JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1989, p. 2717–2723
0095-1137/89/122717-07$02.00/0

Vol. 27, No. 12

Species-Specific Cloned DNA Probes for the Identification of *Campylobacter hyointestinalis*

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Received 13 February 1989/Accepted 28 August 1989

Conventional microbiological methods for isolating and identifying *Campylobacter* species are laborious, tedious, and subjective. Because of the increasing importance of *Campylobacter* species in human and animal diseases and the recent emergence of many new species and atypical strains, we are developing chromosomal DNA probes for rapid and simple identification of *Campylobacter* species, especially those of veterinary importance. We report the cloning and characterization of chromosomal DNA fragments from *Campylobacter hyointestinalis*, an organism isolated from pigs with proliferative enteritis. To obtain *C. hyointestinalis*-specific probes, chromosomal DNA fragments from *C. hyointestinalis* were cloned into plasmid vector pGEM-3Z. Recombinant plasmids were screened for *C. hyointestinalis*-specific inserts by DNA hybridization, using chromosomal DNA from either *C. hyointestinalis* or *C. fetus* which had been ^{32}P labeled. Recombinants which hybridized to *C. hyointestinalis*, but not *C. fetus*, DNA were ^{32}P labeled and screened further for sensitivity and specificity. Three probes were identified that were species specific and capable of detecting 10^4 *C. hyointestinalis* organisms by bacterial spot blotting in 48 h. We anticipate that these probes will be useful for routine species identification and for epidemiological studies.

Campylobacter species are a group of microaerophilic and nonfermentative bacteria. They have a variety of requirements for atmosphere and temperature for optimal growth, which, with the lack of metabolic markers, makes conventional procedures for isolation and identification of the various species cumbersome and time-consuming (22). In addition, *Campylobacter* species are increasingly recognized as important pathogens of humans and other animals. Various species are implicated in food poisoning (3, 22), gastritis (22), proliferative enteritis (12, 17), and other disorders (22). Because of their pathogenic importance and the recent emergence of many new species and atypical strains, more sophisticated methods for species differentiation and identification are needed. A simple, rapid, specific, and sensitive approach to strain differentiation is DNA-DNA hybridization (4, 6). In fact, because of the lack of reliable phenotypic criteria available for species differentiation of *Campylobacter*, the quantitative DNA-DNA hybridization technique has been invaluable in overcoming the confusion of species status (2, 15, 20, 21, 24, 25). DNA probes are now being developed for the diagnosis and epidemiology of a range of bacterial infections, including those caused by *Legionella pneumophila* (13), *Salmonella typhi* (10, 27, 30), and *Campylobacter jejuni* (16, 23, 29). The isolation of *C. hyointestinalis*-specific DNA probes, as described in this paper, represents a first step toward the development of a DNA probe for routine diagnosis and future epidemiological studies of this pathogen.

Proliferative enteritis is an intestinal disease of pigs and other animals which is characterized by proliferation of intestinal epithelial cells. Within such intestinal cells are curved-shaped bacteria resembling *Campylobacter* species (26). Although a body of evidence suggests that the intracellular organism observed within the intestinal cells in swine proliferative enteritis cases is a *Campylobacter* species, its absolute identity remains in doubt (26). Several species of *Campylobacter*, including *C. hyointestinalis* and *C. mucosalis*, have been isolated from the intestinal mucosa and feces of pigs with proliferative enteritis (11, 12, 17), whereas *C. coli* has been isolated from both normal and diseased pigs (12). *C. jejuni* is rarely found in pigs. Whether any of these species of *Campylobacter* is the etiological agent of the disease has not been determined.

Each porcine *Campylobacter* species, with the exception of *C. mucosalis*, has also been isolated from humans with enteritis (5, 7, 8, 22). We report here the cloning of chromosomal DNA fragments from *C. hyointestinalis* as a first step in developing rapid, unambiguous methods for detecting and identifying this swine *Campylobacter* species. These probes will be useful for detecting the organisms in humans as well as for studies to delineate the etiological agent of swine proliferative enteritis.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the bacterial isolates used and their origins. *C. hyointestinalis* 8384-4 was isolated in Minnesota from a pig with proliferative enteritis. No plasmids could be detected in this strain by conventional plasmid isolation procedures (data not shown). *C. mucosalis* 8384-4 and *C. coli* 8384-4 were isolated from the same diseased pig as *C. hyointestinalis* 8384-4. Type and reference strains of *Campylobacter* spp. were included from various reference collections. Cultures were maintained on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) containing 5% sheep blood at 36°C in a microaerophilic atmosphere as described previously (12). For long-term storage, cultures were quick-frozen at −70°C in whole sheep blood.

