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Design and Evaluation of *Bacteroides* DNA Probes for the Specific Detection of Human Fecal Pollution

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Because *Bacteroides* spp. are obligate anaerobes that dominate the human fecal flora, and because some species may live only in the human intestine, these bacteria might be useful to distinguish human from nonhuman sources of fecal pollution. To test this hypothesis, PCR primers specific for 16S rRNA gene sequences of *Bacteroides distasonis*, *B. thetaiotaomicron*, and *B. vulgatus* were designed. Hybridization with species-specific internal probes was used to detect the intended PCR products. Extracts from 66 known *Bacteroides* strains, representing 10 related species, were used to confirm the specificity of these PCR-hybridization assays. To test for specificity in feces, procedures were developed to prepare DNA of sufficient purity for PCR. Extracts of feces from 9 humans and 70 nonhumans (cats, dogs, cattle, hogs, horses, sheep, goats, and chickens) were each analyzed with and without an internal positive control to verify that PCR amplification was not inhibited by substances in the extract. In addition, serial dilutions from each extract that tested positive were assayed to estimate the relative abundance of target *Bacteroides* spp. in the sample. Depending on the primer-probe set used, either 78 or 67% of the human fecal extracts tested had high levels of target DNA. On the other hand, only 7 to 11% of the nonhuman extracts tested had similarly high levels of target DNA. An additional 12 to 20% of the nonhuman extracts had levels of target DNA that were 100- to 1,000-fold lower than those found in humans. Although the *B. vulgatus* probes detected high levels of their target DNA in most of the house pets, similarly high levels of target DNA were found only in a few individuals from other groups of nonhumans. Therefore, the results indicate that these probes can distinguish human from nonhuman feces in many cases.

The need to distinguish between human and nonhuman sources of fecal pollution in water has stimulated the search for a species-specific indicator. At present, we use the detection of fecal coliform bacteria to indicate fecal pollution. However, these bacteria are found in a variety of warm-blooded animals and are not unique to the human intestinal flora. As a result, sewage treatment effluents are not distinguished from farm runoff. Therefore, we cannot identify the source of fecal pollution to implement meaningful risk assessment and effective remediation. Since fecal streptococci are more abundant in nonhumans than in humans, fecal-coliform-to-fecal-streptococcus ratios have been used to distinguish between human and nonhuman feces. However, this relationship deteriorates rapidly once the feces are dispersed into receiving waters, because survival of the predominant species in the two groups differs and mixed wastes are difficult to characterize (13). In addition, it has been shown that some fecal coliforms (7, 37) and fecal streptococci (31) can persist and even grow in certain environments, and free-living coliforms may be indigenous to some tropical waters (38). Clearly, a more source-specific indicator is needed.

It has been suggested that bacteria from the genus *Bacteroides* might be used to distinguish human from nonhuman sources of fecal pollution, for several reasons. *Bacteroides* spp. dominate the human fecal flora, and several species outnumber the coliforms (19, 30). Second, being obligate anaerobes, *Bacteroides* spp. do not survive in oxygenated waters (3, 11). Lastly, the most abundant *Bacteroides* spp. in human feces have been detected only at low levels or not at all in feces from other animals (3, 14).

The need to maintain anaerobic conditions during growth, isolation, and biochemical identification of *Bacteroides* spp. has discouraged their use as an indicator. However, the advent of detection methods that use DNA probes obviates the need to cultivate organisms of interest. By using PCR, specific DNA sequences can be amplified in vitro from a few copies to approximately a million copies in just a few hours, thereby eliminating the time and effort needed to grow fastidious organisms to obtain detectable levels of target DNA. Furthermore, since each species has a unique DNA sequence, choice of the correct probe sequence and use of sufficiently stringent assay conditions can make DNA sequence-based detection very selective. Any other trait used to identify a species, such as a specific enzyme activity or morphological feature, may or may not be present, or expressed, under all environmental conditions. However, the information encoded in the DNA provides a means for detection whether or not it is expressed phenotypically. The focus of the present work, therefore, was to design PCR probes for several *Bacteroides* species thought to be abundant in humans, but not in nonhumans, and to test the potential for these probes to distinguish between human and nonhuman feces.

MATERIALS AND METHODS

Cultures and feces. *Bacteroides* cultures, listed in Table 1, were from the Virginia Polytechnic Institute and State University Collection, Blacksburg, Va., the American Type Culture Collection, Rockville, Md., or the Los Angeles Veterans Administration Hospital. Most strains from the Virginia Polytechnic Institute and State University Collection are verified members of the *Bacteroides* genus on the basis of Johnson's (23) DNA-DNA hybridization results. All strains were maintained in chopped-meat medium (Carr-Scarborough Microbiologicals,

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TABLE 1. *Bacteroides* strains tested and specificity of PCR-hybridization assays

