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PCR–Enzyme-Linked Immunosorbent Assay for Detection and Identification of *Campylobacter* Species: Application to Isolates and Stool Samples

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We report a PCR–enzyme-linked immunosorbent assay which identifies *Campylobacter* species by capture hybridization of a single-stranded 16S rRNA gene amplicon with species-specific probes in a microtiter plate format. Specificities were confirmed for both reference and field strains, but the type strain of *Campylobacter coli* was atypical. The assay was rapid, informative, and usable with stool-extracted DNA.

We have previously described PCRs which identify individual *Campylobacter* species from pure cultures or fecal DNA extracts (3–5). In routine clinical laboratory practice, a further advantage would be to avoid multiple PCRs by generating one amplicon containing sequence polymorphisms suitable for species-specific identification.

DNA was extracted (8) from the reference strains listed in Table 1, 49 *Campylobacter jejuni* and 14 *C. coli* Penner serotype reference strains, 10 field isolates of *C. jejuni*, 8 of *C. coli*, 4 of *C. lari*, 4 of *C. upsaliensis*, 4 of *C. helveticus*, 2 of *C. hyointestinalis*, and 5 of *C. fetus*. These were used to analyze probe specificities.

First-round symmetric PCR was performed with genus-specific primers, C412F and C1228R (5). Second-round asymmetric PCR was performed with 10 μl of the first-round reaction mixture in a 50-μl volume, with a single 5′ end fluorescein-labelled primer, C690F (see below). Conditions were as described previously (5), except that the annealing temperature was raised to 60°C and the primer concentration of 400 nM gave a >5:1 ratio of C690F/C1228R. The PCR–enzyme-linked immunosorbent assay (ELISA) was performed as described previously (6), except that 100 ng of capture probe was immobilized, denaturation of second-round PCR product was unnecessary, and the conjugate was diluted 1:1,000. The optical density (OD) of the final yellow product was measured at 450/620 nm (see Fig. 1): signals were considered negative if the absorbance was below 0.1 OD unit and positive if it was above 0.2 OD unit. Certain first-round PCR products were investigated by sequencing with a Prism DNA cycle sequencing kit, a 373A automated DNA sequencer (Applied Biosystems), and the primers C1228R (5) and C641F (see below).

16S rRNA gene sequences of the following species were aligned by using the CLUSTAL program (2): *C. gracilis* L37787 and L04320, [*Bacteroides*] *ureolyticus* L04321, *Campylobacter* sp. strain CLO L14632, *Campylobacter* sp. strain L04318, *C. coli* L04312 and M59073, *C. concisus* L06977 and L04322, *C. curvus* L06976 and L04313, *C. fetus* subsp. *venerealis* L14633, *C. fetus* subsp. *fetus* L04314, *C. helveticus* U03022, *C. hyointestinalis* M65010 and M65009, *C. jejuni* Z29326 and M59298, *C. jejuni* subsp. *jejuni* L04315, *C. lari* L04316, *C. mucosalis*-like L14629, *C. mucosalis* L06978, *C. rectus* L06973 and L04317, *C. showae* L06975, L06974, and UPTC L14631, *C. sputorum* subsp. *bubulus* L04319, *C. sputorum* subsp. *sputorum* X67775, and *C. upsaliensis* L14628. Two *Campylobacter* genus-specific primers, C641F (5′-GAGAGGCAGATGGAATTGG-3′; sequencing primer) and C690F (5′-AGATACCCTGGTAGTCC ACG-3′; second-round primer), were thereby designed.

