J. Hyg., Camb. (1985), **94**, 309–318
Printed in Great Britain

The development of bacterial flora of premature neonates

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(*Received* 3 *September* 1984; *accepted* 24 *October* 1984)

SUMMARY

The sequential acquisition of bacterial flora by premature neonates was studied during a 10 month period. Mean gestational age of the babies was 29·01 weeks and the mean birth weight was 1·728 kg. *Escherichia coli* and group B streptococci (GBS) colonized the umbilicus of 7 and 6 babies respectively, out of 23 studied, on the first day of life. *E. coli* and staphylococci were the predominant flora on the 6th day and they colonized 12 and 13 respectively. The oral flora was predominantly Gram-positive cocci, mainly *Streptoccocus salivarius* which was isolated from 17 out of 22 babies on the 6th day, viridians streptococci were isolated from 14 babies, *Staphylococcus albus* from 16 babies and group D streptococci from 11 babies. *Candida* spp. also colonized the oral cavities of 17 out of 22 babies on the 6th day. At the end of the first week of life, the faecal flora was predominantly anaerobic represented by *Bifidobacterium* spp., *Bacterioides* spp. and *Clostridium* spp. The commonest facultative faecal flora were *E. coli*, which was isolated from all the babies, and *Strept. faecalis* isolated from 20 babies. Early gut colonization by GBS, *Bacteroides* spp. and *Clostridium* spp. was noticed in more babies delivered vaginally than by caesarean section where colonization by these bacteria was relatively delayed. The use of prophylactic penicillin plus gentamicin in the special neonatal unit probably prevented systemic spread of any of the potential opportunistic pathogens during the study.

INTRODUCTION

There are now many studies reported in the literature on the development of the bacterial flora of full-term neonates. Most of these studies were conducted in a variety of geographical locations outside the continent of Africa. These studies have shown that the bacterial colonization of the various ecological niches of the body, including those of the gastrointestinal tract (GIT) and the umbilicus, occurs soon after birth. The predominant flora in the first 48 h are facultative organisms but thereafter the neonates usually become colonized by the strict anaerobes to the same, or greater, extent as the facultative species (Mata, Mejicanos & Jimenez, 1972; Bullen, Tearle & Willis, 1976; Long & Swenson, 1977; Rotimi & Duerden, 1981*a*). The normal resident bacterial flora during the first 10 days of life varies according to the baby's feeds but it usually becomes stable by the end of the first month (Albert *et al.* 1978; Beerens, Romond & Neut, 1980; Shahani & Ayebo, 1980; Rotimi & Duerden, 1981*a*). Congenital abnormalities, particularly of the GIT, and

the subsequent surgical operations performed to correct them, also alter the type and number of bacterial flora of neonates in the first 2 weeks of life (Rotimi & Duerden, 1982).

The majority of bacterial infections in neonates are usually acquired during or soon after birth. Bacterial colonization of these neonates and the birth canal of their mothers play significant roles in the development of neonatal infections. Because neonates are immunologically immature they are therefore at risk of infections. Premature or low-birth-weight neonates are at special risk (Cotton & Goldberger, 1979; Miller, 1979; Laurenti *et al.* 1980), and some normal bacterial flora may become opportunist pathogens, especially in premature babies, causing sepsis requiring antibiotic treatment and prolonged hospital stay.

In the neonatal unit of the Department of Paediatrics of our hospital, after prematurity, bacteraemia and septicaemia are the commonest cause of morbidity and mortality in our babies. The majority of the incriminated organisms are known endogeneous flora. This study was therefore undertaken to investigate the development of bacterial colonization of premature neonates whose mothers received ante-natal care and delivered at Lagos University Teaching Hospital (LUTH), and subsequently admitted to the special baby care unit (Ward C4) of LUTH because of their prematurity.

