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Duodenal Microflora in Very-Low-Birth-Weight Neonates and Relation to Necrotizing Enterocolitis

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Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in the neonatal period. Small-bowel overgrowth with aerobic gram-negative bacteria has previously been implicated in the development of NEC. This prospective study performed quantitative bacteriology on 422 duodenal aspirates collected from 122 very-low-birth-weight (<1,500-g) newborns, at the time of routine changing of nasogastric tubes. Isolates of *Enterobacteriaceae* were typed by repetitive extragenic, palindromic PCR and pulsed-field gel electrophoresis. One or more samples from 50% of these infants yielded gram-negative bacteria, predominantly *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp., with counts up to 10^8 CFU/g. The proportion of samples with gram-negative bacteria increased with postnatal age, while the percentage of sterile samples declined. Molecular typing revealed marked temporal clustering of indistinguishable strains. All infants had been fed prior to isolation of gram-negative organisms. Antibiotic use had no obvious effect on colonization with *Enterobacteriaceae*. There were 15 episodes of suspected NEC (stage I) and 8 confirmed cases of NEC (2 stage II and 6 stage III) during the study period. Duodenal aspirates were collected prior to clinical onset in 13 episodes of NEC. Seven of these yielded *Enterobacteriaceae*, of which five strains were also isolated from infants without NEC. Very-low-birth-weight infants have high levels of duodenal colonization with *Enterobacteriaceae*, with evidence of considerable cross-colonization with indistinguishable strains. There was no association between duodenal colonization with particular strains of *Enterobacteriaceae* and development of NEC.

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in the neonatal period. Approximately 90% of cases occur in premature infants (43). The incidence of disease varies but may affect up to 5% of admissions to neonatal intensive care units (NICUs) and up to 10% of very-low-birth-weight (defined as <1,500-g) infants. Mortality rates of 9 to 28% have been reported in recent case series (54).

The pathogenesis of NEC is not clearly understood and is likely to be multifactorial. Early theories suggested that circulatory disturbances leading to gastrointestinal ischemia are involved (44). While ischemia may be a factor in term infants who develop NEC, case-control studies in preterm infants have identified prematurity as the only consistent risk factor (54). Immaturity of the gastrointestinal tract is thought to play a crucial role, and immunologic factors, reduced gastric acid secretion, increased intestinal permeability, and poor motility may all be implicated (38).

Most cases of NEC occur following institution of enteral feeding, although the disease occurs occasionally in those who have never been fed by this method. NEC has been previously associated with the use of hypertonic formula or with rapid increases in enteral feeding volumes (34). Human breast milk may provide some protection against development of NEC (37).

The role of infection in the pathogenesis of NEC remains unclear, but there is evidence to suggest that bacteria are involved to some degree in the process. NEC has never been reported in stillborn infants (38), and gross necrosis was not produced in a germ-free animal model (42). The radiological hallmark of NEC is pneumatosis intestinalis, and this intramural gas contains hydrogen, which is derived from bacterial fermentation (22). Increased urinary D-lactate excretion in infants with NEC was thought to be related to increased bacterial activity during the disease (23). NEC may occur in epidemics clustered temporally and geographically, with reduction in cases following institution of infection control measures (12). Although a variety of organisms have been associated with these epidemics, they tend to be those commonly found colonizing the intestine (60). Investigation by standard microbiological methods has not revealed any single causative agent that is consistently associated with NEC (26).

At birth, an infant's gastrointestinal tract is sterile but rapidly becomes colonized with organisms acquired from the mother and the local environment. In the first few days of life, *Enterobacteriaceae* and enterococci are the predominant organisms in neonatal stool samples (48, 63). Bifidobacteria then become predominant in most breast-fed infants, while in formula-fed babies, *Enterobacteriaceae*, *Bacteroides* spp., and clostridia remain at high levels (3, 36). Duodenal-intubation studies in healthy infants have shown the upper small bowel to be sterile or have sparse, predominantly gram-positive flora similar to that of adults (2, 5, 16, 20, 45).

Preterm infants in NICUs develop gastrointestinal flora different from that of healthy full-term infants. Studies of gastric and fecal flora show delayed colonization in preterm infants, with predominantly gram-negative aerobic flora and few anaerobes (8, 11, 24). A number of studies have suggested that this abnormal gastrointestinal colonization may be associated with the development of NEC. Bell et al. (6) found that infants who developed NEC were more likely to have gastric and fecal colonization with aerobic gram-negative organisms than other infants in the same NICU. Dellagrammaticas et al. (21) reported a high incidence of NEC in infants fed transpylorically, associated with jejunal colonization with coliforms. Levels of hydrogen excretion in breath, used to diagnose small-bowel

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bacterial overgrowth, have also been shown to increase prior to clinical onset of NEC (17). Acidification of infant feeds leading to a lowering of gastric pH and reduced gastric colonization with gram-negative enteric bacteria has been associated with a decreased incidence of NEC (14). Neonates treated with vancomycin and aztreonam for presumed sepsis had reduced fecal colonization with *Enterobacteriaceae* and significantly fewer episodes of NEC than those treated with vancomycin and gentamicin (40).

NEC may affect all of the gastrointestinal tract, but it most commonly involves the terminal ileum and proximal colon (34). Clark and Miller (18) proposed that organisms capable of rapid fermentation of excess carbohydrates in the small bowel may contribute to the development of NEC; thus colonization with particular strains of *Enterobacteriaceae* may predispose to disease. We have previously reported changes in fecal flora in the 48 h preceding clinical onset of NEC, with either acquisition of a new strain or a quantitative increase in *Enterobacteriaceae* (31).

The only previous study of small-bowel flora in preterm neonates was in infants fed via transpyloric tubes (21), and prolonged intubation is known to produce qualitative changes in duodenal flora, with increased recovery of *Escherichia coli* and *Klebsiella* spp. (16). Few studies of the bacteria involved in NEC have attempted molecular typing of isolated strains. A previous investigation of fecal flora in infants with NEC used plasmid analysis of gram-negative organisms (26); however, because the number of plasmids carried may be low and they can be readily lost and acquired, this typing method may have a low discriminatory value.

