		W05	NEG	19/22 21/22	1 (−3) 2 (−1)	2 (+1)	567	40
	IV	W09	NEG	22/22 19/22	2 (−2)		567	44
		W20	POS	22/22 19/22	2 (−2)		567	44
		W25	POS	22/22 19/22	2 (−2)		567	44
		W26	POS	22/22 20/22	1 (−1), 2 (−2)	1 (+1)	566	11
		W50	POS	21/22 21/22	1 (−1), 2 (−2)		565	11
		W52	POS	21/22 21/22	1 (−1), 2 (−2)		565	11
		W53	POS	21/22 22/22	2 (−1)	1 (+2)	568	122

of isolates. The high conservation of *cdtABC* among broiler and human *C. jejuni* isolates is consistent with being the chicken meat the most common transmission route of *C. jejuni* to humans via food cross-contamination. The *cdtABC* operon from most of the wild bird isolates showed modifications when compared with the $cdtABC_{81\text{-}176}$ operon highly prevalent among broiler, suggesting that the $cdtABC_{81\text{-}176}$ does not seem to promote efficient colonization among bird other than chicken. Evolutionary analyses of the *cdtABC* sequences grouped some wild bird isolates (W12, W28, W29 and W56) with the human and broiler isolates, which is not surprising since cross-transmission among the three ecological niches studied has been demonstrated (Hald et al., 2016). W12, W28 and W56 isolates belong to clonal complexes ST-354 and ST-446 that have been previously associated with broiler chicken and humans (Cobo-Díaz et al., 2021; Jolley et al., 2018). We do not find isolates of this clonal complexes among the wild bird isolates grouped in clusters II, III or IV. W29, also grouped with human and broiler, was isolated from a sea gull (*L. audouinii*) in the city of Barcelona. Most wild birds that live in urban environment have feeding habits that include feeding in urban refuse dumps.

Among the isolates carrying *cdtABC* operons that did not suffer drastic DNA modifications a higher number of SNPs were detected in *cdtA* as compared with *cdtB* and *cdtC*. Interestingly, *cdtA* was the most commonly missing gene when the prevalence of the three *cdt* genes was determined by PCR. In a previous report, it was shown that addition of CdtB and CdtC to human culture cells has a cytotoxic action as effective as when the three subunits were added, whereas addition of CdtA and CdtB did not induce any damage, suggesting that CdtA is not essential for the CDT action (Smith & Bayles, 2006). The frequency of nucleotide substitution for *cdtA* is similar among the isolates from clusters I and II independently of its origin, whereas *cdtB* and *cdtC* showed low variability among human and broiler and much higher among wild birds. These results suggest a correlation between certain *cdtABC* alleles and efficient colonization of specific hosts.

Analysis of the complete sequence of the *cdtABC* operons provide an explanation to the negative detection of *cdt* genes by PCR. All isolates that were negative for one or more *cdt* genes carries *cdtABC* alleles were deletions or SNPs affect the hybridization with the specific primers used. Our data indicate that PCR amplification fails when at least two SNPs are found within the primer-hybridizing sequence. These results question the convenience of using PCR-genotyping to determine gene prevalence of the *cdt* genes.

Several deletions were found among *cdtABC* operon from wild bird isolates defining five different allelic variants. The deletions D51 and D668 were previously reported in isolates from human patients (AbuOun et al., 2005; Kabir et al., 2011). In our isolate collection, we did not detect the indicated deletions among the isolates from broiler or human patients. In AbuOun et al. (2005), the only *C. jejuni* strains carrying the D51 and D668 were isolated from patients with underlying health problems that could induce an immunocompromised status. In fact, those patients develop *Campylobacter* bacteremia. The same deletions (D51 and D668) together with D9 and D17 were detected in *C. jejuni* isolates from crows (Sen et al., 2018). We detected those deletions not only in crow isolates but also in isolates from different wild birds,

belonging to different clonal complex, and living in different ecological niches.

Our data indicates that isolates circulating among wild birds have specific characteristics, regarding the *cdtABC* operon, which differs from the isolates colonizing broiler and human. These findings suggest that *cdtABC* might be a cellular factor involved in host specificity attending to the high degree of conservation among broiler and the relevant differences detected among the wild bird isolates. Moreover, the fact that many wild bird isolates carry apparently nonfunctional CDT toxin suggest that *C. jejuni* from wild bird strains might be less virulent to humans.

AUTHOR CONTRIBUTIONS

P.G, E.M., Y. I-T., F.N., C.S-O.A, C.M. and C.B. contributed to conceptualization and formal analysis. P.G, Y. I-T., E.M., C.M. and C.B. performed the investigation. C.S-O.A performed the WGS analysis. The original draft was written by C.B. and C.M. All the authors reviewed the manuscript. C.B. and C.M. contributed to project administration and funding acquisition. All the authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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