Reliability of a multiplex PCR assay for the identification of the major *Campylobacter* taxa

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ABSTRACT

The primer pair (C412F/C1228R) constructed previously for the polymerase chain reaction (PCR) identification of the genus *Campylobacter* using an approximate 800 base pair (bp) 16S rRNA gene target segment proved to be useful for the identification of a total of 49 *Campylobacter lari* isolates including urease-positive thermophilic *Campylobacter* (UPTC) organisms (n=25). When the primer pair (CLF/R) developed previously for the PCR identification of *C. lari* species using an approximate 250 bp *glyA* segment was employed, 27 *C. lari* isolates, including all the UPTC isolates, were identified to be PCR-negative (55%). Therefore, this PCR procedure developed for the molecular identification of *C. lari* was shown to be unreliable for *C. lari* identification. Nucleotide sequencing analysis clarified the reason(s) why PCR-negative examples occurred in many *C. lari* isolates, including UPTC isolates. The primer pair target sequences in the *C. lari*-specific PCR-negative isolates apparently varied at the 3′ end region, as compared with *C. lari*-specific PCR-positive isolates. Thus, the multiplex PCR assay developed previously was shown to be unreliable for the molecular identification of *C. lari* subspecies organisms.

KEY WORDS: Campylobacter lari.
Polymerase chain reaction.

Introduction

Thermophilic *Campylobacter* species, primarily *C. jejuni* and *C. coli*, are curved Gram-negative bacteria that are the most recognised causes of acute bacterial diarrhoea worldwide. Although the genus is composed of 17–18 described species, human illness is associated primarily with *C. jejuni* and *C. coli*.[1,2]

Campylobacter lari was first isolated from mammalian and avian species, particularly seagulls of the genus *Larus*.[3,4] *C. lari* has also been shown to be a cause of clinical infection.[5,6] An atypical group of 10 isolates of urease-positive thermophilic *Campylobacter* (UPTC) was first isolated from the natural environment in England in 1985.[7] Thereafter, these organisms were described as a biovar or variant of *C. lari*,[8,9] and Mégraud and his colleagues described four human isolates in France.[8,10] Additional isolates of UPTC have been reported in Northern Ireland,[11–13] The Netherlands[14] and in Japan.[15,16] Thus, these two representative taxa, namely urease-negative (UN) *C. lari* and UPTC, occur within the *C. lari* species.[17]

Regarding molecular discrimination of the *Campylobacter* species, several polymerase chain reaction (PCR)-based assays have been developed with some primer pairs to identify the *Campylobacter* species.[18,19] In addition, multiplex PCR assays have been described to identify the *Campylobacter* species.[20,21] Wang *et al*. described a colony multiplex PCR assay for identification and differentiation of the five major clinically relevant *Campylobacter* spp., *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus* subsp. *fetus*.[20]

Most recently, Yamazaki-Matsune *et al*. described the development of a multiplex PCR assay for the molecular identification of the six common *Campylobacter* taxa associated with human gastroenteritis and/or septicaemia, namely *C. coli*, *C. fetus*, *C. hyointestinalis* subsp. *hyointestinalis*, *C. jejuni*, *C. lari* and *C. upsaliensis*.[22] The assay procedure was developed using a combination of newly designed and published PCR primer sets (e.g., the target gene segment for *C. lari* being an approximate 250 bp amplicon of a serine hydroxymethyltransferase gene [*glyA*]).[22] On evaluation of reliability with a total of 146 *Campylobacter* isolates (n=52 *C. jejuni*, n=21 *C. coli*, n=11 *C. lari*, n=16 *C. upsaliensis*, n=31 *C. fetus*, n=12 *C. hyointestinalis*, n=3 other *Campylobacter* species isolates), the assay correctly identified all isolates as one of the six *Campylobacter* taxa.[22] In relation to *C. lari* species organisms employed in the study, 11 *C. lari* isolates including *C. lari* JCM2530^T were obtained from seagull faeces (n=10) and from an unknown source (n=1).[22] However, no UPTC isolate (a representative taxon within the *C. lari* species) appears to have been used in the study.