The aim of this prospective study was to perform quantitative bacteriological cultures on duodenal aspirates collected from infants with birth weights of <1,500 g, at the time of routine changing of nasogastric tubes. Typing of *Enterobacteriaceae* by repetitive extragenic palindromic (REP) PCR and pulsed-field gel electrophoresis (PFGE), both highly discriminatory stable methods, was performed to determine any association between colonization with particular species or strains of *Enterobacteriaceae* and the subsequent development of NEC. The study was approved by the local ethics committee.

MATERIALS AND METHODS

Collection of samples. Samples were collected from 122 infants admitted to a regional NICU between October 1991 and March 1993. They comprised 67 males and 55 females at 23 to 35 weeks gestation (median, 28 weeks) and birth weights ranging from 540 to 1,580 g (median, 1,100 g). With informed parental consent, duodenal contents were sampled at the time of routine replacement of nasogastric tubes, by advancing the tube through the pylorus using the gastric air insufflation technique described by Schaff-Blass et al. (49). The tube was assumed to be in the duodenum if clear bile-stained aspirate was obtained that tested negative for acid with litmus paper. Following sample collection, the tube was pulled back to lie in the stomach and was used for routine feeding. Approximately 0.1 ml of duodenal aspirate was inoculated into a vial containing 0.9 ml of prereduced glycerol-citrate broth (19). The sample weight was calculated by weighing vials before and after addition of the sample, and vials were stored at −70°C.

Culture of samples. Samples were thawed at room temperature. In an anaerobic cabinet (Wise Anaerobic Work Station; Don Whitley, Shipley, United Kingdom), 50 μl of vial contents was inoculated onto the following media: blood agar base (CM 55; Oxoid, Basingstoke, United Kingdom) containing 5% horse blood, MacConkey agar (CM 7b; Oxoid), Sabouraud's agar (CM 41; Oxoid), heated blood agar, and Columbia blood agar base (CM 331; Oxoid) with 5% horse blood supplemented with hemin, menadione, and sodium bicarbonate. All plates were incubated at 37°C, the first three in air, the fourth in 5% CO_2, and the final under anaerobic conditions. All plates were examined for growth daily for up to 1 week after inoculation. Colonies were counted using an automated colony counter. Those samples in which it was not possible to count individual colonies because of density of growth were diluted 10-fold and 100-fold in brain heart infusion broth (CM 225; Oxoid). Fifty microliters of each dilution was inoculated onto media as before plus Slanetz Bartley media (CM 377; Oxoid), incubated at 37°C in 5% CO_2, as well as Columbia blood agar with kanamycin (100 mg/liter) and vancomycin (7.5 mg/liter). Veillonella agar (Difco Ltd.), Rogosa's agar (CM 627; Oxoid) and Wilkins-Chalgren agar (CM 619; Oxoid) with 5% horse blood, sodium pyruvate (1 g/liter), and nalidixic acid (10 mg/liter), which were all incubated anaerobically at 37°C. All manipulations were carried out in an anaerobic cabinet. The lower limit of detection, assuming a 0.1-g sample, would be 200 CFU/g of sample.

Identification of isolates. Isolates were identified to genus level using standard laboratory methods (4). Three isolates of each colonial type of all *Enterobacteriaceae* were picked for identification to species level, which was done using the Mast-ID system (Mast Diagnostics, Bootle, United Kingdom), in which biochemical test agars are inoculated with a multipoint inoculator (29, 51). API 20E (bioMérieux, Marcy l'Etoile, France) was used in strains for which identification was in doubt. All isolates of *Enterobacteriaceae* were stored on beads (Protect; Technical Service Consultants Ltd., Heywood, United Kingdom) at −70°C.

REP typing and DNA extraction. All isolates of *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. were typed by REP-PCR. Strains were grown on Iso-Sensitest agar (CM 471; Oxoid) and incubated overnight at 37°C. *E. coli* DNA was prepared by suspending three to four colonies in 50 μl of water and heating to 95°C for 5 min. Supernatant (5 μl) was used as the template for PCR. For *Klebsiella* and *Enterobacter* spp., growth from Iso-Sensitest plates was suspended in 500 μl of extraction buffer (0.1 M NaOH, 1 M NaCl, 0.5% sodium dodecyl sulfate) and boiled for 15 min. Three extractions were performed on cell lysates using 500 μl of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma, Poole, United Kingdom), and the mixture was centrifuged at 12,000 × *g* for 2 min. DNA was precipitated with 1 ml of ethanol at −70°C for 30 min, then centrifuged at 12,000 × *g* for 15 min. The pellet was washed in 100 μl of diethyl ether and dried under vacuum at room temperature. The extracted DNA was dissolved in 100 μl of water, and 3 μl of solution was used as template.

PCR amplification. The total 50-μl reaction mixture contained 2 U of *Taq* polymerase (HT Biotechnology, Cambridge, United Kingdom), 200 μM deoxynucleotide triphosphates (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.), 1 mM $MgCl_2$, 10% dimethyl sulfoxide, and 40 pmol of primer. The primer used was REP1R-I (5′-IIICGICGICATCIGGC-3′), either alone or in combination with REP 2-1 (5′-ICGICTTATCIGGCCTAC-3′) as described by Versalovic et al. (59). Amplification was performed in an automated thermal cycler (Hybaid Ltd., Middlesex, United Kingdom) with an initial denaturation (3 min at 95°C) followed by 30 cycles of denaturation (30 s at 90°C), annealing (1 min at 39°C), and extension (8 min at 65°C) with a single final extension (16 min at 65°C). A negative control containing water in place of template DNA was included in each run. PCR product (13 μl) was subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. REP-PCR fingerprints were inspected visually and compared to molecular-size markers run concurrently. Profiles were considered highly similar when all visible bands had the same migration distance. Variations in the intensity and shape of bands were disregarded, and absence of up to two bands was allowed before isolates were considered different.

