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Specific detection of *Campylobacter lari* by PCR

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Abstract

Campylobacter lari is a bacterium associated infrequently with human enteritis. Its differentiation from *C. jejuni* and *C. coli* relies on a few biochemical tests whose efficacy is highly questioned; therefore, the incidence and transmission of this pathogen has not been well studied. Two novel oligonucleotide primers for the polymerase chain reaction (PCR) that specifically amplify a 579-bp segment of the 16S rRNA gene of *C. lari* under optimized conditions were designed. No PCR product was detected when DNA from other strains of *Campylobacter*, *Arcobacter*, *Helicobacter*, *Escherichia*, *Salmonella* or *Listeria* was used as templates. Fast identification of *C. lari* through the present technique may aid the understanding of its incidence and epidemiology. © 1997 Elsevier Science B.V.

Keywords: *Campylobacter lari*; PCR; 16S rDNA

1. Introduction

In 1980, a new group of nalidixic acid-resistant thermotolerant campylobacters was described [1]. Later, these isolates were grouped in a single species named *Campylobacter laridis* [2]. The name was validated in 1984 [3], but changed to *Campylobacter lari* in 1990 [4]. There have been reports claiming the isolation of this organism from seagulls, chickens, cattle, pigs, dogs, cats, crows, monkeys, fur seals, aquatic birds and the environment [5–14]. *Campylobacter lari* has been implicated in two fatal cases of bacteremia [15] and diarrhea [16]; in

sporadic cases with gastrointestinal symptoms [5,16–18] and in a water-borne outbreak [19]. In two years of surveillance, from 1987 to 1989, 21 human isolates of *C. lari* were reported to the Centers for Disease Control and Prevention [10].

The thermotolerant campylobacters, which grow at 42°C, include *C. lari*, *C. jejuni* and *C. coli*. Protein profiles [11] and DNA homology [2,20] have shown that these species share a high degree of relatedness. Few useful biochemical tests have been reported for species differentiation. Anaerobic growth in the presence of 0.1% trimethylamine N-oxide hydrochloride was suggested as a biochemical feature unique to *C. lari*, however, other species were shown to grow under these conditions [20]. At present, the differentiation of thermotolerant species is achieved

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by testing isolates for hippurate hydrolysis, indoxyl acetate hydrolysis and resistance to nalidixic acid [21]. *C. jejuni* is positive for hydrolysis of hippurate and indoxyl acetate and is susceptible to nalidixic acid. *C. coli* differs from *C. jejuni* in that it is negative for hippurate and indoxyl acetate hydrolysis. *C. lari*, meanwhile, does not hydrolyze hippurate or indoxyl acetate and is resistant to nalidixic acid [21]. However, the inconsistency of the results and the presence of atypical strains are two major drawbacks of biochemical tests for species identification [9,10,15,22–24]. Consequently, reliable identification of *C. lari* remains a challenge [9].

Previous research has shown that primers for use in the polymerase chain reaction (PCR) that amplify a segment of the 16S or 23S rRNA gene are suitable for rapid detection of *Campylobacter* spp. [25–29]. Broadly reactive nucleic acid amplification systems for the three thermotolerant *Campylobacter* species have been reported, with specific identification of *C. lari* requiring an additional hybridization step [6,30,31]. A PCR assay directed against the 23S rRNA gene of *C. lari* has been reported; however, the procedure was evaluated with a very limited number of field strains [32].

The objective of the present study was to design oligonucleotide primers and to optimize conditions for a PCR procedure that targets the 16S rRNA gene of *C. lari*. The specificity of the resulting PCR assay was then tested against a number of field strains of *Campylobacter* species and other less related bacteria.

