Oligodeoxynucleotide Probes for Campylobacter fetus and Campylobacter hyointestinalis Based on 16S rRNA Sequences

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Received 25 January 1991/Accepted 14 June 1991

Deoxyoligonucleotide probes were constructed for the identification of Campylobacter fetus and Campylobacter hyointestinalis based on 16S rRNA sequence data. Probes were targeted to hypervariable regions of 16S rRNA. Specificity of oligonucleotide probes was tested in a colony blot assay with type strains of 15 Campylobacter and Arcobacter species as well as in a slot blot format using genomic DNA extracted from field strains of C. fetus and C. hyointestinalis. Two oligonucleotides were constructed for C. fetus that hybridized with equal specificity with each of 57 biochemically confirmed isolates of C. fetus but not with any other Campylobacter species. The C. hyointestinalis probe reacted with 47 of 48 biochemically confirmed field isolates of C. hyointestinalis. In Southern blot hybridization of BgII digests of genomic DNA, the respective probes reacted within three restriction fragments of either C. hyointestinalis (7.2, 8.2, and 10.1 kb) or C. fetus (7.0, 7.7, and 9.0 kb). This suggests multiple copies of genes encoding 16S rRNA.

The genus Campylobacter is composed of curved to spiral gram-negative bacteria with a characteristic darting motility (45). There are few metabolic characteristics useful in species differentiation. The genus encompasses bacteria of clinical and veterinary interest, including Campylobacter fetus and Campylobacter hyointestinalis. In 1913, McFay-dean and Stockman recognized a "vibrio" (19), designated Vibrio fetus (40), as an etiologic agent of bovine and ovine infertility and abortion. The pathogen was isolated from the placenta of aborting sheep, stomach contents of aborted fetuses, and blood and intestinal contents of infected ewes and cattle (39). The abortifacient species, subsequently designated C. fetus (38), was subdivided into two subspecies on the basis of differences in clinical presentation and tolerance to 1% glycine (46). C. fetus subsp. fetus is transmitted orally, induces abortion in cattle and sheep, and rarely produces septicemia in humans (29). C. fetus subsp. venerealis is exclusively a venereal pathogen of cattle (39) and disrupts placenta, thus producing infertility. C. fetus subsp. fetus grows at 42°C and grows in the presence of 1% glycine, in contrast to the C. fetus subsp. venerealis, which does not replicate at 42°C and does not tolerate glycine (39). C. fetus subsp. fetus shares 16 to 30% DNA homology with C. hyointestinalis, to which it is most closely related (35). C. hyointestinalis produces H2S in triple sugar iron media, in contrast to C. fetus (6, 9, 21). The two species may also be distinguished on the basis of their fatty acid profiles (4). C. hyointestinalis has been described in association with enteritis in swine (6, 9, 16), cattle (3), and humans (4, 5, 20) but has also been reported in healthy cattle (11).

Since members of the genus Campylobacter have few phenotypic features for species differentiation (21), nucleic acid probes offer an alternative to biochemical tests. Nucleic acid probes have been developed for C. jejuni (1a, 15, 23, 24, 30-32), C. coli (24), C. mucosalis (8), C. hyointestinalis (10), and the intracellular Campylobacter-like organism responsible for porcine proliferative enteritis (7).

Although the nucleotide sequences of rRNAs have been conserved through evolution, mutations have occurred as species have diverged (18, 25, 48). Many of these changes exist in hypervariable regions. Oligonucleotide probes complementary to these regions have been synthesized that differentiate very closely related species. Because the phylogeny and diversity of the genus Campylobacter have been examined by 5S and 16S rRNA sequence analyses (18, 27, 42) and 23S rRNA hybridization studies (45), the construction of oligonucleotide probes derived from sequence data is a logical extension of this information. Deoxyoligonucleotide probes specific for 16S rRNA have been reported for the genus Campylobacter (22, 31).

The goal of this study was to sequence the 16S rRNA of C. fetus and C. hyointestinalis and to construct nucleic acid probes to distinguish these species, which may be misidentified by use of conventional biochemical and serological methods.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacteria were grown on brain heart infusion agar with 10% defibrinated bovine blood and incubated microaerobically (10% CO2, 85% N2, 5% O2; 72 h at 37°C).