PFGE. Selected strains of *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. were also typed by PFGE. PFGE was performed on at least one isolate of each REP pattern from every baby. Strains were grown in an orbital incubator (Sanyo Gallenkamp, Loughborough, United Kingdom) at 100 rpm in 5 ml of tryptone soya broth (CM 129; Oxoid) at 37°C for 5 h (or overnight). Cells were pelleted by centrifugation and washed with SE buffer (75 mM NaCl, 25 mM EDTA [pH 8.5]). Cells were resuspended in EC lysis buffer (6 mM Tris-HCl [pH 7.6], 1 mM NaCl, 100 mM EDTA [pH 7.5]), 0.5% Brij 58, 0.5% lauryl sarcosine, and 0.2% deoxycholic acid), added to an equal volume of 2% low-melting-point agarose, and allowed to set in 120-μl block formers. The blocks were then incubated overnight at 55°C in 1 ml of EC lysis buffer containing 1 mg of proteinase K per ml. After washing three times in 5 ml of TE buffer (10 mM Tris HCl plus 1 mM EDTA) with rolling for 30 min at room temperature, the blocks were stored in TE buffer at 4°C for 3 to 5 days. A 2-mm slice from each block was digested with 10 U of *Xba*I in restriction buffer at 37°C overnight. Blocks were loaded into 1.2% agarose gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA [pH 8.0]), and DNA fragments were separated using a contour-clamped homogeneous electric field apparatus (Bio-Rad Laboratories) with 5- to 35-s linear ramping at 6 V/cm for 20 h at 12°C. Gels were analyzed by eye following ethidium bromide staining, using the criteria of Tenover et al. (57).

Antibiotic susceptibility. Antibiotic susceptibility testing was performed on at least one isolate of each PFGE type. Tests were performed by a comparative disc diffusion method (30) on Iso-Sensitest agar using the following antibiotics: ampicillin (10 μg), cephradine (30 μg), cefotaxime (30 μg), aztreonam (30 μg), trimethoprim (5 μg), ciprofloxacin (5 μg), gentamicin (10 μg), amikacin (30 μg), and meropenem (10 μg).

Multiplex PCR. A multiplex PCR was used to screen *E. coli* isolates for the stx_1, stx_2, *eaeA*, and *hlyA* genes, using primer sets described previously (47). At least one isolate of each PFGE type was investigated. Three or four colonies were suspended in 50 μl of sterile water and heated at 95°C for 5 min to lyse the cells. A 2-μl aliquot of this template was amplified in a 25-μl reaction mixture containing 1 U of *Taq* polymerase, 200 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 10 pmol concentrations of each primer, with PCR cycles as described previously (47). Samples were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide. *E. coli* P1407 (serotype O157) was used as a positive control.

Statistical methods. Results were evaluated using the chi-square test, independent *t* test, and Mann-Whitney test.

RESULTS

A total of 422 samples from 122 infants were examined. Between 1 and 10 samples were collected from each infant (median, three samples). Culturing revealed 108 (25.6%) samples to be sterile, 158 (37.4%) samples yielded gram-positive organisms (150 samples contained gram-positive organisms alone and 8 samples contained gram-positive organisms plus yeasts), 150 (35.5%) yielded gram-negative organisms (41 with gram-negative organisms alone, 103 with gram-negative combined with gram-positive organisms, 4 with gram-negative organisms and yeasts, and 2 with gram-negative plus gram-positive organisms and yeasts), and 6 (1.4%) samples grew only yeasts. No anaerobes were isolated, despite using techniques which have been successfully used to isolate anaerobic organisms from fecal samples.

The organisms isolated are shown in Table 1. The gram-negative organisms isolated were predominantly *E. coli*, *Klebsiella* spp., and *Enterobacter* spp., comprising 95.6% of all gram-negative isolates. The majority of the 150 samples with gram-negative bacteria had one species present; only 8 samples from six infants had more than one species of *Enterobacteriaceae*. During the study period, 61 infants (50%) were colonized with gram-negative organisms in one to seven samples (median, two samples).

Samples in which gram-negative organisms were isolated were collected between days 4 and 240 of postnatal life (median, day 31). Those with gram-positive organisms were collected between 2 and 304 days of life (median, 20), and sterile samples were collected at days 2 to 180 (median, 19). The percentage of samples with gram-negative organisms increased with age while the percentage of sterile samples declined (Fig. 1).

Quantitative counts of *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. at different postnatal ages are shown in Fig. 2.

TABLE 1. Bacterial species isolated from duodenal aspirates

Species	Number of samples containing the organism (n = 422)	Number of infants from which samples were obtained (n = 122)
Escherichia coli	89	35
Klebsiella pneumoniae	22	15
Klebsiella oxytoca	24	12
Enterobacter cloacae	11	8
Enterobacter agglomerans	5	2
Enterobacter spp.	2	2
Citrobacter freundii	1	1
Morganella morganii	1	1
Rahnella aquatalis	2	1
Serratia liquefaciens	1	1
Pseudomonas spp.	1	1
Haemophilus parainfluenzae	1	1
Coagulase-negative staphylococcus	170	83
Staphylococcus aureus	57	36
Enterococcus spp.	79	48
Streptococcus spp.	19	17
Corynebacterium spp.	1	1
Bacillus spp.	1	1
Yeasts	20	12

Counts of gram-negative organisms isolated from individual infants ranged from 53.1 CFU/g to 1.48×10^8 CFU/g, but mean counts in different postnatal age groups were similar (Table 2).

