APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2003, p. 2651–2656 Vol. 69, No. 5
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.5.2651–2656.2003

Use of *Bifidobacterium dentium* as an Indicator of the Origin of Fecal Water Pollution

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Received 22 October 2002/Accepted 20 January 2003

A new, simple, and specific protocol to discriminate between human and animal fecal pollution is described. The procedure is based on the detection of certain *Bifidobacterium* species in the samples. Two 16S rRNA gene-targeted probes are described. One of these probes (BDE) has as its target a region of the 16S rRNA gene of *Bifidobacterium dentium*, a *Bifidobacterium* species of exclusively human origin. The other probe (BAN) is based on the sequence of a region of 16S rRNA gene for several *Bifidobacterium* species related with animal origins. The specificity of both probes was evaluated by using 24 *Bifidobacterium* species, and their threshold detection limit was established by DNA-DNA hybridization. DNA-DNA hybridization with the BDE probe showed it to be specific for *B. dentium*, whereas that with the BAN probe showed it to be specific for *B. animalis*, *B. asteroides*, *B. coryneforme*, *B. cuniculi*, *B. globosum*, *B. magnum*, *B. minimum*, and *B. subtile*. A simple and specific protocol was also developed for the detection of their target species in environmental samples (sewage and feces). DNA-DNA hybridization with the BAN probe was only positive for samples from cattle and goats. Thus, this probe is not suitable for the identification of any animal fecal pollution. Whereas all samples with human fecal pollution showed a positive DNA-DNA hybridization result with the BDE probe, none of those with animal fecal pollution did. Therefore, this finding supports the potential use of this probe in detecting fecal pollution of human origin.

Fecal pollution of aquatic environments causes their degradation and may affect human industries and activities related to water, such as bathing in recreational water, shellfisheries, and the supply of drinking water. Pathogens associated with fecal pollution may cause disease in humans. Despite efforts to minimize fecal input into the water cycle, the problem persists because of inefficient sewage treatment plants, leaking septic systems, agricultural runoff, and wildlife (2). In order to control the discharge, minimize its impact, and evaluate the risk to human health, it is important to identify the source of the pollution. The health risk associated with human exposure to water polluted with human feces is greater than that associated with human exposure to water polluted with animal feces (8, 26). However, some microorganisms of animal intestinal flora may be transmitted to humans and so cause disease (26).

Determining the origin of fecal pollution is also important in order to protect water supplies, to carry out epidemiological studies or, in the legal context, to decide who is responsible for having contaminated the environment (8, 21).

The most widely used fecal indicator microorganisms (coliforms, fecal coliforms, *Escherichia coli*, and enterococci) are found in both human and animal feces and thus do not allow source differentiation. New indicators have therefore been proposed that may provide information about both the presence and origin of fecal pollution. Several microbial and biochemical approaches to fecal source identification have been investigated. Biochemical indicators include fecal sterols, such as coprostanol, and washing powder components, such as fluorescent whitening agents, sodium tripolyphosphate, and long-chain alkylbenzenes (26). Some microbial indicators have also been proposed, including antibiotic-resistant enterococci phenotypes, *Bacteroides fragilis* bacteriophages, F-RNA bacteriophage subgroups, *Rhodococcus coprophilus*, *B. fragilis*, and *Bifidobacterium* spp. (26).

Bifidobacteria constitute a major part of the intestinal microflora in humans, as well as some animals (16, 17, 20). These gram-positive rods are strict anaerobes, have rigorous nutrient requirements, and grow poorly at temperatures below 30°C. Because of these characteristics the genus *Bifidobacterium* has been proposed as a microbial indicator (18, 22). Another characteristic of this genus is the different ecological distribution of its species. Some *Bifidobacterium* species are of human origin, whereas others are exclusively found in animals (3, 24). The detection of human-related *Bifidobacterium* species in a polluted sample could therefore indicate the human origin of the fecal pollution.

Several studies have proposed rapid identification methodologies for environmental *Bifidobacterium* strains in order to discriminate between human and animal origins. Scardovi et al. (25) analyzed the electrophoretic mobility of the fructose-6-phosphate phosphoketolase enzyme and concluded that its mobility varies according to the origin of the strain. Gavini et al. (6) found that growth at 45°C in Trypticase-phytone-yeast broth seemed to provide good discrimination between human and animal strains. Whereas the animal strains were able to grow at 45°C, most of the human strains could not. Mara and Oragui (15) described a new selective medium, human bifid-sorbitol agar, which was able to isolate sorbitol-fermenting strains. These strains were isolated mainly from human samples. These methodologies, based on the culture of bifidobacteria, may be limited by their anaerobic physiology. The use of

