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Rapid Identification of *Campylobacter* spp. by Melting Peak Analysis of Biprobes in Real-Time PCR

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We describe rapid PCR-biprobe identification of *Campylobacter* spp.. This is based on real-time PCR with product analysis in the same system. The assay identifies enteropathogenic campylobacters to the species level on the basis of their degree of hybridization to three 16S ribosomal DNA (rDNA) biprobes. First-round symmetric PCR is performed with genus-specific primers which selectively target and amplify a portion of the 16S rRNA gene common to all *Campylobacter* species. Second-round asymmetric PCR is performed in a LightCycler in the presence of one of three biprobes; the identity of an amplified DNA-biprobe duplex is established after determination of the species-specific melting peak temperature. The biprobe specificities were determined by testing 37 reference strains of *Campylobacter, Helicobacter*, and *Arcobacter* spp. and 59 Penner serotype reference strains of *Campylobacter jejuni* and *C. coli*. From the combination of melting peak profiles for each probe, an identification scheme was devised which accurately detected the five taxa pathogenic for humans (*C. jejuni*/*C. coli, C. lari, C. upsaliensis, C. hyointestinalis*, and *C. fetus*), as well as *C. helveticus* and *C. lanienae*. The assay was evaluated with 110 blind-tested field isolates; when the code was broken their previous phenotypic species identification was confirmed in every case. The PCR-biprobe assay also identified campylobacters directly from fecal DNA. PCR-biprobe testing of stools from 38 diarrheic subjects was 100% concordant with PCR–enzyme-linked immunosorbent assay identification (13, 20) and thus more sensitive than phenotypic identification following microaerobic culture.

Campylobacter is the most common bacterial cause of human gastrointestinal infection worldwide. Incidence rates are estimated at 1% of the population per year in both the United Kingdom and United States, representing a major public health and economic burden (2, 11, 19, 25). The species most frequently isolated from humans are *Campylobacter jejuni* and *C. coli; C. upsaliensis, C. hyointestinalis, C. lari*, and *C. fetus* have also been implicated, though rarely (13). *Campylobacter sputorum* subsp. *sputorum* and *Campylobacter sputorum* subsp. *bubulus* occasionally cause disease in humans, while five species (*C. concisus, C. curvus, C. gracilis, C. rectus*, and *C. showae*) are not gastrointestinal pathogens but are found in association with the oral cavity. *C. mucosalis, C. helveticus*, and *Campylobacter sputorum* subsp. *faecalis* are commensals or pathogens of animals (21, 24).

Rapid diagnosis of *Campylobacter* infections is complicated by their slow growth rate and by difficulties in phenotypic identification to species level due to the fact that few informative biochemical tests can be applied to them. Furthermore, the frequency of non-*C. jejuni*, non-*C. coli* campylobacter infections is probably underestimated, since the isolation methods used (incubation at 42°C on selective antibiotic-containing media) are inhibitory to these species. Genotypic methods may therefore offer an attractive alternative to culture for accurate identification of the range of *Campylobacter* spp. involved in human infection (13).

We have previously described a PCR–enzyme-linked immunosorbent assay (PCR-ELISA) where identification of a genus-specific PCR product was carried out by capture with species-specific oligonucleotides immobilized on a microtiter plate (20). The present study is based on the LightCycler, a temperature-controlled microvolume fluorimeter which provides rapid real-time PCR and product analysis in a single closed-tube system (35). This real-time PCR platform can support several different sequence-specific fluorescent probe detection systems such as hybridization probes (34), TaqMan probes (17), Molecular Beacons (26), and biprobes (3). The term "biprobe" was coined by the user community subsequent to issue of a patent for the system (Roderick Fuerst [Bio/Gene Ltd.], personal communication). In the present report a biprobe assay is described where the hybridization of a biprobe to a target sequence results in intercalation of a double-stranded DNA-specific fluorophore (SYBR Green I). Due to the close proximity of SYBR Green I to the biprobe (which is labeled with the red fluorophore Cy5), there is increased light emission from Cy5, a phenomenon termed fluorescent resonance energy transfer (FRET) (23). The Cy5-labeled probe is blocked with biotin at the 3′ end to prevent its acting as a primer. Conversely, when the bound biprobe melts from the target sequence, a decrease in light emission from Cy5 is observed. By continually monitoring the emission peak of Cy5 (675 nm) during the melt cycle, the temperature at which the biprobe detaches from the single-stranded PCR product is determined and visualized as a defined melting peak. The melting peak data are characteristic of a particular probe-target sequence because mismatches between probe and target affect the kinetics of melting, producing different melting peaks for each species of interest. In the present study we developed