All babies had been started on enteral feeding prior to isolation of gram-negative organisms from duodenal aspirates. Of 15 samples collected from those infants who had not been fed enterally, 8 were sterile, 5 yielded coagulase-negative staphylococci (CONS), and two had mixed growth including CONS, enterococci, and yeasts. The majority of infants were fed either formula or a combination of formula plus unpasteurized

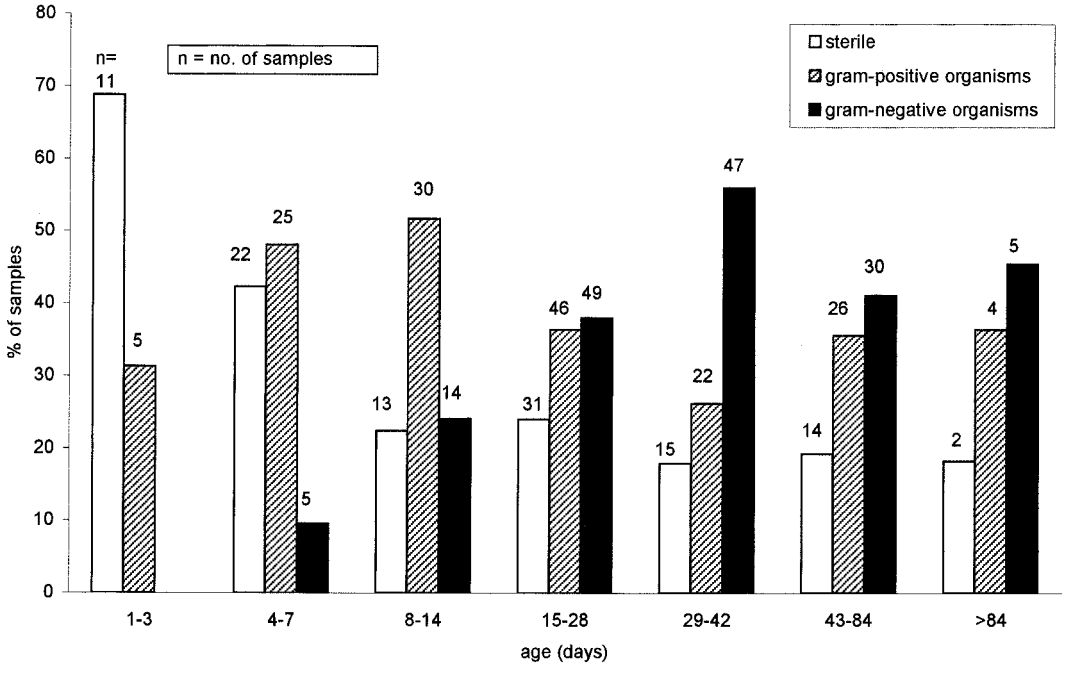

FIG. 1. Duodenal aspirate culture by organism type in relation to age.

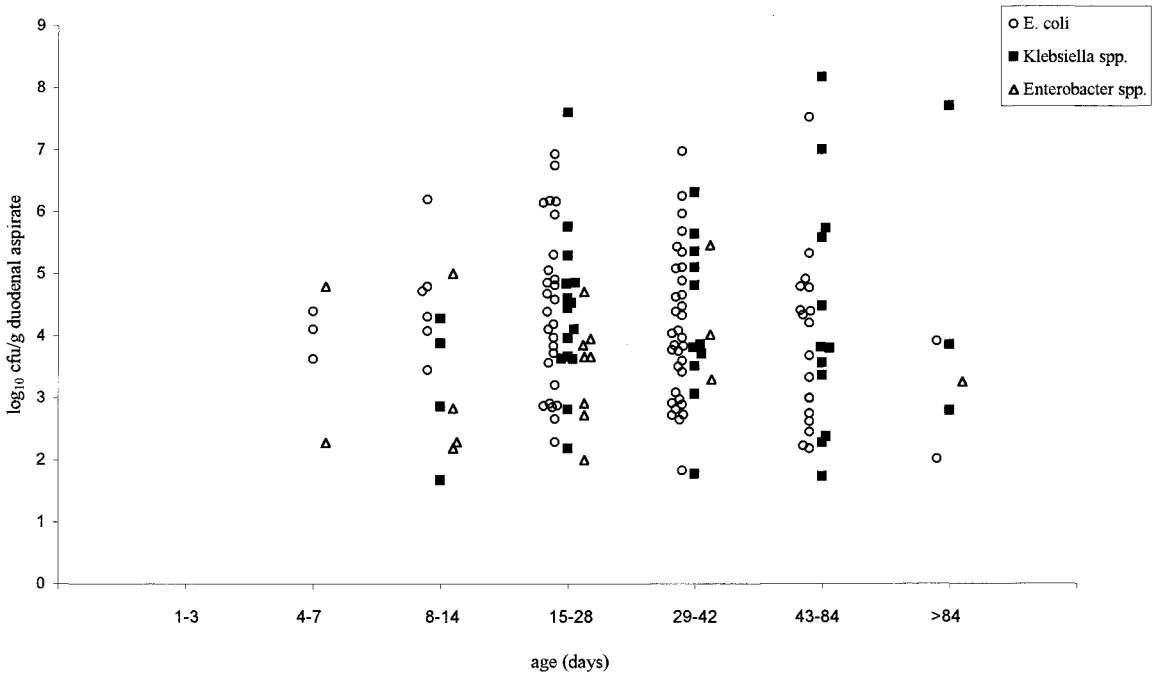

FIG. 2. Quantitative counts of *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. from duodenal aspirates at different postnatal ages.

maternally expressed breast milk. In the group colonized with gram-negative bacteria, 32 infants were fed a combination of breast milk and formula compared to 25 in the group with no gram-negative colonization ($P > 0.2$). Four babies were exclusively fed breast milk, only one of which had *Enterobacteriaceae* isolated from duodenal aspirates.

Infants that were colonized with gram-negative bacteria on one or more occasions were compared to those from which no gram-negative organisms were isolated in duodenal aspirates (Table 3). There were no significant differences in gestational age, birth weight, multiple births, maternal antenatal steroids, Apgar scores, respiratory distress, use of surfactant, intermittent positive-pressure ventilation, umbilical artery catheters, or patent ductus arteriosus between the two groups. There were more cesarean section deliveries in the group that was never colonized with gram-negative organisms ($P = 0.05$). In the gram-negative colonization group, one infant had an exchange transfusion and one a plasma exchange. Infants that were colonized with gram-negative bacteria had a significantly longer stay in NICUs than those that were not colonized.

