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A Membrane-Array Method to Detect Specific Human Intestinal Bacteria in Fecal Samples Using Reverse Transcriptase-PCR and Chemiluminescence

KIM, PYOUNG IL, BRUCE D ERICKSON, AND CARL E. CERNIGLIA*

Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, 3900 NCTR Rd., Jefferson, AR 72079, U.S.A.

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Abstract A membrane-based oligonucleotide array was used to detect predominant bacterial species in human fecal samples. Digoxygenin-labeled 16S rDNA probes were generated by PCR from DNA that had been extracted from fecal samples or slurries. These probes were hybridized to an array of 120 oligonucleotides with sequences specific for 40 different bacterial species commonly found in human feces, followed by color development using an alkaline phosphatase-conjugated antibody and NBT/BCIP. Twenty of the species were detected by this method, but *E. coli*, which was present at ~1×10^5 CFU per gram feces, was not detected. To improve the sensitivity of this assay, reverse transcriptase-PCR was used to generate probes from RNA extracted from fecal cultures. Coupled with a chemiluminescence detection method, this approach lowered the detection limit for *E. coli* from ~1×10^6 to $\leq1\times10^5$. These results indicate that the membrane-array method with reverse transcriptase-PCR and chemiluminescence detection can simultaneously identify bacterial species present in fecal samples at cell concentrations as low as $\leq1\times10^5$ CFU per gram.

Key words: Human intestinal microflora, membrane array, RT-PCR, chemiluminescence detection

The human intestinal microflora plays a significant role in the digestion of food, metabolism of endogenous and exogenous compounds, immunopotentiation, vitamin and other essential nutrient production, and prevention of colonization by pathogens in the gastrointestinal tract [2, 4–6, 8–11, 33]. This bacterial population is composed of approximately 400 bacterial species, with 30–40 species accounting for up to 99% of the commonly detected intestinal microflora [8–9, 28]. Conditions that cause changes in the composition of the intestinal microflora may adversely affect the health of an individual. For example, researchers have reported antimicrobial effects of plant extracts on the human intestin bacteria [15, 18]. However, traditional culture and biochemical techniques for determining the composition of the microflora are labor-intensive, time-consuming, and of limited use for many bacterial species that are present. For rapid, specific, and sensitive detection of a broad range of bacterial species, 16S rRNA probe hybridization has become widely adopted for analysis of samples from various ecosystems, including water [1, 17, 24], sludge [16], and fecal samples [13, 19–21, 26, 27, 29–35, 37]. Recently, Wang *et al.* [37, 38] reported a membrane-array and microarray method for the simultaneous detection of human intestinal bacteria by using PCR-generated 16S rDNA probes and species-specific oligonucleotides [34–35]. This method has now been expanded to include 40 of the predominant species, using additional oligonucleotides and a microarray format [36, 38]. The purpose of this study was to improve the sensitivity of the method to detect bacterial species present in lower numbers. To this end, we developed a reverse transcriptase-PCR method for generating 16S rDNA probes and used chemiluminescence detection to improve the sensitivity of the assay.

Materials and Methods

Fecal Samples and Culture Conditions

Bacterial strains assayed in this study are listed in Table 1 [38]. Fresh fecal samples were obtained from healthy volunteers, and transferred to an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI, U.S.A.) under 85% N_2, 10% H_2, and 5% CO_2. To select a medium for

*Corresponding author
Phone: 870-543-7341; Fax: 870-543-7307;
E-mail: ccerniglia@nctr.fda.gov

Table 1. Oligonucleotide numbers, target bacterial species, and sequences for the 120 oligonucleotides.

