DEVELOPMENT OF GUT COLONISATION IN PRE-TERM NEONATES

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SUMMARY. Twenty-eight pre-term babies of low birth weight were monitored for developing microflora in throat, stomach and faeces during the first 3 weeks of life. The flora at all levels of the gastrointestinal tract differed from that of healthy breast-fed and artificially fed full-term babies. Colonisation of throat and stomach was delayed beyond 4 days of life in 87% and 60% of babies respectively. Only 10% of babies had “normal” oral flora throughout the period of study. Flora of the stomach was sparse, and resembled faecal flora. Faecal flora was established more rapidly than throat or stomach flora, and 70% of babies were colonised during the first 4 days of life. Initially Bacteroides spp. were predominant (57% babies), but Escherichia coli and other aerobic gram-negative bacilli gradually increased in frequency. Colonisation by gram-positive bacteria was slow. Clostridium spp. were present in only 10% of babies during the first 4 days of life. Most strains were transient. Colonisation with C. butyricum (30%), C. perfringens (35%) and C. difficile (25%) was maximum after the first 2 weeks of life. Lactic-acid-producing bacteria usually appeared late in the third week of life. Parenteral feeding immediately after birth was associated with delayed colonisation by a restricted number of species. Parenteral antibiotics (penicillin or gentamicin or both) restricted colonisation with normal oral flora, the lactic-acid-producing bacteria and penicillin-sensitive clostridia, but had little effect on E. coli even when the colonising strain was sensitive to the aminoglycoside in the regimen. Systemic spread of bacteria via the blood stream was not detected in any babies.

The pattern of colonisation of the enteric tract in pre-term infants in the special-care nursery studied, differs from that of healthy full-term babies; this merits consideration when the results of bacteriological tests on this vulnerable group of infants are being interpreted.

INTRODUCTION

The development of the enteric microflora of the healthy full-term neonate is now...
well documented. Rapid contamination of external and internal surfaces occurs when
the fetus is exposed to the environment. The gastrointestinal tract, sterile during
fetal life, becomes colonised within 24 h of birth. Predominant strains fluctuate during
the first 2–3 weeks of life and stabilise according to diet, usually by 1 month of age
(Albert et al., 1978; Beerens, Romond and Neut, 1980; Shahani and Ayebo, 1980;

Premature (pre-term) neonates of low birth weight differ in many respects from
their full-term healthy counterparts. Advances in management have resulted in
survival of babies born as early as 25 weeks gestation. These infants, who are already
compromised by deficiencies in humoral and cellular immune responses (Colten and
Goldberger, 1979; Miller, 1979; Quie and Mills, 1979; Stiehm, Winter and Bryson,
1979; Laurenti et al., 1980) are also exposed to increased hazards of bacterial
contamination because of a prolonged hospital stay and the increased handling
required during special care. In addition, such infants may initially be fed
intravenously and receive antibiotic therapy for prolonged periods. Breast milk from
the mother, if available, usually requires some supplementation.

All these factors would be expected to influence the development of a stable enteric
microflora in low-birth-weight pre-term neonates, but the microflora of such a group
of infants has not been extensively studied. The aim of this investigation was to
identify micro-organisms colonising the gut of 28 pre-term neonates admitted to the
Special Care Nursery of the Royal Children’s Hospital (RCH) Melbourne during
April 1979–October 1980, and to monitor aerobic and anaerobic enteric flora as it
developed during the first 3 weeks of life.

MATERIALS AND METHODS

Patients. There were 28 babies (21 males, 7 females) of mean gestational age 30 weeks (range
25–36 weeks) and mean birth weight 1 125 g (range 560–1500 g). All were born during the period
April 1979–October 1980 in urban or country hospitals and transferred to the RCH 1–8 days
after birth. In all cases, the major reason for transfer was prematurity and clinical conditions
related to prematurity.

