Expression and analysis of a cytolethal distending toxin (*cdt*) gene operon in *Campylobacter lari*

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ABSTRACT

The present study examines the expression of cytolethal distending toxin (*cdt*) gene encoding a cytotoxin in *Campylobacter lari* (n=6 urease-negative [UN] *C. lari*; n=4 urease-positive thermophilic *Campylobacter* [UPTC]). When reverse transcription polymerase chain reaction (RT-PCR) was carried out with 10 *C. lari* isolates using a primer pair to amplify the *cdtB* gene transcript segment, an approximate 260 bp RT-PCR amplicon was generated with all the isolates. In addition, *cdtA*, *cdtB* and *cdtC* gene operon was identified to be polycistronicly transcribed in the *C. lari* cells. The *cdtB* gene translation in the *C. lari* cells was also confirmed by Western blot analysis. Thus, the *cdt* gene operon in *C. lari* organisms, including UN *C. lari* and UPTC, was expressed at the transcriptional and translational levels in the cells. The present results suggest that all three *cdt* genes may be functional in the cells.

KEY WORDS: Campylobacter lari.
Gene expression.
Polymerase chain reaction.

Introduction

Thermophilic *Campylobacter* species, primarily *C. jejuni* and *C. coli*, are the most recognised causes of acute bacterial diarrhoea worldwide.[1] *C. lari* is an atypical thermophilic *Campylobacter* species first isolated from mammalian and avian species, particularly seagulls (genus *Larus*).[1,2] *C. lari* is also an infrequent cause of clinical infection.[3,4] In addition, a group of organisms of urease-positive thermophilic *Campylobacter* (UPTC) was isolated from the natural environment in England in 1985.[5] Thus, at least two representative taxa, namely urease-negative (UN) *C. lari* and UPTC occur within the *C. lari* species.[6]

Among several *Campylobacter* cytotoxins, the cytolethal distending toxin (CDT) has been characterised in detail,[7–10] and the *cdt* genes of *C. jejuni* have been cloned and characterised.[9] Cytolethal distending toxin causes progressive cellular distension and ultimately death in several cell lines,[9,11] and causes cell cycle blockage in several cell types.[12] Cytolethal distending toxin B is thought to be a toxic component (features of type I deoxyribonucleases) while CDTA and CDTC are believed to be carriers of CDTB to target cells.[13,14]

This group has shown genetic heterogeneity of the *cdtB* gene of *C. lari* isolates after analysis of the *cdtB* gene fragments (approximately 720 bp in length) from 24 *C. lari* isolates, including eight UPTC isolates.[15] In addition, the authors have cloned, sequenced and analysed full-length *cdt* genes, including the putative promoter and terminator structures from UN *C. lari* isolates, and compared their molecular characteristics with those of other thermophilic *Campylobacter* organisms.[16] Moreover, the complete genome sequence and analysis of the human pathogen *C. lari* RM2100 has been demonstrated.[17] In the study, the *C. lari* RM2100 isolate contained all three *cdt* genes; however, *cdtB* was truncated at the 5′ end with a suboptimal ribosome binding (RB) site. It was suggested that *cdtB* in this strain is a pseudogene and that this strain possesses no CDT activity.[17]

The present study aims to examine the transcriptional expression of the *cdtB* gene and the *cdt* gene operon (*cdtA*, *cdtB* and *cdtC* genes) among *C. lari* isolates comprising UN *C. lari* and UPTC organisms, and examine the translational expression of the *cdtB* gene in C. *lari* cells.

Materials and methods

C. lari isolates (n=10 UN *C. lari*, n=6 UPTC) from different sources in Asia, Europe and North America used in the present study are shown in Table 1. The isolates were cultured on Mueller-Hinton broth medium at 37°C for 48 h in an aerobic jar on Blood Agar Base No.2 (Oxoid, Hampshire, UK) containing 7% (v/v) defibrinated horse blood (Nippon Bio-Test, Tokyo, Japan) and *Campylobacter* selective medium (Virion, Zurich, Switzerland). An atmosphere of 5% (v/v) O_2 and 10% (v/v) CO_2 was produced by BBL Campypak microaerophilic system envelopes (Becton Dickinson, NJ, USA).