Species	Strain[a]	Source[b]	Assay results[c]		
			D	T	V
B. caccae	3452A	VPI	−	−	−
	2308	VPI	−	−	−
	8608	VPI	−	−	−
	C7-8	VPI	−	−	−
B. distasonis	8503	ATCC	+	−	−
	C30-45	VPI	+	−	−
	C18-7	VPI	+	−	−
	6779	LA-VA	−	−	−
	B1-20	VPI	+	−	−
	85A-14	VPI	−	−	−
	T3-25	VPI	+	−	−
	55A-14	VPI	±	−	−
	0052	VPI	+	−	−
B. eggerthii	B8-51	VPI	−	−	−
	X3-31-1A	VPI	−	−	±
	51A-52	VPI	−	−	±
B. fragilis	25285	ATCC	−	−	−
	4509B	VPI	−	−	−
	2044	VPI	−	−	−
	2393	VPI	−	−	−
	4225	VPI	−	−	−
	2256	VPI	−	−	−
	7397	LA-VA	−	−	−
	2556-1	VPI	±	−	−
	1582	VPI	−	−	−
	3277	VPI	−	−	−
	2552	VPI	−	−	±
	3392	VPI	−	−	−
	4076	VPI	−	−	−
B. merdae	T4-1	VPI	−	−	−
B. ovatus	0038	VPI	−	+	−
	C2-26	VPI	−	+	±
	3049	VPI	−	+	−
	4101	VPI	−	+	−
	R3-39	VPI	−	−	−
	2828	VPI	−	−	−
B. stercoris	B5-21	VPI	−	−	−
	C8-19	VPI	−	−	−
	C51-6	VPI	−	−	−
B. thetaiotaomicron	29148	ATCC	−	+	−
	3443	VPI	−	+	−
	2808B	VPI	−	+	−
	8702	LA-VA	−	+	−
	0940-1	VPI	−	+	−
	0633-1	VPI	−	+	−
	3164A	VPI	−	+	−
	C11-15	VPI	−	+	−
	J19-34B	VPI	−	+	−
B. uniformis	8492	ATCC	−	−	−
	T1-1	VPI	−	−	−
	3537	VPI	−	−	−
	0909	VPI	−	−	−
	C20-25	VPI	±	±	−
	R5-33	VPI	−	−	−
	C7-17	VPI	−	−	−
B. vulgatus	8482	ATCC	−	−	+
	2277	VPI	−	−	±
	6598B	VPI	−	−	±
	4496	VPI	−	−	±
	29327	ATCC	−	−	+
	31376	ATCC	−	−	+
	2365	VPI	−	−	+
	4025	VPI	−	−	+
	4506-1	VPI	−	−	+
	C1-13	VPI	−	−	+
	6168	VPI	−	−	+

[a] The first strain listed for each species is the type strain.

[b] Source culture collections were as follows: VPI, Virginia Polytechnic Institute; ATCC, American Type Culture Collection; LA-VA, Los Angeles Veteran's Administration Hospital. Most strains from Virginia Polytechnic Institute were characterized by Johnson (23).

[c] D, *B. distasonis* assay; T, *B. thetaiotaomicron* assay; V, *B. vulgatus* assay. Results of PCR-hybridization assays with 10^{-7} g of test DNA are reported as − if hybridization signals were less intense than the internal positive control and + if they were at least as strong as the control. Those reported as ± were + with 10^{-7} g but − with 10^{-9} g of test DNA (D or T) or 10^{-10} g of test DNA (V).

Stone Mountain, Ga.) and were subcultured in TYG medium (18) containing 100 μg of kanamycin per ml, 100 μg of gentamicin per ml, and 7.5 μg of vancomycin per ml. Cultures were grown and stored in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, Mich.).

Human fecal samples were collected directly upon voiding into commode specimen collection system vessels (Sage Products, Inc., Crystal Lake, Ill.). Fresh feces from nonhumans were collected as soon after voiding as possible, by scooping with disposable plastic spoons into plastic zip-lock bags. These were sealed and immediately placed on ice for transport to the laboratory. Samples were refrigerated at 4°C and processed within 24 h of collection.

Extraction, purification, and quantification of DNA for PCR. To prevent cross-contamination, disposable labware was used whenever possible and glassware was soaked in a 10% (vol/vol) bleach solution (0.55% [vol/vol] sodium hypochlorite) (35) for several hours before being washed and reused.

A 1-g sample of feces was dispersed in 25 ml of 50 mM sodium phosphate (pH 7.5) by kneading in a plastic zip-lock bag. A bacterial fraction was prepared by differential centrifugation as described by Salyers et al. (40). Bacteria from feces or from *Bacteroides* cultures were resuspended in 50 mM Tris-HCl–50 mM Na-EDTA (pH 8.0), and DNA was extracted by proteinase K lysis followed by hexadecyltrimethylammonium bromide (CTAB) and phenol-chloroform-isoamyl alcohol extractions as described by Wilson (47). Inhibitors present in DNA extracts from fecal bacteria were removed by glass purification, essentially as described by Boom et al. (6) with modifications introduced by Carter and Milton (8). Celite AFA (Fluka, Ronkonkoma, N.Y.) or SpinBind cartridges (FMC BioProducts, Rockland, Maine) were used as an adsorbent, and 3.6 M guanidinium thiocyanate was used as the chaotropic salt.

Fecal extracts contained UV-absorbing impurities, precluding the estimation of DNA levels from A_{260} measurements. Therefore, DNA concentrations were determined by a fluorescent-dye-binding assay. A DNA assay kit (Pierce, Rockford, Ill.) was used as specified by the manufacturer, except that the fluorometer was zeroed against each aliquot of assay buffer before sample addition, as described by Gallagher (12). Since binding of the fluorescent dye (Hoechst 33258) to DNA depends on the base composition of the DNA, which varies greatly among bacterial species (29), DNA concentration estimates were verified by slot blot hybridization. A universal 16S rDNA sequence, 5′-CGT GCC AGC AGC CGC GGT AAT ACG-3′ (25), was labeled with digoxigenin and used as a probe as described below. Hybridization was quantified by densitometry, and the concentration of DNA in fecal extracts was estimated by comparing signal intensities with those of *Bacteroides* DNA preparations of known concentration. Values for DNA levels estimated by the hybridization assay and the fluorescent-dye-binding assay varied by no more than a factor of 2.