DNA isolation. For the preparation of *Campylobacter* chromosomal DNA, thick lawns of bacteria were grown on 5 to 10 plates (150-mm diameter) of Mueller-Hinton broth containing 1.5% agarose and 5% sheep blood. Bacteria were harvested in TES buffer (30 mM Tris hydrochloride, 5 mM EDTA, 50 mM NaCl [pH 8.0]) and centrifuged at 12,000 × *g* for 30 min at 4°C. The pellet was washed twice in TES buffer

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TABLE 1. *Campylobacter* strains used and their origins

Bacterial species and strain[a]	Investigator[b]	Source	Origin
C. hyointestinalis			
CH8384-4	Gebhart	Pig intestine	Minnesota
ATCC 35217^T		Pig intestine	Minnesota
P9681-81	Gebhart	Pig intestine	Pennsylvania
423	Bryner	Pig intestine	Iowa
Wisc 699	Gebhart	Pig intestine	Wisconsin
F675	Gebhart	Bovine feces	Minnesota
8705	Werner	Bovine feces	Iowa
25,048	Firehammer	Bovine feces	Montana
F1412	Gebhart	Human feces	Minnesota
N3145	Hill	Deer feces	Australia
Type and reference strains			
C. fetus subsp. *fetus* ATCC 27374^T		Sheep fetus	
C. fetus subsp. *fetus* ATCC 33246		Human blood	
C. mucosalis CM8384-4	Gebhart	Porcine intestine	
C. mucosalis ATCC 43264^T		Porcine intestine	
C. coli ATCC 33559^T		Pig feces	
C. coli CC8384-4	Gebhart	Pig intestine	
C. jejuni ATCC 33560^T		Bovine feces	
C. jejuni F962	Gebhart	Hamster intestine	
C. laridis ATCC 35221^T		Gull cloacal swab	
C. fetus subsp. *venerealis* ATCC 19438^T		Bovine vaginal mucus	
C. cinaedi CL-167	Fennell	Human feces	
C. fenneliae CL-441	Fennell	Human feces	
C. cryaerophila ATCC 43157^T		Porcine fetus	
C. sputorum biovar fecalis ATCC 33709^T		Sheep feces	
C. sputorum biovar sputorum H-1	Gebhart	Human mouth	
C. sputorum biovar bubulus ATCC 33562^T		Bull sperm	
C. concisus ATCC 33237^T		Human gingival sulcus	
"*C. upsaliensis*" F813	Gebhart	Canine feces	
C. pylori ATCC 43504^T		Human gastric antrum	

[a] T, Type species.

[b] Strains were isolated and supplied by the following investigators: C. J. Gebhart, Department of Veterinary Pathobiology, University of Minnesota, St. Paul; J. H. Bryner, National Animal Disease Center, Ames, Iowa; B. D. Hill, Animal Research Institute, Yeerongpilly, Ipswich, Australia; D. Werner, National Animal Disease Center, Ames, Iowa; B. D. Firehammer, Department of Veterinary Science, Montana State University, Bozeman; and C. L. Fennell, Harborview Medical Center, Seattle Public Health Hospital, Seattle, Wash.

and suspended in 5 ml of lysis buffer (25% sucrose in 50 mM Tris hydrochloride [pH 8.0], 50 mM EDTA, 10 mM NaCl). Lysozyme was added to a final concentration of 2 mg/ml, and the tubes were mixed by tipping. After 10 min on ice, 20 μl of proteinase K (25 mg/ml) and 2 ml of 10% Sarkosyl in 50 mM Tris–50 mM EDTA–50 mM NaCl–20% sucrose were added, and the mixture was incubated for 3 h at 55°C. Ammonium acetate (7.5 M) was added to a final concentration of 2.5 M, and the tubes were incubated on ice for 20 min. After addition of 100 μl of phenylmethylsulfonyl fluoride (2-mg/ml stock in ethanol) to inactivate the proteinase K, the solution was centrifuged at 12,000 × *g* for 20 min. DNA was precipitated from the supernatant with 2 volumes of ethanol, dried, and suspended in TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) to a concentration of 500 ng/μl.

Library construction and screening. Genomic libraries were constructed as described by Maniatis et al. (18) and were screened by using a modification of the method of Grunstein and Hogness (14). Briefly, *Hin*dIII-digested chromosomal DNA from *C. hyointestinalis* was ligated (T4 DNA ligase; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) into plasmid vector pGEM-3Z (Promega Biotech, Madison, Wis.), which was dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Recombinant plasmids were then used to transform JM109 *E. coli* (Promega Biotech), and transformants were selected by growth on plates containing 100 μg of ampicillin per ml (18). The number of transformants was determined by incorporation of isopropyl-β-D-thiogalactopyranoside into the media to detect *lacZ* inactivation with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as the substrate, as recommended by the manufacturer (Promega Biotech). Colonies were transferred by replica plating to nylon filters (Hybond N, 0.45 μm; Amersham Corp., Arlington Heights, Ill.) or by applying clones to multiple nylon filters with a Cathra Repliplate inoculator with 5-mm pins (Cathra Replicator System; Cathra, Inc., St. Paul, Minn.).