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

Species	Source[a]
Campylobacter spp.	
C. jejuni subsp. *jejuni*	NCTC11351^T
C. jejuni subsp. *doylei*	NCTC11951^T
C. coli	NCTC 11350
C. lari	NCTC11352^T
C. upsaliensis	NCTC11541^T
C. helveticus	NCTC12470^T
C. fetus subsp. *fetus*	NCTC10842^T
C. fetus subsp. *venerealis*	NCTC10354^T
C. hyointestinalis subsp. *hyointestinalis*	NCTC11608^T
C. sputorum subsp. *sputorum*	NCTC11528^T
C. sputorum subsp. *faecalis*	NCTC11415^T
C. sputorum subsp. *bubulus*	NCTC11367^T
C. gracilis	NCTC12738^T
C. concisus	NCTC11485^T
C. rectus	NCTC11489^T
C. curvus	NCTC11649^T
C. showae	NCTC12843^T
C. mucosalis	NCTC 11001
C. lanienae	NCTC13004^T
[*Bacteroides*] *ureolyticus*[b]	NCTC10941^T
Helicobacter spp.	
H. canis	NCTC12739^T
H. pullorum	NCTC12824^T
H. rappini	NCTC12461^T
H. acinonyx	NCTC12686^T
H. cinaedi	NCTC12423^T
H. fennelliae	NCTC11612^T
H. mustelae	NCTC12198^T
H. pylori	NCTC11637^T
H. hepaticus	ATCC51448^T
H. muridarum	NCTC12714^T
H. pamatensis	ATCC51478^T
H. nemestriae	NCTC12491^T
Arcobacter spp.	
A. cryaerophilus	NCTC11885^T
A. skirrowii	NCTC12713^T
A. butzleri	NCTC12481^T
A. nitrofigilis	NCTC12551^T
Wollinella succinogenes	NCTC11488^T

[a] NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection.

[b] Species *incertae sedis* which is genotypically *Campylobacter* (28).

an assay that would identify *Campylobacter* species both from field isolates and from DNA extracted from fecal samples.

MATERIALS AND METHODS

Bacterial strains and clinical fecal samples. Reference strains used for assay development and assessment of biprobe specificities are listed in Table 1. To evaluate the assay, 46 *C. jejuni* and 13 *C. coli* Penner serotype reference strains were tested. In addition, 110 field isolates already identified to species level by phenotype were tested; these were blind-coded and comprised 26 *C. jejuni*, 12 *C. coli*, 13 *C. lari*, 24 *C. upsaliensis*, 13 *C. fetus*, 11 *C. helveticus*, 10 *C. hyointestinalis*, and 1 *C. lanienae* isolate.

Thirty-eight fecal samples from gastroenteritis patients from a large-scale study (13) were tested. From 24 of these samples, campylobacters had been identified to the species level by PCR-ELISA (15 *C. jejuni*, 2 *C. coli*, 2 *C. hyointestinalis*, and 5 *C. upsaliensis* isolates). From 19 of these 24, *Campylobacter* had been identified to the genus level by selective culture. The remaining 14 clinical samples were negative both by *Campylobacter* PCR-ELISA and culture: in 9 samples no pathogenic organisms were detected, while 1 sample was positive for *Salmonella*, 1 for *Shigella*, 1 for *Escherichia coli*, 1 for *Cryptosporidium*, and 1 for *Giardia*.

