Genetic heterogeneity of the cytolethal distending toxin B (*cdtB*) gene locus among isolates of *Campylobacter lari*

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Thermophilic *Campylobacter* species, primarily *C. jejuni* and *C. coli*, are curved Gram-negative bacteria that are the recognised cause of acute bacterial diarrhoea around the world.[1,2] *C. lari* is a relatively recently discovered thermophilic *Campylobacter* species first isolated from mammalian and avian species, particularly seagulls of the genus *Larus*.[1,3] *C. lari* has also been shown to be a cause of clinical infection.[4–7]

An atypical group of isolates of urease-positive thermophilic campylobacters (UPTC) was first isolated from the natural environment in England in 1985.[8] Thereafter, these organisms were described as a biovar or variant of *C. lari*.[9,10] Subsequent reports described four human isolates in France.[9,11] Some additional isolates of UPTC have also been reported in Ireland,[12–14] in The Netherlands[15] and in Japan.[16,17]

The possible association of UPTC with human disease remains unclear. Two representative taxa, namely urease-negative (UN) *C. lari* and UPTC, occur within the species of *C. lari*.[18]

Although several *Campylobacter* species' cytotoxins have been identified,[19,20] only the cytolethal distending toxin (CDT) has been characterised in detail.[21,22] The *cdt* genes of *C. jejuni* have been cloned and characterised by Pickett *et al*.[21] However, in relation to the *cdt* genes, no reports have yet appeared for *C. lari*.

Therefore, the aim of the present study is to clone, sequence and analyse the *cdtB* gene of *C. lari* isolates and compare the sequences obtained with those of other thermophilic campylobacters.

Twenty-four isolates of *C. lari* (UN *C. lari* [n=16] and UPTC [n=8]) were used in the present study (Table 1), together with three reference strains (JCM2530T, NCTC12892 and NCTC12893). The test organisms were isolated from different sources in several countries. The organisms were cultured on blood agar containing defibrinated horse blood (Nippon Bio-Test, Tokyo, Japan) and supplemented with campylobacter-selective medium (Nissui, Tokyo, Japan), under microaerophilic conditions at 37°C for two days.

Template DNA was prepared by boiling in water at 95°C for five minutes. The PCR mixture contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 400 µmol each dNTP, 1 µmol each primer, and 1 unit of *Thermus aquaticus* (*Taq*) DNA polymerase (Takara Bio, Shiga, Japan).

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Table 1. Isolates of *C. lari* analysed in the present study.

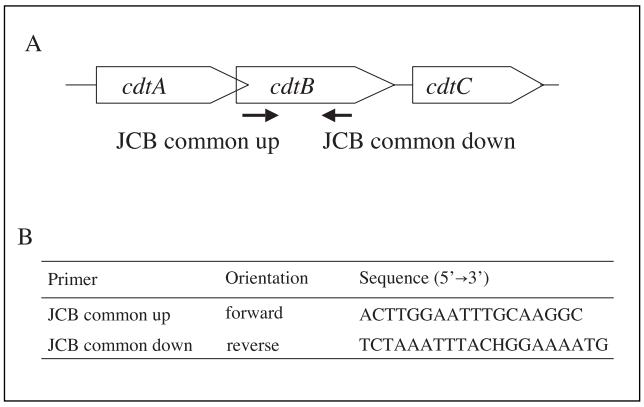
Isolate	Source	Country	Accession number
UN *C. lari* JCM2530T	Seagull	Japan	AB 266778
UN *C. lari* 28	Mussel	N. Ireland	AB 266779
UN *C. lari* 48	Mussel	N. Ireland	AB 266780
UN *C. lari* 84C-1	Human	N. Ireland	AB 266781
UN *C. lari* 84C-2	Human	N. Ireland	AB 266782
UN *C. lari* 99	Sea water	N. Ireland	AB 266783
UN *C. lari* 170	Seagull	Japan	AB 266784
UN *C. lari* 264	Mussel	N. Ireland	AB 266785
UN *C. lari* 274	Mussel	N. Ireland	AB 266786
UN *C. lari* 288	Black-tailed gull	Japan	AB 266787
UN *C. lari* 293	Seagull	Japan	AB 266788
UN *C. lari* 296	Human	Canada	AB 266789
UN *C. lari* 298	Human	Canada	AB 266790
UN *C. lari* 299	Human	USA	AB 266791
UN *C. lari* 381	Mussel	N. Ireland	AB 266792
UN *C. lari* 2316A3	NA	NA	AB 266793
UPTC CF89-12	River water	Japan	AB 266794
UPTC CF89-14	River water	Japan	AB 266795
UPTC NCTC12892	River water	England	AB 266796
UPTC NCTC12893	River water	England	AB 266797
UPTC A1	Seagull	N. Ireland	AB 266798
UPTC A3	Seagull	N. Ireland	AB 266799
UPTC 89049	Human	France	AB 266800
UPTC 92251	Human	France	AB 266801

NA: not available.