The genomic library was screened differentially by hybridization to ^{32}P-labeled whole chromosomal DNA from *C. hyointestinalis* and from *C. fetus*, a closely related species. DNA from each of these two organisms was labeled with [α-^{32}P]dCTP by the random primer method (7) to specific activities of 1×10^8 to 5×10^8 cpm/μg, using a commercial kit (Pharmacia, Inc., Piscataway, N.J.). After radiolabeling, sonicated salmon sperm DNA was added to a final concentration of 100 μg/ml. Unincorporated label was removed by spun-column chromatography (Sephadex G-25; Isolab, Inc., Akron, Ohio), and the labeled DNA was denatured immediately before use by boiling for 5 min.

A variety of recombinant plasmids, containing variously sized inserts, which hybridized with *C. hyointestinalis* whole chromosomal DNA, but not with *C. fetus* DNA, were harvested. These recombinants were labeled with [^{32}P]dCTP and hybridized with DNA from 29 strains, including each porcine *Campylobacter* species, a variety of *C. hyointesti-*

nalis isolates, and each of the other described *Campylobacter* species (Table 1). The entire recombinant plasmid was used as a probe and was labeled by the random primer method described above to a specific activity of at least 10^8 cpm/μg of DNA.

For hybridizations to bacterial DNA, purified DNA samples (0.1 to 100 ng) were applied to nylon membranes (Amersham) with a Minifold apparatus (Schleicher & Schuell, Inc., Keene, N.H.). The DNA was denatured by treatment with 0.4 M NaOH–1.5 M NaCl for 7 min. Filters were neutralized in 0.5 M Tris hydrochloride (pH 7.2)–1.5 M NaCl for 3 min, washed in 2× SSC (0.3 M NaCl plus 0.03 M trisodium citrate), air dried, and fixed by UV irradiation for 4 min. Membranes were hybridized for 16 h at 42°C with 1×10^5 to 2×10^5 cpm of ^{32}P-labeled probe per ml of hybridization buffer. Hybridization buffer consisted of 50% formamide, 4× SSPE (0.72 M NaCl, 40 mM sodium phosphate buffer [pH 7.0], 4 mM EDTA), 7% polyethylene glycol, 5% sodium dodecyl sulfate (SDS), and 0.5% nonfat dry milk. After hybridization, the membranes were washed twice with 2× SSC–0.1% SDS (42°C; 20 min), three times with 0.1× SSC–0.1% SDS (42°C; 20 min), and once with 0.1× SSC–1% SDS (50°C; 20 min). Membranes were exposed to Kodak X-OMAT AR film at −80°C for 16 to 18 h with one Wolf Blue III intensifier.

For hybridizations to total bacteria, organisms were grown on Mueller-Hinton agar containing 5% sheep blood, harvested, and diluted into TES to a density of about 10^8 CFU/ml. Dilutions were prepared in Mueller-Hinton broth and plated on Mueller-Hinton agar with 5% sheep blood to obtain viable counts. Tenfold dilutions (10^8 to 10^5 CFU/ml) in TES, 100 μl each, were applied to nylon membranes, using a Minifold dot blot apparatus, corresponding to concentrations of 10^7 to 10^4 CFU per spot. One replica membrane was made for each probe to be tested. Filters were air dried and hybridized as described above.

For Southern blotting, 1- to 2-μg samples of DNA from *C. hyointestinalis* and the other porcine *Campylobacter* species were digested with *Hin*dIII and electrophoresed on a 0.8% agarose gel in Tris acetate buffer (pH 8.0). DNA was transferred to nylon membranes by capillary blotting in 20× SSPE (28) and hybridized with the *C. hyointestinalis*-specific probes as described above.

Detection of *C. hyointestinalis* in porcine fecal and mucosal specimens. One probe, pQHC01, was used to detect *C. hyointestinalis* by DNA-DNA hybridization in fecal and mucosal specimens. A fecal sample obtained from a healthy pig and containing no *Campylobacter* species was seeded with 10-fold dilutions of *C. hyointestinalis* or *C. coli* organisms in phosphate-buffered saline. A portion of each of the feces was passed through 0.8-μm membrane filters (Millipore Corp., Bedford, Mass.) to remove selectively normal enteric bacteria and fecal debris which may cause nonspecific binding of the probe. Nylon membranes were inoculated by using the dot blot Minifold apparatus, with 200 μl each of dilutions of pure cultures of *C. hyointestinalis* or *C. coli* (10^7 and 10^6 CFU per spot), feces spiked with *C. hyointestinalis* or *C. coli* (approximately 10^6 and 10^5 CFU per spot), and 0.8-μm filtrates of spiked feces (approximately 10^5 and 10^4 CFU per spot). Membranes were treated and hybridized with the *C. hyointestinalis*-specific probe pQHC01 as described above.

