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Colony Multiplex PCR Assay for Identification and Differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*

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A multiplex PCR assay was used to simultaneously detect genes from the five major clinically relevant *Campylobacter* spp. Those genes selected were *hipO* and 23S rRNA from *Campylobacter jejuni*; *glyA* from each of *C. coli*, *C. lari*, and *C. upsaliensis*; and *sapB2* from *C. fetus* subsp. *fetus*. The assay was evaluated with 137 clinical and environmental isolates and was found to be rapid and easy to perform and had a high sensitivity and specificity for characterizing isolates, even in mixed cultures.

Pathogens belonging to the genus *Campylobacter* have fastidious growth requirements, making conventional detection and identification procedures problematic. As a consequence, rapid and reliable detection procedures are required. Methods based on DNA probe technology have been developed for these organisms; however, they are generally of low sensitivity in food products (9, 10, 15, 18). A number of genetically based detection and typing methods have been developed for detecting *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* in clinical, environmental, and food samples. Such assays have used 23S rRNA PCR-restriction fragment length polymorphism (8, 20), sequence analysis of the GTPase gene (22), multiplex PCR for *C. jejuni* and *C. coli* (7), DNA-based PCR (*ceuE*) detection (11), variable region analysis of 16S rRNA (17), dot blot hybridization using a digoxigenin-labeled *C. fetus*-specific oligonucleotide probe (3), and DNA hybridization. A combination of PCR and hybridization methods has also been used (4, 5).

A colony multiplex PCR was developed and optimized to simultaneous identify the 23S rRNA from *Campylobacter* spp.; the *hipO* gene (hippuricase) from *C. jejuni* subsp. *jejuni*; the *glyA* gene (serine hydroxymethyltransferase) from *C. coli*, *C. lari*, and *C. upsaliensis*; and the *sapB2* gene (surface layer protein) from *C. fetus* subsp. *fetus*. The multiplex PCR protocol was capable of detecting the type strains and clinical isolates from all five species with a high degree of specificity.

A total of 137 strains of various species of enterobacteria were evaluated, and of these 124 were campylobacters: 70 from *C. jejuni* subsp. *jejuni*; 21 from *C. coli*; 7 from *C. lari*; 6 each from *C. upsaliensis*, *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, and *C. hyointestinalis*; and 1 each from *C. sputorum* biovar bubulus and *C. sputorum* biovar fecalis. In addition, three *Arcobacter butzleri*, three *A. butzleri*-like, three *Helicobacter pylori*, two *Escherichia coli*, and two *Aeromonas hydrophila* isolates were also examined. All isolates were obtained from the culture collection of the National Laboratory for Enteric Pathogens, and these included both clinical isolates and well-established laboratory isolates. The *Campylobacter* isolates were grown on Mueller-Hinton agar (Oxoid, Nepean, Ontario, Canada) supplemented with 10% sheep blood and incubated at 37°C in a microaerobic atmosphere containing 5% O_2, 10% CO_2, and 85% N_2.

DNA template preparation. DNA was prepared by the whole-cell procedure. Each PCR template was prepared by using approximately half a loopful of culture transferred to 1 ml of brain heart infusion broth (Oxoid). The optical density was adjusted to give a reading of 0.3 at 600 nm. The optimized whole-cell DNA preparations from all *Campylobacter* species were further diluted 1:500 in distilled water and were heated at 100°C for 10 min in a 0.5-ml Eppendorf tube. Templates were used immediately for PCRs or were kept at 4°C for up to 1 month.