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TABLE 1. Specificity of DNA-DNA hybridization with the BDE and BAN probes

Strain	Specificity with probe[a]:	
	BDE	BAN
B. adolescentis DSM 20083^T	−	−
B. angulatum DSM 20098^T	−	−
B. animalis DSM 20104^T	−	+
B. asteroides DSM 20089^T	−	+
B. bifidum DSM 20456^T	−	−
B. boum DSM 20432^T	−	−
B. breve DSM 20213^T	−	−
B. catenulatum DSM 20103^T	−	−
B. choerinum DSM 20434^T	−	−
B. coryneforme DSM 20216^T	−	+
B. cuniculi DSM 20435^T	−	+
B. dentium DSM 20084^T	+	−
B. globosum DSM 20092^T	−	+
B. indicum DSM 20214^T	−	−
B. infantis DSM 20088^T	−	−
B. longum DSM 20219^T	−	−
B. magnum DSM 20222^T	−	+
B. minimum DSM 20102^T	−	+
B. pseudocatenulatum DSM 20438^T	−	−
B. pseudolongum DSM 20099^T	−	−
B. pullorum DSM 20433^T	−	−
B. subtile DSM 20096^T	−	+
B. suis DSM 20211^T	−	−
B. thermophilum DSM 20210^T	−	−

[a] Hybridization temperature, 60°C; probe concentration, 2.5 pmol/ml. −, negative hybridization; +, positive hybridization.

molecular rather than culture-based methods to detect them could overcome the problems associated with growing strict anaerobes.

One of the most widely used molecular approaches in ecological and taxonomic studies is the use of the rRNA molecule and its gene as a target (4, 9, 13, 31). The use of primers or probes based on the ribosomal DNA (rDNA) sequence has been useful in the detection and identification of certain species of *Bifidobacterium* in mixed populations, which could be difficult and not always feasible by phenotypic characterization (5, 20, 23, 27). Yamamoto et al. (29) also developed an identification methodology for five *Bifidobacterium* species present in the human intestine based on the use of 16S rRNA-targeted oligonucleotide probes. Subsequently, Langendijk et al. (12) and Kaufmann et al. (10) defined *Bifidobacterium* genus-specific probes for use in fluorescence in situ hybridization and colony hybridization. Wang et al. (28) designed species-specific 16S rDNA-targeted primers for the detection and quantification of predominant anaerobic bacteria in human and animal fecal samples.

The aim of the present study was to develop and evaluate a simple and efficient molecular methodology for discriminating between human and animal fecal pollution by using specific probes based on the 16S rRNA gene of human- and animal-related *Bifidobacterium* species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in the present study are listed in Table 1. *Bifidobacterium* strains were grown anaerobically at 37°C on Columbia blood agar (Difco, Detroit, Mich.) supplemented with 5 g of glucose and 0.5 g of L-cysteine-HCl/liter before sterilization and reinforced clostridial medium (Oxoid, Hampshire, England). The incubation periods were between 48 and 72 h, depending on the strain.

TABLE 2. Characteristics of the specific probes

Probe	Sequence	T_m[a] (°C)	Target site[b]
BDE	5′-ACG TCA CGG TGG GAA CTC A-3′	60	1132–1151
BAN	5′-CCG GTT CAC AGG TGG T-3′	52	1037–1053

[a] T_m, melting temperature.
[b] Corresponds to *E. coli* numbering of 16S rRNA gene (4).

Sampling procedures. Twenty-nine samples of urban sewage were collected from the entrance to three wastewater treatment plants and from two hospital sewage collectors. Additionally, 12 samples of animal sewage were taken from collectors of pig, cattle, and chicken slaughterhouses. All samples were transported at 4°C to the laboratory. A total of 28 human fecal samples were analyzed in the present study. Human fecal samples were obtained from 8 healthy children and 20 healthy male and female adults between 23 and 57 years old. Seventeen animal fecal samples were obtained from farm animals. All samples were kept at −80°C prior to analysis.

Design of 16S rDNA-targeted probes. Total 16S rRNA gene sequences of 21 *Bifidobacterium* species and 8 partial 16S rRNA gene sequences were retrieved from the EMBL and GenBank data libraries. Multiple alignments with all of the sequences were performed by using the GCG sequence analysis software package (University of Wisconsin, Madison). Since *Bifidobacterium dentium* has been described as exclusively of human origin (3, 24), one target region was selected for the definition of a species-specific oligonucleotide probe, BDE (Table 2). Another target region was selected for the design of an animal group-specific oligonucleotide probe (BAN). The sequence of this region was identical for a large number of animal strains of *Bifidobacterium*, whereas it was different for the recognized human strains (3). The characteristics of the BAN probe are shown in Table 2. The GenBank program BLAST (1) was used to ensure that the proposed probes were complementary with the target species but not with other species. The probes were synthesized, and their 5′ ends were labeled with digoxigenin (DIG; MGW-Biotech).