Bacterial strains were obtained from the American Type Culture Collection (ATCC) and Virginia Polytechnic Institute (VPI) collections. ATCC Campylobacter strains evaluated in this study include C. coli ATCC 35359, C. concisus ATCC 33237, C. fetus subsp. fetus ATCC 27374, C. fetus subsp. venerealis ATCC 19438, C. hyointestinalis ATCC 35217, C. lari ATCC 35221, C. jejuni ATCC 33560, C. mucosalis ATCC 43264, C. sputorum biovar bubulus ATCC 33562, C. sputorum biovar sputorum ATCC 35980, C. sputo-rum biovar faecalis ATCC 33709, Helicobacter cinaedi ATCC 35683, Helicobacter fennelliae ATCC 35684, Arcobacter cryaerophilus ATCC 43158, and Arcobacter nitrofi-gilis ATCC 33309 (45).

Field isolates of C. fetus (n = 57) and C. hyointestinalis (n = 48), which were part of the National Animal Disease...
Center (NADC) collection and were submitted by clinical laboratories, were examined in this study. Isolates were identified as *C. fetus* on the basis of morphology, motility, oxidase and catalase activity, glycite tolerance, and failure to generate H₂S. Isolates of *C. hyointestinalis* were confirmed by morphology, motility, oxidase and catalase reactions, H₂S production, tolerance of glycite, and differential growth at 42°C. Nalidixic acid resistance and cephalothin susceptibility were also used in species characterization. Two strains of *C. jejuni* (NADC 18292 and NADC 1990) were included as negative controls.

16S rRNA sequence analysis. RNA was isolated and partially purified by a modification of the procedure of Lane et al. (17), as described previously (27). Complete 16S rDNA sequences were determined for *C. hyointestinalis* (ATCC 35217 and NADC 2006), *C. fetus subsp. fetus* (ATCC 27374 and VPI H641), and *C. fetus subsp. venerealis* (ATCC 19438), as described (27). These sequences were compared with published partial sequences of *C. concisus*, *C. fetus subsp. fetus*, *C. jejuni*, *C. coli*, *C. lari*, *C. sputorum*, *Helicobacter pylori*, *Helicobacter mustelae*, *Bacteroides gracilis*, *Bacteroides ureolyticus*, *Wolinella recta*, *Wolinella succinogenes*, *Escherichia coli*, *Citrobacter freundii*, and *Proteus vulgaris* and with the unpublished sequence of *Flexispira rappini* (28). A program for data entry, editing, sequence alignment, secondary structure comparison, homology matrix generation, and dendrogram construction for 16S rDNA data was used (27). Nucleic acid sequences which were selected for use as probes were identified by alignment of 16S RNA sequence data, identification of common bases, and selection of regions where mismatches occurred (27).

**Immobilization of nucleic acid on solid supports.** Oligonucleotides were synthesized (Synthecell, Gaithersburg, Md.) and end-labelled with [γ-32P]ATP by the T4 polynucleotide kinase reaction (33).

For colony blot hybridization, a nylon membrane (GeneScreen Plus; Dupont) was gently pressed over bacterial colonies (3 to 4 days old) grown on brain heart infusion agar containing 10% defibrinated bovine blood. After a minimum of 1 h, the membrane was removed denatured (0.5 M NaOH, 1.5 M NaCl), neutralized (1 M Tris, 3 M NaCl, pH 5.5), and UV irradiated to covalently bind the DNA to the membrane filters (2). Prehybridization was carried out for 3 h at 37°C in 6× SSC (0.90 M NaCl plus 0.090 M sodium citrate)-5× Denhardt’s solution (0.5% Ficoll, 0.1% polyvinylpyrrolidone, 0.2% bovine serum albumin)-0.5% SDS-100 µg of sonicated denatured calf thymus DNA per ml (36). Hybridization was carried out at 37°C for 18 h in 6× SSC-0.5% sodium dodecyl sulfate-100 µg of sonicated denatured calf thymus DNA per ml (36). The hybridization solution contained 10⁶ cpm of the appropriate end-labelled 32P-oligonucleotide probe. After incubation, filters were washed once briefly in 2× SSC-0.1% sodium dodecyl sulfate at room temperature, followed by two high-stringency washes (1 h each), as follows. For the *C. fetus* probe (17-mer), stringency washes were performed in 1× SSC at 37°C, whereas the 29-mer specific for *C. fetus* utilized higher-stringency conditions (0.1× SSC, 47°C). For the *C. hyointestinalis* probe, the high-stringency washes were performed in 6× SSC (50°C).