PCR and hybridization assays. 16S rDNA sequences were obtained from GenBank or EMBL and were aligned by DNA STAR (DNA STAR, Inc., Madison, Wis.). Variable regions in the *Bacteroides* gene sequences were identified by

comparison with the corresponding regions in *Escherichia coli* 16S rDNA (32). Variable regions from type strains for the three target *Bacteroides* species were aligned with each other and with those of related species in the gene banks, and unique sequences were analyzed by OLIGO version 3.4 or 4.0 (National Biosciences, Inc., Plymouth, Minn.) to select suitable segments for use as PCR primers and hybridization probes. In addition, sequences for the primers and probes were selected such that all three assays would have similar annealing and hybridization temperature optima. Prospective primer and probe sequences were compared with all sequences in GenBank and EMBL by DNA STAR to ensure that they were as specific as possible for the intended target *Bacteroides* species. PCR primers used to detect *Bacteroides distasonis* were from variable regions 2 and 3 (position 180, 5′-AAT ACC GCA TGA AGC AGG-3′; and position 463, 5′-GAC ACG TCC CGC ACT TTA-3′). A second upstream primer for *B. distasonis* (position 176, 5′-GAC TAA TAC CGC ATG AAG CAG-3′ was also tested (see Results). PCR primers used to detect *B. thetaiotaomicron* were from variable regions 1 and 3 (position 72, 5′-GGG GCA GCA TTT CAG TTT-3′; and position 477, 5′-CAT ATG GTA CAT ACA AAA TTC CAC A-3′), and those for *B. vulgatus* were from variable regions 4 and 6 (position 577, 5′-AAG GGA GCG TAG ATG GAT G-3′; and position 1002, 5′-GGC TTT CAC CGT AAT TCA TC-3′). Internal sequences used for hybridization probes to detect the desired PCR products were 5′-GGG ATG AAG GTT CTA TGG ATC GTA-3′ (position 420) for *B. distasonis*, 5′-ATA ACT CGG GGA TAG CCT TTC G-3′ (position 149) for *B. thetaiotaomicron*, and 5′-GGA TAT CTT GAG TGC AGT TGA GGC-3′ (position 649) for *B. vulgatus*. A second hybridization probe for *B. thetaiotaomicron* (position 190, 5′-ATG GTA TAA TCA GAC CGC ATG GTC-3′) was used to distinguish between PCR products from *B. thetaiotaomicron* and *B. ovatus* (see Results). The *B. ovatus* 16S rDNA sequence was a gift from Bruce Paster, Forsyth Dental Laboratory, Boston, Mass., and has since been entered into GenBank. All oligonucleotides were synthesized with a model 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.) and were purified with Applied Biosystems oligonucleotide purification cartridges as specified by the manufacturer.

PCRs were conducted with core reagent kits (PE Xpress, Norwalk, Conn.) as specified by the manufacturer. After the $MgCl_2$ concentration was optimized, reaction mixtures containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM each deoxynucleoside triphosphate, 0.6 U of Ampli *Taq* DNA polymerase, and both primers at 0.5 μM were used in a total volume of 25 μl. The reaction mixtures were incubated for 2 min at 95°C and then subjected to 30 cycles at 95°C for 1.5 min, 56°C for 1.5 min, and 72°C for 1.5 min. "Hot-start" PCR, used to test the specificity of the standard procedure as described in Results, was performed with Ampli-Wax as specified by the manufacturer (PE Xpress).

Several steps were taken to avoid and detect any carryover contamination of PCR products into new reactions. Tubes containing PCR products were opened and their contents were analyzed in a room fully separated from the laboratories where extracts were prepared and PCR assays were assembled. After opening PCR tubes, no reagents used prior to amplification were handled until the next day (after showering and changing clothes). In addition, at least one reagent blank, containing no added target DNA, was run with each set of reactions to detect contamination. In most experiments, the blank tubes were left uncapped during assay setup to maximize exposure to any target DNA in the environment. Nevertheless, all reagent blanks tested negative.

Oligonucleotide hybridization probes were labeled at their 3′ ends with a Genius 5 kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Dot blot hybridizations and chemiluminescent detection were performed as specified by the manufacturer (5), except that prehybridization incubations were carried out for 4 to 6 h, hybridization incubations were carried out for 15 to 18 h, and CSPD (Tropix, Bedford, Mass.) was used as the chemiluminescent substrate at 1% in 0.1 M diethanolamine–1.0 mM $MgCl_2$–0.02% sodium azide. To detect only the intended PCR products, hybridization with the internal probes was performed at the highest temperature possible without sacrificing sensitivity. Blots of PCR products from serial dilutions of *Bacteroides* DNA were tested to determine this optimum hybridization temperature. Detection was similar at 52 and 54°C (7 to 11°C below the melting temperatures for the three probes) but was reduced at 57°C (4 to 6°C below the melting temperatures). Therefore, 54°C was used for all experiments whose results are presented below.

RESULTS

Specificity and sensitivity of PCR-hybridization assays. Unique sequences from the 16S rRNA genes of *B. distasonis*, *B. thetaiotaomicron*, and *B. vulgatus* were used to prime PCR amplification of DNA from these target *Bacteroides* species. PCR products were immobilized on nylon membranes in a dot blot format, and a third unique oligonucleotide, whose sequence was internal to the PCR primers, was labeled with digoxigenin and used as a hybridization probe to identify reactions yielding the correct product.

Sixty-six *Bacteroides* strains from culture collections (Table 1), representing 10 related species, were tested to determine the specificity and sensitivity of the assays. Results obtained with extracts from the type strains for each species are shown in Fig. 1. For each, the PCR was conducted with test DNA alone (top row in each panel) or test DNA plus positive control DNA from the type strain whose sequence was used to design the primers (bottom row in each panel). The latter identifies test samples that inhibit PCR. Serial dilutions of positive control DNA were assayed to determine the least amount that could be detected reliably, and these amounts were used for internal controls (data not shown). Therefore, the positive control DNA should amplify and yield a hybridization signal. However, if the test sample contains inhibiting substances, the positive control DNA will not be amplified and no signal will be seen at that position on the blot. Extract c appears to inhibit amplification of *B. vulgatus* DNA on the blot shown (Fig. 1C, bottom row). However, PCR product was readily detected on a separate blot with another aliquot from the same reaction, indicating that the control DNA did amplify (data not shown). Since the adjacent spot (d) is more intense than the control on the blot shown in Fig. 1C, it is likely that reactions c and d were loaded at the same position by mistake. The internal control DNA was amplified in the presence of the other 65 strains as well (Fig. 1, bottom row in each panel, and data not shown). Therefore, no inhibition was detected when extracts from cultured cells were used, and all negative results are valid.