The present study aims to clarify whether or not the multiplex PCR assay procedure developed by Wang *et al*. and by Yamazaki-Matsune *et al*.[20,22] is reliable for the molecular identification of more than 40 *C. lari* isolates, including the atypical *C. lari* UPTC taxon.

Materials and methods

Bacterial isolates and culture conditions

A total of 49 *C. lari* isolates (n=24 UN *C. lari*, n=25 UPTC) were used in the study (Table 1). Culture conditions have

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

No.	Isolate no.	Source	Country	Obtained from
1	UN *C. lari* JCM2530[T]	Seagull	Japan	JCM
2	UN *C. lari* 26	NA	N. Ireland	Our collection
3	UN *C. lari* 28	Mussel	N. Ireland	Our collection
4	UN *C. lari* 34	NA	N. Ireland	Our collection
5	UN *C. lari* 48	Mussel	N. Ireland	Our collection
6	UN *C. lari* 155	NA	N. Ireland	Our collection
7	UN *C. lari* 160	NA	N. Ireland	Our collection
8	UN *C. lari* 170	Seagull	Japan	Our collection
9	UN *C. lari* 175	Seagull	Japan	Our collection
10	UN *C. lari* 176	Black-tail gull	Japan	Our collection
11	UN *C. lari* 254	NA	N. Ireland	Our collection
12	UN *C. lari* 264	Mussel	N. Ireland	Our collection
13	UN *C. lari* 274	Mussel	N. Ireland	Our collection
14	UN *C. lari* 293	Seagull	Japan	Our collection
15	UN *C. lari* 295	Human	NA	Our collection
16	UN *C. lari* 296	Seagull	USA	CDC
17	UN *C. lari* 298	Human	Canada	NA
18	UN *C. lari* 299	Human	USA	CDC
19	UN *C. lari* 300	Seagull	USA	CDC
20	UN *C. lari* 448	Mussel	N. Ireland	Our collection
21	UN *C. lari* 1277	NA	N. Ireland	Our collection
22	UN *C. lari* 84C-1	Human	N. Ireland	Our collection
23	UN *C. lari* 84C-2	Human	N. Ireland	Our collection
24	UN *C. lari* ATCC35221	Herring gull	USA	ATCC
25	UPTC NCTC12893	River water	England	NCTC
26	UPTC NCTC12894	Sea water	England	NCTC
27	UPTC NCTC12895	Mussel	England	NCTC
28	UPTC NCTC12896	Mussel	England	NCTC
29	UPTC CF89-12	River water	Japan	Our collection
30	UPTC CF89-14	River water	Japan	Our collection
31	UPTC A1	Seagull	N. Ireland	Our collection
32	UPTC A2	Seagull	N. Ireland	Our collection
33	UPTC A3	Seagull	N. Ireland	Our collection
34	UPTC 89049	Human	France	F. Megraud*
35	UPTC 92251	Human	France	F. Megraud*
36	UPTC 14	Mussel	N. Ireland	Our collection
37	UPTC 15	Mussel	N. Ireland	Our collection
38	UPTC 87	Sea water	N. Ireland	Our collection
39	UPTC 99	Sea water	N. Ireland	Our collection
40	UPTC 136	Scollop	N. Ireland	Our collection
41	UPTC 142	Oyster	N. Ireland	Our collection
42	UPTC 237	Oyster	N. Ireland	Our collection
43	UPTC 467	Mussel	N. Ireland	Our collection
44	UPTC 475	Mussel	N. Ireland	Our collection
45	UPTC 482	Mussel	N. Ireland	Our collection
46	UPTC 487	Mussel	N. Ireland	Our collection
47	UPTC 494	Mussel	N. Ireland	Our collection
48	UPTC 497	Mussel	N. Ireland	Our collection
49	UPTC 504	Mussel	N. Ireland	Our collection

JCM: Japan Collection of Microorganisms; NA: not available; CDC: Centers for Disease Control and Prevention; N., Northern;
*Universite Victor Segalen, Bordeaux, France; ATCC, American Type Culture Collection; NCTC, National Centre for Type Cultures, UK.