Sample preparation. A boiled colony suspension in sterile water was prepared from 24- to 48-h blood agar plate cultures. Archived DNA extracts stored at −20°C were tested retrospectively; DNA was extracted from these fecal specimens as previously described (4, 12).

PCR and biprobe melting peak analysis. Symmetric PCR was performed on a RoboCycler (Stratagene, La Jolla, Calif.) using previously described *Campylobacter* genus-specific primers (16) C412F (GGA TGA CAC TTT TCG GAG C) and C1228R (CAT TGT AGC ACG TGT GTC).

Heminested asymmetric PCR and melting peak analysis were performed in glass capillaries in a LightCycler apparatus (Bio/Gene Ltd., Kimbolton, United Kingdom) (34, 35) using 1 μl of first-round PCR product in a total volume of 10 μl. Asymmetric PCR is used to produce excess copies of the strand complementary to the biprobes. The reaction mixture contained 2.5 pmol of the single primer C690F (AGATACCCTGGTAGTCCACG), 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.5 mg of bovine serum albumin/ml, 200 μM each deoxynucleoside triphosphate, 0.4 U of Platinum *Taq* DNA polymerase (Life Technologies Ltd., Paisley, United Kingdom), SYBR Green 1 (Bio/Gene Ltd.) at 0.01%, and 5 pmol of biprobe labeled at the 5′ end with Cy5 and at the 3′ end with biotin. This type of nucleic acid detection system is subject to a patent held by the Defence Evaluation Research Agency, Farnborough, United Kingdom and Bio/Gene Ltd. (3). Primers and probes were HPSF (highly purified salt-free) grade, synthesized by MWG-Biotech UK Ltd., Milton Keynes, England. Thermal cycling and fluorescence acquisition conditions comprised an initial denaturation cycle at 95°C for 10 s and 40 amplification cycles (with a temperature transition rate of 20°C/s) of 95°C for 0 s; annealing at 60°C for 10 s, and extension at 74°C for 30 s. Fluorescence readings were taken after each cycle following the extension step. This was followed by melting analysis of the probe-PCR product duplex consisting of 95°C for 0 s, then cooling to 40°C before the temperature was raised to 99°C at a rate of 0.1°C/s with continuous fluorescence acquisition.

RESULTS

Design of biprobes and assay development. The available EMBL DNA sequences coding for the 16S rRNA gene of *Campylobacter* were aligned using the Clustal multiple alignment program (10). A series of probes for species of interest were then designed, targeting variable regions between nucleotide positions 690 and 1228. The probes were 20 to 25 bases in length. There were four design parameters: hybridization to sites away from either end of the PCR product, melting temperatures above that of the primer, mismatching of bases central to the probe itself, and minimum hairpin loop formation. Probes were designed to have up to four base mismatches with the species (sequence) of interest.

The exact effect of mismatched bases on the melting behavior of a given probe cannot be predicted from the sequence alone, due to secondary structure and other steric effects. Performance was therefore assessed against a panel of reference strain sample DNAs (Table 1) to identify probes which gave discrete melting peaks, with good separation of the melting temperatures for the species in question. No single probe produced discrete melting peaks discriminatory for all the species of interest. However, combining data for three probes allowed identification of *C. jejuni*/*C. coli, C. lari, C. upsaliensis, C. helveticus, C. hyointestinalis, C. fetus*, and *C. lanienae*.

The sequence and labeling of the chosen combination of probes were as follows: biprobe A, Cy5-GCA CCC CAA CAA CTA GTG TAC A-biotin; biprobe B, Cy5-CAG CAC CTG TCA CTA ATT TCT TG-biotin; biprobe C, Cy5-GCA AGC TAG CAC TCT CTT ATC TCT-biotin. During end point analysis of the melting kinetics of a given biprobe/target, a decrease in light emission from Cy5 was observed, and this was recorded by continually monitoring the emission peak of Cy5 during the melt cycle. Data collected were presented in the