Probe pQHC01 was also hybridized with intestinal mucosal scrapings, mixed *Campylobacter* cultures, and isolated *Campylobacter* species collected from pigs with and without proliferative enteritis. Nylon membranes were spotted with mucosal scrapings obtained from the ileum of a healthy pig (50 μl of a 1:10 dilution in phosphate-buffered saline), mucosal scrapings from the ileum of a pig with naturally occurring proliferative enteritis (50 μl of a 1:10 dilution in phosphate-buffered saline), and *C. coli* (10^7 CFU per spot), *C. mucosalis* (10^7 CFU per spot), and *C. hyointestinalis* (10^7 CFU per spot) isolated from the mucosa. In addition, a suspension of the mixed *Campylobacter* culture was prepared from the primary isolation plate and spotted onto the membrane (about 10^7 CFU per spot). The mixed *Campylobacter* culture obtained from the pig with proliferative enteritis contained *C. hyointestinalis*, *C. mucosalis*, and *C. coli*. The mixed *Campylobacter* culture from the healthy pig contained only *C. coli*. Membranes were hybridized as described above.

RESULTS

Library construction and screening. The strategy for constructing a recombinant library containing *C. hyointestinalis* chromosomal DNA fragments was to generate a limit restriction digest composed predominantly of fragments 0.5 to 10 kilobases (kb) in size that could be ligated directly into a suitable plasmid vector. *Hin*dIII was one of the few restriction enzymes that digested *Campylobacter* DNA to the desired size range. *Hin*dIII-digested *C. hyointestinalis* DNA (90 ng) was ligated into dephosphorylated pGEM-3Z (30 ng) and used to transform *Escherichia coli* JM109. Approximately 9,900 ampicillin-resistant colonies were obtained, of which >95% were white after induction with X-Gal and isopropyl-β-D-thiogalactopyranoside. The average insert size was 1.0 ± 0.3 kb (mean ± standard error) based on an analysis of 15 randomly selected colonies.

It has been shown previously (12) that, within the genus *Campylobacter*, *C. fetus* is the species with the greatest DNA homology to *C. hyointestinalis* (30%). Therefore, *C. fetus* chromosomal DNA was used as a control probe in colony blotting experiments to eliminate those clones which contained DNA that would hybridize to *Campylobacter* species other than *C. hyointestinalis*. To identify candidate *C. hyointestinalis*-specific clones rapidly, a portion of the library on replicate membranes was screened with total DNA from either *C. hyointestinalis* or *C. fetus*. The majority of clones showed hybridization to *C. hyointestinalis* but little or no hybridization to *C. fetus* DNA. Seventy-two colonies were selected and screened by this method. Of the 64 clones which gave distinct signals when hybridized to *C. hyointestinalis* ^{32}P-labeled DNA, 60 showed no hybridization to *C. fetus* DNA.

Eight putative *C. hyointestinalis*-specific clones were selected for further analysis on the basis of specificity, signal intensity, and insert size. Because the signal intensity may have been related to insert size, several clones were included which showed only a slight hybridization signal to *C. hyointestinalis* to ensure inclusion of smaller inserts in the screening procedures. Purified plasmid DNA was isolated from these eight clones, labeled with [α-^{32}P]dCTP by the random primer reaction (7), and hybridized to 10-fold dilutions of purified DNA from *C. fetus* and to all of the *Campylobacter* species found in swine, i.e., *C. hyointestinalis*, *C. coli*, *C. mucosalis*, and *C. jejuni*, in the dot blot hybridization assay.

Three probes containing variously sized inserts were found which hybridized to 0.1 ng of homologous DNA from *C. hyointestinalis* and were designated pQHA01, pQHC01, and pQHL01, respectively. Probes pQHA01 and pQHC01