DNA extraction from pure cultures. DNA extraction from bifidobacteria was performed by using a previously described protocol (11). Reinforced clostridial medium was inoculated with a single colony and grown under anaerobic conditions up to a concentration of ca. 10^8 CFU/ml. Then, a 1-ml aliquot was harvested, and the cells were washed twice with sterile distilled water by centrifugation. Cells were finally suspended in 0.2 ml of solution A (100 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl; pH 8.3) and 0.2 ml of solution B (2.5 mM $MgCl_2$, 10 mM Tris-HCl, 1% Tween 20, 1% Nonidet P-40; pH 8.3). Proteinase K (Merck, Darmstadt, Germany) was added up to a final concentration of 120 μg/ml. After incubation at 55°C for 1 h, 0.2 ml of InstaGene Matrix was added to each of the extractions, and these were incubated at 55°C for 15 min. Proteinase K was inactivated at 100°C for 8 min. Cell debris was removed by centrifugation at 16,000 × *g*, and 2 μl of the supernatant was used for PCR analysis.

DNA extraction from samples. Before extracting DNA from fecal samples, the feces were suspended in an appropriate buffer (Ringer solution, 0.05 M potassium phosphate buffer [pH 7.0] or 0.25 M glycine buffer [pH 10.0]) in order to obtain a 1:10 dilution. The suspensions were homogenated for 1 h at 4°C. Then, 0.1-ml aliquots of the suspensions were centrifuged at 16,000 × *g* for 3 min. The pellets obtained were washed three times by suspending them in 1 ml of distilled water and centrifuging them at 16,000 × *g* in order to reduce the amount of PCR inhibitors.

Sewage samples were centrifuged at 250 × *g* for 15 min. Then, 1 ml of the supernatant was centrifuged at 16,000 × *g* for 3 min. In order to extract total DNA, pellets obtained from sewage and feces were processed as follows (16, 30). First, the pellets were suspended in 250 μl of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 μl of 10% sodium dodecyl sulfate (SDS) and then freeze-thawed. The extractions were incubated at 100°C for 5 min and then placed on ice for 5 min. Benzyl chloride (150 μl) was added to each extraction, and then the extractions were incubated at 50°C for 30 min, with vigorous agitation every 5 min. This product lyses the cellular wall of plants, fungi, and bacteria and, like phenol, is able to extract proteins and cellular debris from the watery phase of the extraction (30). Then, 150 μl of 3 M sodium acetate was added, and the mixtures were cooled on ice for 15 min. After centrifugation at 16,000 × *g* for 10 min, the supernatant was collected and DNA was obtained by