MATERIALS AND METHODS

Subjects

Twenty three neonates (7 males, 16 females) of mean gestational age 29·01 weeks (range 24–36 weeks) and mean birth weight 1·728 kg (range 0·75–2·4 kg), born at LUTH between March 1982 and May 1983 were studied. Twelve, nine and two babies were born by normal vaginal delivery (NVD), caesarean section and forceps respectively. One baby died 3 days after birth following end-to-end anastomosis of the oesophagus to correct his tracheo-oesophageal fistula. During the period of study 2 infants received only formula feed while 9 received both formula and breast milk. In the remaining 12, parenteral fluids were started at birth and gradually replaced with milk depending on the infant's tolerance. Prophylactic penicillin and gentamicin, were administered parenterally to 15 babies during the first week of life. No antifungal agents were administered to the babies at any period during their stay in the ward.

Specimens

Specimens were collected from the mouth, umbilicus and rectum (faeces) by a set of three albumen-coated cotton-wool swabs (Exogen Ltd, Dumbarton Road, Glasgow). The swabs were broken into bijoux bottles containing Amies transport medium and were processed within 30 min of collection in the laboratory. The set of swabs were collected on days 1, 2, 3 and 6 of life. Umbilical swabs were not collected from 2 babies on the 6th day because their umbilical stumps were not accessible.

Culture of specimens

The specimens were seeded onto a variety of sets of freshly prepared selective and non-selective media which included: blood agar (BA; Columbia Agar base

(Oxoid) with 7·5% human blood), MacConkey Agar (Oxoid), mannitol-salt agar (Oxoid), BM kanamycin-vancomycin agar (Holbrook, Ogston & Ross, 1978), chocolate agar, mitis-salivarius agar (Oxoid), Sabouraud agar (Oxoid), Rogosa agar (Oxoid, pH 5·8), neomycin-blood agar (Oxoid Blood Agar base no. 2 with 7·5% defibrinated horse blood and neomycin 75 μg/ml), reinforced clostridial medium (Oxoid) to which 0·75% New Zealand Agar and 0·1% cotton blue (pH 5·0) were added (Willis *et al.* 1973), cycloserine cefoxitin egg fructose agar (CCFA; George *et al.* 1979), cooked meat broth, brain heart infusion broth (Oxoid) and thioglycollate broth (Oxoid).

Inoculation of the plates was carried out in a standard manner so that a semi-quantitative assessment of the density of growth could be made on solid media as previously described (Rotimi & Duerden, 1981*a*). All media meant for anaerobic culture were pre-reduced in anaerobic jars 1 h before use and were not used after 3 days of preparation. Anaerobic incubation was in anaerobic jars (Oxoid) equipped with a room-temperature 4 g palladium catalyst satchet. Anaerobiosis in the jars was obtained by use of the Gaspak gas regenerating kit system (Oxoid). Each jar was biologically controlled by including *Pseudomonas aeruginosa* seeded onto Simmon's citrate slope in the jars; the medium usually turns blue if the jar has failed. Incubation was at 37 °C for minimum period of 48 h and extended to 72 h when necessary. Media for aerobic incubation was incubated in air plus 10% CO_2 (except MacConkey) at 37 °C for 24 h.

Identification of isolates

All aerobes were identified by standard methods (Cowan, 1974) and atypical enterobacteria by the API 20E system (API system, S.A., La Balme Les Grottes, Montalier, France). The anaerobes were identified by colonial and cellular morphologies, antibiotic resistance test, dye tolerance test, biochemical test and sugar fermentation pattern described by Duerden *et al.* (1980), Rotimi, Faulkner & Duerden (1980) and Willis (1977). *Clostridium difficile* was identified by Gram's stain reaction, characteristic smell and yellow colonies on CCFA medium that fluoresced under short-wave ultraviolet light. *Campylobacter* sp. were not looked for because other studies have show that they are not a component of the normal flora of neonates (Blakey *et al.* 1982).

