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Sequencing and analysis of the 16S rDNA of thermophilic *Campylobacter lari* and their reliability for molecular discrimination

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In 1980, a new thermophilic *Campylobacter* organism, referred to as nalidixic acid (NAL)-resistant thermophilic *Campylobacter* (NARTC), which is urease-negative, was first described by Skirrow and Benjamin.[1] Although Benjamin *et al*.[2] first proposed the name *C. laridis* for the NARTC, it was later revised to *C. lari*.[3] Thereafter, *C. lari* organisms have been isolated from various elements of the natural environment, including from wild birds and animals, and also recovered from humans.

An atypical group of urease-positive thermophilic campylobacters (UPTC) was first isolated from the natural environment in England in 1985.[4] Subsequently, this organism has been reported in four patients in France.[5,6] After the existence of these organisms had been confirmed, isolated UPTC were reported in Northern Ireland,[7–10] The Netherlands[11] and Japan.[12,13]

To discriminate these organisms, Megraud *et al*. first identified three human clinical isolates of UPTC as *C. lari* by means of a hybridisation dot-blot assay, as well as by biochemical characterisation, and they described their finding as a variant of *C. lari*.[5] Owen *et al*.[14] suggested that UPTC (n=8) and NAL-sensitive *Campylobacter* (NASC, n=4) strains belonged within *C. lari*, possibly as biovars based on the numerical analysis of high-resolution polyacrylamide gel electrophoresis (PAGE) of proteins combined with computerised analysis of the profiles.

In 1991, Vandamme *et al*.[15] described a true *C. lari* species of a representative UPTC strain (CCUG18267) and two urease-negative (UN) *C. lari* strains (CCUG23947^T and CCUG12774) by means of hybridisation studies employing DNA–23S rRNA and DNA–DNA. In 1995, Alderton *et al*.[16] reported a classification analysis based on the 16S rRNA sequence of an organism formerly described as strain RMIT32A^T and a group of similar bacteria (a new *C. hyoilei* species), two strains of UPTC CCUG18267 and UN *C. lari* CCUG23947^T (EMBL/GenBank, accession numbers, L14631 and L04316). Although, the 16S rRNA sequences showed 99.2% similarity between these two strains, many internal unidentified bases have been detected in the sequences of the nearly full-length 16S rDNA from both the strains. When Wesley *et al*.[17] performed an analysis of the *Arcobacter*-specific and *A. butzleri*-specific 16S rRNA-based DNA probes, the 16S rRNA sequences from the two *C. lari* strains (CCUG18267 and CCUG23947^T) were also employed.

In studies of *C. lari*, based on 16S rDNA sequence information, the 16S rDNA sequences containing internal unidentified bases have been used. However, sequencing and comparison of 16S rDNA have resulted in the identification and discrimination of many bacterial taxa.

The present study aims to determine and employ the sequences of nearly full-length 16S rDNA, which do not contain internal unidentified bases, from over 10 *C. lari* isolates from various countries and sources, and to evaluate the reliability of 16S rRNA sequence data for molecular discrimination of *C. lari*, including UPTC and UN *C. lari*.

The 15 isolates of UPTC and UN *C. lari* used to clone, sequence, analyse and compare the 16S rDNA in the present study are shown in Table 1. The 12 isolates of UPTC were isolated from the natural environment, including from seagulls and humans in England, France, Northern Ireland and Japan. The remaining three isolates of UN *C. lari* were isolated from a seagull and two humans in Northern Ireland and Japan.

Polymerase chain reaction (PCR) amplification using a primer pair of fD1 and rD1,[18] TA cloning and sequencing of the 16S rDNA from UPTC and UN *C. lari* isolates were carried out using procedures described previously.[19] The fD1 and rD1 sequences correspond to the nucleotude position (np) 8 through to 27 and np 1492 through to 1510 of the 16S rDNA of *Escherichia coli*, respectively.[20,21] These sequences corresponding to the PCR primers were excluded from the sequences of the nearly full-length 16S rDNA (approximate 1400 bp) and further similarity analysis. In the present study, multiple TA-cloned PCR products were sequenced for accuracy.