Total cellular RNA was extracted and purified from *C. lari* cells using RNA Protect bacteria reagent and the RNeasy Mini Kit (Qiagen, Tokyo, Japan). First, reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using the primer pair f-/r-*cdtB*RT2 shown in Figure 1, with the Qiagen OneStep RT-PCR kit. In the present study, the

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authors designed the degenerate primer pair *in silico* for RT-PCR amplification of the *cdtB* gene transcript segment of the *C. lari* isolates based on sequence information of the partial *cdtB* gene fragments from the 24 *C. lari* isolates determined previously.[15] This primer pair was expected to generate a RT-PCR product of the *cdtB* transcript segment of approximately 260 bp. An additional three primer pairs were designed (Fig. 1) for the RT-PCR amplifications of the partial *cdtA* messenger RNA (mRNA; f-/r-*cdtA*RT), *cdtA-B* mRNA (f-/r-*cdtB*RT) and *cdtB-C* mRNA (f-/r-*cdtC*RT) in order to confirm the polycistronic transcription of the entire *cdt* gene operon in the UN *C. lari* JCM2530^T and UPTC CF89-12 cells.

Nucleotide sequence alignment analysis to design the primer pair was carried out using CLUSTAL W software (1.7 program)[18] incorporated in the DDBJ.

Amplified RT-PCR products were separated by 1% (w/v) agarose gel electrophoresis in 0.5 × TBE at 100 V and detected by ethidium bromide staining.

The *cdtB* gene encoding a mature protein was amplified by PCR using genomic DNA of *C. jejuni* strain Co1-008, and primers constructed based on the sequence determined previously,[19] including artificial *Eco*RI and *Sal*I restriction sites, were 5′-GCGAATTCAACCTGGAAAACTTTAACGTTGGCACCTGG-3′ and 5′-GTCGTCGTCGACCTAAAATTTTCTAAAATTTAC-3′, respectively. The PCR fragment was digested with *Eco*RI and *Sal*I restriction enzymes and cloned into the *Eco*RI-*Sal*I-digested pET-28a vector (Merck KGaA, Darmstadt, Germany). *Escherichia coli* strain BL21 (DE3) was transformed with the

Table 1. *Campylobacter lari* isolates analysed in the present study.

Isolate	Source	Country	Accession number
UN *C. lari* JCM2530^T	Seagull	Japan	AB292351
UN *C. lari* 84C-1	Human	N. Ireland	AB292352
UN *C. lari* 84C-2	Human	N. Ireland	AB292353
UN *C. lari* 28	Mussel	N. Ireland	NA
UN *C. lari* 170	Seagull	Japan	NA
UN *C. lari* 264	Mussel	N. Ireland	AB292354
UN *C. lari* 296	Human	Canada	NA
UN *C. lari* 298	Human	Canada	AB292355
UN *C. lari* 299	Human	USA	NA
UN *C. lari* 448	Mussel	N. Ireland	AB292356
UPTC CF89-12	River water	Japan	AB509347
UPTC CF89-14	River water	Japan	AB509348
UPTC A1	Seagull	N. Ireland	AB509354
UPTC A3	Seagull	N. Ireland	AB509356
UPTC 89049	Human	France	AB509357
UPTC 92251	Human	France	AB509358

NA: not available.