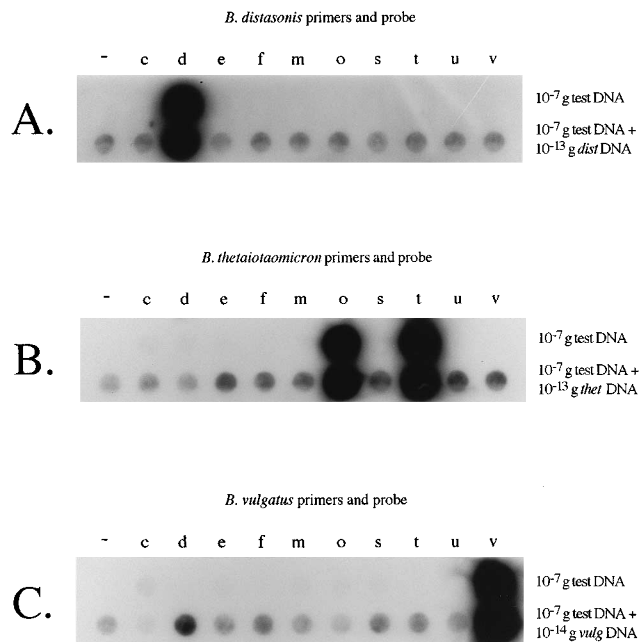

FIG. 1. Specificity of PCR-hybridization assays with *Bacteroides* cultures. Unique sequences from the 16S rRNA genes were used to prime PCR amplification of DNA from target *Bacteroides* species. For each, the PCR was run with test DNA alone (top row in each panel) or test DNA plus positive control DNA (bottom row in each panel) to identify test extracts that inhibit the reaction. PCR products were immobilized on nylon membranes with a dot blot manifold, and digoxigenin-labeled probes were used to identify reactions yielding the correct products by hybridization and chemiluminescent detection. Results from the type strain for each of the 10 species tested (listed in Table 1) are shown: −, no test DNA; c, *B. caccae*; d, *B. distasonis*; e, *B. eggerthii*; f, *B. fragilis*; m, *B. merdae*; o, *B. ovatus*; s, *B. stercoris*; t, *B. thetaiotaomicron*; u, *B. uniformis*; v, *B. vulgatus*. (A) Assay for *B. distasonis*; (B) assay for *B. thetaiotaomicron*; (C) assay for *B. vulgatus*.

The assays for *B. distasonis* and *B. vulgatus* are very selective: only *B. distasonis* strains (Fig. 1A, d on blot) or *B. vulgatus*

TABLE 2. Source of feces used in this study

Species	No. of collection sites[a]	No. of samples tested
Humans	7	9
House pets		
Cats	3	4
Dogs	3	3
Rabbits	1	1
Cattle		
Dairy	3	9
Beef	3	8
Swine		
Hogs	4	14
Pot-bellied pigs	1	2
Horses		
Donkeys	1	1
Horses	3	8
Ponies	1	2
Goats and sheep		
Goats	2	4
Sheep	4	9
Poultry		
Chickens	2	5
Turkeys	1	1

[a] Collection sites include nine different houses (for humans and house pets), nine different farms (for most of the cattle, hogs, horses, sheep, and chickens), the Cincinnati mounted police stables and a riding stable (for horses), and two local petting zoos (two calves, two pot-bellied pigs, one donkey, two ponies, four goats, two sheep, one chicken, and one turkey).

A. Wash Fecal Bacteria

- Disperse feces into phosphate buffer
- Pellet debris (low speed centrifugation)
- Rewash debris to free trapped bacteria
- Pellet bacteria from supernates
- Rewash bacteria

B. Extract DNA from Cells

- Lyse cells with SDS + proteinase K
- Precipitate carbohydrates with NaCl + CTAB
- Extract precipitate with phenol:chloroform
- Collect nucleic acids by isopropanol precipitation

C. Purify DNA

- Absorb DNA to glass in guanidinium thiocyanate
- Wash away inhibitors
- Elute DNA

PCR

FIG. 2. Outline of procedures used to extract and purify DNA from feces for PCR. (A) Fractionation of feces to yield a washed bacterial pellet by the method of Salyers et al. (40). (B) Extraction of DNA from the bacterial pellet as described by Gallagher (12). (C) Purification of DNA from the extract by adsorption to glass by the method of Boom et al. (6), with modifications by Carter and Milton (8). Abbreviated versions of this scheme yielded extracts that inhibited PCR in many cases.

strains (Fig. 1C, v on blot) were readily detected. DNA from either two or four nontarget *Bacteroides* species did amplify weakly in the initial screen with 10^{-7} g of DNA (Table 1, columns D and V). However, this amount is at least 10^6 times as much DNA as is needed to detect the intended target from the respective type strain. No PCR products were detected when 100-fold less template DNA from these nontarget species was used. The assay for *B. thetaiotaomicron* is less selective since four of the six *B. ovatus* strains tested were readily detected (Fig. 1B, t and o on blot; Table 1, column T). When the sequence for the 16S rDNA from *B. ovatus* became available, it was discovered that it is very similar to that of *B. thetaiotaomicron*, differing at just one position in the upstream primer and one in the hybridization probe. Therefore, this cross-species detection is not surprising. A second hybridization probe was designed that hybridizes more selectively with PCR products from *B. thetaiotaomicron*. Unfortunately, it was less sensitive than was the original probe for detecting its target species in human feces, as discussed in the next section.

All three assays are very sensitive: target DNA was detected when only 10^{-13} g of *B. distasonis* or *B. thetaiotaomicron* DNA or 10^{-14} g of *B. vulgatus* DNA was used. This level of sensitivity indicates that several copies of the target DNA are needed for detection, because eubacteria have several copies of the 16S rRNA gene per genome (43), and the extraction method used in this study yielded approximately 10^{-13} g of DNA per one to five bacteria (data not shown).