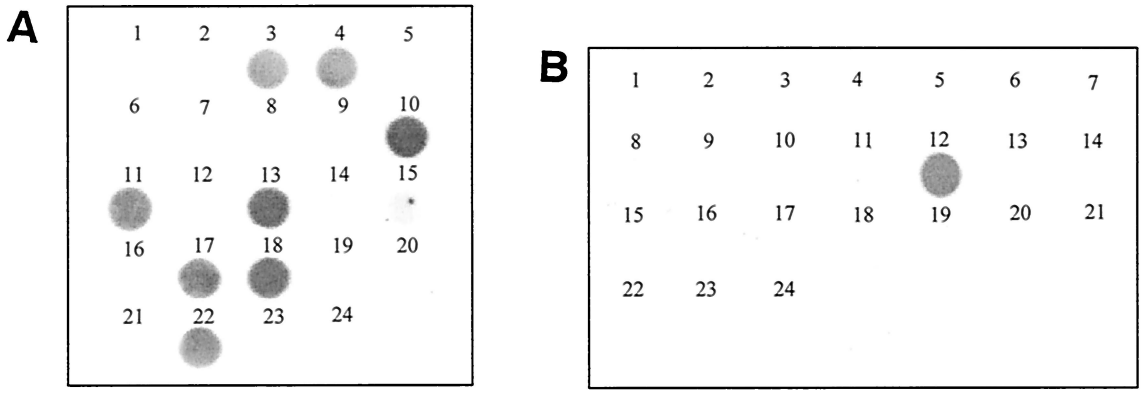

FIG. 1. DNA-DNA hybridization of *Bifidobacterium* type strains with the BAN (A) and BDE (B) probes. *Bifidobacterium* strains: 1, *B. adolescentis* DSM 20083^T; 2, *B. angulatum* DSM 20098^T; 3, *B. animalis* DSM 20104^T; 4, *B. asteroides* DSM 20089^T; 5, *B. bifidum* DSM 20456^T; 6, *B. boum* DSM 20432^T; 7, *B. breve* DSM 20213^T; 8, *B. catenulatum* DSM 20103^T; 9, *B. choerinum* DSM 20434^T; 10, *B. coryneforme* DSM 20216^T; 11, *B. cuniculi* DSM 20435^T; 12, *B. dentium* DSM 20084; 13, *B. globosum* DSM 20092^T; 14, *B. indicum* DSM 20214^T; 15, *B. infantis* DSM 20088^T; 16, *B. longum* DSM 20219^T; 17, *B. magnum* DSM 20222^T; 18, *B. minimum* DSM 20102^T; 19, *B. pseudocatenulatum* DSM 20438^T; 20, *B. pseudolongum* DSM 20099^T; 21, *B. pullorum* DSM 20433^T; 22, *B. subtile* DSM 20096^T; 23, *B. suis* DSM 20211^T; 24, *B. thermophilum* DSM 20210^T.

isopropanol precipitation. Finally, the DNA was suspended in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

PCR amplification. Each PCR mixture (50 μl) was composed of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.1% Triton X-100, each deoxynucleoside triphosphate at a concentration of 200 μM, the *Bifidobacterium* genus-specific primers lm26 and lm3 (10) at a concentration of 0.3 μM, 2 μl of bacterial DNA extraction, and 2 U of *Taq* DNA polymerase (Eppendorf, Hamburg, Germany). The PCR was carried out in a Perkin-Elmer thermal cycler (Perkin-Elmer, Norwalk, Conn.). The following amplification program was used: one cycle consisted of 95°C for 2 min; this was followed by 35 cycles of 94°C for 1 min, 67°C for 3 min, and 72°C for 4 min, with a final cycle of 72°C for 7 min. The amplification products were subjected to gel electrophoresis in 0.8% agarose, followed by ethidium bromide staining. Contamination of the samples with PCR inhibitors was checked by PCR amplification by using a mixture containing DNA extracted from *B. dentium* DSM 20084, the genus-specific lm26 and lm 3 primers, and 1 μl of the environmental sample (16).

DNA-DNA hybridization with DIG-labeled probes. The 16S rRNA gene amplimers were vacuum blotted onto nylon membranes (Hybond-N; Amersham Pharmacia Biotech) by using the Minifold I (Schleicher & Schuell, Dassel, Germany). DNA was fixed to the membranes by UV cross-linking. Prehybridization and hybridization with the DIG-labeled probe, BAN, or BDE were performed as described by the manufacturer (Boehringer Mannheim) at 60°C. The washing steps after hybridization were performed with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 60°C and 2× SSC–0.1% SDS at room temperature. The hybridization temperature was previously established as 60°C as follows. Three experimental temperatures (T_m, T_m −5°C, and T_m, +5°C) were used to hybridize dot blot membranes with the target species and possible interfering species. The optimum temperature for each probe was established as 60°C. Detection of the DIG-labeled DNA-DNA hybrids was performed with the DIG luminescence detection kit for nucleic acids (Boehringer), as described by the manufacturer. In order to confirm the presence of blotted 16S rRNA gene onto the nylon membranes, hybridization with a *Bifidobacterium* genus-specific probe (Bif) was performed as described earlier (19).

Threshold detection by specific hybridization. In order to determine the threshold of the *Bifidobacterium* detection by the hybridization procedure described above, a set of decimal dilutions was performed from a pure culture of the target species at a concentration of ~10^8 CFU/ml. The target species for the BDE probe was *B. dentium* DSM 20084, whereas that for the BAN probe was *B. globosum* DSM 20092^T. DNA extraction by using the benzyl chloride protocol was performed from each dilution, and the 16S rRNA gene was amplified with lm26 and lm3 primers as described above. The amplified 16S rRNA gene was fixed onto nylon membranes, and hybridization with the appropriate probe was performed according to the protocol described above. The detection limit of the hybridization procedure was also evaluated by using mixed cell suspensions. Decimal dilutions of a pure culture of the target species, obtained as previously described, were performed. Then, 1 ml of a culture, at a concentration of ca. 10^8 CFU/ml, of a potentially interfering species was added to each dilution to obtain the proportions 1:1, 1:10, 1:10^2, 1:10^3, 1:10^4, and 1:10^5. Interfering species were *B. globosum* DSM 20092^T for the BDE probe and *B. dentium* DSM 20084 for the BAN probe.

