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Identification of *Campylobacter fetus* by PCR-DNA Probe Method

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A PCR method for rapid identification of *Campylobacter fetus* subsp. *fetus* was evaluated. A fragment of the gene coding for 16S rRNA was amplified from crude cell lysates of 18 *C. fetus* strains and 30 strains representing other *Campylobacter* species and subspecies. The amplicons were probed by dot blot hybridization with a digoxigenin-labeled *C. fetus*-specific oligonucleotide probe. The probe reacted only with *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* and may be useful for rapid identification in clinical laboratories.

Until recently, *Campylobacter fetus* subsp. *fetus* was primarily regarded as an animal pathogen that caused bovine and ovine abortion and sterility. However, since 1980, *C. fetus* has been implicated in four reported outbreaks of human disease in North America, three of which were suspected to be caused by contaminated foods (raw milk, raw calf liver, and cottage cheese) (1, 5, 9). The *C. fetus* outbreak in which contaminated cottage cheese was epidemiologically implicated occurred in a nursing home in Ohio in 1992 and 1993 (5a) and caused two deaths. Clearly, *C. fetus* subsp. *fetus* has the potential to cause sporadic and epidemic food-borne disease. Therefore, there is a need to rapidly and reliably identify this organism in clinical specimens and foods.

Because of their relatively sparse metabolic activity, few biochemical tests are useful for differentiating *Campylobacter* spp. (8). *C. fetus* is differentiated from *C. hyointestinalis*, the species most closely related by DNA-DNA hybridization (10), primarily by the lack of production of H_2S in triple sugar iron agar (2). Biochemically atypical strains further complicate identification.

Wesley et al. (11) designed a 29-mer DNA probe (IVWCF2) based on the gene coding for 16S rRNA (16S rDNA) that discriminated between *C. fetus* and *C. hyointestinalis*. We evaluated the specificity of their 29-mer probe for *C. fetus* by dot blot hybridizations with a PCR-amplified 600-bp fragment of 16S rDNA as the template.

The *Campylobacter* strains used in the investigation are listed in Table 1. *C. jejuni*, *C. coli*, and *C. lari* (thermophilic campylobacters) were incubated for 3 days at 42°C on heart infusion agar with 5% rabbit blood (BBL Microbiology Systems, Cockeysville, Md.) in a microaerobic atmosphere of approximately 5% oxygen, 7.5% carbon dioxide, 7.5% hydrogen, and 85% nitrogen. *C. concisus*, *C. rectus*, and *C. sputorum* subsp. *sputorum* were incubated for 5 days at 36°C on heart infusion agar with 5% rabbit blood under anaerobic conditions (GasPak Plus; BBL Microbiology Systems). The other *Campylobacter* species were grown under the same conditions as described for the thermophilic campylobacters but at 36 instead of 42°C. Crude cell lysates, prepared as described below, were the source of target DNA for PCR amplification of a 600-bp fragment of 16S rDNA. The cells from one agar plate (15 by 100 mm) were harvested in 1 ml of sterile water and were lysed by boiling for 20 min. The suspended cell debris was separated by centrifugation at 10,000 × *g* for 5 min, and the supernatant was used as the source of template DNA for PCR amplification.

Two oligonucleotide primers, CampC5 and P3MOD201C, were used to amplify the 600-bp 16S rDNA fragment. These primers were synthesized by standard phosphoramidite chemistry, using an Applied Biosystems 392/4 or 380B DNA synthesizer at the Biotechnology Core Facility, Centers for Disease Control and Prevention. Primer CampC5 (5′-GGC TGA TCT ACG ATT ACT AGC GAT-3′) was designed from *Campylobacter* 16S rDNA sequences available from GenBank (Los Alamos, N.Mex.), and P3MOD201C (5′-GCG CGC ATT AGA TAC CCT AGT AGT CC-3′) was modified from the P3MOD primer of Wilson et al. (12). DNA probe IVWCF2 (5′-CTC AAC TTT CTA GCA AGC TAG CAC TCT CT-3′) of Wesley et al. (11) was synthesized as described for the primers and end labeled according to the method of Maniatis et al. (6) with [γ-^{32}P]ATP and polynucleotide kinase. The probe was also labeled with digoxigenin by using the Digoxigenin Labelling System (Genius System; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to configure the assay to a nonradioisotopic format.