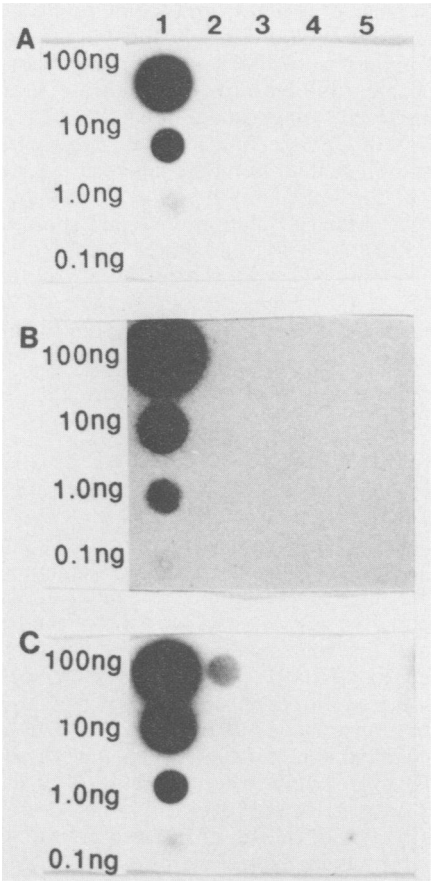

FIG. 1. Spot blot hybridization of *C. hyointestinalis* probes with chromosomal DNA from *C. fetus* and swine *Campylobacter* species. Three nylon membranes were spotted with the indicated amounts of chromosomal DNA from the following species: *C. hyointestinalis* 8384-4 (lane 1); *C. fetus* ATCC 27374 (lane 2); *C. mucosalis* 8384-4 (lane 3); *C. coli* 8384-4 (lane 4); and *C. jejuni* 962 (lane 5), as described in Materials and Methods. The membranes were hybridized with plasmids pQHA01 (A), pQHC01 (B), and pQHL01 (C), as described in the text.

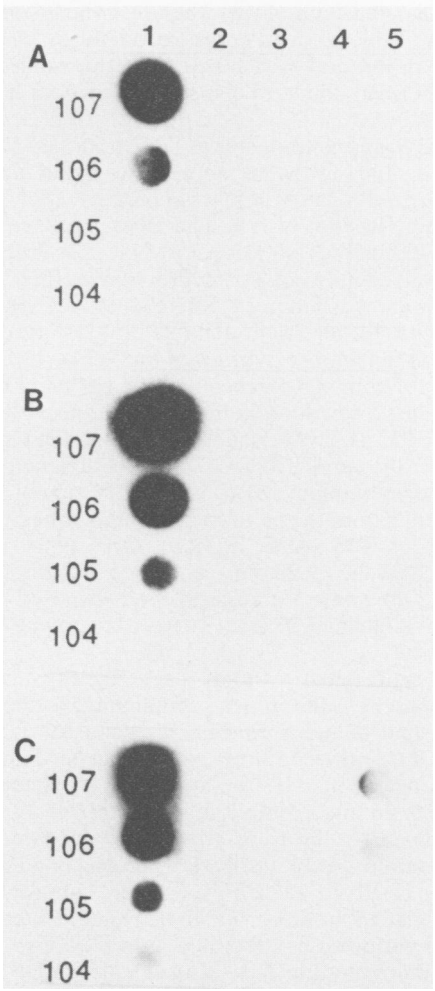

FIG. 2. Spot blot hybridizations of *C. hyointestinalis* probes with lysed bacteria. Three nylon membranes were spotted with the indicated number (CFU) of bacteria from the following species: *C. hyointestinalis* 8384-4 (lane 1); *C. fetus* ATCC 27374 (lane 2); *C. mucosalis* 8384-4 (lane 3); *C. coli* 8384-4 (lane 4); and *C. jejuni* 962 (lane 5), as described in Materials and Methods. The membranes were hybridized with plasmids pQHA01 (A), pQHC01 (B), and pQHL01 (C), as described in the text.

contained inserts of 0.4 and 1.6 kb, respectively. Probe pQHL01 contained two inserts of 3.1 and 2.8 kb. pQHA01 and pQHC01 gave no cross-hybridization with *C. fetus*, *C. mucosalis*, *C. coli*, or *C. jejuni* at a concentration of 100 ng of heterologous DNA (Fig. 1A and B). Probe pQHL01 hybridized slightly with *C. fetus* DNA at a concentration of 100 ng, 1,000 times the concentration at which it showed detectable hybridizations to *C. hyointestinalis* DNA, but not at all with DNA from *C. coli*, *C. mucosalis*, or *C. jejuni* (Fig. 1C). For all three *C. hyointestinalis* probes, there was a 100-fold difference in sensitivity between *C. hyointestinalis* and *C. fetus* and between *C. hyointestinalis* and the other three porcine species of *Campylobacter*.

Bacterial dot blots. Similar hybridizations were done with the same set of probes, using 10-fold dilutions of bacteria ranging from 10^7 to 10^4 CFU per spot (Fig. 2). Probes pQHA01 and pQHC01 detected 10^6 and 10^5 *C. hyointestinalis* bacteria per spot, respectively, and did not cross-hybridize with *C. fetus*, *C. coli*, *C. mucosalis*, or *C. jejuni*. Probe pQHL01 detected as few as 10^4 *C. hyointestinalis* bacteria per spot (Fig. 2C). There was some hybridization with *C. jejuni* at 10^7 and 10^6 bacteria per spot. We found that this was due to the presence of large amounts of plasmid DNA in *C. jejuni* which hybridized with the probe (data not shown). For all three probes, there was at least a 100-fold difference in sensitivity between *C. hyointestinalis* and *C. fetus* bacterial spots.

