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Quantitative study of the aerobic and anaerobic faecal flora in neonatal necrotising enterocolitis

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SUMMARY Faeces from 24 neonates with proved necrotising enterocolitis (NEC), from 12 with clinically suspected NEC, and from 41 control infants were quantitatively cultured under aerobic and anaerobic conditions. An important difference in colonisation with *Klebsiella* was found between the NEC groups and the control group. Although the cause of NEC is unknown, colonisation with *Klebsiella* seems to increase the risk.

Necrotising enterocolitis (NEC) is found especially among preterm infants. Its principal clinical signs are distended abdomen, retention of food, and faecal blood loss. A typical radiological appearance is gas in the intestinal wall (pneumatosis intestinalis) and evidence suggests that this gas originates from bacteria.[1] NEC is usually found in the terminal ileum, but it may extend to other parts of the intestine. It is associated with necrosis of the intestinal wall and in many cases leads to perforation and peritonitis. The incidence is usually reported as 1·0–1·5/1000 live born infants, with a mortality of about 30%. Published figures show that the incidence of NEC is higher and the prognosis more unfavourable in infants of low birthweight and young gestational age.[2] Although NEC is an important problem in neonatal intensive care units (ICU), its cause is unknown. Several factors probably play a part in its aetiology—intestinal mucosal injury (caused by ischaemia, mechanical damage, or toxic substances), enteral feeding, and the presence of bacteria.[3] Whether certain bacterial species increase the risk of NEC is uncertain and a prospective controlled study of the faecal flora in NEC was undertaken to investigate this.

Methods

Patients. Three groups of patients were studied. Group 1 comprised 24 children in whom NEC was diagnosed radiologically or at operation, or both, or at necropsy. Group 2 comprised 12 children who showed clinical signs of NEC—distended abdomen, retention of food, and facael blood loss—but in whom the diagnosis could not be proved. Group 3 comprised 41 control infants, matched for birthweight and gestational age, who were cared for in the same ICU during the study period. A subgroup of the controls was considered separately in the analysis of results. This was group 4, comprising 19 children whose faeces were cultured within 7 days before or after NEC was diagnosed in another child in the ICU.

Collection of material and culture. Between 20 September 1980 and 1 January 1982, faeces were collected from children who were at high risk from NEC. These were infants aged less than 1 month, born at a gestational age of less than 34 weeks, and with a birthweight of less than 2000 g, who were admitted to the ICU. Faecal cultures were made on the first day of illness in groups 1 and 2. In group 3 this was done on about day 10, which is the usual age of onset of NEC.

After collection, a small amount of faeces was transferred to a bottle containing 3 ml glycerol broth, stored at 4°C, and used within 24 hours for quantitative cultures under aerobic and anaerobic conditions.[4] The amount of faeces was determined by weighing the bottle of glycerol broth before and after adding the faeces.

After homogenisation, 10-fold serial dilutions in saline solution were prepared[5] and 0·1 ml of each dilution (10^{-2} to 10^{-9}) spread on the following nutrient media: 5% sheep blood agar (twice), MacConkey agar, Rogosa agar, and brucella agar (BBL) enriched with haemin and vitamin K. Two variants of the latter nutrient medium were used: 1 to

which kanamycin (75 mg/l) had been added, and 1 which also contained vancomycin (7·5 mg/l).

The sheep blood agar and the MacConkey medium were incubated aerobically at 37°C and read after 24 and 48 hours. The other media were incubated anaerobically at 37°C (Gas Pak system) and read after 48 to 72 hours and again after 7 days. Aerobic Gram negative cultures were worked up to species level (API 20 E). Staphylococci were divided into strains which did or did not produce coagulase. Streptococci were divided into enterococci and others. The anaerobic flora was identified by Gram staining and colony type. Anaerobic spore forming Gram positive rods (clostridia) were further typed with the aid of biochemical reactions and, when necessary, by gas chromatography.[5] Isolations of *Klebsiella* were typed according to Riser *et al.*[6]

Toxin assay. After starting the culture the faecal samples were stored at −70°C. Later the cytotoxic effect of the filtered supernatants of these faecal samples was determined on human embryonic lung fibroblasts. Neutralisation tests were performed with antitoxin against *Clostridium sordellii* (Wellcome).[7]

Statistical analysis. Results were compared for the three groups of patients using Student's *t* test and the Fisher exact test. A difference was described as significant when a two sided tail probability was $P<0·05$.