Oligo #	Bacterial strain	Oligo sequence
1	*Bacteroides thetaiotomicron* ATCC 29148	Aatacccgatggtataatcagaccgcatggtttgttatta
2	"	Cattaggcagttggtgaggtaacggctcaccaaaccttcg
3	"	Cagctacctggtgacaggatgctaatcccaaaagcctctc
4	*B. vulgatus* ATCC 8482	Aaggaataaagtcgggtatggataccgtttgcatgtactt
5	"	Agatgaattacggtgaaagccgtaagccgcaaggcatctg
6	"	Tgttgtcagttactaacaggttatgctgaggactctgaca
7	*B. fragilis* ATCC 23745	Ccgatagcataatgattccgcatggtttcattattaaagg
8	"	Tcgtaaacttcttttatataagaataaagtgcagtatgta
9	"	Gaaggcagctagcgggtgaccgtatgctaatcccaaaat
10	*B. distasonis* ATCC 8503	Gcgggacgtgtcccgttttgtatgtaccttatgaataagg
11	"	Ttcggaccgaggtggaaacacctttctagcaatagccgt
12	"	Aggccacctggcgacagggagcgaatccccaaaccacg
13	*Clostridium clostridioforme* ATCC 29048	Agtgccgcatggcagtgtgtgaaaaactccggtagtgtga
14	"	Gaagcaagtctgaagtgaaaacccagggctcaaccctggc
15	"	Cccctgacggccggtaacgcggccnttcttcgggacaggg
16	*C. leptum* ATCC 29065	Ctctgttcttagtgacgataatgacggtagctaaggagaa
17	"	Tctatgggcttaacccataaactgcgcttgaaactgtctt
18	"	Caaagccgcgaggtggagcaaaaccctaaaagcagtcc
19	*Faecalibacterium prausnitzii* ATCC 27768	Gtcgaacgagcgagagaggagcttgctttctcaagcgagt
20	"	Cctgcgacgcgcatagaaatatgtgtttcttcgggaccag
21	"	Gagaagcaagaccgcgaggcgagcaaaactcagaaacttcg
22	*Ruminococcus productus* ATCC 27340	Taagacggatttcttcggattgaagtctttgtgactgagc
23	"	Ggaagagcaagtctgatgtgaaaggctggggcttaacccca
24	"	Cctctgaccgtc ccgtaacggg ganttcccct cggggcaga
25	*Ruminococcus obeum* ATCC 29174	Aacctttcattgaagcttcggcagatttggtctgtttcta
26	"	Gtcccttaaccggatctttccttcgggacaggggagacag
27	"	Cctatccccagtagccagcagtccggctgggcactctgag
28	*R. bromii* ATCC 27255	Gaatgctaataccgcatgacatatcggaaccacatggttc
29	"	Cttcttttattaaggacgaaaaatgacggtacttaatga
30	"	Taatacccgaagtcagtagtccaacctcgtgaggacgctg
31	*R. callidus* ATCC 27760	Catggattcgcatgtttctgtgatcaaagatttatcgcttaga
32	"	Tgaagaggacgataatgacggtactcttttagaaagctc
33	"	Aaagccggtcgtctaaccttcgggaggatgccgtctaagg
34	*R. albus* ATCC 27210	Ccaattggaaacgattgttaatacctcataacataacgaat
35	"	Agagggaagcaaaacagtgatgtggagcaaaacccta
36	"	Cctgtgttctaaccgcaaggaggaagcagtcgaaggtgg
37	*Bifidobacterium longum* ATCC 15687	Cttgatggcggggtaacggcccaccatggctttgacgggt
38	"	Ggcttgacatgttcccgacgatcccagagatggggtttcc
39	"	Agccggtggcctaacccttgcgggagggagccgtctaatg
40	*B. adolescentis* ATCC 15703	Ggatcggctggagcttgctccggccgtgagagtggcgaa
41	"	Ctccagttggatgcatgtccttctgggaaagattctatcggt
42	"	Caacgggatgcgacctcgtgaggggggagcggatccctt
43	*B. infantis* ATCC 15697	Tcgaacgggatccatcgggctttgcttggtggtgagagtg
44	"	Ccagttgatcgcatggtcttctgggaaagctttcgcggta
45	"	Caacgggatgcgacgcggcgacgcggagcggatccctga
46	*Eubacterium biforme* ATCC 27806	Aagagaaaaacgacattcatagggaatgatgagtgagtgat
47	"	Gtgatatgttactaacattgagttgaggactcatatcaga
48	"	Agagcggcaagcctgtgaaggcaagcgaatctcataaagga
49	*Collinsella aerofaciens* ATCC 25986	Gcccgaaaggacgggtaataccggatacccc gggggtgc
50	"	Cggcaggccgggggtcgaagcgggggctcaacccccgaa
51	"	Atgggtgaagcgggggagacccgtggccgagaggagcccata
52	*Lactobacillus acidophilus* ATCC 4356	Tgaaccaacagattcacttcggtgatgacgttgggaaacgct
53	"	Gcaatccgtagagatacggagttcccttcggggacacta
54	"	Acagtacaacgaggagcaagcctgcgaaggcaagcg

Table 1. Continued.

Oligo #	Bacterial strain	Oligo sequence
55	*Escherichia coli* ATCC 25922	Acaggaagaagcttgctctttgctgacgagtggcgga
56	"	Ggaagggagtaaagttaataccttgctcattgacgttac
57	"	Catccacggaagttttcagagatgagaatgtgccttcgg
58	*Enterococcus faecium* ATCC 19434	Tgatttgaaaggcgctttcgggtgtcgctgatggatggac
59	"	Gaagaacaaggatgagagtaactgttcatcccttgacgg
60	"	Gaagtacaacgagttgcgaagtcgcgaggctaagctaat
61	*Bacteroides uniformis* ATCC 8492	Gcatgaacttagcttgctaagtttgatggcgaccggcg
62	"	Atggcatagttcttccgcatggtagaactattaaagaa
63	"	Acgggaataaagtgaggcacgtgtgccttttgtatgtac
64	*B. ovatus* ATCC 8483	Tagtttgttggcggggtaacggcccaccaagactacgatg
65	"	Ggtcaatgggcgagagcctgaaccagccaagtagcgtg
66	"	Caacagaatatattggaaacagtatagccgtaaggctgt
67	*B. caccae* ATCC 43185	Gaaagattaatatccgatagcatatatttcccgcatggg
68	"	Aagtggtccacgtgtggacttttgtatgtaccatatgaat
69	"	Aatgaattatggggaaacccatacgccgcaaggcatntg
70	*Clostridium perfringens* ATCC 13124	Gaaggttttcggatcgtaaagctctgtctttggggaagat
71	"	Tgcattactcttaatcgaggaaatcccttcggggacaagg
72	"	Caacgagcgcaacccttgtcgttagttactaccattaagt
73	*C. butyricum* ATCC 19398	Gcataagattgtagtaccgcatggtacagcaattaaagg
74	"	Tactctgtaatggaggaagccacttcggtggcaggaaga
75	"	Tcggtacaatgagatgcaacctcgcgagagtgagcaaaa
76	*C. ramosum* ATCC 25582	Tgcctcaaagcactggtagaggatggacttatggcgcat
77	"	Aagaagaacggcggctacaggaaatggtagccgagtga
78	"	Actcataaaggctccagagatggagagatagctatatga
79	*C. difficile* ATCC 9689	Ttgccaagccgtaaggtggagctaatcccttaaagctac
80	"	Atgctaatacgggataatatatttgagaggcatctcttga
81	"	Gcttgacatcccaatgacatct ccttaatcggagagttcc
82	*C. indolis* ATCC 25771	Gacggcgatgcaagtctggagtgaaagcccggggctcaac
83	"	Gaccggtccgtaacggggccttcccttcggggcattccag
84	"	Aacaaagggaagcaaaggagtgatctggagcaaaccccaa
85	*Fusobacterium russii* ATCC 25533	Tcattgcatgatgaagtcatgaaagctataagcgctgtga
86	"	Taagggctcagagatgagcttgtgctccttcgggagaaag
87	"	Gaacagagagtggcgaagctgtgaagtggagcaaatctc
88	*F. nucleatum* ATCC 25586	Tctacttgaatttgggttttttaacttcgatttgggtggcg
89	"	Tgatattatgattatagggcatcctagaattatgaaagct
90	"	Aggaatgagacagagatgtttcagtgtcccttcggggaa
91	*Bifidobacterium catenulatum* ATCC 27539	Ggtagtcggcggggtaacggcccaccgagcctacgacg
92	"	Atgccggatgctccgactcctcgcatggggtgtcgggaa
93	"	Gacatgttcccgacagccgtagagatacggtctcccttc
94	*B. angulatum* ATCC 27535	Tcgaacgggatcggctggagcttgctccggccgtgagag
95	"	Tgctccagtccatcgcatggtggtctgggaaagattttat
96	"	Acatgttcccgacagccccagagatggggcctcccttcgg
97	*Eubacterium rectale* ATCC 33656	Agcactttatttgatttccttcgggactgattattttgtg
98	"	Ccttctgaccggtacttaaccgtaccttctcttcggagcag
99	"	Gtaaacaaagggaagcaaagctgtgaagccgagcaaatc
100	*E. eligens* ATCC 27750	Gcatttacgaacagattatttcggtatgaagttcctttatg
101	"	Cttgtactgggggatagcagctggaaacggctggtaatac
102	"	Cgcacaatgttgcatgacatggtgtgaaaaactccggtgg
103	*E. limosum* ATCC 8486	Ggttttgaatgatccttcgggtgaaattagaactggaaag
104	"	Ttatggttttgtcgcatggcgagatcatgaaaactccggtg
105	"	Ctgacgagcctagagataggaagtttccttcgggaacaga
106	*Eggerthella lenta* ATCC 25559	Aggtcgagcgatgaaaccgccctcgggcggacatgaag
107	"	Tgctccggacaaccttgggaaaccgaggctaatatccgca
108	"	Gacgtgaagccggggaaacccggtggctgagaggagcgt