The DNA sequences of the nearly full-length 16S rDNA of all UPTC and UN *C. lari* isolates determined in the present study (Table 1) were compared with those of the other thermophilic campylobacters (i.e., UPTC CCUG18267 and *C. lari* CCUG23947^T, LMG7607, LMG11251, LMG11760, LMG14338, *C. jejuni* CCUG11284, *C. jejuni* doylei CCUG24567, *C. coli* CCUG11283, *C. hyoilei* RMIT32A and

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Table 1. Isolates of UPTC and UN *C. lari* used in the present study and accession numbers of the nuclenotide sequence data of the 16S rDNA accessible in the DDBJ/EMBL/GenBank.

Isolate	Organism	Source	Country	NAL (30 μg/mL)	Accession No
CF89-12	UPTC	River water	Japan	S	AB066098
CF89-14	UPTC	River water	Japan	S	AB181356
NCTC12892	UPTC	River water	England	S	AB181357
NCTC12893	UPTC	River water	England	S	AB181358
NCTC12894	UPTC	Sea water	England	S	AB181359
NCTC12895	UPTC	Mussel	England	S	AB181360
NCTC12896	UPTC	Mussel	England	S	AB181361
A1	UPTC	Seagull	N. Ireland	S	AB181362
A2	UPTC	Seagull	N. Ireland	S	AB181363
A3	UPTC	Seagull	N. Ireland	S	AB181364
89049	UPTC	Human	France	S	AB181365
92251	UPTC	Human	France	S	AB181366
JCM2530T	UN *C. lari*	Seagull	Japan	R	AB181368
84C-1	UN *C. lari*	Human	N. Ireland	R	AB181367
84C-2	UN *C. lari*	Human	N. Ireland	R	AB181369

S: sensitive; R: resistant

Table 2. Sequence similarities and numbers of nucleotide sequence differences of the 16S rDNA sequences of UPTC, UN *C. lari* and other thermophilic campylobacters.

	% Sequence similarity and numbers of different bases [a]																
Taxon	UPTC CF89-12	UPTC CF89-14	UPTC NCTC 12892	UPTC NCTC 12893	UPTC NCTC 12894	UPTC NCTC 12895	UPTC NCTC 12896	UPTC A1	UPTC A2	UPTC A3	UPTC 89049	UPTC 92251	*C. la* 84C-1	*C.la* 84C-2	*C. la* JCM 2530	*C.la* LMG 7607	*C. la* LMG 11251
UPTC CF89-12		99.8	99.5	99.4	99.9	99.8	99.9	99.4	99.5	99.5	99.6	99.6	98.4	98.5	98.4	98.5	97.3
UPTC CF89-14	3		99.6	99.3	99.9	100	99.9	99.4	99.7	99.3	99.8	99.7	98.5	98.6	98.5	98.6	97.4
UPTC NCTC12892	7	6		99.7	99.6	99.6	99.7	99.2	99.4	99.1	99.4	99.4	98.7	98.9	98.7	98.9	97.7
UPTC NCTC12893	8	10	4		99.4	99.3	99.4	99.0	99.1	99.0	99.1	99.1	98.5	98.6	98.5	98.6	97.4
UPTC NCTC12894	1	2	6	8		99.9	99.9	99.4	99.6	99.4	99.7	99.7	98.5	98.6	98.5	98.6	97.4
UPTC NCTC12895	3	0	6	10	2		99.9	99.4	99.7	99.3	99.8	99.7	98.5	98.6	98.5	98.6	97.4
UPTC NCTC12896	2	1	5	9	1	1		99.5	99.7	99.4	99.7	99.7	98.5	98.7	98.5	98.7	97.5
UPTC A1	9	6	11	15	8	8	7		99.7	99.4	99.7	99.7	98.5	98.3	98.5	98.4	97.1
UPTC A2	7	4	9	13	6	4	4	4		99.3	99.9	99.8	98.3	98.5	98.3	98.6	97.2
UPTC A3	7	9	13	15	8	10	9	6	9		99.2	99.2	98.3	98.2	98.3	98.2	97.0
UPTC 89049	6	3	9	13	5	3	4	5	1	10		99.9	98.4	98.5	98.4	98.7	97.3
UPTC 92251	6	4	9	13	4	5	4	4	3	11	2		98.4	98.5	98.4	98.7	97.3
C. lari 84C-1	23	22	18	22	22	22	21	22	24	24	23	23		99.9	99.9	99.6	98.5
C. lari 84C-2	21	20	16	20	20	19	19	23	22	25	21	21	2		99.8	99.8	98.7
C. lari JCM2350[T]	22	22	18	22	22	21	20	22	24	24	23	23	1	3		99.7	98.4
C. lati LMG7607	21	20	16	20	20	20	19	22	20	26	19	19	5	3	4		98.4
C. lari LMG11251	38	37	33	37	37	37	36	41	39	43	38	38	21	19	22	22	
C. lari LMG11760	2	1	5	9	1	1	0	7	5	9	4	4	21	19	21	19	36
C. lari LMG14338	20	19	15	19	19	19	18	23	21	25	20	20	4	2	3	1	21