A schematic representation of the *cdtB* gene and its genetic loci for *C. lari* RM2100 (GenBank Accession No. AAFK00000000)[23] including the locations of a primer pair for the *cdtB* (JCB common up and JCB common down [Asakura primer])[24] employed in the present study for PCR amplification is shown in Figure 1. This primer pair was designed to generate a product of approximately 700 bp (equivalent to a 90% segment of the *cdtB* structural gene of *C. lari* RM2100, AAFK00000000) of the *cdtB* gene with *C. jejuni*, *C. coli* and *C. fetus* isolates.[24] The polymerase chain reaction (PCR) was performed in 50-µL reaction volumes, for 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, followed by a final extension of 72°C for 5 min.

Amplified PCR products were separated by 1.0% (w/v) agarose gel electrophoresis in 0.5x TBE at 100 V and detected by ethidium bromide staining. PCR products amplified by the constructed primer pair for the partial *cdtB* gene were purified using a QIA quick PCR purification kit (Qiagen, CA, USA) and inserted in the pGEM-T vector using the pGEM-T Easy Vector System (Promega, Tokyo, Japan).

Sequencing of the cloned *cdtB* gene fragment was performed (Hitachi DNA autosequencers SQ-5500L and SQ-5500EL) after a dideoxy nucleotide sequencing reaction, using a Thermo Sequenase premixed cycle sequencing kit (Amersham Pharmacia Biotech, Tokyo, Japan). Sequence analysis of the PCR amplicons was carried out using the GENETYX-MAC (version 9) computer software.



Primer	Orientation	Sequence (5'→3')
JCB common up	forward	ACTTGGAATTTGCAAGGC
JCB common down	reverse	TCTAAATTTACHGGAAAATG

Fig. 1. Schematic representation of the *cdtB* genes of *C. lari*, including A) the locations of a primer pair for the amplification of the *cdtB* gene fragment of *C. lari* and B) nucleotide sequences of the primer pair.[24]

Nucleotide sequences of approximately 720 bp of the partial *cdtB* gene fragments from 24 *C. lari* isolates were compared to each other and to accessible sequence data of other thermophilic campylobacters (*C. lari* RM2100, AAFK00000000; *C. jejuni* RM1221, CP000025; *C. coli* RM2228, AAFL00000000), employing CLUSTAL W software (1.7 program),[25] which was incorporated in DDBJ. A phylogenetic tree was constructed by the unweighted pair group method, using arithmetic means analysis (UPGMA) available on the GENETYX-MAC program (version 9.0).

In the present study, the primer pair (JCB common up and down, Asakura primer)[24] amplified PCR products of approximately 720 bp in length with all 24 isolates of *C. lari* (UN *C. lari* [*n*=16] and UPTC [*n*=8]) (data not shown). For PCR cloning of the partial *cdtB* fragments, PCR products were purified and inserted in the pGEM-T vector using the TA cloning procedure. The nucleotide and deduced amino acid sequence data of the partial and possible open reading frame (ORF) of the *cdtB* gene fragments cloned and sequenced from 24 isolates of *C. lari* are accessible in the DDBJ/EMBL/GenBank nucleotide sequence database (Table 1).

The nucleotide sequences of the partial *cdtB* gene fragments (approximately 720 bp) of 16 UN *C. lari* isolates showed 81–100% similarity to each other. Those of eight UPTC isolates showed 74.7–99.6% similarity to each other. In addition, those of the *cdtB* gene fragments showed 74.7–100 % similarity among 16 UN *C. lari* isolates and eight UPTC isolates. Moreover, the nucleotide sequences of the *cdtB* gene fragments of 25 *C. lari* isolates, including *C. lari* RM2100, showed 63.9–88.0% sequence similarity to those of *C. jejuni* RM1221 and *C. coli* RM2228.

When the deduced amino acid sequences of the partial and possible ORFs of the *cdtB* fragments of 24 *C. lari* isolates were determined, the partial and possible ORFs were estimated to consist of 241–242 amino acids. The deduced amino acid sequence alignments were also examined for partial and possible ORFs of the *cdtB* fragments of 24 *C. lari* isolates, as well as those of *C. lari* RM2100, *C. jejuni* RM1221 and *C. coli* RM2228. The partial and possible ORFs of the *cdtB* gene fragments from 25 *C. lari* isolates, including *C. lari* RM2100, showed 78.2–99.6 % amino acid sequence similarity to each other, and 66.6–78.5% amino acid sequence similarity to those of the ORFs of *C. jejuni* RM1221 and *C. coli* RM2228.