Table 1. Continued.

Oligo #	Bacterial strain	Oligo sequence
109	*Lactobacillus fermentum* ATCC 9338	Attgattgatggtgcttgcacctgattgattttggtcgcca
110	"	Aacaacgttgttcgcatgaacaacgcttaaaagatggctt
111	"	Tgttaaagaagaacacgtatgagagtaactgttcatacgt
112	*Enterococcus faecalis* ATCC 27274	Tgccgcatggcataagagtgaaaggcgctttcgggtgtc
113	"	Caaggacgttagtaactgaacgtcccctgacggtatctaa
114	"	Gaagtacaacgagtcgttagaccgcgaggtcatgcaaatc
115	*Finegoldia magna* ATCC 14955	Cgcgtggacaacctgcctatgacagtgggatagcctcggg
116	"	Gtttaataagtcgaatgttaaagatcggggctcaaccccg
117	"	Agcattggaaactgataaacttgagtagtggagaggaaa
118	*Ruminococcus gnavus* ATCC 29149	Agcaccttgacggatttcttcggattgaagccttggtgac
119	"	Gcataagcgcacagtaccgcatggtacggtgtgaaaaac
120	"	Aactgtcaggctagagtgtcggagaggaaagcggaattc

growth of the fecal bacteria, brain heart infusion broth supplemented with vitamin K and hemin (BHI; Remel, Lenexa, KS, U.S.A.) and Gibson's medium [12] were used. Two other media for intestinal bacteria were Medium 1 [5 g starch, 3 g casein, 5 g beef extract, 0.6 g pectin, 0.6 g xylose, 5 g dextrose, 0.4 g L-cysteine, 1 ml vitamin K (10 mg ml^{-1}), 0.005 g hemin, 2 g Tween 80, 2 g KH_2PO_4, 10 g $NaHCO_3$, 4.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.45 g $CaCl_2 \cdot H_2O$, per liter] and Medium 2 [5 g starch, 3 g casein, 5 g peptone, 3 g soytone, 5 g dextrose, 2.5 g yeast extract, 0.4 g L-cysteine, 1 ml vitamin K (10 mg ml^{-1}), 0.005 g hemin, 2 g Tween 80, 2 g K_2HPO_4, 10 g $NaHCO_3$, 4.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.45 g $CaCl_2 \cdot H_2O$, per liter] were tested. Fecal samples (20 g) were suspended in 40 ml of medium, filtered through sterile cheesecloth, and transferred to serum bottles for incubation at 35°C in the anaerobic chamber. Each day, 50% of the culture was removed, transferred to vials, and frozen in 10% glycerol. Fresh medium was added to the remaining culture in a volume equal to that removed. Classical culture analysis of fecal samples or fecal cultures was performed by 10-fold dilution series in phosphate buffered saline (PBS, pH 7.4) and plating on selective and nonselective media. Media used for plating studies included brain heart infusion agar supplemented with vitamin K and hemin (BHI), Brucella blood agar with vitamin K and hemin (Brucella), Bacteroides bile esculin agar (BBE), phenylethyl alcohol agar (PEA), and kanamycin-vancomycin-laked blood agar (LKV) for a wide variety of anaerobic bacteria. Bile esculin azide agar (BEA) was used for *Enterococci* and MacConkey agar for *E. coli*. All plate media were pre-reduced overnight in the anaerobic chamber, and cultures were spread under anaerobic conditions. BHI, Brucella, BBE, PEA, and LKV plates were incubated anaerobically at 35°C, while duplicate BEA, MacConkey, and duplicated BHI plates were incubated aerobically at 35°C. Bacterial isolates were identified using the VITEK (bioMerieux, Durham, NC, U.S.A.) and MIDI (MIDI, Inc., Newark, DE, U.S.A.) systems.