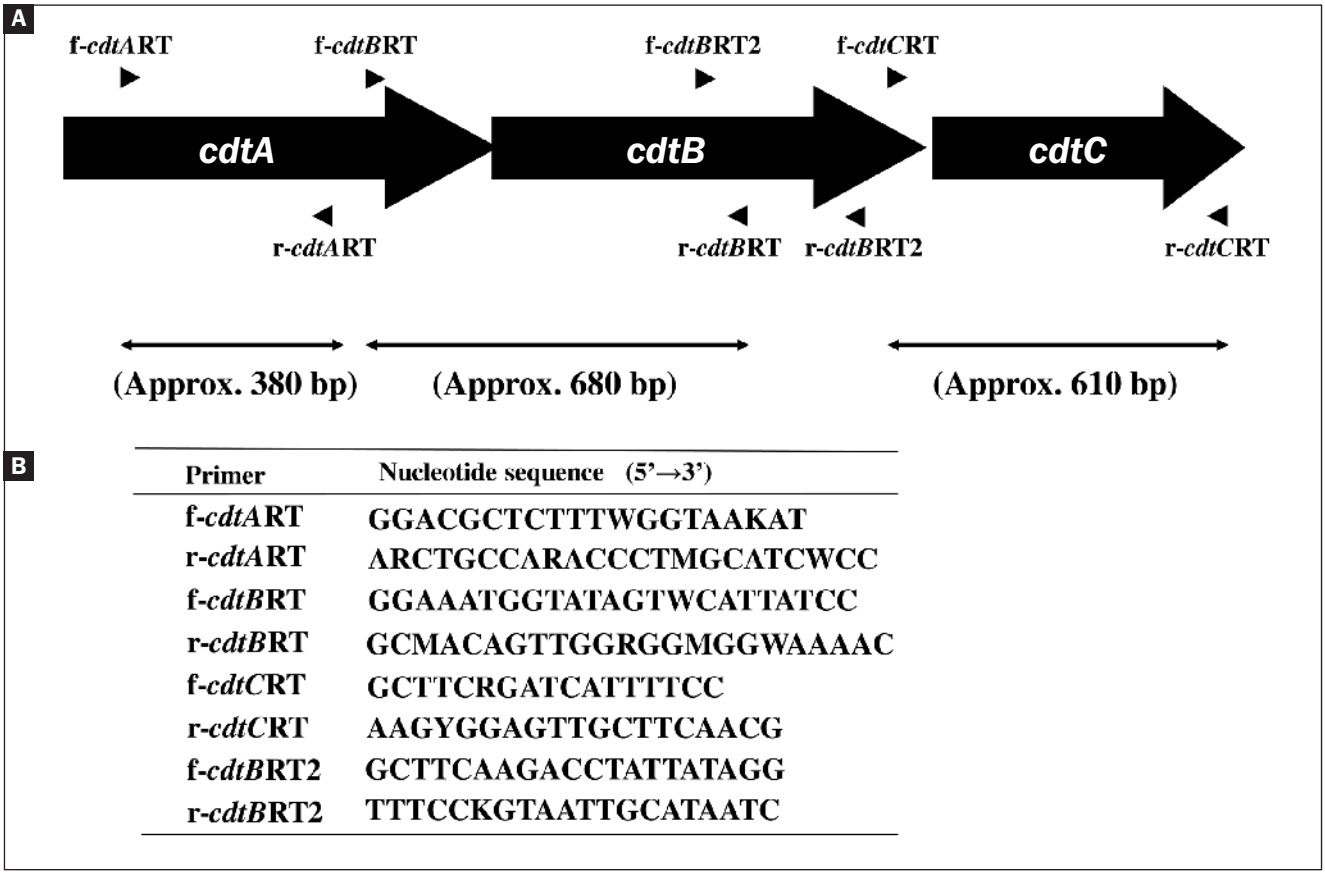

Fig. 1. A schematic representation of the *cdt* genes of *C. lari* including **A)** the locations of the four primer pairs for the RT-PCR amplifications of the *cdt* gene transcript segments, and **B)** nucleotide sequences of the primer pairs. In parentheses are the expected approximate sizes shown to be generated for the RT-PCR products.