Eight capture probes were designed to target variable regions; accession numbers of sequences bearing perfect matches to these probes are given in Table 2. Four probes (lar, fet, hyo, and ups) were designed to capture amplicons from *C. lari*, *C. fetus*, *C. hyointestinalis*, and *C. upsaliensis*, respectively. Probe ups/hel was designed to capture amplicons from *C. upsaliensis* and *C. helveticus*. Thus, capture with both ups and ups/hel would indicate *C. upsaliensis*; capture with the latter alone would indicate *C. helveticus*. A positive reaction with probes jej/col 1 and jej/col 2 should indicate *C. jejuni*, that with col and jej/col 2 should indicate *C. coli*, that with jej/col 1 alone should indicate the urease-positive thermophilic campylobacter group (UPTC), and that with jej/col 2 alone should indicate oral campylobacters (Table 2).

To evaluate the probes, amplicons from the 21 reference strains of *Campylobacter*, the 16 reference strains of *Helicobacter*, the 4 reference strains of *Arcobacter* (Table 1), 63 Penner serotype reference strains (see above), and 37 *Campylobacter* field isolates (speciated by phenotype) were hybridized with the eight probes in a microtiter plate format. All capture probes except col hybridized with both their respective type strains and the corresponding field isolates (Table 1; Fig. 1). No capture was observed for the remaining species of *Campylobacter*, *Helicobacter*, and *Arcobacter*. Probe col reacted with the type strain but not with the serotype reference strains or field isolates of *C. coli*. Probe jej/col 1 reacted with all strains and isolates of *C. jejuni* and also *C. coli* (except NCTC 11366^T).

To further analyze these two apparent anomalies, we sequenced amplicons from 52 of 63 Penner serotype reference strains of *C. coli* and *C. jejuni*. All had a similar sequence between positions 732 and 1198, falling into three groups which varied only at positions 837, 1010, and 1019. Nineteen *C. jejuni* reference strains (Penner serotypes HS 1 to 6, 10, 13 to 18, 22, 31, 37, 40, 44, 62, and 64) and 12 *C. coli* reference strains (HS 5, 14, 25, 26, 28, 30, 34, 39, 47, 48, 51, and 54) had G, T, and A at positions 837, 1010, and 1019, respectively (EMBL accession no. AJ006477). Nineteen *C. jejuni* strains (HS 7 to 9, 12, 19, 21, 23, 27, 29, 32, 33, 36, 38, 41, 42, 53, 58, 60, and 63)

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TABLE 1. Reference strains of *Campylobacter*, *Arcobacter*, and *Helicobacter* species

Species	Source[a]
Campylobacter spp.	
C. jejuni subsp. *jejuni*	NCTC 11351^T
C. jejuni subsp. *doylei*	NCTC 11951^T
C. coli	NCTC 11366^T
C. lari	NCTC 11352^T
C. upsaliensis	NCTC 11541^T
C. helveticus	NCTC 12470^T
UPTC	NCTC 11845
C. fetus subsp. *fetus*	NCTC 10842^T
C. fetus subsp. *venerealis*	NCTC 10354^T
C. hyointestinalis	NCTC 11608^T
C. sputorum subsp. *sputorum*	NCTC 11528^T
C. sputorum subsp. *fecalis*	NCTC 11415^T
C. sputorum subsp. *bubulus*	NCTC 11367^T
C. gracilis	NCTC 12738^T
[*Bacteroides*] *ureolyticus*[b]	NCTC 10941^T
C. concisus	NCTC 11485^T
C. rectus	NCTC 11489^T
C. curvus	NCTC 11649^T
C. showae	NCTC 12843^T
C. mucosalis	NCTC 11001
Helicobacter spp.	
H. felis	NCTC 12436^T
H. canis	NCTC 12739^T
H. pullorum	NCTC 12824^T
H. rappini	NCTC 12461^T
H. acinonyx	NCTC 12686^T
H. cinaedi	NCTC 12423^T
CLO-3[c]	NCTC 12462
H. fennelliae	NCTC 11612^T
H. mustelae	NCTC 12198^T
H. pylori	NCTC 11637^T
H. hepaticus	ATCC 51448^T
H. muridarum	NCTC 12714^T
H. bilis	ATCC 51630^T
H. pamatensis	ATCC 51478^T
H. nemestriae	NCTC 12491^T
Arcobacter spp.	
A. cryaerophilus	NCTC 11885^T
A. skirrowii	NCTC 12713^T
A. butzleri	NCTC 12481^T
A. nitrofigilis	NCTC 12551^T
Wollinella succinogenes	NCTC 11488^T