RESULTS

Tables 1–3 summarize the bacterial species or group of organisms isolated from the umbilical stump, oral cavity and faeces according to age of premature babies at the time of sampling. The percentage of babies sequentially colonized by the isolates according to methods of delivery is shown in Table 4.

Umbilical flora

Samples were obtained from a total of 23 babies on days 1, 2 and 3 of life and from 20 on day 6 because one baby died before the 6th day and post-operative dressings made the umbilicus of the other two inaccessible.

Escherichia coli was the most common species of bacteria isolated from the babies reaching maximum frequency by the day 2 of life (Table 1); 12 each out of 23 and

Table 1. *The bacterial flora of the umbilicus of premature neonates*

Species or group of organisms	No. of babies colonized at the following ages			
	Day 1 ($n = 23$)	Day 2 ($n = 23$)	Day 3 ($n = 23$)	Day 6 ($n = 22$)
E. coli	7	12	12	12
Klebsiella spp.	1	4	2	4
Strept. faecalis	2	5	4	6
Strept. faecium	0	0	1	1
Pseudomonas spp.	1	1	3	3
Proteus spp.	0	2	2	2
Neisseria spp.	0	0	1	1
Group B strept.	6	6	1	0
Viridans strept.	1	1	0	0
Staph. epidermidis	3	7	7	8
Staph. aureus	2	2	5	5
Eubacterium spp.	0	0	2	2

20 babies were colonized respectively on days 3 and 6. It represented 36·8 % of the total umbilical isolates on day 1 and remained the predominant isolate throughout the first week. The next commonest isolate was *Staphylococcus epidermidis*; it was isolated from 8 of the 20 babies by the end of the first week. The other bacterial species isolated from the babies by the sixth day, in order of frequency, were *Streptococcus faecalis* (6 out of 20 babies), *Staph. aureus* (5 out of 20 babies), *Klebsiella aerogenes* (4), *Ps. aeruginosa* (3), *Proteus* spp. and *Eubacterium* spp. (2 each). *Strept. agalactiae* (group B beta-haemolytic streptococci; GBS) was isolated from a relatively high number of babies (6 out of 23) on the first two days of life but its isolation rate declined to one and zero on the third and sixth days respectively.

Mouth flora

The commonest oral isolate was *Strept. salivarius* which was isolated from a consistently high number of babies right from day 1 to day 6. Thirteen out of 23 babies were colonized on the first day and the number colonized on day 6 increased to 17 out of 22 babies (Table 2). *Candida* spp. was also a common colonizer of the babies. A very high proportion of the babies (17 out of 22) were colonized by the yeast on day 6 of life. The next commonest organism was *Staph. epidermidis*, which colonized 16 of the 22 babies on day 6. Colonization of the babies by viridans streptococci was relatively delayed but by the sixth day 14 of the babies had become colonized by these bacteria. GBS was isolated from 6 babies within the first 24 h of birth and from 7, 4 and 3 on the second, third and sixth days respectively. No anaerobic organisms were isolated on the first day but by the third day 6 babies were each colonized by *Veillonella* spp. and *Bifidobacterium* spp. and 3 babies by *Eubacterium* spp. *Pseudomonas* spp. and *Proteus* spp. were transient colonizers.

Faecal flora

E. coli was the commonest facultative species of bacteria isolated from faecal samples of the 23 and 22 babies studied on the first 3 days of life and on the sixth