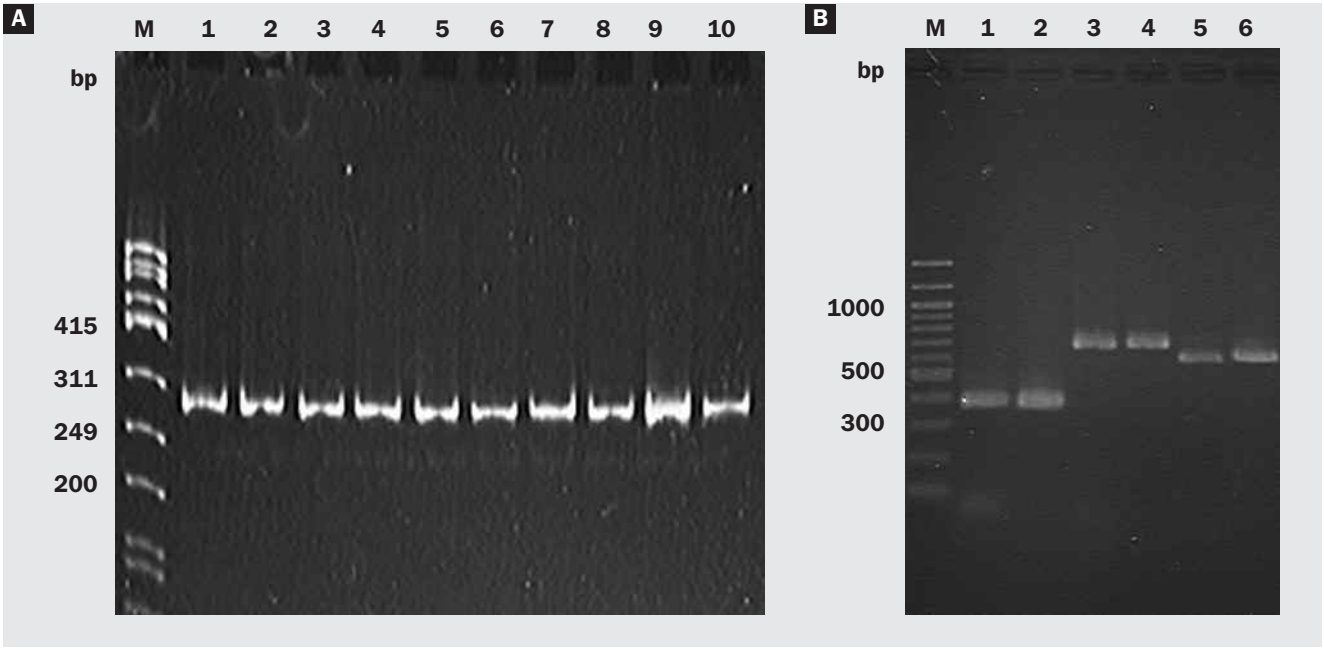

Fig. 2. RT-PCR profiles of the *cdt* genes transcripts performed using the primer pair f-/r-*cdt*BRT2 with the 10 *C. lari* isolates. **A)** Lane M, ϕX174DNA/*Hin*fI digest markers, lane 1: UN *C. lari* JCM2530^T, lane 2: 28, lane 3: 170, lane 4: 296, lane 5: 298, lane 6: 299, lane 7: UPTC CF89-12, lane 8: CF89-14, lane 9: A1, lane 10: 89049. Additional RT-PCR analyses of the *cdtA* (lanes 1 and 2), *cdtA-B* (lanes 3 and 4) and *cdtB-C* (lanes 5 and 6) transcripts were carried out using the three primer pairs f-/r-*cdt*ART, f-/r-*cdt*BRT and f-/r-*cdt*CRT. **B)** Lane M: 100 bp DNA ladder, lanes 1, 3 and 5: UN *C. lari* JCM2530^T, lanes 2, 4 and 6: UPTC CF89-12.

pET-28a-CjCDTB, and CjCDTB expression was induced by 0.1 mmol/L IPTG. The recombinant protein was purified by the HisTrap HP column (GE Healthcare, Buckinghamshire, England), following the manufacturer's instructions.

Polyclonal antisera to *C. jejuni* CDTB was generated in rabbits (New Zealand White; Kitayama Labes, Nagano, Japan) by injecting animals with 100 µg protein on days 0, 14, 21, 28, and 35 subcutaneously and intramuscularly. Protein (1 mL) in phosphate-buffered saline (pH 7.4) was emulsified with an equal volume of Freund complete adjuvant and Freund incomplete adjuvant in the first and second to fifth immunisations, respectively. Rabbit sera were screened for anti-CDTB by Ouchterlony double gel diffusion, as described previously.[20]

The antibody to *C. jejuni* CDTB was purified on the HiTrap Protein A HP column (GE Healthcare), following the manufacturer's instructions. Specificity of the polyclonal antibody was confirmed by Western blotting using rCDTB and cell lysate of *C. jejuni* strains Co1-008 and 81-176.

Soluble fractions from UN *C. lari* JCM2530^T and UPTC CF89-12 were subjected to mini-slab gel electrophoresis using 10% polyacrylamide gel containing 1% (w/v) sodium dodecyl sulphate (SDS) with Tris-glycine buffer at 24 mA for 2 h. Proteins were transferred to a PVDF membrane (Immobilon, Millipore, MA, USA), with cooling at 90 V for 80 min in basic buffer at room temperature for 30 min.[21] Membranes were examined for reactivity with rabbit polyclonal anti-*C. jejuni* CDTB antibodies.

Results

In a previous study, PCR amplicons encoding a *cdt* gene operon were identified in six UN *C. lari* isolates, using a PCR primer pair (f-/r-Cl*cdt*all) constructed *in silico*, as well as three closely spaced and putative open reading frames (ORFs) for *cdtA*, *cdtB* and *cdtC*, two putative promoters and a hypothetically intrinsic ρ-independent transcription terminator found in the operon.[16] In addition, using similar methodology including an inverse (I) PCR procedure, a *cdt* gene operon in the UPTC isolates (DDBJ/EMBL/GenBank accession numbers AB509347–AB509358 for the 12 UPTC isolates) was also identified. Overall, it was suggested that the *cdtA*, *cdtB* and *cdtC* genes in the 18 *C. lari* isolates (n=6