Primers and PCR sensitivity. Oligonucleotides, ranging from 18- to 24-mers, were selected from the published DNA sequences of the various *Campylobacter* species (Table 1) using Oligo software (version 3.4). Synthesis of oligonucleotides was carried out at the DNA Core Facility in the National Microbiology Laboratory, Winnipeg, Canada. The six pairs of primers were designed to identify the genes *hipO* from *C. jejuni*; *glyA* from *C. coli*, *C. lari*, and *C. upsaliensis*; *sapB2* from *C. fetus* subsp. *fetus*; and the internal control 23S rRNA. The primer sequences used in the multiplex PCR are outlined in Table 1. The colony PCR sensitivity was determined by both PCR and plate count methods; in brief, 10-fold serial dilutions of up to 10^{-14} were made in triplicate in brain heart infusion broth from 1- ml cultures with starting optical density readings of 0.3 at 600 nm. From these diluted samples, 100-μl aliquots from *C. jejuni*, *C. coli*, *C. lari*, *C. fetus* subsp. *fetus*, *and C. upsaliensis* isolates were plated onto Mueller-Hinton agar. Duplicate samples of each were used to perform standard colony counts and to evaluate the sensitivity of the PCR assay using agarose gel electrophoresis relative to actual plate counts within the range

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TABLE 1. Primer sequences used in the multiplex PCR assay and the expected sizes of the products

Primer	Size (in bp)	Sequence (5′–3′)	GenBank accession no.	Target gene	Gene location (bp)
CJF	323	ACTTCTTTATTGCTTGCTGC	Z36940	*C. jejuni hipO*	1662–1681
CJR		GCCACAACAAGTAAAGAAGC			1984–1965
CCF	126	GTAAAACCAAAGCTTATCGTG	AF136494	*C. coli glyA*	337–357
CCR		TCCAGCAATGTGTGCAATG			462–444
CLF	251	TAGAGAGATAGCAAAAGAGA	AF136495	*C. lari glyA*	318–337
CLR		TACACATAATAATCCCACCC			568–549
CUF	204	AATTGAAACTCTTGCTATCC	AF136496	*C. upsaliensis glyA*	63–82
CUR		TCATACATTTTACCCGAGCT			266–247
CFF	435	GCAAATATAAATGTAAGCGGAGAG	AF048699	*C. fetus sapB2*	2509–2532
CFR		TGCAGCGGCCCCACCTAT			2943–2926
23SF	650	TATACCGGTAAGGAGTGCTGGAG	Z29326	*C. jejuni* 23S rRNA	3807–3829
23SR		ATCAATTAACCTTCGAGCACCG			4456–4435

of 10^2 to 10^{13} CFU/ml. It appears that a range of 10^8 to 10^{13} CFU/ml was most effective for all the *Campylobacter* colony multiplex PCR assays.

Multiplex PCR conditions. Each multiplex PCR tube contained 200 μM deoxynucleoside triphosphate; 2.5 μl of 10× reaction buffer (500 mM Tris-HCl [pH 8.3], 100 mM KCl, and 50 mM $[NH_4]_2SO_4$); 20 mM $MgCl_2$; 0.5 μM *C. jejuni* and *C. lari* primers; 1 μM *C. coli* and *C. fetus* primers, 2 μM *C. upsaliensis* primers; 0.2 μM 23S rRNA primer (Table 1); 1.25 U of FastStart *Taq* DNA polymerase (Roche Diagnostics, GmbH, Mannheim, Germany), and 2.5 μl of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 μl. DNA amplification was carried out in a Perkin-Elmer thermocycler using an initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), ending with a final extension at 72°C for 7 min. The six primer sets were evaluated individually for primer specificity using the reference strains *C. jejuni* NCTC 11168, *C. coli* NCTC 11353, *C. lari* NCTC 11352, *C. upsaliensis* ATCC 43954, *C. fetus* subsp. *fetus* ATCC 27374, *C. fetus* subsp. *venerealis* ATCC 19438, *C. hyointestinalis* ATCC 35217, *C. sputorum* biovar fecalis ATCC 33711, *A. butzleri* ATCC 49616, and *A. butzleri*-like CDCD2887. Only the corresponding strains showed the expected PCR amplification products. Reproducibility of the multiplex PCR assay was evaluated using 137 clinical and environmental samples.