[a] NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; ^{T} denotes a type strain.
[b] Species incertae sedis which is genotypically *Campylobacter* (7).
[c] CLO-3, *Campylobacter*-like organism.

and one *C. coli* strain (HS 20) had A at positions 837 and 1010 and T at position 1019 (accession no. AJ006478). *C. jejuni* HS 43 uniquely had G at position 837, A at position 1010, and T at position 1019 (accession no. AJ006479).

The new assay format (without probe col) was tested on DNA extracted from 40 clinical fecal samples and 10 fecal samples from healthy volunteers as described previously (3). The clinical samples comprised 28 from which presumptive *C. jejuni* or *C. coli* had been cultured by standard methods (4), 10 *Campylobacter* culture-negative samples (5 positive for *Salmonella*, 1 for *Shigella*, 1 for *Aeromonas*, 1 for *Cryptosporidium*, and 2 for *Giardia*), and 2 samples in which no pathogenic organisms were detected. Amplicons from the 28 *Campylobacter* culture-positive samples hybridized with jej/col 1 and jej/col 2, indicating *C. jejuni* or *C. coli*, and were assigned to one of these species as previously described (4). The 10 samples positive for other enteropathogens showed no hybridization. One culture-negative sample reacted with ups and ups/hel, and the other reacted with hyo. Results for these two samples were confirmed with individual species-specific PCR assays for *C. upsaliensis* and *C. hyointestinalis* (4, 5). For the 10 samples from healthy volunteers, 6 negative results were obtained with all probes, while 2 were weakly positive with jej/col 1 alone and 2 were weakly positive with jej/col 2 alone, consistent with the presence of nonpathogenic campylobacters.

The data for probes jej/col 1, jej/col 2, and col and the sequencing studies described above indicate that the *C. coli* type strain is not representative of that species. This and the fact that there are no clear-cut differences between *C. jejuni* and *C. coli* explain why probe col did not hybridize with any other *C. coli* strains and highlight the need for caution when using sequence data derived from type strains alone (see also reference 1).

This PCR-ELISA is fast, informative, and can be customized

TABLE 2. *Campylobacter* capture probes, their sequences, and homologies

Capture probe	Sequence[a]	Sequence identity with:	EMBL/GenBank accession no.[b]
jej/col 1	tttGCGGTACA CTTAATGC GTT	*C. jejuni*	Z29326, M59074, L04315, L14630
		UPTC	L14631
jej/col 2	taaGCTCGGC CGAACCG TTA	*C. coli*	L04312
		C. jejuni	Z29326, M59074, L04315, L14630
		C. rectus	L06973
		C. showae	L06974
		C. curvus	L06976
		C. gracilis	L04320, L37787
col	aaaCCCTGACT AGCAGAG CAA	*C. coli*	L04312
lar	taaGCTCACCC GAAGTGT TAG	*C. lari*	L04316, M92310
ups	aaaCTACAGA ATTTGTTG GATATC	*C. upsaliensis*	L14628
ups/hel	taaGCTCGACC GAATCGT TAG	*C. helveticus*	U03022
		C. upsaliensis	L14628
hyo	ataCTCTAAGA TGTTATTA GGATAT	*C. hyointestinalis*	M65009, M65010
fet	aaaCTAAGA GATTAG TTGGAT ATC	*C. fetus*	M65011, M65012 L04314, L14633

[a] Bases in lowercase letters represent nonhybridizing spacer regions.
[b] Species identified only to genus level have not been included.