2. Materials and methods

The bacterial strains used in this experiment are listed in Table 1. *Campylobacter* spp. were cultured (37°C for 72 h) under microaerophilic conditions in tryptic soy agar (Difco) supplemented with 0.6% yeast extract (Difco) and 5% laked horse blood (Unipath) (TSAB). *Arcobacter* spp. were cultured for 4–5 days, and *Helicobacter pylori* were cultured for up to 6 days under similar conditions. Enterobacteriaceae were cultured aerobically on TSA (24–48 h at 37°C). *Listeria monocytogenes* was cultured on modified Oxford medium (Difco) (at 37°C for 48 h). Cells were harvested in sterile distilled deionized

water and the DNA was extracted by boiling for 10 min [26,27].

The alignment and comparison of the 16S rRNA gene sequences from *Campylobacter* spp. was done with a computer program (PC/Gene, IntelliGenetics, Mountain View, CA, USA). Sequences were electronically retrieved from GenBank. Species included in the analysis and their accession numbers are: *C. lari* (L04316), *C. jejuni* (L04315, L14630, M59298), *C. coli* (L04312, M59073), *C. fetus* subsp. *fetus* (M65012), and *C. fetus* subsp. *venerealis* (M65011). Short sequences, where mismatches occurred, were selected for the generation of a set of specific primers for *C. lari* (Fig. 1). The forward primer, CL55 (5'-ATG CAA GTC GAA CGA TGA AGC GAC-3'), corresponded to nucleotides 55 to 78 and the reverse primer, CL632 (5'-CCA CTC TAG ATT ACC AGT TTC CC-3'), corresponded to bases 612 to 634. The calculated melting temperature for CL55 was 68°C, and for CL632 it was 67°C. Primers were synthesized by Life Technologies (Grand Island, NY, USA).

The PCR amplification mixture consisted of 5 µl of GeneAmp PCR 10× buffer, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), 100 µM (each) of deoxyribonucleotides (dATP, dTTP, dCTP, dGTP), 1 mM MgCl₂, 50 ng of each primer and 5 µl of the appropriate DNA sample. Negative controls consisted of all reagents except the DNA template [33]. PCR amplification was carried out in 50 µl aliquots. Samples were incubated in a DNA thermal cycler (Perkin-Elmer Cetus) for 2 min at 94°C, to denature the DNA, and then were cycled 25 times at 94°C for 30 s, 65°C for 30 s and 72°C for 30 s. Final extension was performed at 72°C for 5 min. Amplified products were detected by electrophoresis in 1.3% agarose in 0.5× Tris–borate–EDTA buffer at 80 V for 30 min. Gels were stained with ethidium bromide. A UV transilluminator (Gel-Doc System) with a Molecular Analyst computer program (Bio-Rad Laboratories, Hercules, CA, USA) was used for visualization.

PCR-amplified products from *C. lari* ATCC 35221 and from *C. lari* NADC 4899 were sequenced at Scott Ritchey Research Center (Auburn University) using internal primers (forward, CL-U 5'-ATG ACA CTT TTC GGA GCG-3'; reverse, CL-d 5'-CCA TTG CGC AAT ATT CCC-3') and a non-radioac-