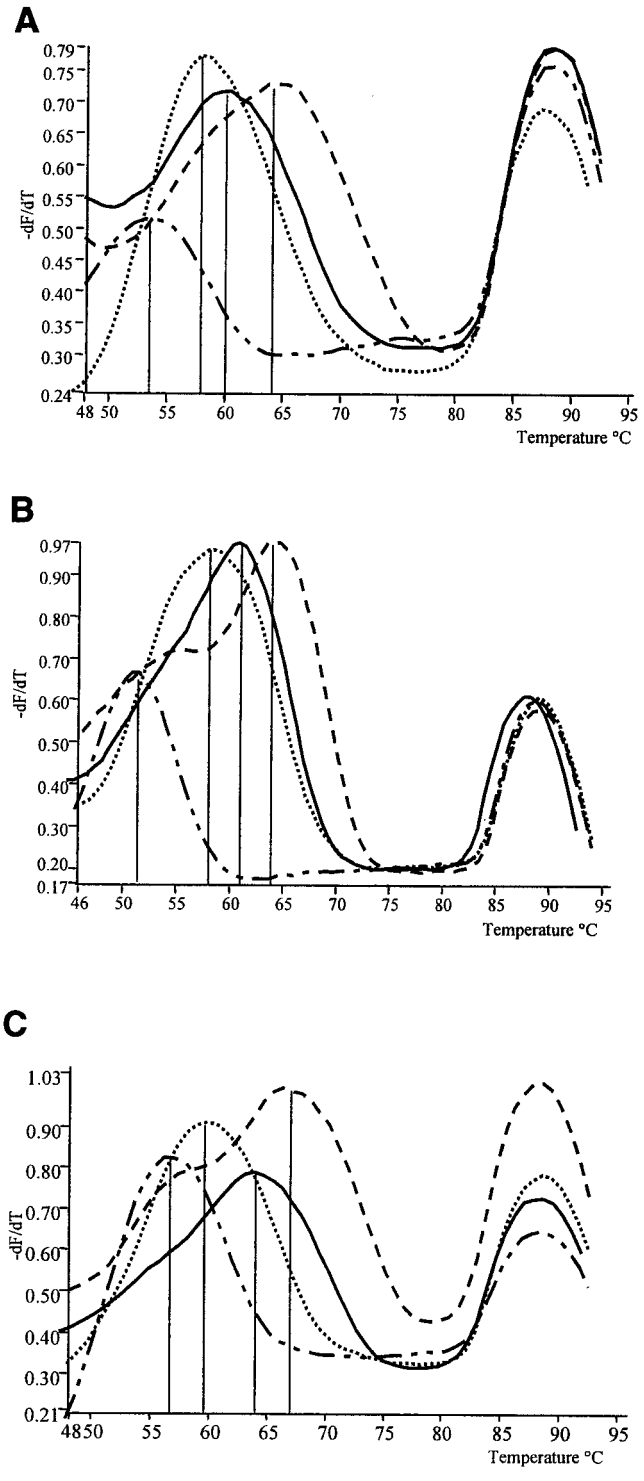

FIG. 1. Biprobe fluorescence data. Shown are negative first-derivative plots, reflecting the change in fluorescence divided by the change in temperature ($-dF/dT$). The melting peaks seen in the range from 48 to 75°C correspond to the decrease in FRET and hence the decrease in light emitted at the Cy5 wavelength, as the biprobe melts from the single-stranded target DNA. The secondary peak seen at 86°C acts as a positive amplification control and corresponds to residual double-stranded PCR product. (A) Melting peaks obtained with biprobe A. *C. jejuni*/*C. coli* gave a peak at 64°C (dashed line), *C. jejuni* gave a peak at 58°C (dotted line), *C. lari*/*C. upsaliensis* gave a peak at 60°C (solid line), and *C. helveticus* gave a peak at 54°C (irregularly dashed line). (B) Melting peaks obtained with biprobe B. *C. hyointestinalis* gave a peak at 64°C (dashed line), *C. jejuni*/*C. coli*/*C. lari*/*C. helveticus* gave a peak at 62°C (solid line), *C. fetus*/*C. lanienae* gave a peak at 58°C (dotted line), and *C. upsaliensis* gave a peak at 51°C (irregularly dashed line), (C) Melting peaks obtained with biprobe C. *C. fetus* gave a peak at 67°C (dashed line), *C. jejuni*/*C. coli*/*C. lanienae* gave a peak at 64°C (solid line), *C. jejuni*/*C. coli*/*C. lari*/*C. helveticus* gave a peak at 60°C (dotted line), and *C. upsaliensis* gave a peak at 56°C (irregularly dashed line).