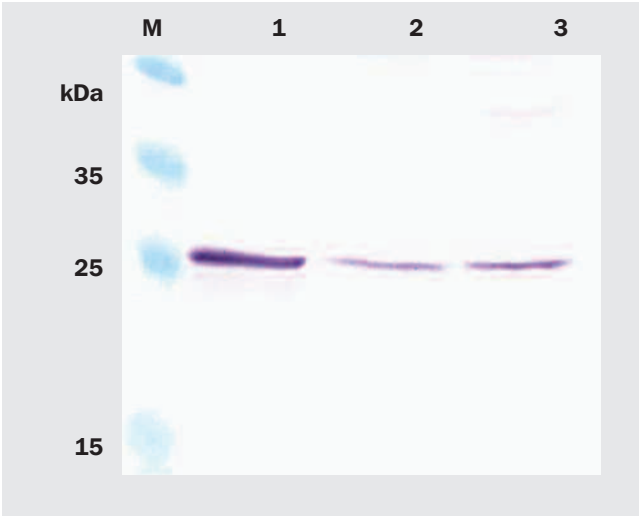

Fig. 3. Western blot analyses of soluble extracts from the UN *C. lari* JCM2530^T and UPTC CF89-12 cells with rabbit polyclonal anti-recombinant CjCDTB antibodies. *C. jejuni* was also analysed for comparison. Lane M: Page Ruler Prestained Protein Ladder (Fermentas Life Sciences, Tokyo, Japan), lane 1: *C. jejuni* 81-176, lane 2: UN *C. lari* JCM2530^T, lane 3: UPTC CF89-12.

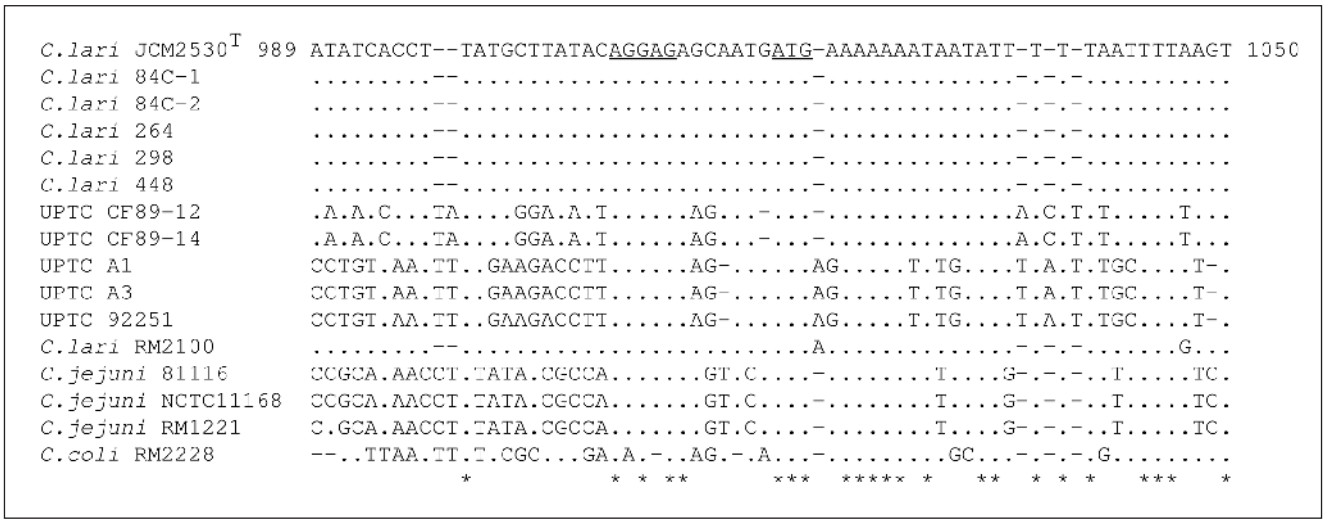

Fig. 4. Nucleotide sequence alignment analysis of the probable RB sites for the *cdtB* genes with the 12 *C. lari* isolates including *C. lari* RM2100 (AAFK01000004). Numbers at the left and right refer to the nucleotide sequence positions of *C. lari* JCM2530^T *cdt* operon (AB292351). Probable RB site and initiation codon for the *cdtB* gene in the UN *C. lari* JCM2530^T are underlined. Corresponding nucleotide sequences from *C. jejuni* 81116 (CP000814), NCTC11168 (AL111168), RM1221 (NC_003912) and *C. coli* RM2228 (AAFL01000003) are also aligned for comparison. Dot indicates identical bases, changes are indicated, dashes are deletions, identical positions in all cases are marked with asterisks.