Table 1
Bacteria tested with *C. lari* primers

Bacteria	No. of strains	Origin	Source	Reactivity with <i>C. lari</i> primers
<i>C. lari</i>	15	ATCC ^a : 35221, 35222, 35223, 43675 NADC ^b : 3121, 3126, 4899, 4900, 4901, 4902, 4903, 4904, 4905, 4906, 4907	Human, herring gull, dog, chicken, sediment	+
<i>C. jejuni</i>	23	ATCC: 33560, 35918, 35920, 35922, 49349 NADC: 2810, 2811, 2812, 2856, 3128, 3129, 3130, 3131, 3299, 3300, 3379, 3380, 3381, 5161, 5162, 5163, 5164, 5165	Human, bovine, ovine oysters, tofu, monkey	–
<i>C. coli</i>	8	ATCC: 33559, 43473, 43484 NADC: 3521, 3522, 3744, 3745, 3748	Human, porcine, bovine chicken	–
<i>C. fetus</i>	11	ATCC: 27374, 19438 NADC: 3753, 3754, 3755, 3756, 3757, 5075, 5077, 3051, 3052	Human, equine, bovine sheep	–
<i>C. hyointestinalis</i>	11	NADC: 2006, 2007, 2008, 2009, 2018, 3514, 3515, 3516, 3896, 3897, 3898	Human, bovine	–
<i>C. sputorum</i>	5	NVSL ^c : 96-202, 94-148, 94-172, 94-526, 94-1006	Bovine	–
<i>Arcobacter butzleri</i>	24	ATCC: 49615 NADC: 3487, 3488, 3489, 3490, 3491, 3500, 3501, 3502, 3503, 3504, 4034, 4035, 4036, 4037, 4038, 4489, 4490, 4491, 4492, 4494, 4495, 4496, 4497	Human, animal, turkey porcine	–
<i>A. cryoaerophila</i>	1	ATCC: 43158	Bovine	–
<i>A. skirrowii</i>	15	NADC: 3524, 3699, 3700, 3701, 3702, 3703, 3704, 3705, 3707, 3708, 3709, 3710, 3712, 3714, 3715	Bovine, lamb	–
<i>Helicobacter pylori</i>	1	ATCC: 43504	Human	–
<i>Escherichia coli</i>	7	ATCC: 15144; FML ^d	Chicken, porcine	–
<i>Salmonella</i> spp.	3	FML	Chicken	–
<i>L. monocytogenes</i>	7	ATCC: 15313; FML	Rabbit, meat, milk	–

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tive, fluorescent dye terminator (ddNTPs) nucleotide sequencer (Applied Biosystems Division, Perkin Elmer, Foster City, CA, USA).

3. Results and discussion

C. lari has been infrequently implicated in human diarrhea. Its close relatedness with *C. jejuni* and *C.*

coli and the lack of definite biochemical testing methods for species identification have prompted our search for a PCR protocol that would specifically identify *C. lari*.

Amplification of the 16S rRNA gene by PCR has been used as a tool in phylogenetic studies of pathogenic bacteria [34] and the technique may also be applied to clinical settings for the diagnosis of *Campylobacter* spp. Thus, the time required for the

A [5'-3']	
	51 82
<u>C.lari</u>	<u>ATACATGCAAGTCGAACGATGAAGCGACT-AGC</u>
<u>C.jejuni</u>TTTT-AGC
<u>C.coli</u>TTCT-AGC
<u>C.fetus</u>GA.T.TTAAGAGAG

B [3'-5']	
	608 638
<u>C.lari</u>	<u>GCTTGGGAAACTGGTAATCTAGAGTGGGGGA</u>
<u>C.jejuni</u>A..G.....AGGNA
<u>C.coli</u>A.....A.....AGGGA
<u>C.fetus</u>A.....A.....AGGGA

Fig. 1. Alignment and comparison of sequences encoding 16S rRNA of *Campylobacter* spp. Nucleotides that differ from *C. lari* are the only ones indicated. Deletions are indicated by dashes. N=not determined. Underlined sequences indicate the position of primers (A=forward primer. B=reverse primer).

identification of the etiological agent may be considerably reduced and treatment decisions and disease prognoses may be highly improved. The 16S rRNA gene contains conserved and hypervariable regions. Oligonucleotide primers targeting those hypervariable areas may yield PCR assays that are specific for species identification. After analyzing the sequences of the 16S rRNA genes from thermotolerant campylobacters, we developed a set of primers that were specific for *C. lari* identification. The optimized technique was tested with 131 bacterial strains comprising both ATCC and field isolates of thermotolerant campylobacters and other Gram-negative and Gram-positive bacteria.

Amplified products of 579-bp (predicted size) were obtained from all *C. lari* isolates (Fig. 2). No product was observed when either *C. jejuni* ($n=23$ isolates) or *C. coli* ($n=8$ isolates) was used as the DNA template. In addition, no amplification was seen with DNA from other *Campylobacter*, *Arcobacter*, *Helicobacter*, *Escherichia*, *Salmonella* and *Listeria* strains used in the experiment (Table 1).