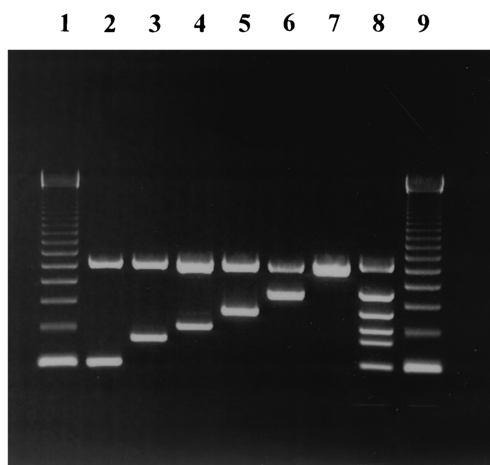

FIG. 1. Amplification fragments of multiplex PCR detection and identification. Lanes 1 and 9: 123-bp ladder (Bethesda Research Laboratories Inc., Gaithersburg, Md.); lane 2: *C. coli* NCTC 11353, 126-bp fragment; lane 3: *C. upsaliensis* ATCC 43954, 204-bp fragment; lane 4: *C. lari* NCTC 11352, 251-bp fragment; lane 5: *C. jejuni* NCTC 11168, 323-bp fragment; lane 6: *C. fetus* subsp. *fetus* ATCC 27374, 435-bp fragment; lane 7: *C. sputorum* biovar fecalis ATCC 33711, 650-bp fragment of 23S rRNA (which occurred in all *Campylobacter* spp., *Arcobacter* and *Helicobacter* isolates tested); and lane 8: PCR-positive control with DNA mixture.

Figure 1 illustrates the PCR-amplified products with the *Campylobacter* reference strains as templates following 1.5% agarose gel electrophoresis. In the assay, six bands were detected from a mixture of DNA containing each of the five *Campylobacter* spp. (Fig. 1, lane 8). The amplicons from the control strains were subjected to further confirmation and characterization by digestion using restriction endonucleases with cleavage sites within the amplicon. The restriction enzymes used and the predicted product sizes are shown in Table 2. Enzyme fragments with the anticipated sizes were obtained in each case (data not shown).

All 124 *Campylobacter* samples were identified by using biochemical assays and 16S rRNA-PCR (14). Complete agreement was obtained with the species-specific primers used in the present assay for all isolates examined. The amplicon for the *Campylobacter* 23S rRNA primers was present in all tested *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates but failed to amplify *E. coli* and *A. hydrophila* isolates (Table 3). The sensitivity range of the colony multiplex PCR in number of CFU per milliliter was 10^8 to 10^{13} for *C. jejuni*, 10^6 to 10^{13} for *C. coli* and *C. upsaliensis*, 10^7 to 10^{13} for *C. lari*, and 10^2 to 10^{13} for *C. fetus* subsp. *fetus*.

TABLE 2. Predicted sizes of restriction fragments and enzymes used for restriction fragment length polymorphism analysis of amplified products of multiplex PCR

Gene	PCR amplicon size (in bp)	Enzyme	Expected size of restriction fragments (bp)
hipO	323	*BsrD*I	109, 214
C. coli glyA	126	*Alu*I	11, 36, 79
C. lari glyA	251	*Apo*I	79, 172
C. upsaliensis glyA	204	*Dde*I	31, 173
C. fetus subsp. *fetus-sapB2*	435	*Bcl*I	130, 305
C. jejuni 23S rRNA	650	*Hha*I	212, 438

TABLE 3. PCR results by multiplex PCR analysis of *Campylobacter* strains[a]