RESULTS

Specificity of probes. Figure 1 shows the DNA-DNA hybridization results for 24 *Bifidobacterium* species when the probes BAN (Fig. 1A) and BDE (Fig. 1B) were used. DNA-DNA hybridization with the BAN probe showed it to be specific when the hybridization temperature was 60°C, and it was positive for the following strains: *B. animalis* DSM 20104^T, *B. asteroides* DSM 20089^T, *B. coryneforme* DSM 20216^T, *B. cuniculi* DSM 20435^T, *B. globosum* DSM 20092^T, *B. magnum* DSM 20222^T, *B. minimum* DSM 20102^T, and *B. subtile* DSM 20096^T (Table 1). DNA-DNA hybridization with the BDE probe showed it to be specific for *B. dentium* DSM 20084 (Table 1) when the hybridization temperature was 60°C.

Threshold detection by specific hybridization. Figure 2 shows that the target species used for the BAN probe, *B. globosum* DSM 20092^T, was detected by DNA-DNA hybridization at a concentration of ca. 10^4 cells/ml (Fig. 1A). The BDE target species, *B. dentium*, was detected up to a concentration of ca. 10^3 cells/ml (Fig. 1B). In addition, the BAN probe was able to detect *B. globosum* when it was in a proportion of 1:10^2 with *B. dentium* (Fig. 1C), whereas the BDE probe was able to detect *B. dentium* when it was in a proportion of 1:10^4 with *B. globosum* (Fig. 1D).

Detection of target species in environmental samples. The results obtained from human and animal sewage and fecal samples are shown in Table 3. Use of the PCR technique with the *Bifidobacterium* genus-specific primers lm26 and lm3 enabled 16S rRNA gene amplification from all of the human sewage samples. These primers are located at both ends of the sequence of the bifidobacteria 16S rRNA gene, and their product length is 1.35 kb. Of the 29 samples of human sewage amplified by PCR, 27 gave a positive signal for DNA-DNA hybridization with the BDE probe, whereas none did so for hybridization with the BAN probe. Only one sample of urban

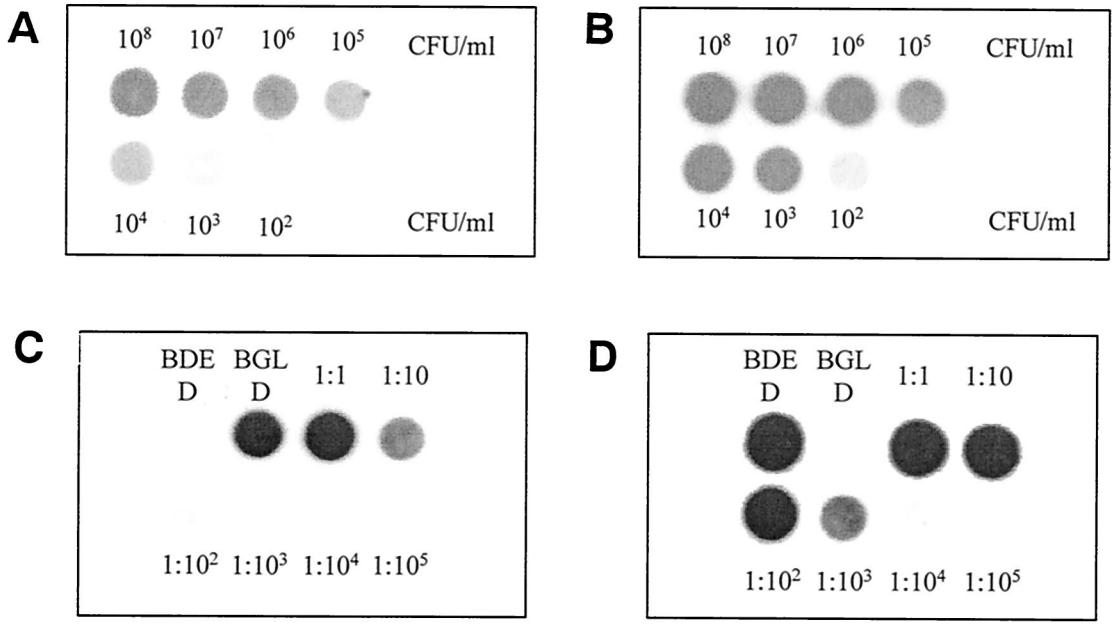

FIG. 2. Threshold detection by specific hybridization with the BAN probe with a pure culture of *B. globosum* DSM 20092^T (A) or a mixed suspension of *B. globosum* DSM 20092^T and *B. dentium* DSM 20084 (C) and with the BDE probe with a pure culture of *B. dentium* DSM 20084 (B) or a mixed cell suspension (D).

sewage and one sample of hospital sewage did not show a positive signal when hybridizing with the BDE probe.