Table 2. *The bacterial flora of the mouth of premature neonates*

Species or group of organisms	No. of babies colonized at the following ages			
	Day 1 ($n = 23$)	Day 2 ($n = 23$)	Day 3 ($n = 23$)	Day 6 ($n = 22$)
E. coli	10	13	12	13
Klebsiella spp.	3	3	6	8
Strept. faecalis	3	6	10	11
Strept faecium	0	0	1	0
Pseudomonas spp.	5	3	1	1
Proteus spp.	2	2	0	0
Neiseria spp.	1	5	8	9
Group B strept.	6	7	4	3
Viridans strept.	6	10	12	14
Strept. salivarius	13	17	17	17
Staph. epidermidis	6	13	14	16
Staph. aureus	0	0	0	1
Veillonella spp.	0	5	6	6
Bifidobacterium spp.	0	3	6	7
Lactobacilli	0	1	2	2
Eubacterium spp.	0	2	3	3
Candida spp.	3	11	17	17

day respectively (Table 3). Its isolation rate was high right from the first day (18 out of 23 babies), through the second and third days (22 out of 23), to the sixth day (22 of 22 babies). *Strept. faecalis* was also commonly isolated; it was found in 20 of the 22 faecal specimens of the babies on the sixth day. Over three-quarters of the babies were colonized by *Staph. epidermidis* by the third day onwards. *Candida* spp. was consistently isolated from a high number of the babies and by the third day over 50% of the babies were colonized by candida. Colonization of the babies on the first day by *Klebsiella* spp. was low and only 12 babies were colonized on the third day but about 59% of the babies had been colonized on the sixth day. Other aerobes, isolated in small numbers from a few babies, were GBS, viridans streptococci, *Proteus* spp., *Neisseria* spp. and *Pseudomonas* spp.

Anaerobes were qualitatively and quantitatively (growth density) more common in the faeces than aerobes. *Bifidobacterium* spp. were the commonest anaerobes isolated, colonizing about 95% of the babies by the end of the first week of life. *Bacteroides* spp. were the next commonest anaerobes isolated. About 80% of the babies were colonized by the sixth day. More than one species of *Bacteroides* were isolated from these babies; *B. vulgatus* from 15 out of 22 babies and *B. thetaiotaomicron* from 12. *Clostridium* spp. were also well represented. *Cl. perfringens*, *Cl. butyricum* and *Cl. difficile* were isolated from 13, 6 and 5 babies respectively. The colonization rate of lactobacilli was low on all the four sampling days. About 33% of the babies were colonized by anaerobic streptococci at the end of the sixth day.

Influence of method of delivery on colonization

During the first 3 days of life, there was an obvious influence of the method of delivery on the acquisition of faecal flora by the babies. There was general delay in the colonization of the babies delivered by caesarean section (CS) by the

Table 3. *The bacterial flora the faeces of premature neonates*

Species or group of organisms	No. of babies colonized at the following ages			
	Day 1 ($n = 23$)	Day 2 ($n = 23$)	Day 3 ($n = 23$)	Day 6 ($n = 22$)
E. coli	18	18	22	22
Klebsiella spp.	4	8	12	13
Strept. faecalis	9	13	19	20
Strept. faecium	2	2	9	12
Pseudomonas spp.	1	1	1	2
Proteus spp.	1	0	3	3
Neisseria spp.	0	0	2	2
Group B strept.	7	5	3	3
Viridans strept.	4	3	3	3
Staph. epidermidis	7	11	16	17
Staph. aureus	1	1	2	3
Bifidobacterium spp.	12	20	20	21
Lactobacilli	1	3	3	3
Bacteroides vulgatus	0	3	12	15
B. thetaiotaomicron	0	4	10	12
B. distasonis	0	0	1	1
B. ovatus	0	0	1	1
Cl. perfringens	4	10	13	11
Cl. difficile	0	3	5	5
Cl. butyricum	0	5	5	4
Eubacterium spp.	0	3	4	4
Anaerobic strept.	0	1	6	6
Candida spp.	10	12	12	12

bacteroides and the clostridia (see Table 4). Colonization by *B. vulgatus* and *B. thetaiotaomicron* started on the third day whereas about 30% of the babies delivered vaginally were already colonized by these species on the second day. *Ps. aeruginosa* and *Neisseria* spp. were not isolated from any of the CS babies. *Cl. difficile* was isolated from *ca.* 38 and 50% of the CS babies on the 3rd and 6th day respectively while colonization of the vaginally delivered babies by this species was *ca.* 7% on the respective days. There was no significant influence of methods of delivery on the isolation rates of faecal coliforms and enterococci. Only babies born vaginally acquired candida and anaerobic streptococci.