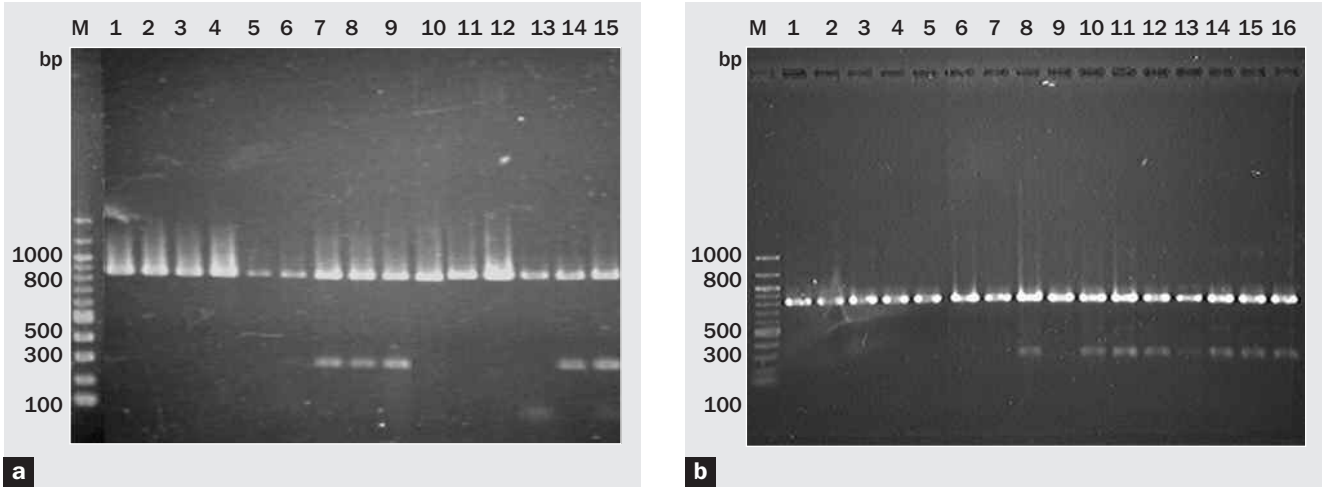

Fig. 1. Agarose gel electrophoresis profiles of PCR products amplified using two sets of primer pairs (C412F/C1228R and CLF/R) with *C. lari* isolates. Lane M: 100 bp DNA ladder (New England BioLabs Japan, Tokyo). **a)** Lane 1: UPTC 92251; lane 2: UPTC 14; lane 3: UPTC 15; lane 4: UPTC 87; lane 5: UPTC 99; lane 6: UN *C. lari* 155; lane 7: UN *C. lari* 254; lane 8: UN *C. lari* 296; lane 9: UN *C. lari* 300; lane 10: UPTC 467; lane 11: UPTC 494; lane 12: UPTC 497; lane 13: UPTC 504; lane 14: UN *C. lari* 84C-1; lane 15: UN *C. lari* ATCC35221. **b)** Lane 1: UPTC CF89-14; lane 2: UPTC 89049; lane 3: UPTC 136; lane 4: UPTC 237; lane 5: UPTC 482; lane 6: UPTC NCTC 12895; lane 7: UPTC NCTC 12896; lane 8: UN *C. lari* 48; lane 9: UN *C. lari* 160; lane 10: UN *C. lari* 170; lane 11: UN *C. lari* 448; lane 12: UN *C. lari* 264; lane 13: UN *C. lari* 298; lane 14: UN *C. lari* 295; lane 15: UN *C. lari* 34; lane 16: UN *C. lari* 84C-2. An approximate 800 bp amplicon provides molecular identification of the genus *Campylobacter*; an 250 bp amplicon provides molecular identification of *C. lari* organisms.

been described previously,[15] and *C. lari* identification was carried out using API Campy (bioMerieux, Tokyo, Japan).