Bacterial dot blots were then performed against various strains of *C. hyointestinalis* isolated from a variety of geographical sources. Ten different strains of *C. hyointestinalis*, including the ATCC 35217 type strain, were applied to membranes at a concentration of 10^6 CFU per spot with appropriate controls. The *C. hyointestinalis* probes reacted strongly with all 10 *C. hyointestinalis* strains tested, but not with 2 different strains of *C. fetus*. Hybridization was not observed between any probe and chromosomal DNA from the *E. coli* cloning host JM109 (data not shown). Nor was hybridization observed between radiolabeled pGEM-3Z and any of the *C. hyointestinalis* strains tested (data not shown).

In the same manner, the probes were tested against type or reference strains of all other described *Campylobacter* species. Each probe reacted strongly with all strains of the

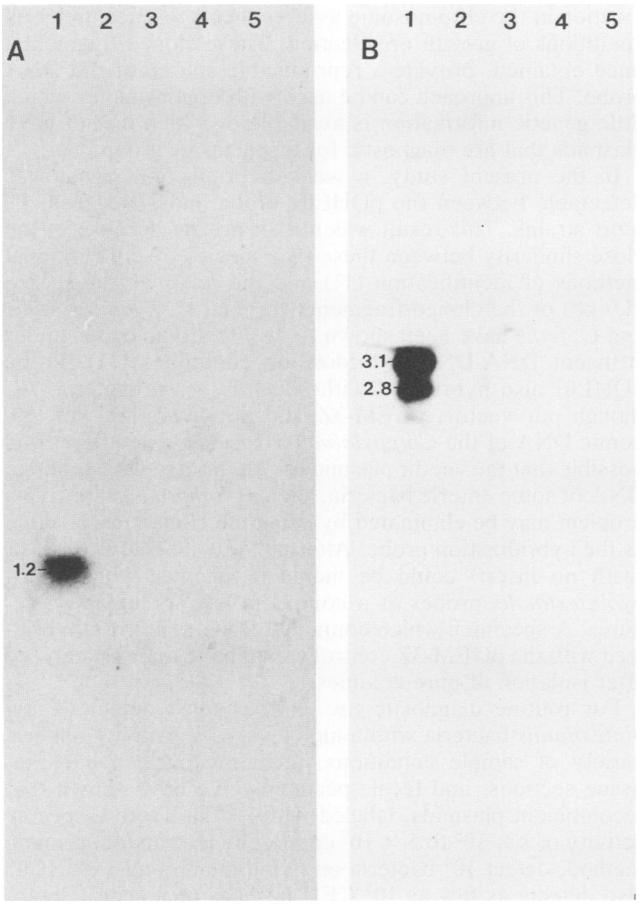

FIG. 3. Southern blot analysis of chromosomal DNA from various *Campylobacter* species. Samples, 2 μg, of *Hin*dIII-digested DNA from the indicated species were electrophoresed, blotted, and hybridized with plasmids pQHC01 (A) or pQHL01 (B) as described in Materials and Methods. Lanes: 1, *C. hyointestinalis* 8384-4; 2, *C. fetus* ATCC 27374; 3, *C. mucosalis* 8384-4; 4, *C. coli* 8384-4; 5, *C. jejuni* 962. Size markers determined from *Hin*dIII fragments of bacteriophage lambda DNA.