PCR amplification was performed with 100-μl reaction volumes which contained 10 μl of the target DNA and 90 μl of the amplification cocktail. The amplification cocktail was composed of 15 pmol of each primer (CampC5 and P3MOD201C), 200 μM each deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP), 1× GeneAmp PCR buffer (Perkin-Elmer Cetus, Norwalk, Conn.), and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). The amplification was done in a PC-100 Thermal Controller (MJ Research, Watertown, Mass.) under the following temperature cycling conditions: cycles 1 to 5 of 2 min at 96°C, 30 s at 37°C, and 30 s at 72°C and cycles 6 to 30 of 30 s at 96°C, 30 s at 52°C, and 1 min at 72°C. PCR amplification was completed with a 10-min final extension at 60°C. The amplified product was detected by agarose gel electrophoresis (2.5% agarose in 1× Tris-borate–EDTA electrophoresis buffer at 4 V/cm) in the presence of ethidium bromide.

Dot blot hybridization was performed essentially as described by Kafatos et al. (4). After prehybridization, 10^7 cpm of the end-labeled ^{32}P-IVWCF2 probe was added and the membrane was incubated overnight at 37°C in a shaking water bath. The membrane was rinsed in 6× SSC (1× SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate) at 4°C and

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TABLE 1. Results of dot blot hybridization of *Campylobacter* reference strains and 18 *C. fetus* subsp. *fetus* isolates with the 29-mer *C. fetus*-specific 16S rRNA-based DNA probe

Campylobacter sp. (no. of strains tested)[a]	Reactivity of ^{32}P-labeled DNA probe after hybridization to target DNA in 6× SSC at 37°C and posthybridization wash in 3 M TMAC[b] at:				Reactivity of digoxigenin-labeled 29-mer probe under optimized conditions[c]
	45°C	60°C	66°C	70°C	
C. jejuni (2)	+	+	−	−	−
C. jejuni subsp. *doylei* (2)	+	−	−	−	−
C. coli (2)	+	+	−	−	−
C. lari (2)	+	−	−	−	−
C. fetus subsp. *fetus* (18)[d]	+	+	+	+	+
C. fetus subsp. *venerealis* (2)	+	+	+	+	+
C. hyointestinalis (3)	+	−	−	−	−
C. sputorum subsp. "*fecalis*" (2)	+	−	−	−	−
C. sputorum subsp. *sputorum* (2)	+	−	−	−	−
C. sputorum subsp. *bubulus* (2)	+	−	−	−	−
C. upsaliensis (2)	+	+	+	−	−
C. mucosalis (2)	+	+	+	−	−
C. concisus (2)	+	−	−	−	−
C. curvus (1)[e]	+	−	−	−	−
C. rectus (1)[e]	+	−	−	−	−
C. helveticus (1)	+	−	−	−	−
Campylobacter-like organisms (2)	+	−	−	−	−

[a] The type strain was always included; additional strains represent clinical isolates.
[b] TMAC, tetramethylammonium chloride.
[c] Hybridization was done in 1% (wt/vol) Genius blocking reagent in 0.1 M maleic acid (pH 7.5)–0.15 M sodium chloride at 67°C; posthybridization washing was done in 1× SSC–0.1% SDS at 67°C.
[d] Includes 15 clinical isolates from the Ohio (1992–1993) outbreak.
[e] *C. curvus* and *C. rectus* were formerly called *Wolinella curva* and *Wolinella recta*, respectively.

washed twice (20 min each) in 3 M tetramethylammonium chloride sequentially at 37, 45, 60, 61, 62, 64, 65, 66, 67, 70, 73, and 75°C in a shaking water bath, as described by Wood et al. (13). The membrane was then autoradiographed with XAR-Omat Kodak film and an intensifying screen at −70°C. Nonradioactive dot blot hybridization was performed with digoxigenin-labeled IVWCF2 by the Genius System protocol (Boehringer Mannheim Biochemicals). After hybridization, the membrane was rinsed twice for 5 min with 2× SSC–0.1% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature and washed twice for 15 min with 1× SSC–0.1% (wt/vol) SDS at 60, 62, 64, 65, 66, 67, and 68°C. The hybridized DNA was detected colorimetrically by the Genius System protocol.