Results

The 3 groups were compared for several clinical variables (Table 1). There were no important differences between the NEC and control groups, even for such factors as use of umbilical vessel catheters and the presence of a patent ductus arteriosus, which have been related to NEC by some authors.[8 9] An important difference was found only twice—in group 2, in which gestational age exceeded that in group 1, and in which antibiotics were used less often than in group 3. In 99% of all patients the antibiotics given were a combination of amoxycillin and gentamicin. One child in group 1 received cephamandol instead of amoxycillin. In a few children the initial treatment was changed. Three children from the NEC and control groups were also treated with cloxacillin. One child from each group was given chloramphenicol. In group 3 amoxycillin was replaced by cephamandol in 3 children and by penicillin in 1.

The results of the faecal cultures are given in Table 2. The table lists only the most frequently isolated bacterial species as there were too few isolations of other species to permit statistical analysis. Table 2 shows that the only difference between group 1 and group 3 was in the frequency of isolation of aerobic Gram negative rods—in particular, *Klebsiella*. We found an important difference in colonisation with *Klebsiella* between groups 1 and 4, but this difference just escaped significance ($P=0·07$).

Table 1 *Clinical details of neonates with necrotising enterocolitis (NEC) (group 1), clinically suspected NEC (group 2), and controls (group 3)*

	Group 1 (n=24)	*Group 2 (n=12)*	*Group 3 (n=41)*
Male:female ratio	1:1	1:2	1:1·05
Birthweight (g) (mean±SD)	1262±382	1386±345	1329±288
Gestational age (weeks), (mean ± SD)	29·4±2·8	31·4±2·7*	30·3±2·0
Age at faecal culture (days), (mean ± SD)	12·3±6·9	10·8±6·8	11·2±2·6
Age at start of enteral feeding (days), (mean ± SD)	2·3±2·1	1·9±1·4	1·9±1·3
No (%) given antibiotic medication‡	20 (83)	7 (58)†	36 (88)
No (%) treated by umbilical vessel catheter	4 (17)	7 (25)	5 (12)
No (%) treated by arterial line	17 (71)	8 (67)	33 (80)
No (%) given exchange transfusion	1 (4)	2 (17)	4 (10)
No (%) given ventilatory support	14 (58)	5 (42)	21 (51)
No (%) with hyaline membrane disease	7 (29)	3 (25)	16 (39)
No (%) with patent ductus arteriosus	9 (38)	5 (42)	12 (30)

*Significant difference between group 1 and group 2 ($P<0·05$) with Student's *t* test; †significant difference between group 2 and group 3 ($P=0·04$) with Fisher's exact test; ‡antimicrobial drugs given before or during collection of bacterial samples.

Table 2 *Survey of bacterial species isolated from faeces of neonates with necrotising enterocolitis (NEC) (group 1), clinically suspected NEC (group 2), and controls (group 3). Results expressed as number (%) of infants*

	Group 1 (n=24)	*Group 2 (n=12)*	*Group 3 (n=41)*
Gram negative aerobic	22 (92)	9 (75)	24 (59)*
Escherichia coli	8 (33)	3 (25)	12(29)
Enterobacter	6 (25)	3 (25)	9 (22)
Pseudomonas	3 (13)	2 (17)	6 (15)
Klebsiella	17 (71)	7 (58)	15 (37)†
Gram positive aerobic	17 (71)	9 (75)	36 (88)
Enterococcus	14 (58)	6 (50)	23 (56)
Staphylococcus epidermidis	13 (54)	7(58)	25 (61)
Gram negative anaerobic	5 (21)	3 (25)	9 (22)
Bacteroides	4 (17)	2 (17)	8 (20)
Gram positive anaerobic	15 (63)	9 (75)	26 (63)
Clostridium	14 (58)	8 (67)	25 (61)

*Significant difference between group 1 and group 3 ($P=0·005$); †significant difference between group 1 and group 3 ($P<0·01$).