Oligonucleotide Probes and Preparation of the Membrane Array

Three 40-mer oligonucleotides specific for each of 40 predominant intestinal bacteria (120 oligonucleotides total) (Table 1) were used as described by Wang *et al.* [38]. The first consideration for probe design was to choose the region specific to each species, then consider the hairpins and dimers of the oligonucleotides. The oligonucleotides were resuspended in TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to 250 ng μl^{-1}. Xylene cyanol (1 mg ml^{-1}) was added for visibility. The resuspended oligos were heated in a boiling water bath for 2 min, then immediately cooled in ice-water. Each oligonucleotide (0.5 µl) was applied to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.) in a 12×10 array. After air drying and UV-crosslinking, the membranes were heated at 80°C for 2 h and stored in a desiccator at room temperature.

Genomic DNA Extraction and PCR Amplification of 16S rDNA

The bacterial genomic DNA was extracted from feces or fecal cultures using the QIAamp® DNA Stool Mini Kit (QIAGEN, Valencia, CA, U.S.A.). Two grams of feces were resuspended in 8 ml of PBS, and DNA was extracted from 400 µl of the fecal slurry. PCR amplification was performed using a GeneAmp 9700 PCR system (Perkin Elmer, Norwalk, CT, U.S.A.). Digoxygenin (DIG)-labeled probes from the 16S rDNA were generated in a 50 µl PCR reaction mixture that consisted of 33.2 µl H_2O, 5 µl 10× BSA buffer (1 ml 10× buffer is composed of 0.5 ml 1 M Tris-HCl, pH 8.5, 0.2 ml 1 M KCl, 30 µl of 1 M $MgCl_2$, 0.27 ml of water, and 5 mg BSA), 2.5 µl dNTP mix (2 mM each dNTP), 2.5 µl DIG dNTP mix from the PCR DIG Probe Synthesis Kit (Roche, Indianapolis, IN, U.S.A.), 2.4 µl primers Amp-F and Amp-R, 0.4 µl Taq DNA polymerase, and 4 µl bacterial DNA. The sequences of Amp-F and Amp-R were 5'-GAGAGTTTGATYCTGGCTCAG-3' and 5'-AAGGAGGTGATCCARCCGCA-3', respectively [37-

38]. The PCR program was 95°C for 3 min followed by 35 cycles of 95°C for 10 sec, 53°C for 10 sec, and 72°C for 70 sec, with a final 4 min extension at 72°C. The DIG-labeled PCR product was heated in a boiling water bath for 3 min and immediately cooled in ice-water before use.

Hybridization and Detection by Color Development

The membrane-bound oligonucleotides were hybridized with the DIG-labeled 16S rDNA PCR products [37]. The membranes were pre-hybridized in a petri dish including DIG Easy Hyb solution (Roche, Indianapolis, IN, U.S.A.) at 42°C for 2 h with gentle shaking. The membrane was then transferred to fresh DIG Easy Hyb solution which was pre-heated to 42°C and contained the DIG-labeled 16S rDNA PCR products, and hybridized overnight with gentle shaking. The membranes were washed twice for 5 min with 2× SSC and 0.1% SDS at room temperature, and twice for 15 min with 0.1× SSC and 0.1% SDS. Bound DIG-labeled probe was visualized with an alkaline phosphatase-conjugated anti-digoxygenin antibody and NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) using the DIG Wash and Block Buffer Kit and the DIG Nucleic Acid Detection Kit (Roche, Indianapolis, IN, U.S.A.) according to the instructions of the manufacturer.

RNA Extraction and DIG-labeled RT-PCR of 16S rRNA

One gram (wet weight) of fecal sample was homogenized in 9 ml of PBS. Large particles from the fecal suspension (1.5 ml aliquot) were removed at low-speed centrifugations (200 ×*g* for 10 min). This centrifugation step was repeated three times, and then upper phases were centrifuged at 7,000 ×*g* for 10 min. The pellet was washed four times with PBS and twice with water, and then was used for RNA extraction. Total bacterial RNA was extracted from the sample using the Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, U.S.A.). Several different RNA concentrations were tested for optimal RT-PCR amplification. RNA samples were adjusted to 25 ng μl^{-1} with RNase-free H_2O. The RT-PCR mixture (50 µl) was composed of 31.8 µl water, 5 µl 10× PCR buffer (500 mM Tris-HCl, pH 8.3, 400 mM KCl, 80 mM $MgCl_2$, and 10 mM DTT), 2.5 µl dNTP mix (2 mM each of dNTP), 2.5 µl of DIG dNTP mix, 1 µl of RNase inhibitor (20 U μl^{-1}), 2.4 µl of primers Amp-F and Amp-R, 1 µl of Taq DNA polymerase, 1 µl of AMV reverse transcriptase, and 1 µl of bacterial RNA. The RT-PCR conditions were 48°C for 50 min, 95°C for 2 min, 35 cycles of 95°C for 10 sec, 53°C for 10 sec, and 72°C for 70 sec, and finally one cycle of 72°C for 4 min.

Hybridization and Chemiluminescence Detection

One-half µl of the 120 species-specific oligonucleotides were applied to a nylon membrane (Schleicher & Schuell, Keene, NH, U.S.A.) in a 12×10 array. The membranes were pre-hybridized, hybridized with the RT-PCR probe, washed, and reacted with Wash and Block buffer, as described above. To detect the bound probe by luminescence, the CSPD reagent (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) (Roche, Indianapolis, IN, U.S.A.) was diluted in detection buffer 1:100, and then added to the membrane. Hyperfilm™ X-ray film (Amersham Biosciences, Piscataway, NJ, U.S.A.) was exposed to the membrane for 5 h at room temperature before development.