[a] The values on the upper right are the levels of sequence similarity, and the values on the lower left are the numbers of different bases based on 16S rDNA sequences.

C. upsaliensis CCUG14913) that were available from the EMBL/GenBank nucleotide sequence databases, although some of the sequences included some internal unidentified bases. The sequences of the nearly full-length 16S rDNA were aligned using the CLUSTAL W 1.7 program incorporated in the DDBJ.[22] A phylogenetic tree was constructed by the unweighted pair group method with the arithmetic means (UPGMA) available on the GENETYX-MAC program (version 9; Software Development, Tokyo, Japan).

Levels of similarity of the aligned sequences of the nearly full-length 16S rDNA from the 12 UPTC and three UN *C. lari* isolates examined in the present study are shown on the upper right in Table 2. The 12 UPTC isolates shared 99–100% 16S rDNA sequence similarity with each other and 98.2–98.9% sequence similarity with the three UN *C. lari* isolates. This suggests that the 12 UPTC isolates share a higher 16S rDNA sequence similarity with each other than they do with the three UN *C lari* isolates. Table 2 also indicates that the three UN *C. lari* isolates also share a high 16S rDNA sequence similarity (99.8–99.9%) to each other.

In relation to the 15 *C. lari* isolates examined, two other UPTC isolates (CF89-14 isolated from river water in Japan[12] and NCTC 12895 isolated from mussels in England[14]) shared

an identical 16S rDNA sequence. Thus, the maximum sequence diversity of the nearly full-length 16S rDNA was shown to be 1.8% among the 15 isolates of *C. lari* examined.

A phylogenetic tree constructed using the UPGMA demonstrated that the 13 UPTC isolates, including a reference strain of UPTC CCUG18267 examined, formed some minor clusters showing genetic hypervariability separate from the three UN *C. lari* isolates, as well as the four reference UN *C. lari* isolates LMG7607, LMG11251, LMG14338 and CCUG23947^T.

Surprisingly, however, UN *C lari* LMG11760 (isolated from a human in Canada[23]) belonged to a cluster of UPTC isolates, as shown by an identical nucleotide sequence of the nearly full-length 16S rDNA to UPTC NCTC12896 (isolated from mussels in England[14]) sequenced in the present study. This is the second case of an identical 16S rDNA sequence between two *C. lari* isolates, in addition to the case between the two UPTC isolates described above.

This is the first demonstration of two UPTC (NCTC 12896) and UN *C. lari* isolates sharing an identical nucleotide sequence of the nearly full-length 16S rDNA. Moreover, this UN *C. lari* strain (LMG11760) was an NASC *C. lari*. In relation to NAL susceptibility, all 12 UPTC isolates used in the present study were sensitive to NAL, and all three UN *C. lari* isolates were resistant to NAL (Table 1). It is interesting to note that 14 NASC *C. lari* isolates, including 13 UPTC, formed a cluster showing genetic hypervariability that was distinct from the seven NARTC UN *C. lari* isolates.

Then, the level of nearly full-length 16S rDNA sequence diversity was examined among the 19 *C. lari* isolates (including the four reference strains) that did not contain internal unidentified bases in the sequences, to discover whether or not other isolates share identical or nearly identical 16S rDNA sequences. The number of nucleotide sequence differences were demonstrated to be zero to 39 nucleotides (one nucleotide difference [$n=10$], two differences [$n=7$], three differences [$n=7$]) and the approximate 1400 bp difference between two of the 19 *C. lari* isolates.

When Gorkiewicz *et al*.[23] compared the 16S rDNA sequences of all known taxa of the genus *Campylobacter*, including six *C. lari* isolates, the four UN *C. lari* (LMG7607, LMG11251, CCUG23947^T and LMG14338) examined in the present study, the two atypical *C. lari* of NASC *C. lari* LMG11760 (UN *C. lari*) and a Japanese UPTC CF89-12, the 16S rDNA sequencing analysis enabled specific identification of most *Campylobacter* species. However, the exception was a lack of discrimination among the taxa, thermophilic *C. jejuni*, *C. coli* and *C. lari*.