Detection of target *Bacteroides* species in human and nonhuman feces. To evaluate the potential for PCR-hybridization assays to distinguish between human and nonhuman sources of fecal pollution, feces were collected from a variety of animals and tested with the *Bacteroides* primers and probes. As listed in Table 2, nonhuman sources were either house pets or farm animals. These animals live in close proximity to humans and therefore are likely to have frequent contact with human microorganisms. In addition, the farm animals are kept in highly crowed conditions, so they have the potential to make a large impact on fecal pollution. In each case, several samples collected from several different locations were analyzed. For example, in the case of dairy cattle, manure was collected from four cows each at two different farms, one in Ohio and one in Illinois. The ninth sample was from a calf at a petting zoo in Cincinnati.

Although a standard DNA extraction procedure (Fig. 2B) was sufficient to prepare template from *Bacteroides* cultures, extracts prepared in a similar way from feces inhibited the PCR in most cases. No product was detected in assays of these inhibitory extracts spiked with positive control DNA (data not shown). Additional extractions with phenol and/or chloroform removed the offending substance(s) from a few extracts but not from most. It has been suggested that phenolic compounds from plants may bind to nucleic acids and prevent their use as substrates for PCR (22, 49). Since the bulk of manure is plant material, the most logical approach would be to remove this material before lysing the bacteria and exposing their DNA. Therefore, a bacterial fraction was prepared from whole feces by differential centrifugation before lysis (Fig. 2A). This procedure removed large particles, leaving a fraction enriched for target bacteria. Results of PCR assays that compared extracts of whole human feces with those of bacterial fractions from equal proportions of the same feces demonstrated that loss of target *Bacteroides* species during fractionation was negligible (data not shown). Unfortunately, this modification did not eliminate inhibition in most cases. Likewise, addition of 1% polyvinylpyrrolidone to the wash buffer to bind and remove phenolic compounds (22) did not eliminate the inhibition. Pas-

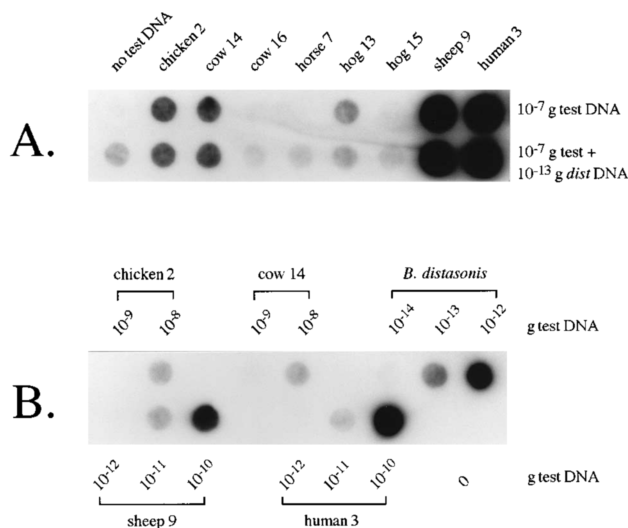

FIG. 3. Specificity of PCR-hybridization assays with DNA from feces. Examples from assays with *B. distasonis* primers and probes are shown. (A) Purified DNA (100 ng) was used as template for PCR, and reactions yielding the correct PCR products were identified by hybridization on dot blots, as described in the legend to Fig. 1. (B) Serial dilutions from each extract that tested positive in the assay shown in panel A were reassayed to determine relative abundance of the target DNA.

sage of the final extract through Chelex-Sephadex columns (1) eliminated inhibition in some cases but not in most. Glass purification of DNA from the final extract was often needed to yield PCR-compatible DNA (Fig. 2C). Hybridization of equal proportions of the extract before and after glass purification with a universal 16S rDNA probe demonstrated that average recovery was 106% ± 36% (n = 24; data not shown). Therefore, all samples were processed as outlined in Fig. 2A to C to allow comparison of results.

A 10- to 100-ng sample of each glass-purified test DNA was assayed both alone (Fig. 3A, top row) and in the presence of positive control DNA (bottom row) by PCR and dot-blot hybridization. The internal control DNA was amplified in all cases (bottom row), demonstrating that the test samples did not inhibit PCR significantly. Target DNA was detected in at least six of the nine human fecal samples tested, as illustrated by the example shown in Fig. 3A. In addition, target DNA was detected in 30 to 50% of the nonhuman fecal samples. However, in most cases, the hybridization signals from nonhuman samples were weak compared with those from human samples. Only 10% were as strong as seen for the sheep fecal sample in Fig. 3A, indicating that the target *Bacteroides* species make up a lower proportion of the fecal flora in these nonhumans.

To quantify this observation, serial dilutions from each positive sample were prepared and assayed for target DNA by PCR and dot blot hybridization. As shown in Fig. 3B, only 10^{-11} g of DNA was needed to detect *B. distasonis* DNA from the human and sheep that gave strong hybridization signals in the original assay with 10^{-7} g (Fig. 3A). On the other hand, 1,000-fold more chicken and cow DNA, which gave much weaker signals in the original assay, was needed (Fig. 3). Therefore, the target *Bacteroides* species appear to be less common in most of the nonhumans.

In all, fecal samples were collected from 9 humans and 70 nonhumans and screened by the crude assay illustrated in Fig. 3A. All positive extracts were then diluted in series and assayed as illustrated in Fig. 3B, and the results are summarized graphically in Fig. 4. For most of the human fecal extracts tested, only 10^{-11} g (0.01 ng) of DNA was needed to detect *B. distasonis* or *B. thetaiotaomicron* and only 10^{-12} g (0.001 ng) of DNA was needed to detect *B. vulgatus*. Levels of target *Bacteroides* species were considered high if the assay was positive when using up to 10-fold more DNA than was needed for detection when using human feces. Therefore, levels of *B. distasonis* or *B. thetaiotaomicron* were considered high if either were detected when using only 10^{-10} g (0.1 ng) of DNA from fecal extracts, and levels of *B. vulgatus* were considered high if it was detected when using only 10^{-11} g (0.01 ng) of DNA. On the other hand, levels were considered low if detection required 100- to 1,000-fold more DNA than was needed for detection when using human feces. Therefore, levels of *B. distasonis* or *B. thetaiotaomicron* were considered low if 10^{-9} to 10^{-8} g (1 to 10 ng) of DNA was needed, and levels of *B. vulgatus* were considered low if 10^{-10} to 10^{-9} g (0.1 to 1 ng) was needed.