Determination of Detection Limit

To determine *E. coli* cell numbers in the fecal samples, fresh feces or fecal cultures were suspended in PBS and a 10-fold dilution series was prepared. One-tenth ml aliquots were spread onto MacConkey agar plates and incubated at 35°C, and colonies were counted after 16–20 h. For pure cultures, *E. coli* ATCC 25922 was grown overnight in Luria Bertani broth at 35°C. The cells were then pelleted and resuspended in PBS. Bacterial cell numbers were determined by direct counting using a Petroff-Hausser counting chamber (A. Daigger, Wheeling, IL, U.S.A.). The cell concentration was adjusted as needed, and 0.2 ml of cell suspension containing *E. coli* cells (10^4–10^8) was added to 0.2 ml of fecal slurry before nucleic acid extraction. DIG-labeled rDNA probes were amplified as described, and then hybridized to the three *E. coli* oligonucleotides spotted on the nitrocellulose strips. Three oligonucleotides specific for *Bacteroides ovatus* were added on the nitrocellulose strips as a positive control. The same oligonucleotides were spotted onto nylon membrane strips and probed with RT-PCR products generated from RNA preparations extracted from fecal slurries. The bound probes were visualized by color development for the PCR reactions and chemiluminescence for the RT-PCR reactions, as described above.

RESULTS

Selection of Growth Medium

A fed-batch culture approach was used to propagate fecal cultures in four different media for seven days, and the complexity of the culture was estimated by plating onto selective and nonselective media on days 0, 4, and 7 (Fig. 1). No changes in total anaerobic populations in Gibson's medium, Medium 1, and Medium 2 were observed, as colonies were enumerated on anaerobic BHI and Brucella agar plates. Total facultative anaerobic populations increased in Gibson's medium and Medium 2, but remained unchanged in BHI broth and Medium 1, as determined on aerobic BHI plates. *E. coli* populations in BHI medium and Medium 1 were decreased by 1.75 log and about 2 log, respectively.

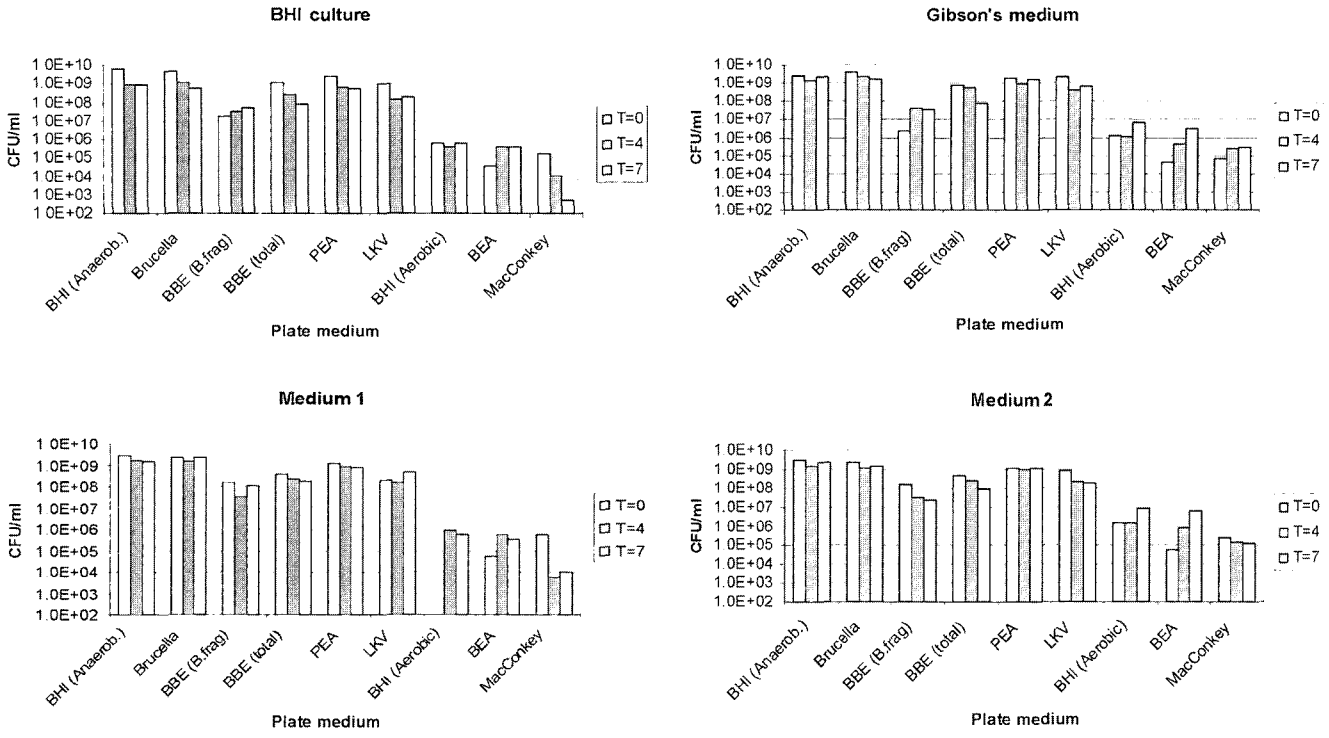

Fig. 1. Fed-batch test of human intestinal microflora in four media under anaerobic conditions.
After culture for 0, 4, and 7 days, bacterial colonies were counted on selective and nonselective agar plates. BBE (B. frag) and BBE (total) mean *Bacteroides fragilis* group and total colonies counted from Bacteroides Bile Esculin plates, respectively. Duplicate supplemented Brain Heart Infusion (BHI) agar plates were spread; one set was incubated anaerobically and the other aerobically. *E. coli* were determined from MacConkey plates.