When PCR with primers lm26 and lm3 was used to amplify the 16S rRNA gene from human feces (infants and adults) all of the samples gave the expected amplimer of 1.35 kb. Of the 20 samples of adult feces analyzed, 5 showed positive hybridization for *B. dentium* with the BDE probe. Four of the eight samples of infant feces were also positive. No human feces sample showed a positive result when hybridizing with the BAN probe.

All of the samples of animal feces were positively amplified with the *Bifidobacterium* genus-specific primers, except for two samples of pig feces, one sample from a pig slaughterhouse, and one sample from a cattle slaughterhouse. Moreover, the 16S rRNA gene of *B. dentium* was not amplified by PCR with the lm 26 and lm 3 primers when 1 μl of these samples was added to the PCR mixture. Amplified 16S rRNA gene was positive for hybridization with the genus-specific probe Bif, except for samples from rabbit and donkey feces. Samples from cattle and goat feces were positive when hybridized with the BAN probe.

No sample of animal feces or sewage showed a positive result when hybridized with the BDE probe. Only one sample from cattle slaughterhouse sewage showed a positive result for hybridization with the BDE probe.

DISCUSSION

Previous research has proposed that the genus *Bifidobacterium* be used to indicate the origin of fecal pollution (14, 15). This genus fulfills most of the requirements for being a fecal indicator. Moreover, some species are associated with human feces, whereas others have an exclusively animal origin (3). This duality in the ecology of *Bifidobacterium* species could be useful in distinguishing the origin of fecal pollution.

Several methodologies have been proposed for distinguishing between human and animal species of *Bifidobacterium*, most of them being based on growth on culture medium (6, 15). In the present study, a new identification procedure has been developed that depends on genotypic characteristics rather than growth on culture medium and phenotypic characteristics. Two 16S rDNA-targeted probes were designed. One of these probes, BDE, allowed *B. dentium*, a bifidobacterium species isolated exclusively from humans (3, 24), to be detected. The other probe, BAN, was able to detect a group of *Bifidobacterium* species isolated exclusively from blood-warm animals and honeybees (*B. animalis*, *B. asteroides*, *B. coryneforme*, *B. cuniculi*, and *B. globosum*), as well as two species isolated from sewage of uncertain origin (*B. minimum* and *B. subtile*). When the BDE probe's detection limit by dot blot hybridization was evaluated by using total bifidobacteria DNA extractions without previous PCR amplification of the 16S rRNA gene, the threshold was too high (i.e., ~10^8 CFU/ml [data not shown]). In order to increase the sensibility of the BAN and BDE probes, a PCR amplification step was added before hybridization. The BDE probe's detection threshold in DNA-DNA hybridization then fell to 10^3 CFU/ml. This threshold is similar to that obtained in studies of other bacteria (7). The detection limit of the BAN probe was 10^4 CFU/ml. When the detection limits were evaluated with mixed bacterial suspensions, one target cell in 100 nontarget cells was detected in the case of the BAN probe and one target cell in 10,000 nontarget cells was detected in the case of the BDE probe. A protocol for the detection of the target species of the BAN and BDE probes in environmental samples was defined and evaluated. Bacterial DNA was extracted successfully by using ben-

TABLE 3. Number of positive amplifications and DNA-DNA hybridizations with Bif, BAN, and BDE probes

Sample source	Subcategory	No. of samples	No. of positive amplifications or hybridizations			
			PCR[a]	Probe[b]		
				Bif	BAN	BDE
Urban sewage	Treatment plant 1	9	9	9	0	9
	Treatment plant 2	15	15	15	0	15
	Treatment plant 3	3	3	3	0	2
Hospital sewage	General	1	1	1	0	1
	Trauma	1	1	1	0	0
Human feces	Adults	20	20	20	0	5
	Infants	8	8	8	0	4
Slaughterhouse	Pig	4	3	2	0	0
Sewage	Cattle	4	3	3	2	1
	Chicken	4	4	ND	0	0
Animal feces	Pig	6	4	4	0	0
	Cattle	4	4	4	3	0
	Duck	1	1	1	0	0
	Goat	1	1	1	1	0
	Rabbit	1	1	0	0	0
	Chicken	1	1	1	0	0
	Asian pig	1	1	1	0	0
	Donkey	1	1	0	0	0
	Lamb	1	1	1	0	0

[a] Positive amplifications with the *Bifidobacterium*-specific primers lm26 and lm3.
[b] Positive DNA-DNA hybridizations with specific probes. ND, not determined.

zyl chloride and was used to perform a PCR amplification with lm26 and lm3 primers. The amplified 16S rRNA gene was fixed onto nylon membranes and these were used to perform DNA-DNA hybridization with the BAN and BDE probes. The presence of 16S rRNA gene from bifidobacteria was confirmed by its specific amplification from all of the environmental samples analyzed, except for two samples of pig feces, one sample of sewage from a pig slaughterhouse, and another sample from a cattle slaughterhouse. Although washing steps were introduced, PCR inhibitors could not be completely removed from these samples. The methodologies based on PCR amplification used with fecal samples are known to be difficult to perform due to the presence of inhibitors of the polymerase enzyme reaction (16).