REP-PCR typing and PFGE typing were performed on 89 *E. coli* isolates from 35 infants. These procedures revealed 7 different REP-PCR patterns using REP1R-I primer alone and 10 when using REP1R-I and REP 2-1 together. PFGE was more discriminatory, revealing 15 different patterns. There was evidence of cross-colonization with identical strains isolated from multiple babies, including 1 strain isolated from 12 infants. There was marked temporal clustering of indistinguishable strains (Fig. 3). The strain isolated from 12 infants was the only *E. coli* isolated over a 16-week period, and following this, it was not isolated again. For most of the study period, only one or two different *E. coli* strains were isolated at any given time. All strains tested by multiplex PCR were negative for stx_1, stx_2, *eaeA*, and *hlyA* genes.

TABLE 2. Counts of gram-negative organisms isolated from duodenal aspirates in relation to age

Infant ages (days [range])	Counts for parameter:	
	Samples (*n*) with gram-negative organisms	Microbial cells ($\log_{10}$ CFU/g [mean ± SD])
1–3	0	0
4–7	5	3.84 ± 0.97
8–14	14	3.81 ± 1.24
15–28	49	4.22 ± 1.30
29–42	47	4.17 ± 1.25
43–84	30	4.04 ± 1.59
>85	5	3.48 ± 1.87

There were 46 isolates of *Klebsiella* spp. from 26 infants. Typing revealed *Klebsiella pneumoniae* with five different REP-PCR types and eight PFGE patterns, plus one REP-PCR type isolated from five infants, which failed to produce a pattern on PFGE. Isolates of *Klebsiella oxytoca* produced four REP-PCR and five PFGE patterns, with one strain failing to produce bands on PFGE. There was evidence of cross-colonization with up to six infants colonized with the same strain. As with *E. coli*, most strains showed temporal clustering, being isolated over relatively short time periods and then disappearing (Fig. 4). Two strains of *K. pneumoniae*, however, were isolated from pairs of infants at intervals of 7 and 11 months. In neither case did the admission dates of the involved pair overlap, which suggests some environmental or other source.

Typing of *Enterobacter* spp. revealed four different REP-PCR patterns, with two strains failing to produce bands, and six different PFGE patterns, with one strain producing no pattern. One strain was isolated from five infants, four of which were linked temporally, but the fifth isolate occurred after a nine-month interval (Fig. 5). All other strains were isolated from individual infants or twins.

Once colonized with a particular strain of *Enterobacteriaceae*, many babies remained persistently colonized. Twenty-one infants yielded the same *E. coli* by REP-PCR and PFGE

TABLE 3. Characteristics of infants with and without gram-negative colonization of duodenal aspirates

Characteristic and units quantified[a]	Value for infants with:	
	Duodenal aspirate with gram-negative organisms (n = 61)	Duodenal aspirate without gram-negative organisms (n = 61)
Sex (M/F)	33/28	34/27
Gestational age in weeks (mean ± SD)	28.1 ± 2.3	28.8 ± 2.7
Birth weight (grams [mean ± SD])	1,100 ± 269	1,107 ± 270
Multiple birth	16	19
Cesarean section[b]	36	46
Maternal antenatal steroids	27	28
Apgar median (interquartile range) after:		
1 min	5 (4–7)	5 (4–7)
5 min	8 (7–9)	8 (7–9)
Respiratory distress	44	43
Surfactant	26	31
Intermittent positive pressure ventilation	53	45
Umbilical artery catheter	36	28
Patent ductus arteriosus	19	17
Exchange transfusion	2	0
Median (interquartile range) length of stay on neonatal unit in days[c]	53 (35–87)	30 (19–50)

[a] Unless otherwise indicated, units are number of infants.
[b] $P = 0.05$.
[c] $P < 0.05$.

typing from two or more duodenal samples. One infant had the same strain isolated from seven different duodenal samples collected over a 2-month period, while another had the same strain present in samples collected 7 months apart. Similarly, 10 babies had the same *Klebsiella* spp. from two or more samples, with one infant having indistinguishable strains from six samples over a 1-month period. Three babies had indistinguishable *Enterobacter* spp. from two or more samples.

Antibiotic sensitivity testing of 15 different PFGE types of *E. coli* showed that 9 were sensitive to all antibiotics tested, 5 were resistant only to ampicillin, and 1 strain was resistant to ampicillin, trimethoprim, and all aminoglycosides. All *Klebsiella* spp. were resistant only to ampicillin. All of seven different *Enterobacter* spp. were resistant to ampicillin and cephradine and sensitive to the other antibiotics tested.

Of the 61 neonates that had gram-negative organisms isolated from duodenal aspirates, 11 had received no antibiotics prior to the first instance of isolation. The remaining infants received between 1 and 10 courses of intravenous antibiotics (median, 2). Of the 11 infants that had received no antibiotics, 4 were colonized with a fully sensitive *E. coli* strain, one with an *E. coli* strain resistant to ampicillin, one with a *K. pneumoniae* strain, and five with *Enterobacter* spp. Of the 61 neonates that had no gram-negative organisms isolated, 11 received no antibiotics during the period when samples were collected. The remainder received 1 to 22 courses of antibiotics (median, 2).