For slot blot hybridization, high-molecular-weight genomic DNA was extracted from bacterial cells as described previously (47). Two micrograms of *Campylobacter* genomic DNA was applied to each well of a GeneScreen nylon membrane (Dupont, NEN, Research Products, Boston, Mass.) in a slot blot apparatus (Bio-Rad, Richmond, Calif.). The DNA was denatured, neutralized, and UV irradiated as described for colony blot hybridization. Prehybridization, hybridization, and high-stringency washes were conducted as described above.

Southern blot hybridization was performed as follows. Genomic DNA was digested with *Bgl*III, and restriction fragments were separated in 0.6% agarose gels (60 V, 18 h), blotted onto GeneScreen nylon membranes (New England Nuclear Corp., Boston, Mass.) by the method of Southern (41), and UV cross-linked as described previously (2). To detect the presence of 16S rRNA gene, nylon filters were prehybridized (3 h) and then hybridized (18 h, 37°C) with 5×
10^6 cpm of the appropriate ^32P-end-labelled oligonucleotide probe, as described above. Membranes were washed once in 2x SSC-0.1% sodium dodecyl sulfate at room temperature. For _C. fetus_ (17-mer), two high-stringency washes (1 h each) were completed in 1x SSC at 37°C, whereas the 29-mer required stringency washes at 47°C in 0.1x SSC. Stringency washes for the _C. hyointestinalis_ oligonucleotide were performed in 4x SSC (45°C).

Dried filters were exposed to Kodak X-Omat film with two intensifying screens (~80°C, 2 to 7 days).

**Nucleotide sequence accession numbers.** A total of approximately 1,400 bases each were sequenced for 16S rRNA of _C. fetus_ subsp. _fetus_, _C. fetus_ subsp. _venerealis_, and _C. hyointestinalis_. Nucleotide sequences have been deposited in GenBank (Los Alamos, N.Mex.) with the following accession numbers: _C. fetus_ subsp. _fetus_ (M65012), _C. fetus_ subsp. _venerealis_ (M65011), and _C. hyointestinalis_ (ATCC 35217, M65010; NADC 2006, M65009).

**RESULTS**

Alignment of nucleic acids indicated that a single base mismatch (position 811) differentiated _C. fetus_ subsp. _fetus_ from _C. fetus_ subsp. _venerealis_. In contrast, a total of 28
fetus-specific oligonucleotides hybridized C. fetus-C. hyointestinalis complex on the basis of these data is shown in the resultant dendrogram (Fig. 2).

A region of eight mismatches (from position 1017 to position 1044) was identified, from which oligonucleotide probes specific for C. fetus and C. hyointestinalis were constructed. Two C. fetus-specific probes, a 17-deoxynucleotide probe (5′-CTC-AAC-TTT-CTA-GCA-AG-3′) and a 29-deoxynucleotide probe (5′-CTC-AAC-TTT-CTA-GCA-AG C-TAG-CAC-TCT-CT-3′), were synthesized from this region. To determine their specificity, the probes were tested in a colony blot hybridization format against ATCC type strains of 15 Campylobacter and Arcobacter species and subspecies (45). The two C. fetus-specific oligonucleotide probes hybridized only with the type strain of C. fetus subsp. fetus (ATCC 27374) and C. fetus subsp. venerealis (ATCC 19438; Fig. 3a). No reaction was seen with any of the other Campylobacter type strains, including the phylogenetically closely related C. hyointestinalis.

Specificity of each of the C. fetus probes was evaluated further in a slot blot format against genomic DNA of field strains. The C. fetus-specific deoxynucleotides hybridized with equal specificity with genomic DNA of the 57 field strains of C. fetus of bovine, human, or ovine origin. The C. fetus-specific oligonucleotides hybridized equally well with isolates of the subspecies fetus (ATCC 27374) and the subspecies venerealis (ATCC 19438). No hybridization occurred with genomic DNA of any other Campylobacter species, including C. hyointestinalis (ATCC 35217).