However, Gibson's medium and Medium 2 maintained *E. coli* populations, as determined by cell count on MacConkey plates. From the above results, Gibson's medium and Medium 2 were selected for culture of the intestinal bacteria and further study. Day 0 and day 7 samples of the Gibson's medium and Medium 2 were tested using the membrane array (Table 2). Identification of colony isolates from selective and nonselective plating showed a diverse community including multiple species of *Bacteroides*, several *Bifidobacterium* species, *Collinsella aerofaciens*, *Faecalibacterium prausnitzii*, *Propionibacterium acnes*, *Coprococcus comes*, *Enterococcus* spp., and *E. coli* (data not shown). *E. coli* was readily detected by plating techniques, but was not positive by the membrane-array method, presumably because it was present below the detection limit of the assay.

Reverse Transcriptase-PCR and Chemiluminescence Detection of Bacteria Using the Membrane Array

The published method for the membrane array uses PCR amplification of the 16S rRNA genes to generate nearly full-length 16S rDNA probes [37]. Reverse transcriptase-PCR (RT-PCR) using the same 16S rRNA primers was examined to see if the increase in template molecules would improve detection of the less numerous species. Separate aliquots from the same fecal sample were used to prepare total fecal DNA and RNA. The fecal DNA was used to generate DIG-labeled 16S rDNA probes representing the populations, including *Bacteroides thetaiotaomicron*, *B. vulgatus*, *B. distasonis*, *Ruminococcus obeum*, *R. bromii*, and *R. albus*, as previously described [37]. The fecal RNA preparation was used to generate the same type of 16S rDNA probes, using a reverse transcriptase reaction followed by PCR amplification. Membranes spotted with 120 oligonucleotides were then hybridized with either the PCR-generated probes or the RT-PCR-generated probes. The PCR-generated probes were visualized with NBT/BCIP-mediated color development, while the RT-PCR-generated probes were visualized using a chemiluminescence method (Table 3). For this experiment, 21 species were scored as positive using the PCR method, while 26 species were positive using the RT-PCR method coupled with chemiluminescence visualization. Eighteen species (*Bacteroides thetaiotaomicron*, *B. vulgatus*, *B. distasonis*, *B. uniformis*, *B. ovatus*, *B. caccae*, *Bifidobacterium longum*, *B. angulatum*, *Clostridium clostridioforme*, *C. leptum*, *C. perfringens*, *C. indolis*, *Eubacterium eligens*, *E. rectale*, *Faecalibacterium prausnitzii*, *Ruminococcus productus*, *R. albus*, *R. obeum*) were positive by both the RT-PCR/chemiluminescence method and the PCR/color development method. *Bacteroides fragilis*, *Ruminococcus gnavus*, and *R. bromii* were scored as positive

Table 2. Membrane array for all 40 bacterial species from fecal slurries cultured in Gibson's medium and Medium 2.

Bacterial species	Gibson's medium		Medium 2	
	Day 0	Day 7	Day 0	Day 7
Bacteroides thetaiotomicron ATCC 29148	++	++	++	++
B. vulgatus ATCC 8482	++	+++	++	+++
B. fragilis ATCC 23745	-	-	-	-
B. distasonis ATCC 8503	++	+++	++	++
Clostridium clostridioforme ATCC 29048	+	+	+	++
C. leptum ATCC 29065	++	+	++	+
Faecalibacterium prausnitzii ATCC 27768	++	++	++	+
Ruminococcus productus ATCC 27340	+	+	+	+
Ruminococcus obeum ATCC 29174	+	++	+	++
R. bromii ATCC 27255	-	+	+	+
R. callidus ATCC 27760	-	-	-	-
R. albus ATCC 27210	+	+	+	+
Bifidobacterium longum ATCC 15687	+	+	+	+
B. adolescentis ATCC 15703	-	+	-	+
B. infantis ATCC 15697	-	-	-	-
Eubacterium biforme ATCC 27806	-	-	-	-
Collinsella aerofaciens ATCC 25986	-	+	-	+
Lactobacillus acidophilus ATCC 4356	-	-	-	-
Escherichia coli ATCC 25922	-	-	-	-
Enterococcus faecium ATCC 19434	-	-	-	-
Bacteroides uniformis ATCC 8492	++	+	++	+
B. ovatus ATCC 8483	++	++	++	++
B. caccae ATCC 43185	+	-	+	-
Clostridium perfringens ATCC 13124	+	+	+	+
C. butyricum ATCC 19398	-	-	-	-
C. ramosum ATCC 25582	-	-	-	-
C. difficile ATCC 9689	-	-	-	-
C. indolis ATCC 25771	+	++	+	++
Fusobacterium russii ATCC 25533	-	-	-	-
F. nucleatum ATCC 25586	-	-	-	-
Bifidobacterium catenulatum ATCC 27539	-	-	-	-
B. angulatum ATCC 27535	-	+	-	+
Eubacterium rectale ATCC 33656	+++	+	+++	+
E. eligens ATCC 27750	-	+	-	+
E. limosum ATCC 8486	-	-	-	-
Eggerthella lenta ATCC 25559	-	-	-	-
Lactobacillus fermentum ATCC 9338	-	-	-	-
Enterococcus faecalis ATCC 27274	-	-	-	-
Finegoldia magna ATCC 14955	-	-	-	-
Ruminococcus gnavus ATCC 29149	+	+	+	+

+, Positive signal; -, negative signal.

by color development and negative with chemiluminescence. *Bifidobacterium adolescentis*, *B. catenulatum*, *B. infantis*, *Collinsella aerofaciens*, *E. coli*, *Clostridium butyricum*, *C. ramosum*, and *C. difficile* were positive with the chemiluminescence detection method and negative by color development.