Strain	No. of strains tested	No. of strains that were PCR positive for:					
		C. jejuni	*C. coli*	*C. lari*	*C. upsaliensis*	*C. fetus* subsp. *fetus*	23S rRNA
C. jejuni	70	70	—	—	—	—	70
C. coli	21	—	21	—	—	—	21
C. lari	7	—	—	7	—	—	7
C. upsaliensis	6	—	—	—	6	—	6
C. fetus subsp. *fetus*	6	—	—	—	—	6	6
C. hyointestinalis	6	—	—	—	—	—	6
C. fetus subsp. *venerealis*	6	—	—	—	—	—	6
C. sputorum biovar bubulus	1	—	—	—	—	—	1
C. sputorum biovar fecalis	1	—	—	—	—	—	1
A. butzleri	3	—	—	—	—	—	3
A. butzleri-like	3	—	—	—	—	—	3
H. pylori	3	—	—	—	—	—	3
E. coli	2	—	—	—	—	—	—
A. hydrophila	2	—	—	—	—	—	—
Total	137	70	21	7	6	6	133

[a] —, tested negative.

Case control studies demonstrate that human campylobacteriosis is a food-borne disease with infection most frequently resulting from handling and consuming contaminated poultry meat (21). Indeed, in one study it was reported that 73.2% of 489 meat samples were contaminated with the pathogen (12). Clinically the most important campylobacters are the members of the thermophilic group that includes *C. jejuni*, *C. coli*, *C. lari*, *and C. upsaliensis*, with *C. jejuni* responsible for the majority of human cases (1). *C. fetus* is also recognized as a human and animal pathogen and has been identified in 12.5% of ox liver samples (12). Accurate identification of these organisms is required in order to decide upon appropriate therapeutic measures, to understand the pathology of disease, and to provide clinical and epidemiological data for disease control. A number of protocols (3–8, 11, 17, 20, 22) have been described in the literature for use in the differentiation of the closely related thermophilic *C. jejuni*, *C. coli*, *C. lari*, *and C. upsaliensis* species as well as of *C. fetus*. Most of these methods are based on DNA probe technology or rRNA PCR-restriction fragment length polymorphism. Due to a requirement for restriction enzymes or hybridization steps with species-specific probes, both are relatively complex to perform. An assay combining PCR and DNA hybridization was developed for the rapid detection of *C. fetus* (5), but it is not capable of differentiating *C. fetus* subsp. *fetus* from *C. fetus* subsp. *venerealis*. In a recent study multiplex PCR was used for simultaneously differentiating *C. jejuni*, *C. coli*, and *C. lari* (6). This assay was based on the sequence information of the gene encoding the oxidoreductase subunit of *C. jejuni* (23; GenBank accession no. AL139075), the aspartokinase gene for *C. coli* (13), and 16S rRNA for *C. lari* (16).

Detection by PCR of the *hipO* gene, shown to be highly conserved in *C. jejuni*, provided an effective identification marker for *C. jejuni* (19, 20). In addition, PCR hybridization confirmed that the *Campylobacter glyA* gene can be used as the target to identify and differentiate *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* at the species level (2). Furthermore, the *sapB2* gene of *C. fetus* subsp. *fetus* was recognized as a suitable target for identifying *C. fetus* (4).

In this study, a colony multiplex PCR-based diagnostic protocol was developed to simultaneously detect five genes specific to each of the five pathogenic *Campylobacter* species, while the 23S rRNA probe was included to serve as an internal validation control to monitor PCR conditions and reagents. The 23S rRNA was found in all of the *Campylobacter*, *Arcobacter*, and *H. pylori* species investigated.

The present colony multiplex PCR assay proved to be accurate and simple to perform and could be completed within 3 h. It had the added advantage of detecting the *hipO* gene in *C. jejuni* strains that were hippuricase negative by phenotypic methods and therefore difficult to differentiate from *C. coli* (5, 20). In addition to clinical use, the method has potential as a diagnostic kit for detecting thermophilic campylobacters in complex samples, such as foods in which low pathogen numbers ($<10^3$ CFU/ml) are frequently present. The present PCR assay offers an effective alternative to traditional biochemical typing methods for the identification and differentiation of *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus* isolated from humans and poultry.

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