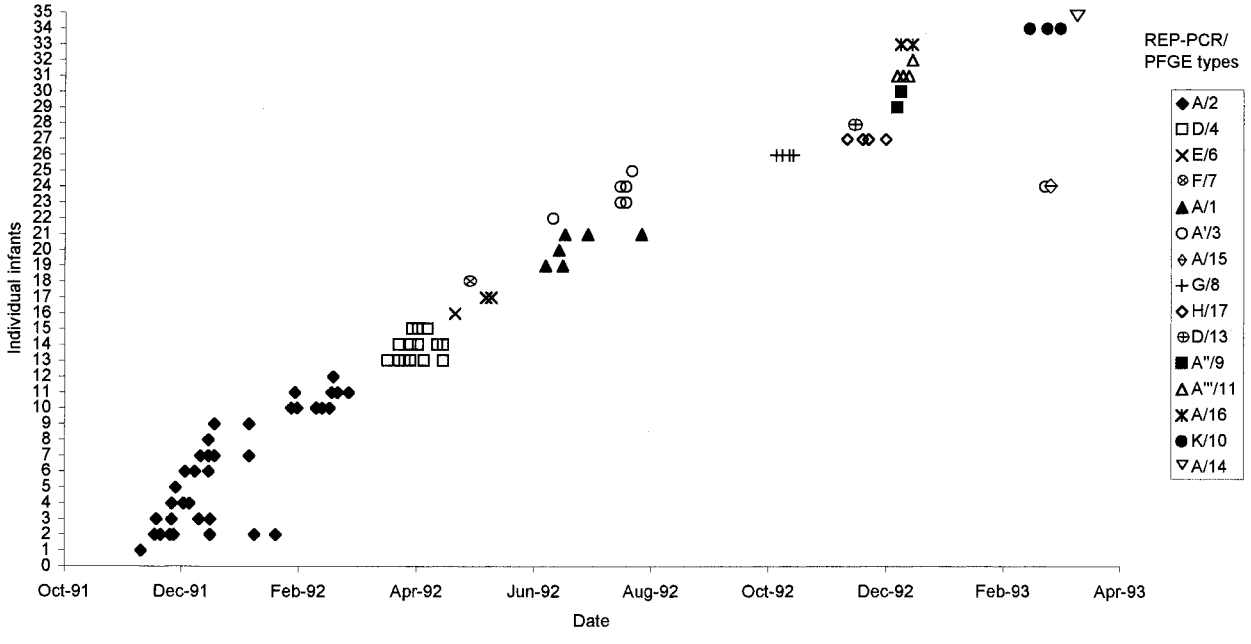

FIG. 3. *E. coli* REP-PCR and PFGE types isolated from duodenal aspirates between October 1991 and March 1993.

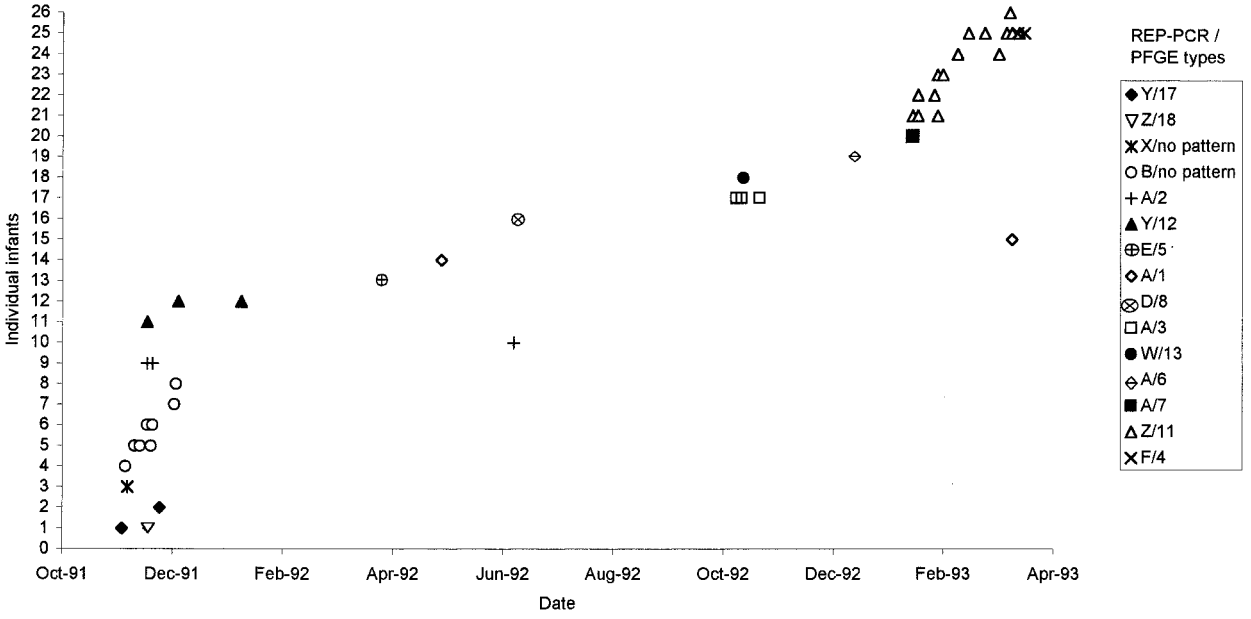

FIG. 4. *Klebsiella* spp. REP-PCR and PFGE types isolated from duodenal aspirates between October 1991 and March 1993.

In both groups the most frequently used antibiotics were ampicillin plus gentamicin and vancomycin plus gentamicin. Other antimicrobial agents used in both groups of infants included benzylpenicillin, flucloxacillin, metronidazole, and cefotaxime. One baby in the gram-negative colonization group received chloramphenicol, while individual infants with no gram-negative organisms were treated with pipericillin, netilmicin, and ceftazidime.

There were 23 episodes of NEC in 20 infants during the study period. Of these, 15 episodes were suspected NEC, classed as stage I by the staging criteria of Bell et al. (7). These infants had clinical signs suggestive of NEC but no specific radiological evidence of disease. The remaining eight episodes were confirmed NEC with either intramural or portal venous gas on X ray or surgical confirmation of disease. Two of these episodes were classified as stage II and six as stage III. Six babies, five of whom had gastrointestinal perforation, underwent surgery. Two of these infants died because of severe, extensive NEC.

Small-bowel aspirates were collected up to 9 days prior to clinical onset of NEC in 13 episodes (5 stage III and 8 stage I). Duodenal samples from one infant with stage III NEC and six with stage I disease grew *Enterobacteriaceae* prior to onset of disease (Table 4). The samples preceding the four other epi-