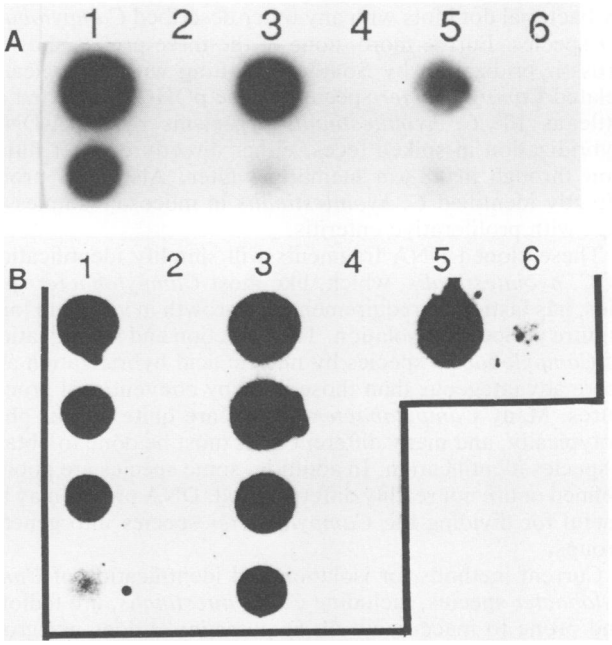

FIG. 4. Hybridizations of plasmid pQHC01. (A) Hybridization to spot blots of porcine fecal specimens with or without added *C. hyointestinalis* or *C. coli* organisms. Lanes: 1, pure culture of *C. hyointestinalis* 8384-4 (10^7 and 10^6 CFU per spot); 2, pure culture of *C. coli* 8384-4 (10^7 and 10^6 CFU per spot); 3, porcine feces spiked with *C. hyointestinalis* (10^5 and 10^4 CFU per spot); 4, porcine feces spiked with *C. coli* (10^7 and 10^6 CFU per spot); 5, 0.8-μm filtrate from the feces in lane 3; 6, 0.8-μm filtrate of the feces in lane 4. (B) Hybridization with intestinal mucosal scrapings from pigs with and without proliferative enteritis, with serial dilutions of the mixed *Campylobacter* culture and with individual isolates obtained from them. *C. hyointestinalis* and *C. mucosalis* were isolated from a diseased pig, and *C. coli* was isolated from a nondiseased pig. Lanes: 1, mixed *Campylobacter* culture harvested from the primary isolation plate (0.8-μm filtrate); 2, *C. mucosalis*; 3, *C. hyointestinalis*; 4, *C. coli*; 5, ground intestinal mucosa from a diseased pig; 6, ground intestinal mucosa from a normal pig. Rows represent fivefold serial dilutions of each sample. Sample application, hybridization, and autoradiography were done as described in Materials and Methods.

homologous species, but not with any other species. A relatively weak hybridization was observed when probe pQHL01 was hybridized with *C. fetus*, and no detectable hybridization between the plasmid probe and chromosomal DNA from any of the other species of *Campylobacter* could be detected even after prolonged (72-h) exposure of the autoradiograph. Thus, each cloned *C. hyointestinalis* chromosomal DNA fragment was specific for *C. hyointestinalis*.

Southern blots. Southern blots of *Hin*dIII-digested DNA from *C. hyointestinalis*, *C. fetus*, *C. mucosalis*, *C. coli*, and *C. jejuni* confirmed that the recombinant clones pQHC01, pQHL01, and pQHA01 specifically hybridized only with *C. hyointestinalis* (Fig. 3). The size of the hybridized band corresponded to the plasmid insert for pQHC01 and pQHA01. However, the intensity of the band obtained with pQHA01 was too faint for photographic duplications. pQHL01 hybridized to two bands, which were of the same size as the two inserts.

Detection of *C. hyointestinalis* in porcine fecal and mucosal specimens. The usefulness of DNA probes for detecting *Campylobacter* species in clinical samples was assessed by using plasmid pQHC01. It detected *C. hyointestinalis* by DNA-DNA hybridization in fecal specimens spiked with *C. hyointestinalis*, but not in specimens spiked with *C. coli* (Fig. 4A). Filtration of the feces through 0.8-μm membrane filters (Millipore) decreased the sensitivity of the technique 10-fold, but did not affect its specificity.

Plasmid pQHC01 also directly identified *C. hyointestinalis* in clinical specimens. It hybridized with intestinal mucosal scrapings, a mixed *Campylobacter* culture obtained from the primary isolation plate, and the *C. hyointestinalis* isolate obtained from the mucosal scrapings from two pigs with proliferative enteritis (Fig. 4B). No hybridization was obtained with *C. mucosalis*, *C. coli*, or the mucosal scrapings from two nondiseased pigs.

DISCUSSION

We have described three species-specific DNA probes useful for identifying *C. hyointestinalis*. The recombinant plasmids pQHC01, pQHL01, and pQHA01, containing inserts of 1.6, 3.1 and 2.8, and 0.4 kb, respectively, hybridized to all tested strains of *C. hyointestinalis* from a variety of mammalian hosts and showed little or no cross-hybridization

by bacterial dot blots with any other described *Campylobacter* species. Furthermore, none of the three probes showed cross-hybridization by Southern blotting with ecologically related *Campylobacter* species. Probe pQHC01 detected as little as 10^5 *C. hyointestinalis* organisms by DNA-DNA hybridization in spiked feces, either directly or after filtration through a 0.8-μm membrane filter. Also, this probe directly identified *C. hyointestinalis* in mucosal samples of pigs with proliferative enteritis.

These cloned DNA fragments will simplify identification of *C. hyointestinalis*, which, like most *Campylobacter* species, has fastidious requirements for growth in vitro and long culture periods for isolation. The detection and identification of *Campylobacter* species by nucleic acid hybridization are more advantageous than those done by conventional procedures. Many *Campylobacter* species are quite similar phenotypically, and many different tests must be done to obtain a species identification. In addition, some species are poorly defined or are not readily differentiated. DNA probes may be useful for dividing the *Campylobacter* species into genetic groups.

