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Application of 16S rRNA Gene PCR To Study Bowel Flora of Preterm Infants with and without Necrotizing Enterocolitis

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The purpose of the present study was to determine the extent to which bacteria not detected by culture contribute to the microbial flora of the bowel of preterm infants with and without neonatal necrotizing enterocolitis (NEC). Fecal samples from 32 preterm infants in special care baby units including samples from 10 infants with NEC were examined by culture and PCR amplification of the 16S rRNA gene (rDNA). The 16S rDNA V3 region was amplified with eubacterial primers, and the amplification products derived from the fecal sample DNA were compared with the products from individual cultured isolates by PCR and denaturing gradient gel electrophoresis (PCR-DGGE), allowing the DNA from uncultured bacteria to be identified. For the 22 infants without NEC weekly samples were examined for a mean of 5.3 postnatal weeks. The total number of types detected by culture combined with PCR-DGGE was 10.1 per infant, of which PCR-DGGE contributed 10.4% of the types identified. Additional types detected by PCR-DGGE were found in 14 (63.6%) of the 22 infants. The majority of the sequences associated with uncultured bacteria showed >90% 16S rDNA sequence identity with sequences from culturable human enteric flora, and all were found in single infants with the exception of sequences indistinguishable by DGGE from seven infants. These sequences showed >90% sequence identity with the 16S rDNA of *Streptococcus salivarius* and may have been derived from upper gastrointestinal or respiratory tract flora. In the present study uncultured bacteria detected by PCR-DGGE were no more frequent in fecal samples from infants with NEC than in samples from infants without NEC, although these findings do not exclude the possibility of unrecognized bacteria associated with the mucosa of the small intestine of infants with NEC.

The enteric flora of preterm infants contributes both to health and to disease, facilitating carbohydrate assimilation (13), interacting with the developing immune system, and providing a reservoir for nosocomial pathogens such as members of the family *Enterobacteriaceae*, yeasts, and coagulase-negative staphylococci (6). The bowel flora has been implicated in the pathogenesis of neonatal necrotizing enterocolitis (NEC) (3, 15), which is the most common intra-abdominal emergency in preterm infants, with a significant associated morbidity and mortality.

Previous studies of the microbial flora of the bowel of preterm infants have relied on the application of cultural methods. Preterm infants in intensive care units have relatively simple bowel flora by comparison with that of full-term infants (2, 10, 24). By molecular methods it is possible to detect the presence of bacteria by identifying their DNA or RNA, irrespective of whether or not the bacteria can be cultured. These methods have been used to determine the extent of microbial diversity in the environment (7, 8) and also to study uncultured bacteria associated with human disease such as the bacterium of Whipple's disease (23).

The purpose of the study described here was to determine the extent to which bacteria, which are difficult or in the present state of knowledge nonculturable, contribute to the microbial flora of the bowel of preterm infants and possibly to the pathogenesis of NEC.

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MATERIALS AND METHODS

The fecal samples that were used in the present study included those collected, stored, and cultured in a previous study (17). The samples and *Enterobacteriaceae* isolates had been stored at −70°C in glycerol broth (4). These samples were collected from 22 infants who had been admitted between September 1991 and January 1992 to the neonatal intensive care unit of Princess Anne Hospital, Southampton, United Kingdom. The samples studied were those collected within 72 h of the day of starting nasogastric feeds and weekly thereafter until discharge from intensive care. The mean age at which feeding was started was 3.1 days, and all infants received their first nasogastric feed in the first week of life. The mean number of weekly samples for each infant was 5.3.

Fecal samples and in one case postmortem tissue samples were also collected between September 1991 and December 1995 from infants with suspected NEC in neonatal units in The General Infirmary, Leeds, United Kingdom (six infants); St. Michaels Hospital, Bristol, United Kingdom (fecal samples from two infants and postmortem small intestine from a third infant); and the Princess Anne Hospital, Southampton (one infant). Fecal samples in glycerol broth and the tissue sample were stored at −70°C. The diagnosis of NEC was based on histopathological examination of a surgical or postmortem tissue sample or was considered probable if there was a radiological report of intramural or intrahepatic gas. The gestational age, basis for diagnosis, and day of sample studied for infants with suspected NEC are presented in Table 1. Nine of the 10 infants had radiologically probable or histological changes consistent with NEC. There was a clinical diagnosis of possible NEC in 1 of the 10 infants following perforation of the colon, although there was no radiological evidence of intramural gas. This infant was managed without surgical intervention, so no tissue samples were available for histology. All of these infants were treated with antibiotics as soon as the diagnosis of NEC was suspected.