form of a negative first-derivative plot of fluorescence/temperature versus temperature ($-dF/dT$ versus T), calculated by LightCycler software. This plot converts the data into discrete melting-peak graphs. The secondary peak observed on such plots corresponds to residual double-stranded PCR product, since Cy5 has a spectral overlap with SYBR Green 1. Such plots, as produced by each probe with the range of reference strain sample DNAs, are shown in Fig. 1. The combined melting-peak profiles constitute an identification scheme as presented in Table 2. Six species (*C. lari, C. upsaliensis, C. helveticus, C. hyointestinalis, C. fetus*, and *C. lanienae*) produced unique combined melting-peak profiles. However, *C. jejuni* and *C. coli* could not be differentiated at the species level. The melting peaks of all strains of *C. hyointestinalis* with biprobe B and the melting peaks of all strains of *C. fetus* with biprobe C showed a "shoulder effect" at the lower temperature range (Fig. 1B and C).

The 46 Penner heat-stable (HS) serotype reference strains of *C. jejuni* produced one of two melting peaks with biprobe A (64 or 58°C) and with biprobe C (64 or 60°C) but a single peak (62°C) with biprobe B (Fig. 1; Table 2). The 13 *C. coli* HS reference strains produced a single melting peak with biprobe A, identical to one of the *C. jejuni* peaks (64°C). With biprobes B and C, *C. coli* strains produced melting peaks identical to those of the *C. jejuni* strains (Fig. 1; Table 2). Combining these results yielded the three possible profiles shown in Table 2. For example, A/B/C peaks of 64:62:64 or 60 could denote *C. jejuni* or *C. coli*. Peaks of 58:62:60 denoted only *C. jejuni*. The distribution of each profile with respect to the HS serotypes is shown in Table 2.

Evaluation of *Campylobacter* field isolates. One hundred ten field isolates were blind tested. The profiles obtained with the three biprobes above were identified according to the scheme outlined in Table 2. Controls for each melting peak were included in each run to remove run-to-run variation. Thirteen *C. lari*, 24 *C. upsaliensis*, 13 *C. fetus*, 11 *C. helveticus*, 10 *C. hyointestinalis*, and 1 *C. lanienae* isolate were all identified by the PCR-biprobe assay and had the characteristic species profiles described in Table 2. Twenty-six *C. jejuni* and 12 *C. coli* field isolates were identified by the assay as *C. jejuni*/*C. coli*. Twenty-five *C. jejuni* field isolates and all 12 *C. coli* field isolates gave melting-peak profiles of 64:62:64 (A/B/C). One *C. jejuni* field isolate gave the A/B/C profile 64:62:60. PCR-biprobe identification therefore showed 100% correlation with phenotypic identification to species level for all tested field isolates.

Evaluation of fecal DNA extracts. Thirty-eight archived fecal DNA extracts were tested. The combined melting-peak profiles of these extracted DNAs were compared with corresponding results for the samples obtained by phenotypic identifica-

TABLE 2. *Campylobacter* sp. identification by combined biprobe melting-peak profiles

Campylobacter sp. (no. of strains tested)[a]	Melting peaks (°C) for biprobe:			Penner serotype reference strain profile[c]
	A	B	C	
C. lari (14)	60	62	60	
C. upsaliensis (25)	60	51	56	
C. helveticus (12)	54	62	60	
C. hyointestinalis (11)	—	64	—	
C. fetus (15)	—	58	67	
C. lanienae (2)	—	58	64	
C. jejuni/*C. coli* (72)	64	62	64	*C. jejuni* HS 1–6, 10, 11, 13, 15–18, 22, 31, 37, 40, 44, 50, 52, 62, 64 *C. coli* HS 5, 14, 25, 26, 28, 30, 34, 39, 47, 48, 51, 54
C. jejuni/*C. coli* (8)	64	62	60	*C. jejuni* HS 23, 35, 43, 63 *C. coli* HS 20
C. jejuni (20)	58	62	60	*C. jejuni* HS 7–9, 12, 19, 21, 27, 29, 32, 33, 36, 38, 41, 42, 43, 53, 55, 57, 58, 60