All of the urban sewage samples presented positive hybridization with the BDE probe, except for one sample of hospital sewage and one sample of urban sewage. Routine washing and disinfection of hospital facilities could be influencing the bacterial population present and may explain the negative result in hospital sewage. The only negative result from urban sewage could be explained by the presence of *B. dentium* below the detection threshold in this sample. Positive hybridization of the BDE probe with almost all of the human sewage samples (27 of 29) supports the use of this probe in identifying human fecal pollution sources. Moreover, none of these samples presented positive hybridization for the BAN probe. None of the target species for the BAN probe were present in urban sewage or, if they were present they were at levels below the detection limit of the procedure. The detection of *B. dentium* in urban sewage by the procedure described here demonstrates that this species is present in such samples. Previous studies had argued that *B. dentium* could not be isolated from sewage samples (24). The use of culture media for the isolation of *B. dentium* may be behind the nonrecovery of this species. Our results demonstrate the presence of 16S rRNA gene from this species in sewage and thus lend support to the observations of Matsuki et al. (16). Although these authors did not detect *B. dentium* in human feces with culture media, they did detect it with species-specific 16S rDNA-targeted primers.

In the present study, 28 human fecal samples were also analyzed. A total of 5 the 20 adult feces samples and 4 of the 8 infant feces samples were positive for DNA-DNA hybridization with the BDE probe. This confirmed the presence of *B. dentium* in human feces. No human fecal sample was positive for the BAN probe. Given the results obtained for the BDE probe in human samples (sewage and feces), it can be concluded that *B. dentium* may be present in adult and infant feces and could be detected in sewage of human origin. Although *B. dentium* may not be present at the detection levels in the fecal samples of all humans, it seems that this species is present in urban sewage, which may be considered to be a pool of fecal samples from a human population.

Only two samples from cattle slaughterhouses were positive for the BAN probe. All animal samples were negative for the BDE probe except for one sample from a cattle slaughterhouse. This sample presented sorbitol-positive yellow colonies when spread on the selective medium human bifid-sorbitol agar (15), suggesting the presence of fecal pollution of human origin. Furthermore, a detailed examination of the sewage pipe network revealed that human sewage from toilet facilities was entering the site from where this sample was taken. These findings support the efficacy of the BDE probe in determining the origin of fecal pollution.

All samples of animal feces, which were amplified by PCR, showed positive hybridization with the Bif probe except for samples from a rabbit and a donkey. DNA degradation during manipulation steps may be the cause of these negative results. Only feces of cattle and goats showed positive hybridization with the BAN probe. Therefore, this probe cannot be used to detect fecal pollution of animal origin. However, none of the animal samples were positive for the BDE probe and, therefore, this probe may be used to detect fecal pollution of human origin.

In conclusion, a new, simple, and specific procedure to discriminate between human and animal fecal pollution has been described. The procedure is based on the detection of the 16S rRNA gene of *B. dentium* with a species-specific probe and does not require the use of a selective culture medium. Although the procedure may be limited by the presence of PCR inhibitors in some environmental samples with a large amount of fecal pollution, it is a reliable method for identifying the source of fecal pollution in water.