<table>
<thead>
<tr>
<th>Item</th>
<th>Number of infants receiving the stated items at the following age-periods (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1–4</td>
</tr>
<tr>
<td>Feeding</td>
<td></td>
</tr>
<tr>
<td>Breast milk</td>
<td>12</td>
</tr>
<tr>
<td>Formula</td>
<td>4</td>
</tr>
<tr>
<td>Parenteral</td>
<td>14</td>
</tr>
<tr>
<td>Antibiotics</td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td>9</td>
</tr>
<tr>
<td>Penicillin + gentamicin</td>
<td>14</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
</tr>
<tr>
<td>Nil</td>
<td>7</td>
</tr>
</tbody>
</table>
COLONISATION OF PRE-TERM NEONATES

Table I lists the composition of feeds in relation to age of infants at the time of collection of each set of specimens. Fourteen babies did not receive oral feeds before 5 days of age. Thereafter, most infants received maternal expressed untreated breast milk, sometimes supplemented with donor breast milk which had been pooled and boiled. A few babies required intermittent periods of parenteral nutrition after 5 days of age. Table I also lists regimens of parenteral antibiotic therapy in relation to age. Twenty-three infants received either penicillin or penicillin and gentamicin during the first 4 days of life. Only six babies still required antibiotics after day 17 of life. All infants were given antifungal therapy during the first 2 weeks of life; this was mycostatin syrup 100,000 units three times daily.

Specimen collection. Specimens were collected within 8 h of admission and twice weekly thereafter. Some collections were omitted when clinically contra-indicated. In total, 143 sets of specimens were collected during the survey; the number of sets collected during each of six 4-day periods is shown in table I.

Throat swabs, gastric aspirate (withdrawn from the stomach via a sterile 5FG feeding tube), and freshly expelled faeces (retained in the napkin), were brought to the laboratory immediately. Blood cultures were collected with the first set of specimens (mean age 2-7 days) and again during the final collection (mean age 21-4 days).

Culture of specimens. All specimens were processed within 1 h of collection. Macroscopic appearances of gastric aspirate and faeces were recorded and pH was determined. Direct smears of gastric aspirate and faeces were prepared and fixed for later staining by Gram's stain (Kopeloff modification; Holdeman, Cato and Moore, 1977). Results of light microscopy of all smears were compared with results of culture to check adequacy of culture media and methods of incubation. All specimens were seeded on to horse-blood agar (HBA; Columbia Agar Base, Oxoid), MacConkey agar (Oxoid) and mannitol salt agar (Oxoid), and incubated aerobically at 37°C. Throat swabs seeded on HBA were incubated in an atmosphere of 5-10% carbon dioxide.

Gastric aspirate and faeces were also cultured on a range of enriched and selective media for isolation of anaerobic bacteria. These were HBA, phenylethyl alcohol blood agar and kanamycin vancomycin laked-blood agar (Sutter, Vargo and Finegold, 1975), sodium azide blood agar and cycloserine cefoxitin egg fructose agar (CCFA; George et al., 1979). In addition, gastric aspirate and faeces were seeded into cooked-meat broth with a Schaedler broth overlay (26.5 gm/L), Columbia agar (0.1 mg/L), vitamin K1 (0.2 ml/L), resazurin (0.4% v/v) and a few iron filings. Faeces were also seeded on to two triple antibiotic blood-agar plates (Steele and McDermott, 1978) and incubated at 42°C and 25°C in a candle jar for the selective isolation of Campylobacter spp.

Throat swabs were not cultured for anaerobic bacteria because other studies have shown oral anaerobes to be rare before the eruption of deciduous teeth (Hurst, 1957; McCarthy, Snyder and Parker, 1965; Rotimi and Duerden, 1981).

All anaerobic plate media were reduced before use and discarded if more than 7 days old. During inoculation, plates were held under a stream of high-purity carbon dioxide (Carba, Melbourne, Australia) to maintain the reduced state. Cooked-meat medium was inoculated under a similar jet of high-purity carbon dioxide.

All platings were carried out in a standardised manner so that a semi-quantitative estimate of colony-forming units (cfu) could be made (Goldmann, Leclair and Macone, 1978). Aerobic plates were examined at 24 and 48 h. Anaerobic plates were incubated in anaerobic jars containing Oxoid gas-generating sachets with freshly dried catalyst, and examined after incubation for 48 h and 7 days at 37°C. Cooked-meat medium was incubated at 37°C for periods up to 60 days. Blood was cultured in 18-ml volumes of pre-reduced supplemented Peptone Broth (Becton Dickinson, Empire Way, Wembley, Middlesex HA9 0PS) capped with vented and Vaspar-plugged tops for aerobic and anaerobic cultivation. Blood cultures were incubated at 37°C for 35 days. Aerobic subcultures were made at 24 h and anaerobic subcultures at 48 h. Subcultures were repeated twice weekly and samples from the broth were analysed by gas-liquid chromatography for short-chain volatile fatty acids.