Fecal samples were cultured on a range of selective and nonselective media as described previously (11). In addition, in an attempt to culture bacteria detected by eubacterial PCR with sequences showing a high degree of 16S rRNA gene (rDNA) identity with *Streptococcus salivarius* 16S rDNA, samples were inoculated onto fastidious anaerobe agar (code 090-A; Lab M, Bury, United Kingdom) supplemented with 5% horse blood and incubated anaerobically for 48 h and onto Columbia blood agar (CM 329; Unipath, Basingstoke, United Kingdom) supplemented either with 5% horse blood and 0.001% (wt/vol) pyridoxal or with 5% sucrose and 0.02% sodium azide and incubated in air at 37°C for 72 h.

The strategy for detecting uncultured bacteria is outlined in Fig. 1. PCR with

TABLE 1. Gestational age, diagnostic criteria, day of sampling, and bacteria detected by culture or PCR-DGGE in infants with NEC

Infant	Gestational age (wk)	Diagnosis[a]	Day of sampling[b]	Bacteria cultured	Presumptive identification of bacteria detected solely by PCR-DGGE
1	24	H	+14[c]	NC[d]	*Escherichia coli*
2	32	Possible H	+5	*Proteus* spp., *Staphylococcus* spp.	ND[e]
3	34	R	+2	Coliform,[f] *Staphylococcus* spp.	ND
4	33	H	+3	*Staphylococcus* spp., *Enterococcus* spp.	ND
5	32	R	+0	*Staphylococcus* spp., *Clostridium* spp.	ND
6	25	H	−9, −2, +1	*Staphylococcus* spp., *Enterococcus* spp., *Escherichia coli*, *Enterobacter cloacae*, coliform, microaerophilic GVR[g]	ND
7	28	H	+4	*Staphylococcus* spp., *Enterococcus* spp.	ND
8	24	H	+3	*Staphylococcus* spp., *Enterococcus* spp.	ND
9	33	H	+2	*Staphylococcus* spp.	ND
10	28	R	+7	*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus* spp.	*Ewingella americana*

[a] H, histological diagnosis of NEC; R, presence of intramural and/or intrahepatic gas.
[b] Values indicate days before or after onset of clinical signs of NEC.
[c] Small intestine.
[d] NC, sample not cultured.
[e] ND, no additional bacteria detected by PCR-DGGE.
[f] Coliform indicates facultative, oxidase-negative lactose-fermenting, gram-negative rod.
[g] GVR, gram-variable rod.

primers complementary to sequences which are highly conserved among bacteria was used to amplify the V3 region in the 16s rRNA gene. Denaturing gradient gel electrophoresis (DGGE) was used to separate DNA fragments of the same length but with different sequences. Preliminary data indicated that this technique is able to detect up to six different bacteria in a mixture when they are present in equal numbers and could detect the DNA of bacteria that composed less than 1% of the mixture, as reported previously (18).

DNA preparation. DNA was extracted from fecal samples in glycerol broth as follows: 10 μl of the fecal suspension was placed in a 1.5-ml Eppendorf tube, mixed with 500 μl of sterile water, and then centrifuged at 400 × *g* for 2 min. The supernatant was discarded, and the pellet was resuspended in 50 μl of water and overlaid with 2 drops of paraffin oil. The sample was then placed in a heat block at 100°C for 20 min and then allowed to cool to room temperature before the addition of 5 μl of a 20-mg/ml solution of proteinase K. The sample was then incubated at 55°C overnight, after which it was centrifuged at 400 × *g* for 1 min. The supernatant was stored at −20°C.

DNA was extracted from bacterial cell suspensions with the GeneReleaser kit (Cambio Ltd., Cambridge, United Kingdom) following the manufacturer's procedures. A total of 2 μl of a visibly turbid cell suspension was added to 10 μl of GeneReleaser in a 0.5-ml microcentrifuge tube, and the mixture was mixed well. The mixture was then overlaid with 2 drops of paraffin oil, and the tubes were heated at high power (800 W) in a microwave oven for 5 min. The resulting extract was used directly in a PCR.