The DNA segments from *C. lari* ATCC 35221 and from *C. lari* NADC 4899 amplified with primers CL55 and CL632 corresponded with the *C. lari* sequence retrieved from GenBank. The sequence alignment indicated that the region of the *C. lari* 16S rRNA gene amplified by the present technique differs from the *C. jejuni* homolog by 8 bases and from the *C. coli* homolog by 10 bases.

The specificity of the PCR assay was demon-

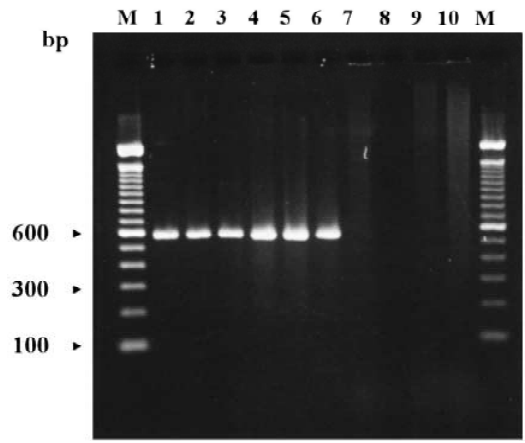


Fig. 2. PCR amplification products from ATCC and clinical isolates of *Campylobacter* spp. in a 1.3% agarose gel. M=100-bp molecular marker; 1=*C. lari* ATCC 35221; 2=*C. lari* ATCC 35222; 3=*C. lari* ATCC 35223; 4=*C. lari* NADC 3125; 5=*C. lari* NADC 4901; 6=*C. lari* NADC 4902; 7=*C. jejuni* ATCC 33560; 8=*C. coli* ATCC 33559; 9=*C. fetus* ATCC 27374 and 10=*C. hyointestinalis* NADC 2008.

strated when isolate NADC 2856 failed to amplify with our technique. This isolate was positive for hippurate hydrolysis and resistant to cephalothin and nalidixic acid. Thus, this isolate phenotypically resembles *C. lari*. However, amplification and sequencing of most of the variable regions (1065 bp) of the 16S rRNA gene showed that NADC 2856 is a *C. jejuni* strain. These findings confirm that biochemical testing for species identification within the thermotolerant group of campylobacter can incur false positive results [9,10,15,22–24]. These results also support previous reports on strains of *C. jejuni* that are resistant to nalidixic acid [31].

To determine the sensitivity of the method, serial ten-fold dilutions of a 72-h broth culture of *C. lari* (ATCC 35223) were made. TSAB plates were seeded in triplicate with 0.1 ml from each dilution. An aliquot (100 μ l) from each dilution was boiled for DNA extraction and the PCR was performed as previously described. The PCR assay detected 6 CFU per 5 μ l of DNA sample, which corresponds to 1.2×10^3 CFU per ml.

The present PCR protocol permitted fast, reliable identification of *C. lari* within 3 h of detection of suspicious colonies on agar plates. Furthermore, the method can also be useful for the rapid detection of

C. lari in food samples. Traditional isolation procedures involving enrichment media may hinder the recovery of *Campylobacter* spp. from food samples [35]. Therefore, reliable molecular techniques, such as the one reported here, may overcome the interference associated with enrichment media. However, controls must be included to show the absence of PCR inhibitors in food samples, to avoid false negatives [24,36].

Although isolation and identification of *C. lari* is important for further epidemiologic studies, few laboratories routinely isolate *Campylobacter* or attempt to speciate the isolates biochemically. The main disadvantage of biochemical testing is the time required and the lack of consistency of results. In conclusion, the differentiation of *C. lari* from *C. jejuni* and *C. coli* is hindered by both its phylogenetic proximity and the lack of reliable biochemical methods. The PCR technique described in this report will aid in the identification of *C. lari* and, subsequently, it will expand our knowledge of its epidemiology.

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