[a] Reference and field isolates.
[b] —, no melting peak.
[c] Heat-stable (HS) Penner serotype designations apply only to *C. jejuni* and *C. coli*.

tion to the genus level (selective culture) and by PCR-ELISA of the extracted DNA (13). As can been seen from Table 3, identifications by the PCR-biprobe assay and the PCR-ELISA were the same. There were five samples where culture was negative but both the PCR-biprobe and PCR-ELISA procedures detected a *Campylobacter* isolate (one *C. hyointestinalis* and four *C. upsaliensis* isolates). Extracts from clinical samples containing no enteropathogens or other enteropathogens such as *Salmonella* or *Cryptosporidium* spp. were negative by all three methods.

DISCUSSION

We describe here a PCR-biprobe assay for detection and identification to the species level of campylobacters from isolates and DNA extracts of fecal samples. The multicopy 16S rRNA gene, which comprises conserved regions interspersed with variable regions, was used as the target for this DNA sequence-based species differentiation. Conserved regions of the gene were targeted by *Campylobacter* genus-specific oligonucleotide primers, while variable regions suitable for identification of individual taxa were targeted for biprobe hybridization. Thus, the oligonucleotides C412F–C1228R (first-round PCR) and C690F (asymmetric second-round PCR) were used as primers to generate the genus-specific amplicon, and this was subsequently identified by its combined melting-peak profiles with the three biprobes. The assay showed no cross-reaction with other *Campylobacter* species or with species of *Helicobacter* or *Arcobacter*. Under test conditions it correctly identified 110 field isolates according to previous phenotypic identification to the species level. It was robust enough to identify *Campylobacter* species directly in DNA extracted from fecal specimens.

Six taxa were differentiated to the species level, namely, *C. lari, C. upsaliensis, C. helveticus, C. hyointestinalis, C. fetus*, and *C. lanienae*. *C. jejuni* and *C. coli* were identified as a single taxon. This was a limitation but was not unexpected, since previous 16S ribosomal DNA (rDNA) sequencing of a 467-nucleotide region of 52 Penner serotype reference strains showed that all belonged to one of three different cross-species groups, between which sequences varied at just three nucleo-

TABLE 3. Comparison of *Campylobacter* sp. identification from fecal DNA extracts by three methods: phenotypic (genus level), PCR-ELISA, and PCR-biprobe assay

No. of samples	Identification by:		PCR-biprobe result	
	Culture	PCR-ELISA	A/B/C profile (°C)[a]	Identification
15	*Campylobacter* sp.	*C. jejuni*/*C. coli*	64:62:64	*C. jejuni*/*C. coli*
2	*Campylobacter* sp.	*C. jejuni*/*C. coli*	58:62:60	*C. jejuni*/*C. coli*
1	*Campylobacter* sp.	*C. hyointestinalis*	—:64:—	*C. hyointestinalis*
1	Negative	*C. hyointestinalis*	—:64:—	*C. hyointestinalis*
1	*Campylobacter* sp.	*C. upsaliensis*	60:51:56	*C. upsaliensis*
4	Negative	*C. upsaliensis*	60:51:56	*C. upsaliensis*
9	Negative	Negative	Negative profile	Negative
1	*Salmonella*	Negative	Negative profile	Negative
1	*Shigella*	Negative	Negative profile	Negative
1	*Escherichia coli*	Negative	Negative profile	Negative
1	*Cryptosporidium*	Negative	Negative profile	Negative
1	*Giardia*	Negative	Negative profile	Negative

[a] —, no melting peak.