Identification of isolates. Aerobic isolates were identified by standard biochemical criteria (Cowan, 1974) and antibiogram. Anaerobic isolates were identified on the basis of colonial and cellular morphology, and volatile fatty-acid products determined by gas-liquid chromatography (GLC) of the cooked-meat medium (Packard GLC, Model No. 427) and an API 20A strip.
Anaerobic gram-positive bacilli suspected morphologically and by GLC profile of being Clostridium spp. were seeded on to Nagler plates and examined for inhibition of lecithinase production in the presence of polyvalent clostridial antitoxin (CSL, Melbourne, Australia) and monovalent Clostridium perfringens antitoxin. C. difficile was specifically identified by Gram's stain, colony morphology, fructose fermentation, yellow fluorescence of colonies on CCFA under short-wave ultraviolet light, and metabolic products on GLC.

Strains of Escherichia coli isolated were sent to Dr Richard Luke, La Trobe University, and tested for ability to produce heat-labile toxin in HeLa cell culture and heat-stable toxin by intragastric inoculation of infant mice.

Identification of viruses. Faecal specimens were processed for electronmicroscopy by preparing a 20% homogenate in phosphate-buffered saline. This was centrifuged at 10 000 g for 20 min at 4°C and the supernate was further centrifuged at 100 000 g for 60 min at 4°C. The deposit obtained was examined with an electronmicroscope after negative staining with 10% ammonium molybdate. Viruses present were identified by morphology. Faecal samples were also inoculated into cells used routinely for isolation of viruses, including monkey kidney, human fetal fibroblast and HeLa cells. Viruses isolated were identified by cytopathogenic effect and serology.

RESULTS

Tables II, III and IV summarise the species of bacteria isolated from 143 throat swabs, specimens of stomach contents and faeces according to age of the baby at the time of sampling. The percentage of specimens yielding species representative of the flora in relation to age is illustrated in the figure.

Throat flora

Twenty-six of the 30 throat specimens cultured during the first 4 days of life yielded no growth (table II). After day 4, only eight specimens yielded no growth, and all were obtained from children on antibiotic therapy. In the earliest days of life,

<table>
<thead>
<tr>
<th>Species or group</th>
<th>Percentage frequency at the following age-periods (days)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0-4</td>
</tr>
<tr>
<td>No growth</td>
<td>87</td>
</tr>
<tr>
<td>Viridans streptococci</td>
<td>7</td>
</tr>
<tr>
<td>Str. pneumoniae</td>
<td>7</td>
</tr>
<tr>
<td>Streptococcus (group A)</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus (group D)</td>
<td>0</td>
</tr>
<tr>
<td>Staph. epidermidis</td>
<td>17</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>0</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
<td>7</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>0</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>0</td>
</tr>
<tr>
<td>Paracolon bacteria</td>
<td>0</td>
</tr>
</tbody>
</table>

n = Total number of samples investigated during the period.
Staphylococcus epidermidis was the most common isolate, reaching maximum frequency (43%) by days 9–12 of life. By contrast, colonisation with *E. coli* was slower reaching maximum frequency (45%) in specimens taken during days 17–20 of life. Colonisation with *Klebsiella* spp. was infrequent during the first 16 days of life but was common thereafter. Colonisation with viridans streptococci was delayed, and other species usually regarded as frequent components of normal oral flora, e.g., *Neisseria*.
spp, *Haemophilus* spp, *Corynebacterium* spp., were not isolated during the period studied. *Candida* spp. were not isolated during this study but all babies were on antifungal therapy. Oral flora increased in complexity with age. It was usual to isolate only one species initially, followed by the appearance of additional species in the next 4 days. By day 20 most specimens yielded a flora comprising 3–4 different species. *Klebsiella* spp. with *Streptococcus pneumoniae* was a common combination, whereas *E. coli* and *Staph. aureus* were never isolated from the same specimen.

**Stomach flora**

*Bacteroides* spp. of the *B. fragilis* group (*B. fragilis*, *B. vulgatus*, *B. theta iotaomicron*, *B. ovatus*, *B. 349A* and *B. distasonis*) were the most common species isolated throughout the period of the study (table III). By days 9–12 of life, 54% of specimens yielded *Bacteroides* spp. Aerobic gram-negative bacilli were also frequently present in stomach contents. By contrast, gram-positive coccii (*Staph. epidermidis*, *Staph. aureus*, viridans streptococci and *Streptococcus* group D) were less frequent. A low level of colonisation was observed for *C. butyricum*. *C. perfringens* was isolated on three
Colonisation of pre-term neonates