Determination of Detection Limits for the Modified Membrane-Array Method

A specific PCR signal can generally be amplified from fewer cells of a bacterial species in pure culture than in a complex mixture of bacterial species. In the fecal samples and fecal cultures examined in this study, *E. coli* could

Table 3. Comparison of membrane array methods using PCR/color deveopment and RT-PCR/chemiluminescence for detection of intestinal microflora.

Probe #	PCR/color development	RT-PCR/chemilumiscence
1-3	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron*
4-6	*B. vulgatus*	*B. vulgatus*
10-12	*B. distasonis*	*B. distasonis*
13-15	*Clostridium clostridioforme*	*Clostridium clostridioforme*
16-18	*C. leptum*	*C. leptum*
19-21	*Faecalibacterium prausnitzii*	*Faecalibacterium prausnitzii*
22-24	*Ruminococcus productus*	*Ruminococcus productus*
25-27	*R. obeum*	*R. obeum*
34-36	*R. albus*	*R. albus*
37-39	*Bifidobacterium longum*	*Bifidobacterium longum*
61-63	*B. uniformis*	*B. uniformis*
64-66	*B. ovatus*	*B. ovatus*
67-69	*B. caccae*	*B. caccae*
70-72	*C. perfringens*	*C. perfringens*
82-84	*C. indolis*	*C. indolis*
94-96	*B. anglulatum*	*B. anglulatum*
97-99	*Eubacterium rectale*	*Eubacterium rectale*
100-102	*E. eligens*	*E. eligens*
118-120	*R. gnavus*	
7-9	*Bacteroides fragilis*	
28-30	*Ruminococcus bromii*	
40-42		*Bifidobacterium adolescentis*
43-46		*B. infantis*
49-51		*Collinsella aerofaciens*
55-57		*E. coli*
73-75		*Clostridium butyricum*
76-78		*C. ramosum*
79-81		*C. difficile*
91-93		*B. catenulatum*

often be detected by selective plating methods, but not by the membrane array with color detection. Previous work suggested that the limit of detection for *E. coli* was approximately 10^6 *E. coli* cells per gram of feces using the membrane array. In Table 3, *E. coli* was not detected by the PCR-color development membrane array, but was detected when the RT-PCR-chemiluminescence approach was used. Serial dilution of the fecal sample used for this experiment and plating on MacConkey agar indicated that *E. coli* was present at ~1×10^5 CFU per gram feces in this sample. This suggests that the limit of detection for *E. coli* using the RT-PCR method with chemiluminescence detection is $\leq1\times10^5$ CFU per gram. In order to measure the limits of detection more directly, 1×10^4, 1×10^5, 1×10^6, 1×10^7, or 1×10^8 *E. coli* cells per gram were added to the fecal samples described above. The samples were then split, with one part used for RNA extraction and the other for DNA extraction. The extracted nucleic acids were used to generate DIG-labeled probes as described above. In Fig. 2, oligonucleotides specific for *E. coli* and *B. ovatus* were spotted onto membrane strips and used to determine the detection limit. *B. ovatus* was chosen as a positive control because it was present in high numbers in these fecal samples. The PCR membrane array with NBT/BCIP color development was able to detect *E. coli* in samples with 1×10^6 or greater cells per gram feces, while the RT-PCR method with chemiluminescence detection could detect *E. coli* in all samples, indicating that the limit of detection was at or below the level of the indigenous *E. coli* ($\leq1\times10^5$ CFU per gram).

DISCUSSION

Classical methods for monitoring the human intestinal microflora require culturing and isolation of the bacterial species, which are time-consuming and difficult due to the complexity of the bacterial population. There are approximately 10^{12} bacterial cells per gram of feces [31], and the different bacterial species, present in numbers, that differ by several orders of magnitude, complicate the analysis. Molecular methods that do not require isolation of individual species