Target *Bacteroides* species were detected either at high levels or not at all in human feces. As shown in Fig. 4, high levels of either *B. distasonis* or *B. thetaiotaomicron* were found in 78% of the fecal extracts from humans and high levels of *B. vulgatus* were found in 67%. It should be noted that not all strains of the three target species were detected with the primers and probes designed for this study (Table 1), which may account for the inability to identify these species in all human fecal samples.

Low levels of the three target *Bacteroides* species were detected occassionally in nonhuman feces, and high levels, similar to those found in most human feces, were found in very few fecal samples from other animals. High levels of target DNA were detected in only 7, 9, or 11% of the nonhuman feces tested for *B. distasonis*, *B. thetaiotaomicron*, or *B. vulgatus*, respectively. However, high levels of target DNA were detected more frequently in some groups of nonhumans than in others. Fecal extracts from 25% of the house pets contained high levels of target DNA from *B. distasonis* (Fig. 4A, solid bars), and fecal extracts from 63% contained similarly high levels of target DNA from *B. vulgatus* (Fig. 4C, solid bars). Therefore, the level and frequency of *B. vulgatus* detection in cats and dogs was similar to that for humans. The latter result is not surprising, because house cats and dogs live in close contact with humans and undoubtedly ingest human-associated microorganisms on a regular basis. High levels of all three target *Bacteroides* species were detected in both samples of sheep feces collected from one of the farms; however, sheep feces from three other locations contained low or undetectable levels. Low levels of the target DNAs were found in 20, 15, or 12% of the nonhumans tested for *B. distasonis*, *B. thetaiotaomicron*, or *B. vulgatus*, respectively. Such low levels were detected most frequently in feces from swine as well as sheep (Fig. 4, hatched bars). None of the three target DNAs were found in feces from any of the 12 adult cows tested; however, at least one was detected in feces from each of the three immature cows tested.

To verify that the correct PCR products were detected by dot blot hybridization, all PCR products were analyzed on Southern blots with the same probes and hybridization conditions as for the dot blots. As shown for *B. distasonis* in Fig. 5A, some extraneous bands were visible on agarose gels used to size fractionate the PCR products. However, only products of the correct size were detected when these patterns were transferred onto nylon membranes and hybridized with the species-specific probes, as illustrated for *B. distasonis* in Fig. 5B. Therefore, the PCR-hybridization assays appear to detect their intended targets selectively.

Attempts to improve the specificity of the PCR-hybridization assays for selective detection of human feces proved futile

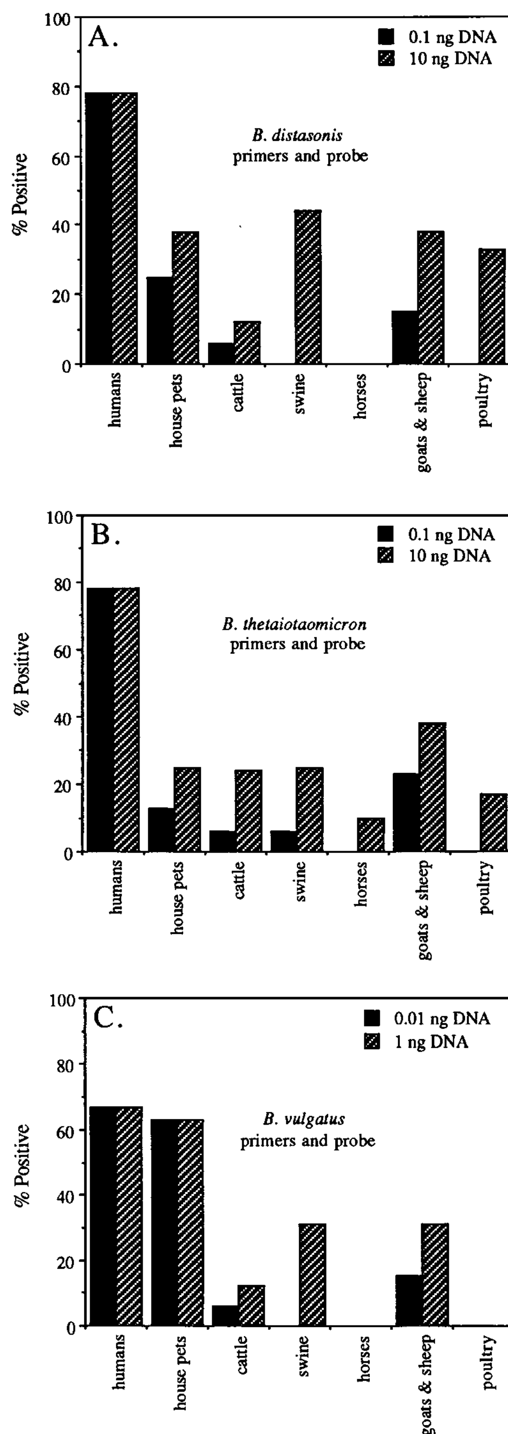

FIG. 4. Compiled results of PCR-hybridization assays with DNA from feces. The percentages of extracts from each group of animals listed in Table 2 that tested positive for *B. distasonis* or *B. thetaiotaomicron* with 10^{-10} g (0.1 ng) of DNA or for *B. vulgatus* with 10^{-11} g (0.01 ng) of DNA are plotted as solid bars. The percentages of extracts that tested positive for *B. distasonis* or *B. thetaiotaomicron* with 10^{-8} g (10 ng) of DNA or for *B. vulgatus* with 10^{-9} g (1 ng) of DNA are plotted as hatched bars. The significance of the amounts of DNA used is described in Results. (A) *B. distasonis* assays; (B) *B. thetaiotaomicron* assays; (C) *B. vulgatus* assays.