tide positions (20). This is a particular feature of the 16S rDNA of these two species and has not been found for any other pair of *Campylobacter* species. Comparison of the 16S sequences of *C. jejuni* and *C. coli* with that of biprobe A indicated that a perfect match of target to probe produced a melting peak at 64°C, while a melting peak at 58°C represented a single-base change from G to A in the target sequence. Similarly, for biprobe C, melting peaks at 64 and 60°C represented a single-base change from A to T in the target sequence. The distribution of the biprobe melting-peak profiles showed complete congruence with the sequence data and confirmed that there are no clear-cut species-specific differences between the 16S rRNA gene sequences of *C. jejuni* and *C. coli*. The shoulder effect was seen with the melting peaks of *C. hyointestinalis* and *C. fetus* analyzed with biprobes B and C, respectively. This is possibly a result of hybridization of the probe to the 16S rDNA target being destabilized in the lower temperature range, by DNA sequences remote from the probe binding site that form secondary structures. Indeed, this shoulder phenomenon has also been observed by other workers (7).

The assay was validated for use with clinical (fecal) samples. Here, PCR-biprobe analysis of 38 fecal DNA extracts was more sensitive than phenotypic identification of isolates to the genus level, in that the former was 100% concordant with identification by PCR-ELISA (13). All the culture-positive and PCR-ELISA-positive samples were also positive by the PCR-biprobe assay (17 *C. jejuni*/*C. coli*, 1 *C. hyointestinalis*, and 1 *C. upsaliensis* isolate). All the culture-negative, PCR-ELISA-negative fecal samples or those containing other enteropathogens were negative by the PCR-biprobe assay. In five diarrheic samples negative by culture, both the PCR-biprobe assay and the PCR-ELISA detected 1 *C. hyointestinalis* and 4 *C. upsaliensis* isolates. These results are explained by the fact that the culture conditions typically used for isolation of campylobacters in human gastroenteritis (*C. jejuni, C. coli*, and *C. lari*) are inhibitory for other campylobacters. Hence the "rare" species detected here by the PCR-biprobe assay (*C. hyointestinalis* and *C. upsaliensis*) would not have been isolated by routine laboratory culture.

Previous studies that have used PCR and/or hybridization for identification of *Campylobacter* to the species level based on 16S rDNA (8, 13, 14, 16, 18, 27, 31), 23S rDNA (5, 6), flagellin genes (22, 32, 33), or genes encoding a protein involved in siderophore transport (9), hippuricase (15), or aspartokinase (15) can distinguish only between *C. jejuni* and *C. coli*. The gene encoding the GTP-binding protein (29, 30) and the *glyA* gene, which encodes serine hydroxymethyltransferase (1), can identify four *Campylobacter* species, but neither has yet been applied to clinical samples. We have previously described a PCR-ELISA with first- and second-round pangenus PCRs that eliminated the need for multiple single-species reactions and then achieved identification by probe hybridization (20). The present work applies a PCR multiple biprobe assay directly to identification of *Campylobacter* spp.. Direct PCR amplification of fecal DNA extracts removes the need for isolation and culture of campylobacters, as demonstrated in earlier PCR applications (12, 22, 32). The biprobe assay can be completed within 1 h of first-round conventional PCR, and data analysis is quick and accurate. Further development of assay-specific software would allow for automatic peak assignment and comparison. Compared to PCR-ELISA, the risk of contamination is reduced by performing the second-round PCR and product identification in a closed-tube system. The assay is flexible and can be tailored by incorporating additional biprobes to identify further species of interest. Our study employed an Idaho Technology LightCycler with the capacity for two-channel detection (SYBR Green and Cy5). Second-generation real-time PCR platforms can perform three- to four-channel detection, which may allow monitoring of all three biprobes described in this report in a single reaction. All such systems have the potential for automated handling and a capacity for higher throughput. PCR-biprobe assays can play a role both in the diagnostic laboratory and in molecular ecology studies of the prevalence and significance of campylobacters in human disease, especially with regard to those species which are not amenable to routine culture and whose role in human disease is inadequately understood.

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