**Table V**

Influence of enteral feeding on faecal flora during the first 4 days of life

<table>
<thead>
<tr>
<th>Result of culture</th>
<th>Number of infants yielding the stated bacteria when feeding was</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>parenteral n = 14 (9A)*</td>
</tr>
<tr>
<td></td>
<td>enteral n = 16 (14A)</td>
</tr>
<tr>
<td>No growth</td>
<td>6 (43%)</td>
</tr>
<tr>
<td><em>Bacteroides</em> spp.</td>
<td>5</td>
</tr>
<tr>
<td>Aerobic gram-negative bacilli</td>
<td>5</td>
</tr>
<tr>
<td><em>Clostridium</em> spp.</td>
<td>3</td>
</tr>
<tr>
<td>Other bacteria</td>
<td>0</td>
</tr>
</tbody>
</table>

* 9 of the 14 parenteral group, and 14 of the 16 enteral group were receiving antibiotics at the time of sample collection.

Occasions during the first 12 days of life, but *C. difficile* was absent throughout. *Candida* spp. and *Propionibacterium acnes* were only occasionally found.

**Faecal flora**

Most of the babies were colonised by bacteria during the first 4 days of life (table IV). *Bacteroides* spp. were the most common species and were isolated from 57% of specimens obtained during the first 4 days of life, but declined in incidence in specimens obtained after day 12. By contrast, *E. coli* and *Klebsiella* spp. were common after day 4 and persisted. Other aerobic gram-negative bacilli were isolated infrequently. None of the strains of *E. coli* tested produced heat-labile or heat-stable enterotoxin. *Campylobacter* spp. were not isolated from any babies. *Clostridium* spp. were isolated with low frequency during the first 16 days of life, and increased in frequency thereafter. At any age, mixtures of clostridial species in a stool specimen were rare. No strains of *C. difficile* produced detectable toxin. Only two babies (twins) yielded mixed clostridial species in a single specimen. Lactobacilli were seldom isolated during the period of the study. In addition to the organisms listed in table IV, *P. acnes* was occasionally cultured. The only viruses identified were rotavirus (3 babies) and enteroviruses (2 babies).

**Influence of enteral feeding on colonisation**

During the first 4 days of life, 16 specimens were obtained from babies who were fed enterally with either fresh maternal breast milk (15) or with formula (1). Another 14 specimens were obtained from babies who did not receive enteral feeds (table V). Colonisation was delayed beyond the fourth day of life in 43% of babies receiving only parenteral feeding compared with 12.5% of those fed orally. This was not due to antibiotic therapy because the incidence of antibiotic therapy was higher in the enterally fed group (88%) than in the parenterally fed group (64%).


**Influence of parenteral antibiotics on faecal flora**

Table VI compares species of bacteria isolated from babies receiving parenteral antibiotics with babies not receiving antibiotics, in relation to age at which each species was first isolated. *Lactobacillus* spp. were never isolated from babies receiving antibiotics. Before 12 days of age, the incidence of *Clostridium* spp. was reduced in infants receiving antibiotic therapy compared with those who did not receive antibiotics.

**Levels of colonisation**

The initial detection of anaerobic bacteria frequently relied upon chromatographic identification of metabolic products in the primary cooked-meat broth. Species detected in this manner were probably present at levels less than \(10^4\) cfu/ml. By contrast, aerobic gram-negative bacilli grew abundantly from throat and from faeces soon after birth, even when sensitive *in vitro* to the antibiotic in the regimen. Five of nine specimens of faeces from infants less than 4 days of age yielded aerobic gram-negative bacilli in numbers greater than \(10^6\) cfu/ml. Four of these babies were on antibiotics. Isolation of a species in decreasing numbers from sequential specimens usually indicated transition to a new dominant strain. Stomach contents consistently yielded colony counts lower than those from throat and faeces.

**Blood cultures**

Micro-organisms were not isolated from specimens of blood obtained at mean age 2.7 days, nor from specimens of blood obtained at mean age 21.4 days.