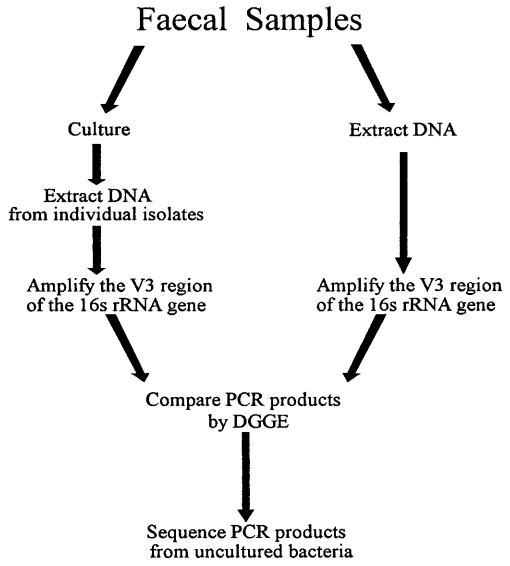

FIG. 1. Strategy for detection of uncultured bacteria from fecal samples from preterm infants.

PCR amplification. The PCRs were performed in a total volume of 50 μl. The reaction mixture contained 0.25 U of Super TAQ polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom), buffer (10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% [vol/vol] Triton X-100, and 0.01% [wt/vol] gelatin), 20 μM (each) deoxynucleoside triphosphate (Pharmacia Biotech, Uppsala, Sweden), and 0.1 μM (each) primers P2 (5′-ATTACCGCGGCTGCTGG-3′) and P3 (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCC TACGGGAGGCAGCAG-3′; a 40-bp GC clamp was attached at the 5′ end of primer P3) (18). For the fecal samples, 5 μl of DNA was added to the PCR mixture, and for the bacterial suspensions, the entire 12-μl product of the GeneReleaser kit was used (other PCR constituents were added directly to the tube of the GeneReleaser product beneath the paraffin oil). The reaction mixtures were overlaid with 2 drops of paraffin oil and were then incubated for 3 min at 94°C. A total of 30 cycles of PCR were performed with a thermal cycler (Omnigene; Hybaid, Teddington, United Kingdom) consisting of a denaturation step for 30 s at 94°C, an annealing step for 1 min at 65°C, and an extension step for 1 min at 72°C. After the final cycle there was a step of 7 min at 72°C.

Control samples that had each of the reaction constituents except genomic DNA were run along with the samples containing DNA. DNA extraction, PCR mixture preparation, and post-PCR analysis were carried out in separate rooms with equipment designated for each area. The preparation of the PCR mixtures was carried out in a class II laminar flow cabinet with filter-protected pipette tips (Aerogard; Alpha, Eastleigh, United Kingdom).

The PCR products generated were visualized by ethidium bromide staining after electrophoresis in a gel containing 2% agarose before commencing DGGE.

DGGE. A Bio-Rad Protean II system (Bio-Rad Laboratories Ltd., Herts, United Kingdom) was used to perform parallel DGGE as described previously (19) with 12% polyacrylamide (acrylamide:bisacrylamide, 37.5:1) in Tris-borate buffer at pH 8.3 (0.09 M Tris, 0.09 M boric acid, and 1.8 mM EDTA). The gradient was made with a gradient maker (Hoeffer SG 50; Hoeffer Pharmacia Biotech Inc., San Francisco, Calif.) with starting stock solutions of 15% (1.05 M urea and 6% deionized formamide) and 55% (3.85 M urea and 22% deionized formamide). The formamide was deionized by passing it through a deionization column [AG 501-x8(D); Bio-Rad]. After polymerization the top portion of the gradient gel was overlaid with a 3% acrylamide stacking gel into which a 20-tooth comb was inserted. A total of 25 μl of PCR product was loaded onto the gel, and electrophoresis was performed at a constant 225 V and a temperature of 60°C. After electrophoresis, the gels were stained with ethidium bromide and were visualized on a UV transilluminator. The products from all of the fecal samples from an individual infant and from each of the cultured isolates were electrophoresed on the same denaturing gradient gel whenever possible, allowing for the direct comparison of bands from samples and cultured isolates.

Sequencing. The bands on the DGGE gel derived from fecal samples that did not line up with any band from any of the cultured bacteria from the corresponding infant were considered to be uncultured and were sequenced. Bands were cut from the gel with a fresh sterile scalpel blade and were placed in 50 μl of water and left at 4°C for 24 h. A total of 4 μl of the resulting solution was added to a PCR mixture under the same PCR conditions and with the primers described above. The PCR products were then run on a DGGE gel to check that they were of one sequence type. A total of 4 μl of the DNA solution from the cut band was then added to a PCR mixture as described above, except that the

primers used were P1 (which is like P3 but without the 40-bp GC clamp) and P2. The PCR products were cleaned with Microcon 100 microconcentrators (Amicon, Beverly, Mass.) following the manufacturer's instructions for PCR products.