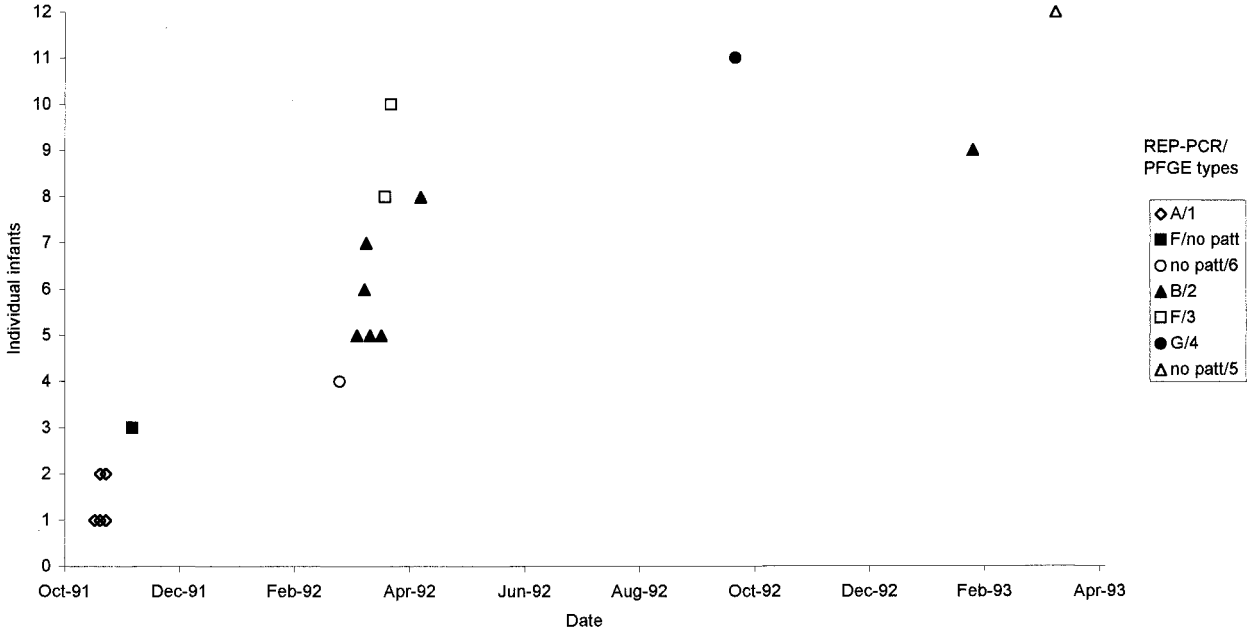

FIG. 5. *Enterobacter* spp. REP-PCR and PFGE types isolated from duodenal aspirates between October 1991 and March 1993.

TABLE 4. Quantitative culture results on duodenal aspirates preceding NEC in infants colonized with gram-negative organisms and mean log count of indistinguishable *Enterobacteriaceae* from infants without NEC

NEC stage	Days from sample to clinical NEC	Duodenal aspirate quantitative culture results (log_{10} CFU/g)	REP-PCR/PFGE types[a]	No. of non-NEC infants with same REP/PFGE type	Gram-negative organisms (mean log_{10} CFU/g)[b]
III	Same day	*E. coli* (5.96) *Enterococcus* spp. (5.88)	A′/3	2	5.70 (*n* = 3)
I	1	*E. coli* (5.44) *Enterococcus* spp. (3.46)	A/2	7	6.18 (*n* = 24)
	2	*E. coli* (3.63) *S. aureus* (4.77)	A′/3	2	5.70 (*n* = 3)
	1	*K. oxytoca* (5.58) CONS (4.34)	Y/12	0	
	9	*K. oxytoca* (1.77) CONS (2.37)	Y/12	0	
	2	*Enterobacter cloacae* (2.29)	F/3	1	2.19 (*n* = 1)
	8	*K. pneumoniae* (2.79)	A/1	1	3.63 (*n* = 1)
		Enterobacter spp. (3.26) *S. liquefaciens* (2.54)	no patt/5	0	

[a] Types correspond to those illustrated in Fig. 3 through 5. no patt, no pattern.
[b] *n*, no. of samples.

sodes of stage III NEC were either sterile or yielded CONS or yeasts. An *E. coli* strain isolated from the infant with confirmed NEC was indistinguishable from strains isolated from one baby with stage I NEC and two with no gastrointestinal disease. Four of the *Enterobacteriaceae* isolated from infants with suspected NEC were also found in those who did not develop the disease. The infants colonized with strains indistinguishable from those found in NEC babies had microbial counts of the same order of magnitude as those in NEC cases (Table 4).

DISCUSSION

This is the first prospective study of small-bowel flora in preterm neonates without prolonged duodenal intubation. Compared to the sparse, mainly gram-positive flora found in the upper small bowel of healthy infants, we found a high incidence of duodenal gram-negative colonization in 122 very-low-birth-weight infants, with *Enterobacteriaceae* counts of up to 10^8 CFU/g recorded. A previous investigation of small-bowel flora in a small number of preterm infants suggested predominantly gram-negative colonization, although the prolonged use of transpyloric tubes in that study may have influenced the results (21).

The gastric insufflation technique (49) used in this study was highly successful in facilitating rapid duodenal intubation. Samples collected by tube aspirates have been criticized, as open tubes are liable to contamination from the mouth and stomach. However a previous study comparing double lumen tubes with the Shiner capsule, designed to protect the inner surface during passage to the site being sampled, found extremely close correlation both quantitatively and qualitatively in organisms recovered from jejunal aspirates (33). A comparison of tube collection and direct sampling at operation in dogs found no significant difference in organisms recovered by the two techniques (25).

We found that samples obtained in the first 3 days of life were predominantly sterile (68.8%), with the proportion of sterile samples declining to around 20% beyond 1 week of age. Colonization with gram-negative organisms occurred beyond 4 days of age in infants that had been fed enterally, increased with age, and was associated with a longer stay on NICUs. Microbial counts of gram-negative organisms varied among individual infants but were similar at different postnatal age ranges.

Maternal flora is assumed to be the major source of early gastrointestinal colonization, as babies delivered vaginally have significantly more aerobic gram-negative bacilli and anaerobic organisms in feces at 48 h than those delivered by cesarean section (36). Plasmid profile analysis has demonstrated transmission of maternal fecal isolates to vaginally delivered infants (56). In preterm infants, greater frequencies of cesarean section delivery, parenteral feeding, and the widespread use of antibiotics are likely to contribute to the delay in colonization with a less complex flora (9, 11, 55).