**DISCUSSION**

The flora of throat, stomach and faeces of 28 pre-term babies of low birth weight
examined during this study differed in numerous respects from the flora described previously in healthy full-term babies (Mata and Urrutia, 1971; Willis et al., 1973; Long and Swenson, 1977; Beerens et al., 1980; Rotimi and Duerden, 1981). A flora that was predominately gram-negative bacilli was established rapidly in faeces, and less rapidly in throat and stomach. Species regarded as normal components of oral flora (Str. salivarius, viridans streptococci and Neisseria spp.) were diminished or absent. The flora of the stomach was qualitatively similar to the flora of faeces but much less abundant. Stomach contents yielded either no growth or low colony counts (<10⁴ cfu/ml) suggesting that in many babies sufficient acid was present to restrict colonisation at this level. Mixed populations of E. coli, Klebsiella spp. and Bacteroides spp. were established early in faeces. The flora resembled that usually associated with formula feeding rather than breast feeding (Bullen, Tearle and Stewart, 1977; Goldmann, 1980). Gram-positive bacteria (aerobic cocci, lactobacilli and clostridia) became more common with increasing age. Even though most babies in this study were receiving some unprocessed breast milk, particularly in the early days of life, acquisition of lactic-acid-producing bacteria was delayed until late in the third week of life.

The method of feeding influenced acquisition of flora. Parenteral feeding in the first 4 days of life was associated with delayed colonisation in some babies, and with a restricted variety of colonising species. This was not due to the coincidental use of antibiotics.

The effect of parenteral antibiotics on the developing flora was complex. The limited sensitivity of the B. fragilis group to penicillin and to aminoglycosides probably accounts for their rapid proliferation during the first week of life in most babies. However, antibiotic therapy did not always succeed in restricting the growth of sensitive species. For example, E. coli was isolated during the first 11 days of life from one-third of infants on therapy, even though, without exception, the strains were sensitive in vitro to the aminoglycoside in the regimen. When antibiotic usage decreased, usually by day 9 of age, there was a rapid increase in the variety of bacterial species identified.

Antibiotic usage might partly explain the observed delayed colonisation by Clostridium spp. until after the second week of life. This pattern of colonisation differed from that described by others for healthy full-term babies both breast-fed and artificially fed. In breast-fed full-term babies, large numbers of clostridia of mixed species are present in faeces sampled within a few days of birth, and decline by the second week of life. In artificially fed full-term babies, clostridia also appear soon after birth and maintain high levels of colonisation during the following weeks (Willis et al., 1973; Hentges, 1980). In the pre-term babies in this study, Clostridium spp. appeared in small numbers soon after birth in faeces from only a few babies. C. butyricum and C. perfringens were more common than C. difficile which was rarely isolated. All three clostridial species increased in number and frequency during the third week of life when antibiotic therapy was discontinued.

There was no evidence of systemic spread of enteric flora as judged by blood-culture results. This is reassuring, because the intestinal mucosa has been shown to have increased permeability during the neonatal period (Berg, 1980), and the low-birth-weight pre-term neonate can neither concentrate inflammatory cells effectively nor kill bacteria with efficiency (Miller, 1979; Quie and Mills, 1979).
The results of this study show that the flora of the premature neonate separated at birth from the mother, housed in a humidicrib and treated from birth with parenteral antibiotics differs from that of breast fed and of artificially fed healthy full-term babies. The enteric flora is established less rapidly than in healthy full-term babies, probably as a combined result of delay in enteral feeding after birth and the early use of parenteral antibiotics. Micro-organisms colonising the throats of premature babies managed in this way are more likely to be species that are regarded as components of normal faeces rather than normal oral flora. The faecal flora is initially less complex than in healthy full-term babies, with a notable absence of clostridial species. The flora of the upper enteric tract begins to "normalise" in the fourth week of life, i.e., to show decreased predominance of aerobic gram-negative bacilli together with an increase in gram-positive bacteria. However the risk of acquiring potential pathogens such as Staph. aureus, Streptococcus (group A) and Str. pneumoniae increases. The flora of the lower enteric tract also begins to "normalise" in the fourth week of life.

These results provide baseline data for interpretation of bacteriological studies in sick pre-term infants in our special-care nursery. Their applicability to other nurseries must be evaluated so that differences between flora of healthy full-term babies and pre-term babies can be assessed locally. Knowledge of these differences will aid interpretation of studies of bacteriological investigation of pre-term babies.

We thank the Nursing and Medical staff of the Neonatal Intensive Care Unit of the Royal Children's Hospital for their help in collecting specimens; staff at the Virology and Bacteriology Laboratories, Royal Children's Hospital; Dr R. K. J. Luke and Dr N. A. Ryan, School of Agriculture, La Trobe University, Melbourne, for testing for enterotoxigenicity of E. coli; and Mrs Gail Serzycki and Mrs Jane Lee for typing the manuscript. The study was made possible by grants from the Felton Bequest, The Royal Children's Hospital Research Foundation and the Royal Children's Hospital Staff Auxiliary.

REFERENCES


COLONISATION OF PRE-TERM NEONATES