These PCR products were sequenced directly with the P1 primer (18) on the ABI 377 automated fluorescent DNA sequencer using dye terminator chemistry. The sequences were analyzed with the FastA program from the Wisconsin Genetics Computer Group suit of programs, Central Laboratory of the Research Councils, Daresbury Laboratory (20), to give sequence alignment and percent identity.

Specific PCR for *S. salivarius* was applied to samples from which sequences had been amplified with a high degree of identity with the 16S rDNA of *S. salivarius*. DNA from the fecal samples was extracted as described earlier, and a specific PCR targeting the glucosyltransferase gene of *S. salivarius* was performed at an annealing temperature of 50°C for 30 rounds of amplification as described previously (1).

RESULTS

The number of bacterial types obtained in cultures of all samples (mean, 5.3 samples per infant) from each of the 22 infants without a diagnosis of NEC during the previously reported study (17) was 8.73 types per infant. Samples stored at −70°C were recultured for the present study, and all but eight types from eight infants were recovered on reculturing. These were *Klebsiella* spp. ($n = 3$), *Bacillus* spp. ($n = 2$), *Bifidobacterium* sp. ($n = 1$), and unidentified gram-positive bacilli ($n = 2$).

PCR-DGGE detected 1.3 additional sequences per infant (30 sequences from 22 infants) which did not correspond to isolates cultured from any of the samples from that infant. Following PCR-DGGE it was possible to culture additional bacteria with sequences identical to 7 of the 30 additional sequences detected by PCR-DGGE. These were identified by sequence identity and by conventional identification methods as *Clostridium* spp. (six isolates from five infants) and *Staphylococcus haemolyticus* (one isolate from one infant). In most of the cases in which PCR-DGGE identified additional bacterial types in a sample, there were large numbers of bacterial types from the same genus (for example, *Clostridium* spp.) or family (*Enterobacteriaceae*). Twenty-three sequences (mean of 1.05 per infant) which did not correspond to bacteria cultured before or after PCR-DGGE were detected. The total number of types detected by culture and PCR-DGGE was 10.1 per infant, of which PCR-DGGE contributed exclusively 10.4% of the types identified. These additional types detected by PCR-DGGE were found in 14 (63.6%) of the 22 infants. Only two of the sequences were identified in samples collected from an infant receiving an antibacterial agent (cefotaxime in both cases). A presumptive identification of the sequences from uncultured bacteria was derived by comparison of the 16S rDNA sequence with the sequences of rDNA in the EMBL or GenBank databases. Seventeen of the 23 sequences showed more than 90% identity with those from the V3 region of 16S rDNA of culturable human enteric flora.

The uncultured bacteria were presumptively identified on the basis of >90% sequence identity with the V3 region of 16S rDNA as *Clostridium* spp. (four sequences distinguished by DGGE from four infants, 5 samples), *Eubacterium* spp. (one infant, 2 samples), *Ewingella americana* (one infant, 2 samples), *Actinomycetes* spp. (one infant, 1 sample), *Bifidobacterium* spp. (one infant, 2 samples), *Hafnia* spp. (one infant, 1 sample), *Lactobacillus* spp. (one infant, 2 samples) and *S. salivarius* (seven infants, 11 samples) (see below). Six sequences from five infants showed less than 90% identity with sequences in the EMBL or GenBank databases. These samples are undergoing further investigation, and the sequences will be submitted to the EMBL database.

The 16S rDNA sequences from seven infants, including two pairs of twins, lined up at the same point on the DGGE gel. All of these sequences showed a high degree of identity (91.5 to 96.4%) with *S. salivarius* 16S rDNA. It was not possible to culture bacteria with medium supplemented with pyridoxal hydrochloride or with sucrose azide selective medium. These infants were in the neonatal intensive care unit during the period from October 1991 to January 1992 and included 5 of 12 infants who received feed supplementation with Lactobacillus GG (a nonpathogenic strain of *Lactobacillus casei*) and 2 of the 10 infants who should not have received supplementation. One of these two infants was probably inadvertently fed Lactobacillus GG (17). All of the samples from which these sequences were identified were collected in the third postnatal week or later. There was an association between the presence of Lactobacillus GG in the sample and the presence of *S. salivarius*-like sequences (9 of 42 samples from which Lactobacillus GG was cultured versus 2 of 75 samples from which Lactobacillus GG was not cultured; chi-square analysis with Yates correction, 7.26; $P < 0.01$). PCR with species-specific primers from the glucosyltransferase gene of *S. salivarius* (*gtfL*) gave an amplification product from the fecal samples from the 7 infants in which PCR-DGGE had detected *S. salivarius*-like sequences and from only 1 other infant (1 of 15 infants in which *S. salivarius* was not detected by PCR-DGGE). This infant had also received feed supplementation with Lactobacillus GG. *S. salivarius* was not detected by culture or PCR with 16S rDNA or *gtfL* primers in a batch of Lactobacillus GG freeze-dried powder (batch 1789-1) which had been stored since the time of the feed supplementation study. This batch had been provided with the batch used for the supplementation study but had been unused.