ACKNOWLEDGMENTS

This research was partially supported by the European project TOFPSW EVK1-2000-22080, the Generalitat de Catalunya (99GR169), and the Centre de Referència en Biotecnologia.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bernhard, A. E., and K. G. Field.** 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl. Environ. Microbiol. **66:**1587–1594.
3. **Biavati, B., B. Sgorbati, and V. Scardovi.** 1992. The genus *Bifidobacterium*, p. 817–832. *In* A. Ballows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes—a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Springer-Verlag, New York, N.Y.
4. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
5. **Dore, J., G. Gramet, I. Goderel, and P. Pochart.** 1998. Culture-independent characterisation of human faecal flora using rRNA-targeted hybridisation probes. Genet. Sel. Evol. **30:**287–296.
6. **Gavini, F., A. M. Pourcher, C. Neut, D. Monget, C. Romond, C. Oger, and D. Izard.** 1991. Phenotypic differentiation of bifidobacteria of human and animal origins. Int. J. Syst. Bacteriol. **41:**548–557.
7. **Hiney, M., M. T. Dawson, D. M. Heery, P. R. Smith, F. Gannon, and R. Powell.** 1992. DNA probe for *Aeromonas salmonicida*. Appl. Environ. Microbiol. **58:**1039–1042.
8. **Jagals, P., and W. Grabow.** 1996. An evaluation of sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution of environmental water. Water SA **22:**235–238.
9. **Kaneko, T., and H. Kurihara.** 1997. Digoxigenin-labeled deoxyribonucleic acid probes for the enumeration of bifidobacteria in fecal samples. J. Dairy Sci. **80:**1254–1259.
10. **Kaufmann, P., A. Pfefferkorn, M. Teuber, and L. Meile.** 1997. Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl. Environ. Microbiol. **63:**1268–1273.
11. **Kellogg, D. E., J. J. Sninsky, and S. Kwok.** 1990. Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction. Anal. Biochem. **189:**202–208.
12. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. F. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61:**3069–3075.
13. **Leblond-Bourget, N., H. Philippe, I. Mangin, and B. Decaris.** 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int. J. Syst. Bacteriol. **46:**102–111.
14. **Lynch, P. A., B. J. Gilpin, L. W. Sinton, and M. G. Savill.** 2002. The detection of *Bifidobacterium adolescentis* by colony hybridization as an indicator of human faecal pollution. J. Appl. Microbiol. **92:**526–533.
15. **Mara, D. D., and J. I. Oragui.** 1983. Sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution. J. Appl. Bacteriol. **55:**349–357.
16. **Matsuki, T., K. Watanabe, R. Tanaka, M. Fukuda, and H. Oyaizu.** 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. **65:**4506–4512.
17. **Matsuki, T., K. Watanabe, R. Tanaka, and H. Oyaizu.** 1998. Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. FEMS Microbiol. Lett. **167:**113–121.
18. **Muñoa, F. J., and R. Pares.** 1988. Selective medium for isolation and enumeration of *Bifidobacterium* spp. Appl. Environ. Microbiol. **54:**1715–1718.
19. **Nebra, Y., and A. R. Blanch.** 2002. The effect of reducing agents on the recovery of injured *Bifidobacterium* cells. J. Microbiol. Methods **49:**247–254.
20. **O'Riordan, K., and G. F. Fitzgerald.** 1997. Determination of genetic diversity within the genus *Bifidobacterium* and estimation of chromosomal size. FEMS Microbiol. Lett. **156:**259–264.
21. **Puig, A.** 1998. Influència de l'hoste en la recuperació de bacteriòfags de *Bacteroides fragilis* en el medi ambient. Ph.D. thesis. Universitat de Barcelona, Barcelona, Spain.
22. **Resnick, I. G., and M. A. Levin.** 1981. Assessment of bifidobacteria as indicators of human fecal pollution. Appl. Environ. Microbiol. **42:**433–438.
23. **Satokari, R. M., E. E. Vaughan, A. D. L. Akkermans, M. Saarela, and W. M. De Vos.** 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67:**504–513.
24. **Scardovi, V., and F. Crociani.** 1974. *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, and *Bifidobacterium angulatum:* three new species and their deoxyribonucleic acid homology relationships. Int. J. Syst. Bacteriol. **24:**6–20.
25. **Scardovi, V., B. Sgorbati, and G. Zani.** 1971. Starch gel electrophoresis of fructose-6-phosphate phophoketolase in the genus *Bifidobacterium*. J. Bacteriol. **106:**1036–1039.
26. **Sinton, L. W., R. K. Finlay, and D. J. Hannah.** 1998. Distinguishing human from animal faecal contamination in water: a review. N. Z. J. Mar. Freshwater Res. **32:**323–348.
27. **Ventura, M., R. Reniero, and R. Zink.** 2001. Specific identification and targeted characterization of *Bifidobacterium lactis* from different environmental isolates by a combined multiplex-PCR approach. Appl. Environ. Microbiol. **67:**2760–2765.
28. **Wang, R. F., W. W. Cao, and C. E. Cerniglia.** 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. **62:**1242–1247.
29. **Yamamoto, T., M. Morotomi, and R. Tanaka.** 1992. Species-specific oligonucleotide probes for five *Bifidobacterium* species detected in human intestinal microflora. Appl. Environ. Microbiol. **58:**4076–4079.
30. **Zhu, H., F. Qu, and L. H. Zhu.** 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. Nucleic Acids Res. **21:**5279–5280.
31. **Zoetendal, E. G., A. D. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854–3859.