Current methods for isolation and identification of *Campylobacter* species, including *C. hyointestinalis*, are tedious and prone to inaccuracy. *C. hyointestinalis* does not grow well on selective media containing antimicrobial substances to suppress the growth of normal, commensal enteric flora (8, 11). These selective media were developed for *C. jejuni* isolation, and some of the antibiotics may inhibit growth of *C. hyointestinalis*. Although a presumptive identification of *Campylobacter* can be made on the basis of colonial morphology, Gram stain, oxidase production, catalase production, and motility, definitive characterization of the organism requires more sophisticated tests (1, 9). These tests are time-consuming, costly, and not always sufficient for differentiating *C. hyointestinalis* from other *Campylobacter* species.

The use of randomly cloned DNA fragments as probes for *C. hyointestinalis* detection and identification is preferable to other identification approaches that use total genomic DNA, endogenous plasmids, rRNA sequences, or immunological probes. DNA hybridization with total genomic DNA has been reported for bacteria, but shows considerable cross-hybridization to heterologous sequences in dot blot assays and by in situ hybridization (13). Whole genomic DNAs from *Campylobacter*-like organisms have been used as probes to investigate the taxonomic relationship of various strains (31). Our approach to obtaining specific DNA probes differs in that we used cloned fragments of chromosomal DNA rather than whole chromosomal DNA (9, 13), a fraction of chromosomal DNA (13), characterized cloned genes (19), or plasmids (32). Similarly, rRNA sequences are highly conserved among related bacteria, so that probes based on them would have to be used under carefully controlled conditions to obtain reproducible, accurate *Campylobacter* species identification. Tests based on the use of plasmids or antibodies may be unreliable due to the variable presence of plasmids among strains or their loss under nonselective bacterial growth conditions or due to the variable expression of immunoreactive proteins associated with changing growth conditions. For example, few porcine *C. hyointestinalis* isolates contain plasmids in vitro (C. Gebhart, unpublished data). Thus, specific chromosomal DNA fragments are the material of choice for use as *Campylobacter* diagnostic probes because they are highly specific and are reliable under a range of conditions. Sequences are selected based on their being unique to a given bacterial species, and their location in the chromosome assures their presence under all conditions of growth or selection. These cloned fragments, once obtained, provide a reproducible source of the DNA probe. This approach can be used with organisms for which little genetic information is available or which do not have plasmids that are diagnostic for a species or group.

In the present study, a weak hybridization signal was detectable between the pQHL01 probe and DNA from *C. fetus* strains. This result was not surprising because of the close similarity between these two species by conventional methods of identification (11) and the relatively large size (5.9 kb) of the cloned fragments. In fact, *C. hyointestinalis* and *C. fetus* have been shown to be 30% homologous under stringent DNA-DNA hybridization conditions (11). Probe pQHL01 also hybridized with lysed *C. jejuni* bacteria. Although our vector, pGEM-3Z, did not hybridize with genomic DNA of the *Campylobacter* species tested here, it is possible that the vector plasmid may hybridize with genomic DNA of some enteric bacteria, such as *Salmonella* spp. This problem may be eliminated by using the cloned insert alone as the hybridization probe. Alternatively, labeled pGEM-3Z (with no insert) could be included together with the *C. hyointestinalis* probes as a control in any screening procedures. A specimen which contained DNA that cross-hybridized with the pGEM-3Z control would have to be reanalyzed after isolation of pure colonies.

For routine diagnostic use, probes must detect *C. hyointestinalis* bacteria with a high level of sensitivity under a variety of sample conditions, including purified bacteria, tissue sections, and fecal specimens. We have shown that recombinant plasmids, labeled with [^{32}P]dCTP to a specific activity of 1×10^8 to 5×10^8 cpm/μg by the random priming method, detect 10^4 bacteria on nylon membranes. pQHC01 also detects as few as 10^5 CFU in feces or mucosal scrapings. This level of sensitivity is adequate for the detection and identification of *C. hyointestinalis* in feces and intestinal mucosa, in which the number of *C. hyointestinalis* is typically $>10^5$ CFU/ml (Gebhart, unpublished observations). It was not necessary to extract and purify the DNA from the strain to be tested, which must be done when the entire chromosome is used in the hybridization.

The species-specific probes could also detect the presence of the homologous strain in mixed cultures, in ground crude porcine intestinal mucosa spotted directly onto nylon membranes, and in filtrates of mucosal material. Although sensitivity decreased 10-fold when feces and mucosa were used, the technique was highly specific. Several species of *Campylobacter* were isolated from these preparations, but the *C. hyointestinalis* probes hybridized only with the material containing the homologous organisms. Since *C. hyointestinalis* or *C. mucosalis* or both are consistently isolated from pigs with proliferative enteritis, but not from normal pigs, these DNA probes may be used as a diagnostic technique to identify the disease presumptively. We believe that the technique will also be sufficiently sensitive for in situ hybridization of intracellular bacteria in intestinal tissue sections.

ACKNOWLEDGMENTS

We thank T. Molitor for a careful review of the manuscript.

This research was supported by U.S. Department of Agriculture research grant USDA-86-CRSR-2-2893.

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