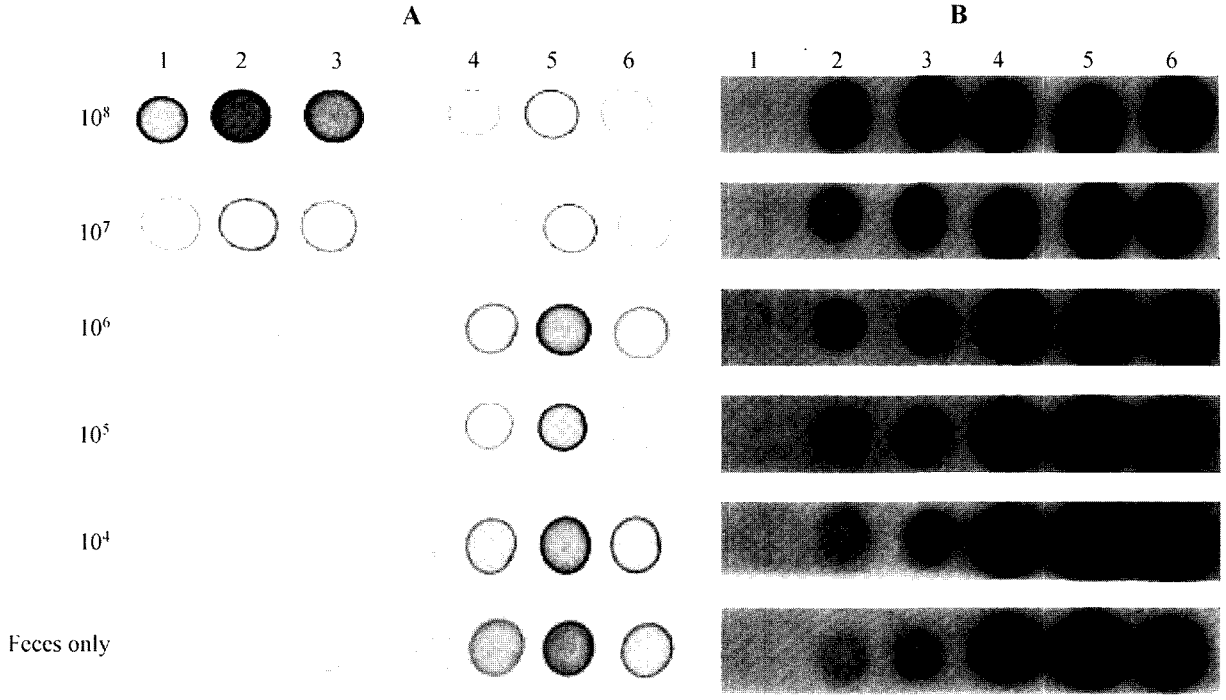

Fig. 2. Tests for the detection limit of *E. coli* from fecal sample using the membrane-array method.
1×10^4 to 1×10^8 *E. coli* cells were added per gram of feces. The feces contained an indigenous population of *E. coli* of ~1×10^5 CFU/gram. For panel A, the DNA was extracted, amplified by PCR, and visualized by NBT/BCIP color development. For panel B, the RNA was extracted, amplified by RT-PCR, and visualized using chemiluminescence. Spots 1-3 are the oligonucleotide probes specific for *E. coli* (oligos 55-57 in Table 1). Spots 4-6 are the oligonucleotide probes specific for *B. ovatus* (oligos 64-66 in Table 1), which were included as a positive control. Different syntheses of oligo 55 (spot 1) were used for panel A and panel B, which may account for the lack of signal from the first *E. coli* probe in all the strips of the chemiluminescence method.

can be useful for characterizing and monitoring the bacterial composition of the intestinal microflora. One of the most promising methods involves the use of oligonucleotide probes to identify the presence of individual bacterial species. These probes have been used in dot-blot assays to detect 16S rRNA molecules directly [7, 14, 23, 25, 36], and in membrane arrays or microarrays to detect PCR-amplified 16S rRNA genes [37-38]. However, because of the great difference in cell numbers between the prevalent bacterial species in the gut and other less common but still potentially important bacteria, these methods may be unable to detect the less prevalent bacterial species. For example, *E. coli* is relatively easy to detect in fecal samples using culture methods because of efficient selective media, even though it is generally present in cell numbers several orders of magnitude lower than the predominant fecal species. In several tests evaluating the sensitivity of the membrane-array method, we used fecal samples with *E. coli* concentrations of ~10^5 cells per gram of feces. The *E. coli* was readily identified by culture, but was not detected using the array. In this study, we modified the membrane-array method such that it improved the sensitivity of the assay, allowing the detection of microorganisms present in significantly lower cell numbers. The PCR amplification step of this method is influenced by the number of cells for each bacterial species that are present in the fecal sample. DNA isolation methods may dilute the sample to the point where there are too few copies of an individual bacterial species genome to be efficiently amplified. Since the 16S rRNA molecules are present in much higher copy number than the corresponding gene sequences, reverse transcriptase-PCR was used to amplify the rRNA sequence and generate the digoxygenin-labeled probe. Results using the labeled probe from the RT-PCR were slightly better than the normal PCR products from the same fecal sample, with most species showing equal or greater spot intensity (data not shown). This difference was enhanced when the method to visualize the signal was changed. The initial work with the membrane-array method involved visualizing the hybridized probe using color formation on the membrane. While this detection method was fast and easy, only a single image was obtained for each membrane, which made it difficult to optimize the signal-to-background ratio. Using the chemiluminescent substrate for the alkaline phosphatase gave better signals from some bacteria that were present in lower numbers in the sample. The detection limit for *E. coli* was 10^6 using the original method, and $\leq1\times10^5$ using RT-PCR and chemiluminescence detection.

It is important to understand that the detection limits for other bacterial species may be higher or lower than that

determined for *E. coli*. Work is in progress to better estimate the detection limits for different types of bacteria. The membrane-array method coupled with the chemiluminescence detection system is a sensitive method for the simultaneous detection of many different bacterial species in complex mixtures such as the human intestinal microflora. The strength of this assay lies in its ability to detect bacterial species that are not readily separated by culture techniques. The 40 species selected for this study represent bacteria that either are among the most prevalent species that have been identified by culturing of fecal samples, or are species that are important for human health. We did not attempt to monitor any species that make up the nonculturable segment of the human intestinal microflora, although the assay could be modified to detect other organisms by designing unique 16S rRNA probes. The results reported for this membrane-array method are qualitative because they report the presence or absence of a bacterial species and do not attempt to directly determine the cell numbers for the different bacteria. Issues of PCR bias when similar sequences are amplified from complex mixtures of bacterial species make quantification by direct amplification problematic [3]. However, changes in the cell numbers for individual species could be investigated by testing different dilutions of the nucleic acids extracted from the fecal samples to determine when the species-specific template drops below the limit of detection of the assay.

The membrane-array assay could be used to monitor the human intestinal microflora under a wide variety of conditions, including exposure to exogenous compounds such as dietary supplements, prebiotic compounds, or antibiotics, as well as probiotic treatment or pathogen exposure. Being able to monitor shifts in the intestinal bacterial population may assist in evaluating the efficacy of a treatment or identifying potential side effects. The membrane array also has several advantages over the related microarray method for monitoring the human intestinal microflora. The membrane assay requires no expensive equipment and can be performed in just about any molecular biology laboratory. The organisms screened by the array can be changed to suit each application without the time and expense required to make new slides. It can also be used in conjunction with microarray studies as a rapid evaluation tool for new probes. Both approaches offer potential solutions for the difficult task of characterizing complex mixtures of bacterial species in a single, rapid assay.

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