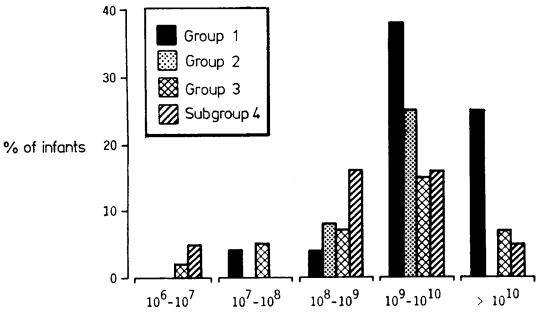

Fig. 1 *Bacterial density of Klebsiella in faeces; percentage of infants in each group colonised with a certain number of organisms/g faeces.*

The density of aerobic Gram negative organisms in the faeces of neonates was generally very high, attaining values exceeding 10^{10}/g. The difference in colonisation with *Klebsiella* between groups 1 and 3 was most prominent at high bacterial densities (Fig. 1).

Table 3 shows how often the density of *Klebsiella* in the faeces exceeded the density of 1 of the other aerobic Gram negative bacterial species. *Klebsiella* was found significantly more often in group 1 than in groups 3 and 4.

Table 3 *Number of faecal samples in which density of Klebsiella (number of organisms/g) exceeded that of other Gram negative aerobic species*

	Group 1 (n=24)	*Group 2 (n=12)*	*Group 3 (n=41)*	*Group 4 (n=19)*
No of samples containing *Klebsiella*	17	7	15	8
No of samples in which *Klebsiella* predominated	13	4	5	2

Significant differences between group 1 and group 3 (P=0·031) and between group 1 and group 4 (P=0·028).

Table 4 *Clostridium species isolated from faeces of 77 neonates in the 3 groups*

	Group 1 (n=24)	*Group 2 (n=12)*	*Group 3 (n=41)*
No of samples from which clostridia were isolated	14	8	25
Clostridium difficile	12	8	19
Clostridium perfringens	3	2	5
Clostridium butyricum	3	—	1
Clostridium paraputrificum	4	1	2
Others	2	—	6
Total	24*	11*	33*

*More than 1 species was isolated repeatedly from faeces of 1 patient.