Template DNA preparation and multiplex PCR procedure

Whole genome DNA for PCR amplification was prepared according to the procedure described by Sambrook and Russell.[23] In the present study, two primer pairs were used for the multiplex PCR procedure, namely C412F/C1228R for the genus *Campylobacter* (DDBJ/EMBL/GenBank Accession Number AL111168)[18,22] and CLF/R for the PCR amplification of *C. lari* species (AF136495).[20,22]

The 16S rRNA gene within the genus *Campylobacter* is a target for the primer pair C412F/C1228R and the predicted size of 816 bp for the PCR amplicon is expected.[22] In addition, the *C. lari glyA* gene is a target for the primer pair CLF/R and the predicted size of 251 bp for the PCR amplicon is expected.[18,22] 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 a total of one unit rTaq DNA polymerase (TaKaRa Bio, Tokyo, Japan). The PCR method was performed in 25 µL volumes at 94˚C for 5 min, and then 30 cycles of 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 90 sec, followed by a final extension at 72˚C for 7 min.

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

Amplification of the glyA *segment*

The *C. lari glyA* gene segment was amplified by the primer pair S1/2.[24] This primer pair is expected to generate an amplicon of approximate 640 bp. The PCR mixtures contained 2.5 µL 10 x buffer, 200 µmol each dNTP, 1 mmol/L $MgSO_4$, 1 µmol each primer, 30 ng whole genome DNA, and 1.25 units KOD DNA polymerase (Toyobo, Osaka, Japan). The PCR was carried out in 25 µL reaction volumes at 94˚C for 20 min, followed by 30 cycles at 94˚C for 15 sec, 42˚C for 30 sec, 68˚C for 1 min, followed by a final extension of 68˚C for 5 min.

Direct nucleotide sequencing was performed using ExoSAP-IT (GE Healthcare, Tokyo, Japan) treatment of the PCR product (0.2 µL) at 37˚C for 30 min and at 80˚C for 15 min. The PCR products were purified using the QIA Quick PCR purification kit (Qiagen, Tokyo, Japan).

The purified fraction was subjected to cycle sequencing with BigDye Terminator (version 3.1; Applied Biosystems, Tokyo, Japan) and with sequencing primers. Sequence analysis was carried out using the Genetyx Windows software (version 9; Genetyx, Tokyo, Japan).

Results and discussion

Multiplex PCR identification of the genus Campylobacter *and* C. lari *species*

Figure 1 shows some of the PCR profiles of the amplicons generated by the primer pair C412F/C1228R constructed for the molecular identification of the genus *Campylobacter*, developed by Linton *et al*.[18] and described by Yamazaki-Matsune *et al*.[22] All 49 *C. lari* isolates produced the approximate 800 bp amplicon expected using the C412F/C1228R primer (Table 1). Thus, this primer pair constructed for the molecular identification of the genus *Campylobacter* proved to be useful for the identification of all 49 *C. lari* isolates, including UPTC organisms (n=25).

When PCR was carried out using the primer pair CLF/R developed to identify *C. lari* species organisms,[20,22] 27 *C. lari* isolates (n=2 UN *C. lari* 155 and 160, n=25 UPTC) did not amplify the approximate 250 bp amplicon expected (Fig. 1). This indicates that the primer pair CLF/R is not reliable for the molecular identification of UPTC isolates and UN *C. lari*.