DISCUSSION

In this study, the bacterial flora of the umbilicus, the mouth and faeces of the premature neonates differed in many respects from the earlier reports of studies conducted by other workers on normal healthy full-term babies (Mata & Urrutia, 1971; Long & Swenson 1977; Rotimi & Duerden 1981*a*) and from a recent report by Blakey *et al.* (1982) on pre-term babies.

The umbilical flora was predominantly a mixed flora of Gram-negative bacilli and Gram-positive cocci. From the first day of life, *E. coli* and *Strept. agalactiae* (GBS) were the predominant species of bacteria isolated. These species are usually associated with severe infections of neonates, particularly premature babies (Reid,

https://doi.org/10.1017/S0022172400061532 Published online by Cambridge University Press

Table 4. *Comparative influence of methods of delivery on faecal flora*

Species or group of organisms	Percentage no. of babies colonized by the representative flora							
	Vaginal delivery ($n = 15$)				Caesarean section ($n = 8$)			
	Day 1	Day 2	Day 3	Day 6*	Day 1	Day 2	Day 3	Day 6
E. coli	87	93	100	100	88	100	100	100
Klebsiella spp.	27	33	47	47	0	38	33	33
Strept. faecalis	40	60	80	86	13	38	75	88
Pseudomonas spp.	7	7	7	14	0	0	0	0
Neisseria spp.	7	13	27	29	0	0	0	0
Staphylococci	47	60	88	86	0	25	50	63
Group B strept.	47	33	13	14	0	0	13	13
Bifidobacterium	67	100	100	100	38	65	63	88
Lactobacilli	7	27	33	36	0	0	38	38
Bacteroides spp.	0	27	73	86	0	0	25	63
Cl. perfringens	20	67	73	67	0	0	25	13
Cl. difficile	0	7	7	7	0	0	38	50
Cl. butyricum	0	0	0	7	0	63	63	50
Anaerobic strept.	0	7	40	43	0	0	0	0
Candida spp.	67	80	80	86	0	0	0	0

* Fourteen babies were studied on Day 6.

1975; Parker, 1977). The early colonization of the umbilicus by these species indicates that they were acquired at or before birth from the birth canals of their mothers. The reduced colonization rate of GBS of the umbilical stumps on the third and sixth days was probably due to the prophylactic penicillin given to the majority of these babies during the first week of life. Fortunately there was no systemic spread by these two organisms during this study. *Staph. aureus* and *Staph. epidermidis* that were previously reported to be the common colonizers of the umbilicus of normal healthy neonates and neonates with congenital abnormalities of the GIT respectively (Rotimi & Duerden, 1981*a*; 1982) were relatively diminished in this study.

The predominant oral bacterial flora in this study were the non-haemolytic *Strept. salivarius* and *Staph. epidermidis*. Finding *Staph. epidermidis* in a large number of babies was not surprising as this species is one of the commonest components of the oral flora (McCarthy, Snyder & Parker, 1965; Rotimi & Duerden, 1981*a*). Blakey *et al.* (1982) in their report also found that *Staph. epidermidis* was common oral flora of their preterm babies. The viridans streptococci were isolated from more than 60% of the babies and this is in agreement with the findings of others who reported that they are common and regular oral commensals from the second day of life (Socransky & Manganiello, 1971; Rotimi & Duerden, 1981*a*), as they, as well as *Strept. salivarius*, proliferate on mucous membranes of the oral cavity and do not require the teeth for survival. The most significant oral anaerobic species isolated from about one third of the babies were the veillonellae and the bifido-bacteria. Another significant finding was the colonization of *ca.* 74% of the babies by *Candida* spp. A similar relatively high rate of oral colonization by candida was reported earlier by Lay & Russell (1972). The present finding

however contrasts the low rate (or complete absence) of colonization of normal full-term and preterm babies previously reported (McCarthy, Snyder & Parker, 1965; Rotimi & Duerden, 1981*a*; Blakey *et al.* 1982). No antifungal agent was administered to any of our babies unlike the babies in the report of Blakey *et al.* (1982) who were all given routine antifungal (Mycostatin) therapy.