(data not shown). The upstream PCR primer for *B. distasonis* had a higher proportion of guanine and cytosine residues at its 3′ end than that recommended in the primer design software instructions (OLIGO 4.0), whereas the other primers conformed to specifications. Therefore, this upstream primer for *B. distasonis* would form strong bonds with nonspecific targets that were complementary only at the 3′ end where polymerization initiates, which could allow extension from nonspecific sites. Unfortunately, a second PCR primer that conformed to the recommended parameters gave results that were indistinguishable from those obtained with the original primer. Second, hot-start PCR, whereby a wax barrier was used to separate the *Taq* polymerase enzyme from other reaction components and prevent annealing and extension before optimum temperatures were attained, was also tested in an attempt to improve PCR specificity. However, all samples that had tested positive by the standard method were also positive when the hot-start technique was used. Finally, the specificity of the assay for *B. thetaiotaomicron* was improved by using a second, more phylogenetically specific hybridization probe. This second probe detected PCR products from the *B. thetaiotaomicron* type strain as well as the original probe had, but it was 10^4-fold less sensitive for detecting PCR products from the *B. ovatus* strains. It also showed a similar decrease in detection for four of the nine strains of *B. thetaiotaomicron*. Although this probe detected less target DNA in nonhumans, it showed a similar decrease in detection of target DNA from human feces. Therefore, specificity was improved, but only at the expense of sensitivity, and the second probe for *B. thetaiotaomicron* was not used further.

DISCUSSION

Assays were developed to identify *B. distasonis*, *B. thetaiotaomicron*, and *B. vulgatus* by PCR amplification of specific 16S rRNA gene sequences followed by hybridization detection of specific PCR products. The assays select for distinctive sequences at two stages, thereby enhancing specificity. Target DNA must have sequences complementary to the primers for amplification by PCR, and the PCR products must contain the third unique sequence for detection by hybridization. Results presented here demonstrate that the PCR-hybridization assays for *B. distasonis* and *B. vulgatus* detect their intended target species selectively, whereas that for *B. thetaiotaomicron* detects both *B. thetaiotaomicron* and *B. ovatus* (Fig. 1; Table 1). Because published results of rRNA-DNA hybridization assays show that 10 *Bacteroides* species, including these 4, are related (24), several strains from each of these species were tested by PCR-hybridization assay with probes for each of the three target species. All three assays detected most strains of their target species (Table 1). On the other hand, related species either were not detected or were detected only when greater than a 10^4-fold excess of DNA was tested. Other members of the *Cytophaga-Flavobacter-Bacteroides* phylum are more distantly related (15, 24, 33) and therefore were not tested.

The potential for these probes to distinguish between human and nonhuman sources of fecal pollution was tested by screening feces from a variety of animals (Table 2). Although a standard extraction procedure was sufficient to prepare PCR-compatible DNA from *Bacteroides* cultures (Fig. 2B), extensive purification of fecal extracts was required to yield DNA suitable for PCR (Fig. 2). Contaminants in fecal extracts, such as bilirubin and bile salts, are known to inhibit PCR (46). Phenolic compounds from plants may interfere as well (22). In addition, reactions with extracts that contain a very low proportion of target DNA have a large excess of oligonucleotide

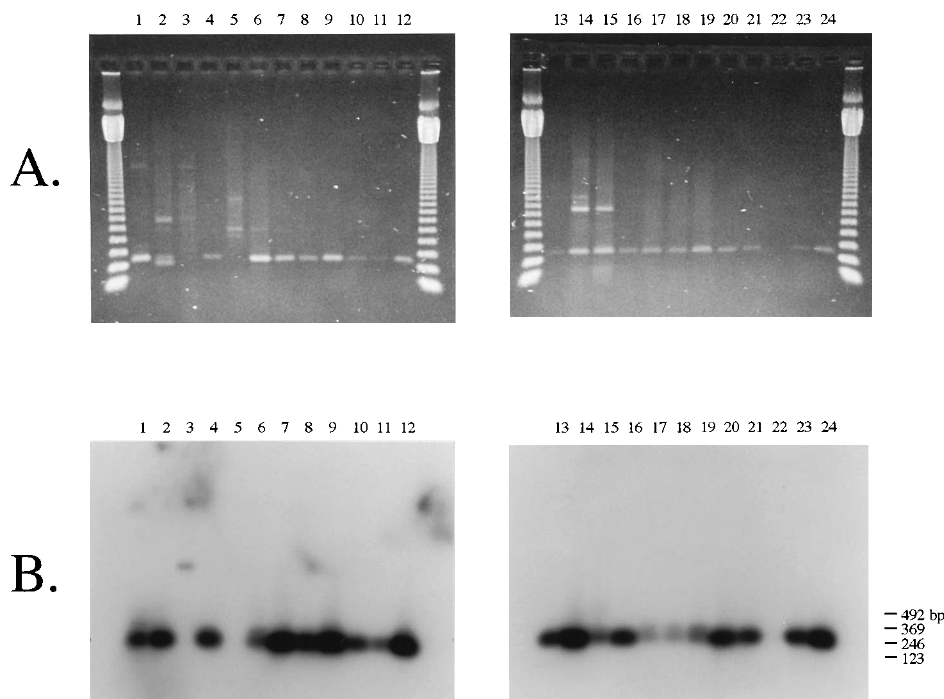

FIG. 5. Southern blot hybridization of PCR products from fecal extracts. PCR products from reactions giving positive results by dot blot hybridization were fractionated on 1.5% agarose gels, transferred to nylon membranes, and probed as for the dot blots. Assays with *B. distasonis* primers and probe are shown. A 123-bp ladder (GibcoBRL Life Technologies, Gaithersburg, Md.) was used for molecular size standards along both sides of the gels. The desired product from *B. distasonis* is 301 bp. (A) Ethidium bromide-stained agarose gels. (B) X-ray film showing hybridizing products detected by chemiluminescence. Lanes: 1, cat 4; 2 and 3, chickens 2 and 3; 4 to 6, cows 6, 12, and 14; 7 to 13, humans 1 to 6 and 9; 14 and 15, hogs 4 and 5; 16, rabbit; 17 to 21, sheep 5 to 9; 22 to 24, *B. distasonis* (0.1, 1, and 10 pg of template DNA). Lanes 3 and 5 are from reactions that also gave no signal by dot blot hybridization.

primers and nonspecific sequences compared with the specific target DNA. Primer-target contacts are less frequent than primer-primer and primer-nontarget contacts, and nonspecific products can form at the expense of target DNA (10). The use of a minimum level of target DNA as an internal positive control detects inhibition of PCR from either cause. However, if the internal control amplifies similarly in the presence and absence of test extract, neither type of inhibition is a problem.