The C. fetus-specific probes were evaluated in Southern blot hybridization of genomic DNA of 13 field isolates of BglII-restricted C. fetus. Nucleic acid sequences homologous with the oligonucleotide probes specific for 16S rRNA genes were localized within no more than three restriction fragments (7.0, 7.7, and 9.0 kb). Hybridization occurred with two common restriction fragments (7.7 and 9.0 kb), whereas reactivity with a third, smaller restriction fragment (7.0 kb) was occasionally noted (Fig. 4).

A 29-base sequence, which was derived from position 1017 to position 1044, was identified as specific for C. hyointestinalis. The resultant synthetic oligonucleotide (5′-C AC-TAA-TTT-GTA-AAC-AAG-CAC-TAT-CT-3′) was tested in a colony blot against reference strains of 15 Campylobacter and Arcobacter species and subspecies. The C. hyointestinalis-specific probe hybridized only with colony blots of the C. hyointestinalis reference strain and not with reference strains of any other Campylobacter species, including the two subspecies of the closely related C. fetus (ATCC 19438, ATCC 27374; Fig. 3b). Specificity of the probe was also evaluated by using genomic DNA from field isolates of C. hyointestinalis in a slot blot screening assay. The probe hybridized with 47 of 48 field isolates of C. hyointestinalis, which were biochemically confirmed as such. The C. hyointestinalis-specific oligonucleotide probe reacted with the type strain of C. hyointestinalis (ATCC 35217) but not with any other Campylobacter sp., including strains of C. fetus subsp. fetus (ATCC 27374) and subsp. venerealis (ATCC 19438). Isolate NADC 1585-G5, which did not hybridize with the probe, was identified as C. hyointestinalis on the basis of the anatomical site of recovery, morphology, and biochemical profiles, although H2S production was weak.

Southern blot hybridization of C. hyointestinalis strains digested with the endonuclease BglII and probed with the C. hyointestinalis-specific oligonucleotide indicated that sequences encoding 16S rRNA genes were localized within no fewer than three restriction fragments: 7.2, 8.2, and 10.1 kb (Fig. 5).

Figure 6 compares Southern blot hybridization patterns obtained with C. hyointestinalis and C. fetus with their homologous oligonucleotide probes. Species differences in restriction fragment length polymorphism are evident.

**DISCUSSION**

C. fetus, which induces abortion in sheep and cattle (38), and C. hyointestinalis, which was first described in the intestines of swine (9), have been shown to be closely related by DNA hybridization analysis (6, 26, 35). 16S rRNA sequence analysis showed that C. fetus subsp. fetus shares nearly 100% homology with the subspecies venerealis and 98% homology with C. hyointestinalis. These data verify the phylogenetic assignments proposed earlier on the basis of partial 16S rRNA sequence analysis (42). Therefore, the C. fetus-C. hyointestinalis cluster represents a spectrum of adaptation to host environments encompassing closely related bacteria which are recovered from the intestinal tract
(\textit{C. hyointestinalis}), those which colonize the intestine en route to the reproductive system (\textit{C. fetus} subsp. \textit{fetus}), and those which exclusively inhabit the urogenital tract (\textit{C. fetus} subsp. \textit{venerealis}).