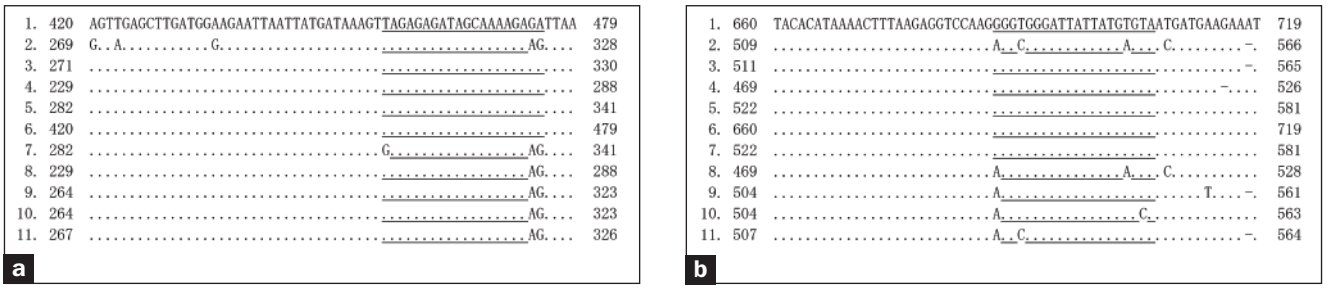

Fig. 2. a) Nucleotide sequence alignment analyses in the vicinity of the primer's base-pairing region within the target *glyA* gene in the *C. lari* isolates examined. 1: UN *C. lari* JCM2530^T; 2: UN *C. lari* 160; 3: UN *C. lari* 298; 4: UN *C. lari* 300; 5: UN *C. lari* ATCC35221; 6: UN *C. lari* 2100; 7: UPTC CF89-12; 8: UPTC92251; 9: UPTC99, 10: UPTC487; 11: UPTC 494. Numbers on the left (5' end) and right (3' end) refer to the nucleotide positions examined. Dots indicate identical bases; changes are indicated; The primer's base-pairing sequence regions[20,22,24] within the target *glyA* gene segment are underlined. **b)** The *glyA* sequence of UN *C. lari* RM2100 is shown for comparison.

***Nucleotide sequence analysis in the vicinity of the primer's base-pairing sequence region within the target gene* glyA**

The present study attempted to perform nucleotide sequence analysis (Table 2) in the vicinity of the primer's base-pairing sequence region within the target *glyA* gene segment amplified using the primer pair S1/S2[24] in the *C. lari* isolates examined. This was undertaken to clarify the reason(s) why no PCR-positive amplicons were observed using the *glyA C. lari*-specific PCR primer CLF/R. Figure 2 shows the nucleotide sequence alignment analysis following nucleotide sequence determination, and clearly demonstrates that the primer-pairing target sequences in the *C. lari*-specific PCR-negative isolates apparently varied at the 3' end regions, as compared with *C. lari*-specific PCR-positive isolates *(C. lari* JCM2530^T, 298, 300, ATCC35221 and RM2100). The present study employed the UN *C. lari* RM2100 strain for nucleotide sequence alignment analysis in the vicinity of the primer's base-pairing sequence region within the target gene *glyA*.

Many UPTC organisms had been isolated[8–16] before the paper by Yamazaki-Matsune *et al*. appeared,[22] following the first description by Bolton *et al*. in 1985 in England,[7] and more recently over 100 UPTC isolates were described in a wild bird community in Sweden.[25] Currently, therefore, in general, UPTC organisms are being described as a representative taxon within the *C. lari* species. As at least three phenotypically distinct variants of *C. lari* have been identified (nalidixic acid-resistant thermophilic *Campylobacter*, nalidixic acid-sensitive variant, and the UPTC biovar), it may prove difficult to establish any molecular identification procedure specific for genetically variable and diverse *C. lari* species organisms.

In conclusion, the multiplex PCR assay developed previously by Wang *et al*.[20] and applied by Yamazaki-Matsune *et al*.[22] for the identification of the five or six major *Campylobacter* taxa has proved unreliable for the molecular identification of at least the *C. lari* species organisms. □

Table 2. Accession numbers of the nucleotide sequence data of the *glyA* accessible in the DDBJ/EMBL/GenBank from the *C. lari* isolates examined in the present study.

Isolate	Accession No.
UN *C. lari* JCM253^T	AB568517
UN *C. lari* 160	AB581533
UN *C. lari* 298	AB581534
UN *C. lari* 300	AB581535
UN *C. lari* ATCC35221	AF136495
UN *C. lari* RM2100	NC_012039
UPTC CF89-12	AB571329
UPTC92251	AB581539
UPTC99	AB581536
UPTC487	AB581537
UPTC494	AB581538

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