Thus, *cdtB* had a high sequence heterogeneity in both nucleotide and deduced amino acid sequences with the 24 *C. lari* isolates examined. An extremely high sequence variability of the *cdtB* was also demonstrated between the 24 *C. lari* isolates and the other two thermophilic campylobacters (*C. jejuni* and *C. coli*).

In relation to the *cdt* gene in campylobacters, Martinez *et al*. found the *cdt* gene variant, a putative shortened *cdtB* gene fragment in two of the 100 *C. jejuni* isolates derived from several sources (humans and animals) and countries, by a multiplex PCR procedure. Although, they observed several point mutations throughout the remaining *cdtA, -B* and *-C* sequences, it has become very clear that *cdt* genes from *C. jejuni* are highly homologous.[26]

In the present study, the partial *cdtB* gene (approximately 720 bp), similar in length in 24 isolates of *C. lari*, was examined using a constructed PCR primer *in silico* (Asakura primer). Thus, essentially all the *C. lari* isolates employed in the present study had the *cdtB* gene. However, high genetic heterogeneity of 74.7–100% nucleotide sequence similarity of the partial *cdtB* gene fragment was identified among all 24 isolates of *C. lari* by cloning and sequencing procedures. This is the first demonstration of the genetic heterogeneity of *cdt* genes among *C. lari* isolates by cloning and sequencing procedures.

A dendrogram showing phylogenetic relationships was constructed using UPGMA, based on the nucleotide sequence information of the partial *cdtB* gene fragments amplified from 24 *C. lari* isolates (Fig. 2) and demonstrated hypervariability among the *cdtB* genes of 24 *C. lari* isolates. This implies that the *C. lari* organisms examined are genetically variable, at least based on the *cdtB* gene information obtained, and separate from the other two thermophilic campylobacters, *C. jejuni* and *C. coli*.

Previously, this group demonstrated genetic hypervariability of 31 isolates of UPTC, as determined by multilocus enzyme electrophoresis typing (MLEE).[14] The present results for the *cdtB* gene from eight UPTC isolates is consistent with the results of the genetic hypervariability obtained by MLEE.

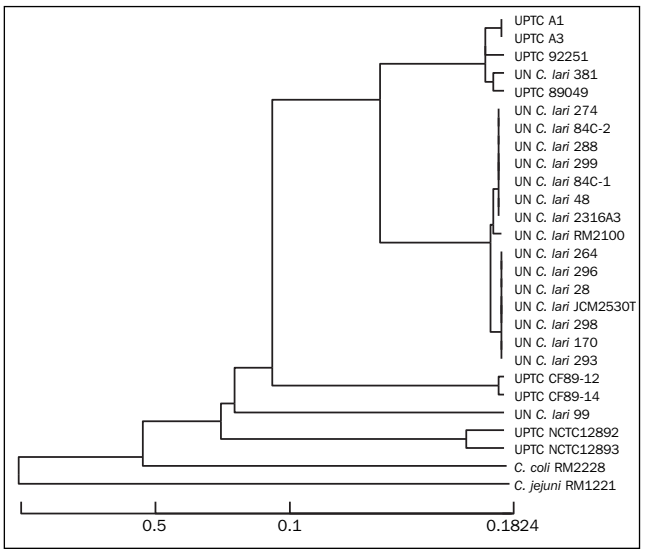


Fig. 2. Phylogenetic tree based on sequence similarity data of the *cdtB* gene fragments from 24 *C. lari* isolates examined and thermophilic isolates of *C. lari* RM2100, *C. jejuni* RM1221 and *C. coli* RM2228. Values in the figure represent evolutionary distances.

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Molecular diagnosis of native mitral valve endocarditis due to *Corynebacterium striatum*

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Corynebacterium striatum is a relatively rare causal agent of infective endocarditis (IE). Its association with IE is complicated by its relatively slow growth, its role as a possible contaminant in such cases, and the phenotypic difficulty in laboratory identification.

The present study reports a case of culture-positive endocarditis due to *C. striatum* in a 77-year-old woman who showed no risk factors for endocarditis. To date, there have been 12 reports of endocarditis due to *C. striatum*, which are discussed and summarised.

Currently, use of molecular microbiological methods has been limited, but such techniques have been adopted in several clinical microbiology laboratories. Molecular methods of identification using the polymerase chain reaction (PCR) and sequencing of 16S ribosomal DNA (rDNA) from the causal agent isolated by blood culture may be very useful in the identification of causal agents in culture-positive endocarditis, which prove difficult to identify using a conventional approach.

The 77-year-old patient presented to hospital with a chronic three-month history of weight loss, fatigue and arthralgia. A history was taken in accordance with the

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