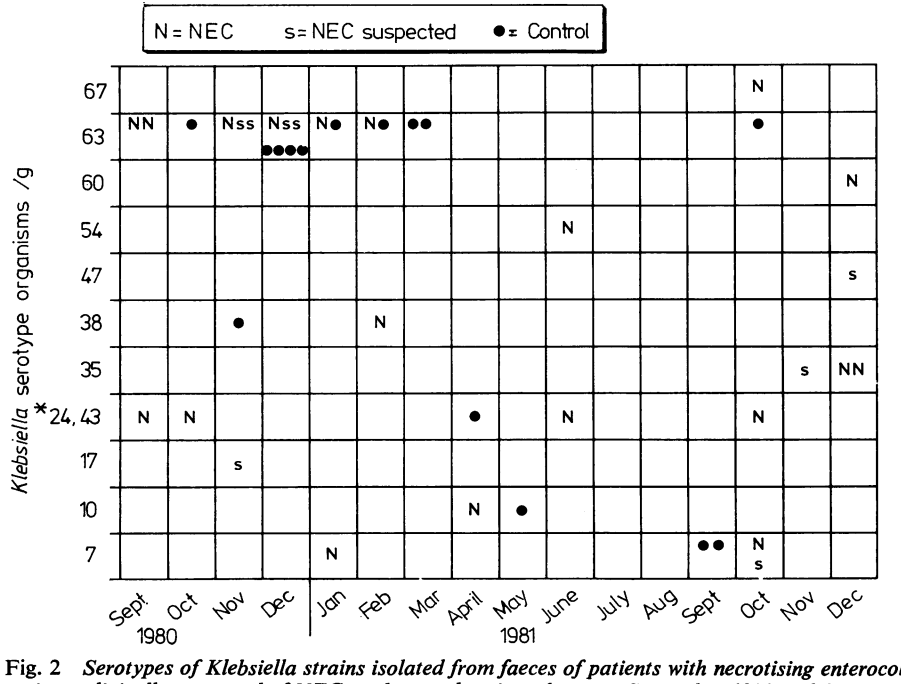

Fig. 2 *Serotypes of Klebsiella strains isolated from faeces of patients with necrotising enterocolitis (NEC), patients clinically suspected of NEC, and control patients between September 1980 and January 1982.*

*Owing to cross reactions it was impossible to establish whether the isolates were of serotype 24 or 43.
In 2 NEC patients and 1 clinically suspect child, 2 different serotypes of *Klebsiella* were isolated.

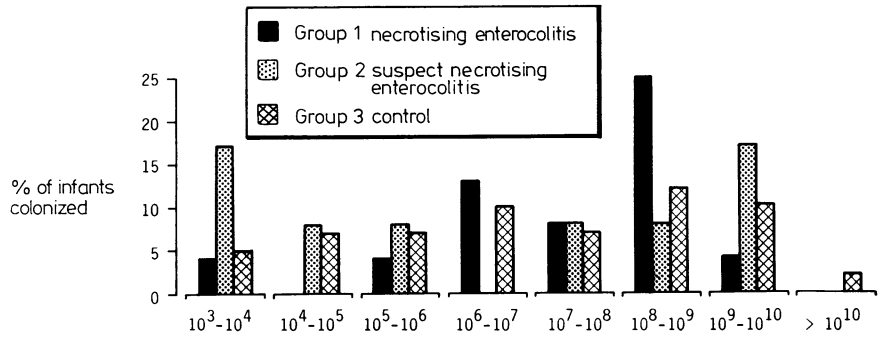

Fig. 3 *Number of clostridial organisms/g faeces.*

Table 5 *Percentage frequency of isolation of bacterial species from faeces of neonates with necrotising enterocolitis (NEC) and a control group. Comparison of 3 studies*

	Frantz et al.[10]			*Bell et al.*[11]			*This study*		
	NEC (n=51)	*Control (n=50)*	*P value*	*NEC (n=27)*	*Control (n=41)*	*P value*	*NEC (n=24)*	*Control (n=41)*	*P value*
Gram negative aerobic				82	51	<0·01	92	59	0·005
Gram positive aerobic				74	71		71	88	
Gram negative anaerobic				30	27		21	22	
Gram positive anaerobic				7	2		63	63	
Klebsiella	38	16	0·03	58	17	<0·005	71	37	0·01
Escherichia coli	36	22		50	29	<0·05	33	29	
Staphylococcus epidermidis	30	30		54	63		54	61	
Enterococcus	10	6					58	56	
α-Streptococcus	24	20							
Group D haemolytic streptococcus				27	17				
Pseudomonas	8	2					13	15	
Candida	16	18					4	17	
'Sterile'	14	24							
Bacteroides							17	20	
Bacteroides fragilis				12	10				
Clostridium							58	61	

Strains were typed to establish whether a particular serotype of *Klebsiella* was predominant during the observation period. Fig. 2 shows an epidemic increase in type 63 during September 1980 to March 1981, but since the epidemic affected children from all the study groups this finding warrants no conclusion about a correlation between a particular serotype and NEC.