UN *C. lari*, *n*=12 UPTC) examined appear to form an operon.

When RT-PCR was carried out with 10 *C. lari* (*n*=6 UN *C. lari*, *n*=4 UPTC) isolates using the primer pair f-/r-*cdtB*RT2 (Fig. 1) to amplify the *cdtB* gene transcript segment, a positive signal of approximately 260 bp was detected with all the isolates (Fig. 2A). Then, RT-PCR amplification of the partial *cdtA*, *cdtA-B*, and *cdtB-C* mRNA transcripts was performed using three different PCR primer pairs (Fig. 1) in order to confirm the polycistronic transcription of the whole *cdt* gene operon in the *C. lari* cells (UN *C. lari* JCM2530^T and UPTC CF89-12). As shown in Figure 2B, the *cdtA*, *cdtB* and *cdtC* gene operon was shown to be transcribed polycistronicly. Thus, *cdt* gene operon transcription was confirmed in the *C. lari* cells.

Approximately 29 kDa recombinant *C. jejuni* (Cj)CDTB was expressed and purified. Antisera against recombinant (rCj)CDTB were induced and polyclonal antibody against (rCj)CDTB was purified (data not shown). Specificity of the polyclonal antibody was confirmed by Western blot analysis (data not shown).

When Western blot analysis was used to identify the CDTB expression at the translational level in the *C. lari* isolates (Fig. 3) it was shown that anti-(Cj)CDTB antibodies identified an immunoreactively positive signal at approximately 25 kDa on UN *C. lari* JCM2530^T and UPTC CF89-12 isolates, as well as on *C. jejuni* 81-176 strains. Thus, *cdtB* gene translation in the *C. lari* cells was confirmed.

Discussion

This is the first demonstration of the expression and analysis of a *cdt* gene operon in *C. lari* organisms. In previous work, the partial *cdtB* genes (approximately 720 bp), similar in length in the 24 *C. lari* isolates (*n*=16 UN *C. lari*, *n*=8 UPTC) examined using a constructed PCR primer pair *in silico* (Asakura primer),[22] showed high genetic heterogeneity of 74.7–100% nucleotide sequence similarity.[15] Thus, the primer pair f-/r-*cdtB*RT2 constructed for the present RT-PCR analysis was based on this highly variable sequence information of the partial *cdtB* gene fragments. Therefore, it may be useful for the RT-PCR analysis of the other *C. lari* isolates. In addition, the other three RT-PCR primers (i.e., f-/r-*cdtA*, f-/r-*cdtB* and f-/r-*cdtC*) were also useful for the RT-PCR analysis of the *cdt* gene operon in UN *C. lari* JCM2530^T and UPTC CF89-12.

Western blot analysis carried out in the present study confirmed that rabbit antiserum against *C. jejuni* CDTB recognised and combined with the counterpart proteins from UN *C. lari* JCM2530^T and UPTC CF89-12, indicating the high immunoreactivity of these proteins between *C. jejuni* and *C. lari* species, confirming *cdtB* gene translation in these *C. lari*. In addition, this result suggests *cdt* gene operon expression at the translational level in the *C. lari* cells.

With regards to probable RB sites complementary to a highly conserved sequence of CCUCCU close to the 3′ end of 16S rRNA and necessary for the translation,[23] it has been shown that the *cdtB* gene of *C. lari* RM2100 is truncated at the 5′ end with a suboptimal RB site.[17] However, in the present study, the authors identified the probable RB sites (nucleotide position [np]: 1009–1013 bp for UN *C. lari* JCM2530^T) for the *cdtB* genes with all 11 *C. lari* isolates (*n*=6 UN *C. lari*, *n*=5 UPTC) examined (Fig. 4) following nucleotide sequence alignment analyses of the regions upstream of the initiation codon for the *cdtB* gene translation.

Although the four human UPTC isolates were described in patients in France in 1988 and 1990,[24,25] whether or not urease-producing UPTC organisms are associated with gastrointestinal or other human disease remains unclear.[6] Therefore, it is especially relevant to clarify the function of CDT, a representative virulence factor, in UPTC cells.

Consequently, the results presented here suggest that the *cdt* gene operon may be transcribed and translated in all the *C. lari* isolates examined, which indicates that these three genes are functional in these organisms. □

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