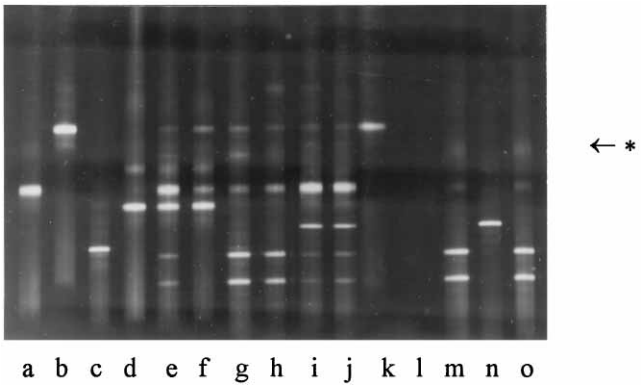

FIG. 2. DGGE analysis of 16S rDNA fragments obtained from six fecal samples (from one infant) and from the bacteria cultured from the samples. Lanes: a, *E. coli*; b, *Enterococcus* spp.; c, Lactobacillus GG; d, *E. cloacae*; e, fecal sample, week 1; f, fecal sample, week 2; g, fecal sample, week 3; h, fecal sample, week 4; i, fecal sample, week 5; j, fecal sample, week 6; k, *Staphylococcus epidermidis*; l, negative control; m, *Klebsiella* spp.; n, *Clostridium* spp.; o, *Klebsiella* spp. *, Sequence data from the band in lane g showed 96.4% identity with the 16S rDNA of *S. salivarius.*

Isolates of some bacterial species, particularly *Enterobacter cloacae*, reproducibly gave multiple, closely located bands by PCR-DGGE. An example of the results for one infant in which DGGE was used to compare 16S rDNA fragments from cultured bacteria with those from fecal samples is presented in Fig. 2. In this case two colonial types of *Klebsiella* spp. and an *E. cloacae* isolate gave two bands. The band in lane g did not correspond to any of those derived from cultured isolates, and when it was sequenced it showed a high degree of identity with the 16S rDNA of *S. salivarius.*

Samples were available from one infant (infant 6) before the onset of NEC. All of the other samples from infants with NEC

were collected either on or after the day of onset of clinical signs of NEC and after the introduction of antibiotic therapy. The identities of the cultured bacterial isolates and of those isolates presumptively identified by PCR-DGGE are presented in Table 1. For the nine infants whose samples were cultured, there was a mean of 2.4 isolates per infant. Bacterial isolates included members of the family *Enterobacteriaceae*, *Enterococcus* spp., coagulase-negative staphylococci, *Clostridium* spp., and *Pseudomonas aeruginosa*. Eubacterial PCR did not detect any bacterial DNA sequences other than those that were cultured from stool samples from eight of the nine infants. PCR-DGGE detected three sequences in addition to those detected by culture of a sample from one infant with NEC. These sequences were closely located on the denaturing gradient gel after electrophoresis, and all showed a high level of identity (90.2 to 95.4%) with the sequence of the 16S rDNA of *E. americana*. The small intestine collected postmortem from an infant who had died of NEC was not cultured; however, application of eubacterial PCR to the tissue sample gave a DNA sequence with a high degree of sequence identity (95%) with that of *Escherichia coli.*

DISCUSSION

Sequencing of the 16S rRNA gene has been extensively used over the last 10 years to develop phylogenetic relationships. PCR with eubacterial 16S rRNA gene primers combined with cloning and sequencing has been used to study genetic diversity in seawater (7, 8), to identify the causative agent of bacillary angiomatosis (22), to identify the taxonomic status of the Whipple's disease bacillus (26), and most recently, to demonstrate uncultured bacteria among cultured bacteria in dental abscess fluid (5). The PCR-DGGE methodology used in the present study can be used to detect bacteria in many types of clinical samples even in the presence of mixtures of bacterial types, so, for example, we used PCR with DGGE to detect the causative agent(s) of infection in culture-negative samples from patients who had received antibiotics.