The primers amplified a 600-bp fragment of 16S rDNA from 47 of 48 *Campylobacter* strains but did not amplify DNA from *Escherichia coli*, *Salmonella* species, and *Listeria monocytogenes*. One *Campylobacter* isolate, *C. coli* D145, did not yield an amplicon until we used DNA isolated from this strain by the conventional detergent lysis–phenol-chloroform extraction procedure (3). This is problematic because the major advantages of PCR amplification of the target DNA (few cells required and rapid sample preparation for PCR) are lost if one has to use purified DNA for the PCR. Our attempts to eliminate substances that may have interfered with the PCR by Chelex-100 (Bio-Rad Laboratories) treatment were unsuccessful. Other investigators have reported similar problems with failure to amplify target DNA from some strains (7). Additional work is needed to determine why some strains of a species pose problems in PCR amplification of crude lysates and to devise appropriate procedures that are rapid and effective for all strains of a species.

The ^{32}P-labeled IVWCF2 probe hybridized only with *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* when the posthybridization washes were done at 70°C in 3 M tetramethylammonium chloride; the same specificity was observed with the digoxigenin-labeled IVWCF2 probe when posthybridization washing was done at 67°C in 1× SSC–0.1% (wt/vol) SDS (Table 1). The probe could not discriminate between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis.*

C. fetus is phylogenetically most closely related to *C. hyointestinalis* on the basis of their complete 16S rRNA sequences (10). Using 16S rRNA sequence information, Wesley et al. (11) designed two *C. fetus*-specific probes (17- and 29-mer oligodeoxynucleotides) and demonstrated their specificity in colony blot, slot blot, and Southern blot hybridizations. The sequences of their probes were chosen from a hypervariable region of 16S rRNA, position 1017 to position 1044, of *C. hyointestinalis* and *C. fetus*. The probes were reported as being specific to *C. fetus* if they did not react with *C. hyointestinalis* DNA. However, homologies in the region of 16S rRNA from which the probes were derived are more relevant to predicting probe cross-reactivity than phylogenetic relationships derived from complete 16S rRNA sequences. When the nucleotide sequence of the probe was compared with those of different *Campylobacter* spp. (obtained from GenBank) in the 29-nucleotide region spanned by the 29-mer probe (IVWCF2), we found that *C. coli* differed from the probe sequence by 4 nucleotides, *C. jejuni* differed by 5 nucleotides, and *C. hyointestinalis* differed by 8 nucleotides. *C. mucosalis* and *C. upsaliensis* differed from the probe by only 1 nucleotide for a 24-mer stretch. This is consistent with our observation that higher-stringency washes were required to eliminate the cross-reactivity of the probe with *C. jejuni*, *C. coli*, *C. mucosalis*, and *C. upsaliensis* than to eliminate the cross-reactivity with *C. hyointestinalis* (Table 1). Also, the perfect sequence homology between the two subspecies of *C. fetus* for the region spanned by the probe explains the inability of the probe to discriminate between the two subspecies. However, in a clinical setting there has not been any immediate need to distinguish *C. fetus* subsp. *fetus* from *C. fetus* subsp. *venerealis* since *C. fetus* subsp. *venerealis* has rarely, if ever, been implicated in human illness (8).

The PCR-based DNA probe method described in this report

has several attractive features for rapid and specific identification of *C. fetus*. Amplification of the target DNA by PCR allows one to start with a sweep of cells (approximately 10^8 to 10^9 cells) from one agar plate and to complete the sample preparation for PCR within a few minutes. In contrast, Wesley et al. used genomic DNA purified by a complex and time-consuming procedure as the target for the probe. We showed that the specificity of the probe was preserved when digoxigenin was used as the label of the probe. The entire assay could be completed within 24 h. This nonradioactive format offers both speed and accessibility for clinical as well as reference and research laboratories. Further simplification of this method should be possible by configuring the hybridization step of the method to a microtitration plate format.

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