The population of the species that form the normal flora of the faeces was predominantly aerobic on the first day of life. Thereafter mixed population of *E. coli*, *Strept. faecalis*, with some anaerobes, particularly the *Bifidobacterium* spp., *Bacteroides* spp. and *Clostridium* spp., became rapidly established. By the end of the first days the predominant faecal flora was anaerobic. The contribution of the *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp., enterococci and Gram-negative aerobic organisms to the development of faecal flora in this report is essentially similar to the findings of various other studies reported by Mata, Mejicanos & Jimenez (1972), Bullen, Tearle & Willis (1976), Hentges (1980) and Rotimi & Duerden (1981*a*), on sequential development of faecal flora of healthy full-term neonates.

In a similar study on pre-term neonates in Australia Blakey *et al.* (1982) found a low rate of clostridial colonization which disagrees with our findings of a high rate of clostridial colonization in the present study. The frequency of clostridial colonization increased daily during the first 6 days of life. *Cl. perfringens* was the commonest species isolated. Five out of 22 babies were colonized by *Cl. difficile*. Analysis of the *Bacteroides* spp. isolated shows that they all belonged to the *B. fragilis* group; *B. vulgatus* being the commonest followed by *B. thetaiotaomicron*. These two species were isolated from *ca.* 76% of the babies. The general high rate of bacteroides colonization agrees with the reports of Blakey *et al.* (1982), who also found rapid colonization of their babies by *Bacteroides* spp. in the first 4 days of life, and of Rotimi & Duerden (1981*b*).

The majority of our babies were routinely given prophylactic penicillin plus gentamicin. The influence of antibiotic administration on the colonization rate of bacteria is not very clear in this study. A direct comparative study was not made with a control group. However, it is believed that the prophylactic antibiotic therapy might have helped to prevent systemic spread of any of the potential opportunist flora, particularly from the gut which is usually vulnerable because of the increased permeability of the gut mucosa during the neonatal period (Berg, 1980). Also the premature neonates, incapable of mobilizing inflammatory cells effectively, and coupled with their poor host defence mechanisms, are susceptible to systemic bacterial invasion (Miller, 1979; Quie & Mills, 1979).

The influence of methods of delivery was significant in the colonization rate of faecal anaerobes, candida and GBS. Early gut colonization by GBS, *Bacteroides* spp. and *Clostridium* spp. was only in babies delivered vaginally. The only baby colonized by GBS in the caesarean section group was colonized late which was most certainly of extra maternal source. *Candida* spp. and anaerobic streptococci were exclusively acquired via the vagina.

The result of the present study shows that premature neonates sequentially develop body flora which is somewhat different in composition from that of the normal healthy full-term babies and from the adult flora. This is highly significant because the knowledge will be useful to guide the empirical antibiotic usage in this

group of babies. Premature babies in this hospital are prone to colonization by potential pathogens such as *Strept. agalactiae* (GBS) and the coliform organisms but fortunately none become systematically invaded by these organisms probably because of the prophylactic antibiotic usage in the special baby care unit. A useful base-line data for interpretation of bacteriological investigations of sick premature neonates in our hospital is provided.

We are grateful to Mr Taiwo Banjo, the resident doctors, sisters and staff nurses of ward C4 (Neonatal Unit) of LUTH for their various help. This study was supported by the College of Medicine Research Grant which is gratefully acknowledged.

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