Control of cross infection and use of antibiotics limit the range of bacteria colonizing the gastrointestinal tracts of preterm infants in intensive care. Even so, reliance on cloning and sequencing, even when they are combined with restriction fragment length polymorphism analysis of cloned genes (5), would have been impractical and prohibitively expensive given the number of samples included in the study. The use of DGGE allowed sequences derived from amplification of sample DNA to be directly compared with sequences derived from bacteria cultured from the corresponding sample, so that the sequences derived from cultured bacteria were not sequenced unnecessarily. DGGE was used to separate mixtures of 193-bp fragments amplified from the V3 region of the 16S rRNA gene. The sequence information from a fragment of this size is insufficient to allow great precision in the construction of phylogenetic trees (27), but it did allow a presumptive identification. This approach was validated by the recovery of additional *Clostridium* species by culture after their presence was detected by PCR-DGGE and by the amplification of a specific *S. salivarius gtfL* gene sequence in samples from all seven infants from which a presumptive identification of *S. salivarius* had been made by PCR-DGGE and in only 1 of the other 15 infants.

The detection of *S. salivarius*-like sequences was associated with culture of Lactobacillus GG from the corresponding sample. It is possible that the batch of Lactobacillus GG freeze-dried powder used to feed the infants from whom these samples were derived (17) was contaminated with an *S. salivarius* strain used in the manufacture of fermented milk products. *S. salivarius* or a related bacterium was not isolated either from the powder or during the study from supplemented feeds which were routinely cultured and was not detected by PCR in Lactobacillus GG freeze-dried powder stored since the time of the supplementation study. A more likely explanation is that colonization of the upper gastrointestinal tract by Lactobacillus GG facilitated colonization by *S. salivarius*, which did not survive in a culturable form in stool samples. There was no evidence that Lactobacillus GG feed supplementation modified the fecal flora, but it may be that there was modification of the bacterial flora in the mouth (following regurgitation of feeds) or small intestine (12). The results of the study suggest that DNA detection methods could be applied to fecal samples to detect microbial colonization of inaccessible sites such as the small intestine. The *S. salivarius*-like gene sequences were the only related sequences found in a large proportion of the infants included in the study.

A range of selective and nonselective media were used in the present study. Most of the "uncultured" bacteria (detected only by PCR-DGGE) were isolated from samples containing large numbers of related bacteria. The uncultured bacteria may have been present in samples but not detected by culture because of overgrowth on selective and nonselective media by more numerous related species. The possibility that the sequences which were detected by PCR-DGGE but which had <90% identity with culturable human enteric flora were derived from the environmental contamination of feeds or the sample cannot be ruled out. Further work with the samples from which those sequences were derived is ongoing.

There is a link between the presence of bacteria in amniotic fluid and preterm birth, and all of the infants included in this study were less than 33 weeks of gestational age. The bacteria associated with preterm birth include bacteria for which specific culture methods were not applied such as *Mycoplasma hominis* and *Ureaplasma urealyticum*. Human gastrointestinal tract colonization with these bacteria has not been described, and none were detected by PCR-DGGE.

Pure cultures of some of the members of the family *Enterobacteriaceae*, particularly *E. cloacae* but also *Klebsiella* spp. (as shown in Fig. 2), consistently gave multiple bands by PCR-DGGE. There are a number of possible explanations for this phenomenon, including the possibility that the multiple 16S rRNA genes in a bacterium may not have the same DNA sequence. This has been described in some bacterial species such as *Mycoplasma* spp. (21) and *Fusobacterium* spp. (25) but not, as far as we are aware, in members of the family *Enterobacteriaceae.*

The pathogenesis of NEC is unclear. Clustering of cases in time and space have led to suggestions that NEC is caused by a transmissible agent (14). Previous studies have failed to consistently implicate a single agent, leading to suggestions that an unrecognized infectious agent contributes to the pathogenesis of NEC (9). A secondary aim of the project was to determine whether uncultured fecal bacteria are associated with NEC. In the present study uncultured bacteria detected by PCR-DGGE were no more frequent in fecal samples from infants with NEC than in samples from infants without NEC, although these findings do not exclude the possibility that unrecognized bacteria are associated with the mucosa of the small intestines of infants with NEC (12).

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