Preliminary fractionation, to remove some sources of interference and nonbacterial DNA before lysis, and glass purification of DNA from the lysate were needed to eliminate inhibition from feces in most cases. Similarly labor-intensive purification schemes have been reported for successful PCR amplification of DNA from clinical, forensic, and environmental samples. Various combinations of ion-exchange and gel filtration resins (44), ultrafiltration (2, 45), organic extractions (4), precipitation methods (41), and gel purification (49) have been reported. Some of these procedures were tested in the present study, as mentioned in Results. A final purification involving adsorption of DNA to glass particles, as in the present work, has been reported by others using PCR to analyze fecal extracts (20, 50), environmental samples (36, 42), and clinical samples (26). However, simpler procedures must be developed before PCR can become a general monitoring or diagnostic tool. Immunomagnetic separation of target organisms from interfering impurities may be one solution (9, 16, 21, 34, 46, 48), but the general utility of this technique remains to be determined.

After eliminating interference, the PCR-hybridization assays for *Bacteroides* species distinguished human from nonhuman feces in most cases. Although the survey was not exhaustive, the results are encouraging. While 67 to 78% of human fecal samples tested contained high levels of the target species, only 7 to 11% of nonhuman fecal samples had similarly high levels. An additional 12 to 20% of the nonhuman fecal samples contained low levels of target DNA. Therefore, the target *Bacteroides* species seem to be more abundant and detected more frequently in humans than in nonhumans.

Although, for the most part, the three target *Bacteroides* species were detected in feces from individual nonhumans, they were more prevalent in some groups than others (Fig. 4). At one extreme, *B. vulgatus* was detected in feces from 63% of the house pets tested. Therefore, the probes for *B. vulgatus* would not be useful to distinguish sewer overflow from suburban runoff as sources of fecal pollution. On the other hand, the probes for *B. thetaiotaomicron* appear to distinguish feces from humans and house pets fairly well. Only 13% of the feces from house pets had high levels of target DNA for these probes, and an additional 12% had low levels. More significantly, all three target *Bacteroides* species were rarely detected in feces from cattle, swine, and poultry. At least 76% of the cattle, 56% of the swine, and 67% of the poultry had levels below detection limits (Fig. 4), or less than 1/1,000 of the level detected in humans. However, because large numbers of these farm animals are confined in small areas for feeding and meat production, they have a much greater impact than house pets on fecal pollution. In addition, one cow produces as much feces as 100 to 300 humans, whereas a hog produces as much as 20 humans (14, 17). Results of the survey indicate that all three sets of probes for *Bacteroides* species would distinguish human feces from the feces of these farm animals. However, since some individual nonhumans did have detectable levels of target *Bacteroides* species and their fecal output greatly exceeds that of

humans, further studies are needed to access whether these assays can be used to distinguish sewage from farm runoff.

The results of using DNA probes to detect *Bacteroides* species in feces agree quite well with those of earlier surveys reported by Geldreich (14) and by Allsop and Stickler (3) using culture techniques. In the earlier studies, members of the *Bacteroides* genus or of the "*Bacteroides fragilis* group" (*B. distasonis*, *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, and *B. vulgatus*) were found at the highest levels in feces from cats and dogs (10- to 100-fold lower than in humans), whereas levels in farm animals were much lower than in house pets or humans (10^3- to 10^5-fold lower than house pets). Similarly, the three target *Bacteroides* species were detected less frequently and generally at lower levels in farm animals than in house pets or humans in the present study.

The use of PCR detection of *Bacteroides* species to identify human sources of fecal pollution has several advantages over other tests for fecal pollution. The results of the survey indicate that the *Bacteroides* probes would be more useful than fecal coliform assays to distinguish sewage from farm runoff. According to data compiled by Geldreich (14), fecal coliform levels in hogs are similar to those in humans, whereas the levels in chickens and cattle are 10- and 50-fold lower, respectively. In contrast, target *Bacteroides* species were rarely detected in hogs, chickens, and cattle, and when detected, the levels were usually 1,000-fold lower than in humans. As mentioned above, the relative levels of fecal coliforms and fecal streptococci can also be used to distinguish sewage from farm runoff. However, this relationship deteriorates rapidly once the feces are dispersed into receiving waters, because survival of the predominant species in the two groups differs (13). Use of a single indicator organism would overcome this problem. Furthermore, DNA-based detection obviates the need to isolate target bacteria in culture. In fact, dead organisms are detected as well as live organisms, as long as the DNA remains somewhat intact. Salyers and coworkers developed specific probes to detect these *Bacteroides* species by hybridization assay (27, 28, 39). However, as the authors point out, hybridization (without prior amplification of the target DNA) is limited as a diagnostic tool by its lack of sensitivity. PCR overcomes the problem of sensitivity by amplification of target DNA in vitro.

Nonetheless, suitably simple procedures must be developed to overcome interference before PCR detection can be used to monitor fecal pollution. In addition, survival of target DNA in the environment must be investigated. The results of preliminary experiments indicate that *Bacteroides* DNA, detectable by PCR-hybridization assay, rapidly disappears after a day or two when whole human feces are dispersed into natural water (data not shown). Therefore, these results show that PCR detection of *Bacteroides* species to monitor human fecal pollution merits further study.

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