Because several species of *Clostridium* have been associated with NEC in the past few years, this group of bacteria was given special attention. No difference in colonisation with clostridia was found between the groups of infants (Fig. 3) even after classification by species (Table 4). *Clostridium difficile* toxin was found in 49 of the 77 children tested, but no intergroup differences were found; nor were there differences in the toxin titres. In each of the 20 cases with a toxin titre exceeding 1/100, *C. difficile* was isolated from the faeces.

Discussion

The cause of NEC is unknown and the role of bacteria in its pathogenesis is probably secondary. Our study focused on whether certain bacterial species are more common in children with NEC. Older, mostly retrospective, studies focused attention on the role of aerobic Gram negative rods because positive blood cultures with these bacterial species were often found in patients with NEC. Prospective controlled studies of the bacterial flora in NEC were undertaken by Frantz *et al.*[10] and Bell *et al.*[11] The results of these studies and our results are summarised in Table 5. An important difference in colonisation with *Klebsiella* was always found between the NEC group and the control group.

Bell *et al.*,[11] who considered the epidemiology in some detail, found a less pronounced difference in colonisation with *Escherichia coli*. To establish the

relation between colonisation with a particular bacterial flora and the development of NEC, they performed a prospective study of the bacterial flora in children in the same ICU during 3 periods of 3 months each. They found the highest incidence (4·7%) during the first period, when *E. coli* and *Klebsiella* accounted for 82% and 88%, respectively, of the Enterobacteriaceae isolated from the stomach and from the faeces. These percentages decreased to 11% and 47% respectively during the second period (during which no NEC was observed), and increased again to 55% and 56% during the third period, when the incidence of NEC increased to 4·4%. *Proteus mirabilis* was predominant during the second period. The authors concluded that the variation in the incidence of NEC correlates with the predominant bacterial flora in a particular ICU. This agrees with the observation of Stanley *et al.*,[12] who found that a decrease in the incidence of NEC coincided with a change in the bacterial flora in an ICU in which *Serratia* superseded the previously predominant *Klebsiella*.

If we confine our analysis of the observed incidences of colonisation by *Klebsiella* to the subgroup of controls (group 4) whose faeces were cultured within 7 days before or after a diagnosis of NEC in another child, the difference between the NEC group and the control group decreases. This suggests that NEC occurred more frequently in the ICU during a period when *Klebsiella* was prevalent and appears to corroborate the conclusions of Bell *et al.*[11] and Stanley *et al.*[12] We did not study the bacterial flora in all children in the ICU during the period of observation, however, and consequently have no comprehensive data on the degree of colonisation. We have meanwhile started a follow up study that does include all children, and this should enable us to examine the epidemiological relation between the occurrence of NEC and colonisation with *Klebsiella*.

In recent years the postulate that clostridia have a role in the pathogenesis of NEC has received much attention in published reports, and *C. perfringens*,[13] *C. butyricum*,[14] and *C. difficile*[15] have been specifically mentioned. Several investigators have shown, however, that these clostridia should be regarded as normal faecal flora in neonates[16–18] and that the production of toxins by *C. difficile* is normal in the first year of life.[19 20] We, too, failed to find differences in colonisation with clostridia or in *C. difficile* toxin production between the different groups studied.

Conclusion

Our findings suggest that colonisation with *Klebsiella* may increase the risk of NEC. Attempts might be made to protect infants in an ICU whose low birthweight further increases this risk. Isolation, selective decontamination, or prophylactic oral antibiotics may merit consideration in this respect, but only the latter method has been studied clinically. Prophylaxis by oral aminoglycosides was reported as successful by some authors[21 22] and as unsuccessful by others.[23 24] In view of the possible development of resistance, prophylactic measures of this sort should be regarded with considerable reservation and should perhaps be confined to periods in which the prevalence of colonisation with *Klebsiella* in a ICU is high. This does imply, however, that the bacterial flora in children in an ICU must be constantly monitored.

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1984	10–14 April	York University
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