Nucleic acid sequencing of the highly conserved 16S rRNA molecules (26, 48) has allowed phylogenetic appraisal of the \textit{Campylobacter} species (18, 27, 42). Selection of nucleic acid sequences which encompass the restriction fragment length polymorphism has resulted in the construction of highly species specific nucleic acid probes (22, 31, 34). In this study, alignment of 16S rRNA nucleic acid sequences of \textit{C. fetus} subsp. \textit{fetus}, \textit{C. fetus} subsp. \textit{venerealis}, and \textit{C. hyointestinalis} indicated a hypervariable region (nucleotide position 1017 to 1046) from which sequences for species-specific oligonucleotide probes were derived. The two deoxyribonucleotides synthesized for \textit{C. fetus} (a 17-mer and a 29-mer) hybridized equally well with \textit{C. fetus} subsp. \textit{fetus} and subsp. \textit{venerealis} because the nucleic acid sequence selected for probe construction did not encompass the single base mismatch which differentiates the two reference subspecies. Whether \textit{C. fetus} is indeed a single species or consists of two subspecies distinguishable by host clinical presentation and resistance to bacitracin, a differential test with questionable reliability, has been reviewed recently (29). The \textit{C. fetus}-specific oligonucleotides did not react with the closely related \textit{C. hyointestinalis} or with type strains of other \textit{Campylobacter} species. The \textit{C. fetus}-specific oligonucleotides hybridized with all of the field strains which were biochemically confirmed as \textit{C. fetus}. The specificity of the \textit{C. fetus} probe was demonstrated when four non-\textit{C. fetus} strains—two bovine isolates of \textit{C. sputorum} biovar bubulus, an ovine strain of \textit{C. jejuni}, and a bovine isolate of \textit{C. hyointestinalis}, which were inadverently included in a screening assay—failed to hybridize.

The \textit{C. hyointestinalis}-specific probe encompassed an 8-base mismatch in the hypervariable region between positions 1017 and 1044. The \textit{C. hyointestinalis}-specific oligonucleotide reacted only with the type strain of \textit{C. hyointestinalis}. No hybridization was detected with the two subspecies of the closely related \textit{C. fetus} nor with any of the additional 14 type strains of \textit{Campylobacter} species evaluated. The \textit{C. hyointestinalis}-specific oligonucleotide hybridized with 47 of 48 field isolates that were biochemically confirmed as \textit{C. hyointestinalis}. The specificity of the \textit{C. hyointestinalis} probe was exemplified when three isolates of \textit{C. fetus} and one strain of \textit{C. jejuni} failed to hybridize. The strains were received by our laboratory tentatively identified as \textit{C. hyointestinalis} and were probing as their identification was being verified simultaneously by conventional techniques. H2S production by \textit{C. hyointestinalis}, despite the contradictory results which may arise (29), is the key biochemical test which distinguishes it from the closely related \textit{C. fetus} subsp. \textit{fetus}. In this study, approximately 7% of the field strains of \textit{C. fetus} subsp. \textit{fetus} and 9% of the strains of \textit{C. hyointestinalis} that were submitted to our laboratory were subsequently found by probe reaction and biochemical reconfirmation to be misidentified.

Multiple copies of 16S rRNA genes have been described for the genus \textit{Campylobacter} (22, 34). In our studies, Southern blot hybridization patterns of \textit{BglII} digests of \textit{C. fetus} and \textit{C. hyointestinalis} with homologous probes emphasized species differences. Following Southern blot hybridization of \textit{BglII} digests of genomic DNA, the \textit{C. fetus}-specific probes consistently hybridized with two restriction fragments (7.7 and 9.0 kb). Occasionally, hybridization with a third fragment was noted (7.0 kb). No correlation could be made between hybridization pattern (number and size of restriction fragments which hybridized with the probe) and host origin or subspecies designation. Southern blot hybridization of \textit{BglII} digests of genomic DNA of \textit{C. hyointestinalis} also indicated that sequences encoding 16S rRNA were localized in three restriction fragments (7.2, 8.2, and 10.1 kb). At least three different hybridization patterns have been detected in 44 field isolates of \textit{C. hyointestinalis} examined to date. The identification of these restriction fragments and their species as a basis for the observed serologic diversity (1).

Nucleic acid hybridizations, including dot blot and Southern blot techniques, have been used in the identification of \textit{Campylobacter} species (24, 26, 31, 35, 43, 44). The application of species-specific deoxyribonucleotide probes with nonradioactive detection techniques (12, 31, 32, 37) should facilitate the identification of \textit{C. fetus} and \textit{C. hyointestinalis} for epidemiological purposes. Furthermore, the observed restriction fragment length polymorphism supports the application of probes based on 16S rRNA sequences in deducing taxonomic affinities (13).

**ACKNOWLEDGMENTS**

The technical support of Sharon K. Franklin and Nancy Benkusky is gratefully appreciated. We thank Annette Bates for typing the manuscript.

**REFERENCES**