The high prevalence of duodenal colonization with *Enterobacteriaceae* found in this study may be related to immaturity of the gastrointestinal tract. Newborn infants, particularly preterm, are thought to have lower rates of gastric acid secretion than adults (38). Gastric acid provides the first defense against colonization of the small bowel, and in achlorhydric patients relatively large numbers of organisms can be found in the jejunum (28). Poor intestinal motility in the preterm infant may also lead to stasis and bacterial overgrowth (10). Preterm infants have impaired defense against bacterial antigens due to reduced numbers of B cells in intestinal mucosa, decreased secretory immunoglobulin A levels, and fewer intestinal T cells (32).

There are well-recognized differences in fecal flora between breast-fed and formula-fed term newborns (3, 63). In this study very few infants were fed exclusively with expressed maternal milk. There was no significant difference in the number of babies fed a combination of human milk and formula in the groups colonized with gram-negative bacteria and those not. However, there was great variation in the amount of breast milk individual infants received. Some babies were predominantly formula fed but received small amounts of maternal breast milk for a few days, while others received predominantly breast milk supplemented with formula.

Molecular typing of *Enterobacteriaceae* in this study has shown considerable cross-colonization with marked temporal clustering of indistinguishable strains, suggesting that rather than acquiring maternal strains, preterm infants develop nosocomial colonization of the gastrointestinal tract. A Japanese study of fecal *E. coli* from full-term infants found frequent

acquisition of hospital-derived rather than maternal strains and suggested that the practice of separating newborn infants from their mothers, to be cared for by nursery staff for up to 72 h, may account for this nosocomial spread (41). Outbreaks of infection caused by *Enterobacteriaceae* in NICUs have suggested that gastrointestinal carriage may act as a reservoir of the epidemic strain with transmission by the hands of personnel (1, 27, 35, 52). *Klebsiella* spp. have been shown to survive on hands for up to 150 min (15). Schreiner et al. (50) showed a high rate of contamination of formula for continuous enteral feeds, probably by cross contamination by staff, and this may provide a route for gastrointestinal colonization by nosocomial strains.

We found that particular strains of *E. coli* and *Klebsiella* spp. were detected for periods of up to 16 weeks and then disappeared, to be replaced by alternative strains. Other strains, including the majority of *Enterobacter* spp. isolated, were found only in individual infants or twins. A survey of fecal *Enterobacteriaceae* in infants discharged from Swedish neonatal units also suggested that some strains, particularly *Klebsiella* spp., had a high propensity to spread (58).

The only difference between babies colonized with gram-negative organisms and those that were not was a higher proportion of vaginal deliveries in the colonized group ($P = 0.05$); thus some of these infants may have acquired maternal organisms at delivery. However, of the five infants with gram-negative duodenal colonization in the first week of life, four had strains previously isolated from other babies, suggesting that early small-bowel colonization may occur with nosocomial strains.

Broad-spectrum antibiotics, which are used frequently in NICUs, have also been shown to have a profound effect on fecal flora, and this effect may contribute to overgrowth in the small bowel (9, 13). We found, however, similar antibiotic usage in infants colonized with gram-negative bacilli and those that were not. Infants that had received no antibiotics were as likely to be colonized with an antibiotic-resistant strain as a fully sensitive isolate. The overall level of antibiotic resistance was in keeping with clinical isolates of *Enterobacteriaceae* from our NICU, where resistance to agents other than ampicillin or cephradine was uncommon.

Enterobacteriaceae have been associated with outbreaks of NEC (60) and implicated in endemic cases of disease. This is the first study to use stable molecular-typing techniques to characterize strains of *Enterobacteriaceae* associated with NEC. REP-PCR is a rapid PCR-based fingerprinting method which utilizes primers for the repetitive extragenic, palindromic sequences found in many bacterial chromosomes. The highly conserved REP sequence includes an inverted repeat and can occur singly or as multiple adjacent copies in the genome (53). REP-PCR typing has been shown to be applicable to a wide variety of bacterial species (59, 61, 62). We found that using REP1R-I primer alone was not as discriminatory as REP1R-I and REP2-1 together and neither of these methods was as discriminatory as PFGE. PFGE has been used for typing a wide variety of bacterial species and found to be highly discriminatory and generally superior to other techniques (39). REP typing is a more rapid technique and may be useful as an initial investigation in an outbreak.

We found no association of particular strains of *E. coli*, *Klebsiella* spp., or *Enterobacter* spp. with NEC, with most of the strains isolated from NEC cases also occurring in asymptomatic infants. Counts of these organisms were similar in infants with NEC and those that did not develop disease. Thus it appears that upper small-bowel colonization with particular strains of *Enterobacteriaceae* is not sufficient to cause NEC and that other factors must contribute to development of the disease.

Panigrahi et al. (46) suggested that gram-positive isolates may prevent adherence of *Enterobacteriaceae* and that this may play a role in the pathophysiology of NEC. Our results reveal that the infants colonized with gram-negative organisms that developed NEC were in most cases also colonized with gram-positive organisms (Table 4). Twelve samples from six asymptomatic infants colonized with the same REP-PCR/PFGE type isolated from NEC cases yielded *Enterobacteriaceae* alone. The remaining samples from these asymptomatic infants grew *Enterobacteriaceae* combined with gram-positive organisms, predominantly enterococci and CONS. Cultures from the upper small bowel may obviously not reflect the situation in the terminal ileum or colon where NEC is most likely to occur.

Bacterial toxins have been proposed as causes of NEC (60). On the basis of a multiplex PCR assay for *stx*$_1$, *stx*$_2$, *eaeA*, and *hlyA*, we found no evidence of toxin-producing *E. coli* strains. The recovery of clonal *Enterobacteriaceae* isolates from both NEC cases and asymptomatic infants suggests that toxin-mediated injury by these organisms is an unlikely cause of NEC, unless there is considerable variation in gene expression. This study was designed to assess the role of small-bowel colonization with *Enterobacteriaceae* in the pathogenesis of NEC. We did not investigate other toxin-producing organisms, and this is an area which may warrant further study.

In summary, we have shown that very-low-birth-weight infants in NICUs have high levels of colonization with *Enterobacteriaciae* in the duodenum. There was evidence of considerable cross-colonization with temporal clustering of indistinguishable strains but no obvious association between colonization with particular strains and development of NEC.

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