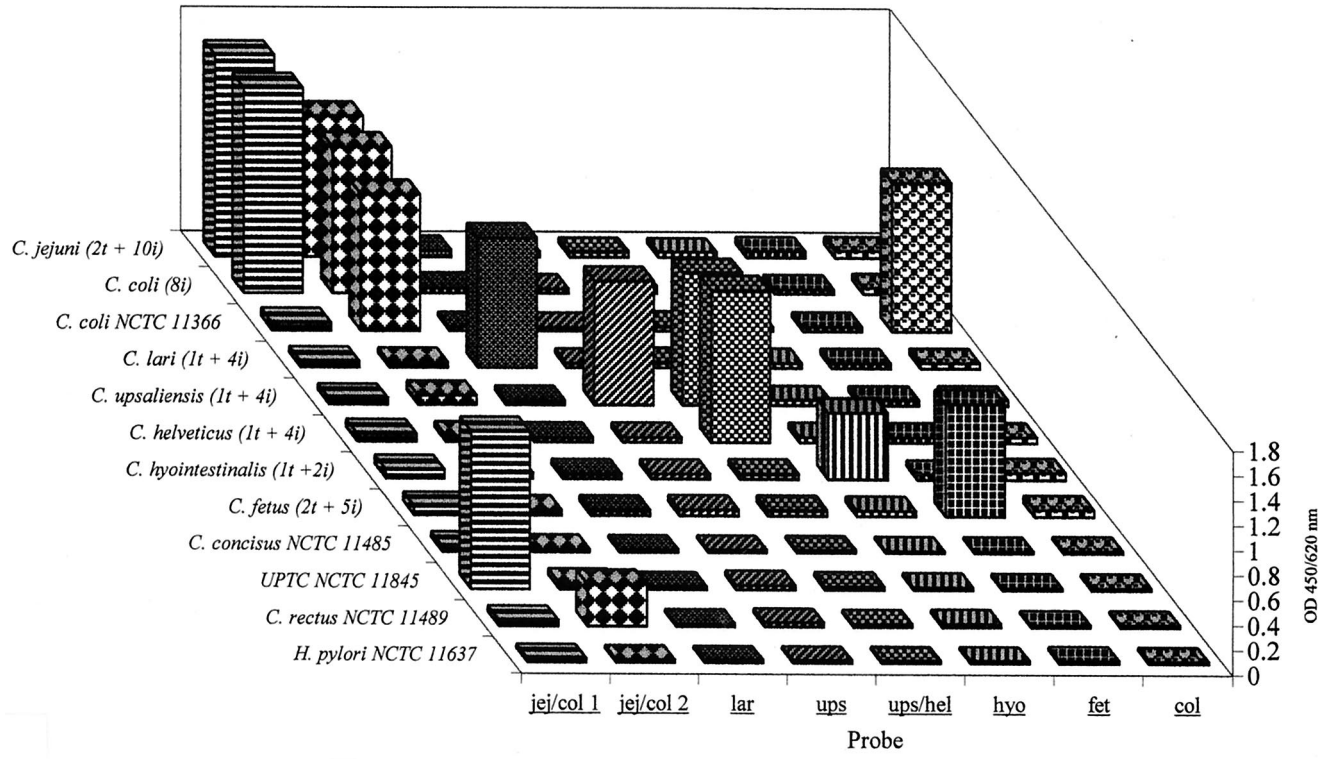

FIG. 1. PCR-ELISA results for 13 strains and 37 field isolates of *Campylobacter*. Values shown for *C. jejuni*, *C. lari*, *C. upsaliensis*, *C. helveticus*, *C. hyointestinalis*, and *C. fetus* are the average for type strains (*t*), described in Table 1, and field isolates (*i*). *C. coli* field isolates behaved differently from the type strain. Values are shown for type strains of *C. concisus* and *C. rectus*, a reference strain of the UPTC group, and a negative control, *Helicobacter pylori*.

by the addition or subtraction of probes to identify species of particular interest. Its microtiter plate format and nonisotopic detection are readily suited to automation. It should find use both in diagnostic laboratories and in molecular ecological studies of campylobacter prevalence in human disease.

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