The results presented here clearly demonstrate that *C. lari* organisms form a major phylogenetic cluster comprising some minor clusters with genetic hypervariability, but UPTC and UN *C lari* cannot be discriminated, based on the 16S rDNA sequence information obtained from the 15 *C. lari* isolates (12 UPTC and three UN *C. lari*) examined.

The present study is the first demonstration of sequencing and analysis of the nearly full-length 16S rDNA, which does not contain any unidentified internal bases, of more than 10 *C. lari* isolates and the occurrence of the identical 16S rDNA sequences between a UPTC and a UN *C lari* isolate. Thus, 16S rDNA sequence information cannot be regarded as reliable in the molecular discrimination of UPTC and UN *C. lari* organisms. □

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Table 1. Laboratory results on admission.

	Result (Normal Value)
White cells (per mm³)	3300 (4-11,000)
Differential count (%)	
Lymphocytes	24 (20-45)
Polymorphs	67 (36-75)
Monocytes	4 (3-9)
Bands	5 (5-11)
Platelets (per mm³)	19,000 (150-400,000)
Liver profile	
ALT (U/L)	907 (10-40)
AST (U/L)	1003 (15-40)
LDH (U/L)	3564 (100-190)
CPK (U/L)	13,992 (38-174)
Albumin (u/L)	27 (34-48)

Table 2. Appropriate use of personal protective equipment for 17 contacts.

	Sometimes	Always	Never
Mask	2	15	0
Gown	3	14	0
Gloves	0	17	0
Face Shield	0	0	17

Alkhumra haemorrhagic fever: case report and infection control details

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A tickborne encephalitis virus of the genus *Flavivirus* was isolated for the first time in Saudi Arabia in 1995.[1] Flaviviruses are single-stranded positive-sense RNA viruses responsible for many serious diseases in humans (e.g., yellow fever and dengue fever). The new isolate, termed Alkhumra virus (ALKV), is one of only three tickborne flaviviruses known to cause haemorrhagic disease in humans; the other two being Kyasanur Forest disease virus and Omsk haemorrhagic fever virus.[2,3] Alkhumra haemorrhagic fever and ALKV take their name from the Alkhumra region of Makkah province (which includes the holy cities of Makkah, Jeddah, and Tayef). Alkhumra is a largely agricultural area 230 km east of Tayef and about 1000 km west of the capital city, Riyadh (Fig. 1).

In 2001 the complete coding sequence of the prototype strain of ALKV was published;[4] however, the mode of disease transmission has not been fully described. Limited observation suggests that human ALKV disease may result from contamination of a skin wound with the blood of infected sheep, from infected tick bites, or by drinking unpasteurised contaminated milk.[5] To date, only 24 symptomatic cases of Alkhumra haemorrhagic fever have been reported, and the mortality rate is 25%.[5] The disease is rare and presently confined to Saudi Arabia, but it may be of wider interest because of the large number of international travellers who visit the country, especially during the Hajj season when close to 1.5 million pilgrims arrive in the holy cities of Makkah and Al Madina.

The risk of nosocomial transmission of ALKV virus is not known and no published infection control recommendations are available for healthcare workers to follow. Here, the first case of ALKV infection imported from the southern region of Saudi Arabia, and which was diagnosed in Riyadh, is reported. In addition, detailed infection control precautions taken to prevent nosocomial transmission of ALKV are presented.

A 25-year-old single male who worked as a police officer in the Ministry of the Interior in Najran presented to the emergency department at King Abdulaziz Medical City (KAMC) with a four-day history of mild fever and subsequent rapid deterioration in his condition. Najran lies in the south-west of Saudi Arabia (Fig. 1), bordered by Yemen to the south and Oman in the east. The patient's symptoms included fever (38.5°C), chills and decreased level of consciousness. Initially, he was admitted to a Ministry of Health hospital after one day of illness but discharged himself against medical advice two days later. His condition deteriorated and he developed haematemesis and melaena. Four days into his illness he was brought to the emergency department and his symptoms included jaundice, gingival bleeding, flapping tremors and confusion. The emergency staff suspected haemorrhagic fever and the patient was

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