Bacteroides species in the normal neonatal faecal flora

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SUMMARY

Bacteroides spp. were isolated from the faeces of neonates and identified by rapid modifications of established conventional methods. Gram-negative anaerobic bacilli were recovered from 12 out of 16 neonates and a heavy growth of *Bacteroides* spp. was obtained from all 12 specimens. Twelve representative isolates from each subject were selected for identification; 141 of the 144 *Bacteroides* isolates belonged to the *B. fragilis* group and three to the *B. melaninogenicus/oralis* group. Eight species were represented within the *B. fragilis* group. *B. vulgatus* (*c.* 46%) and *B. thetaiotaomicron* (*c.* 30%) were the predominant species. *B. fragilis*, the type-species of the group, formed only a small proportion of neonatal faecal flora. The proportional representation of individual species were similar to that found in adults but *B. asaccharolyticus* was not represented.

INTRODUCTION

In adults, *Bacteroides* spp. form a major part of the normal commensal flora of the intestine and rectum and the faecal flora is essentially similar to and representative of the flora of the lower gastro-intestinal tract (Drasar & Hill, 1974). The species of *Bacteroides* isolated from the normal adult faecal flora have been established already (Duerden, 1980; Finegold *et al.* 1975; Moore & Holdeman, 1974). Previous studies (Long & Swenson, 1977; Rotimi & Duerden, 1981) have shown that the rate of isolation of *Bacteroides* spp. from the faeces of normal neonates approaches that in adults but no attempt has been made to identify the species of *Bacteroides* isolated from the normal neonatal faecal flora.

The present study was undertaken to assess the contribution of gram-negative anaerobic bacilli to the normal neonatal faecal flora, to determine the pattern of *Bacteroides* spp. in neonates and to compare it with that found in adults. A recently developed scheme for the rapid identification of gram-negative anaerobic bacilli (Rotimi, Faulkner & Duerden, 1980) was evaluated in this investigation.

MATERIALS AND METHODS

Specimens. Fresh specimens of faeces (*c.* 5 g) from 16 normal healthy neonates were collected in 6 ml of VMG II transport medium (Møller, 1966) in sterile universal containers. They were transported quickly to the laboratory and

processed within an hour of collection. The subjects were babies 5–6 days old born at the Jessop Hospital for Women, Sheffield. None of them was given any antimicrobial chemotherapy. They were all breast fed and all had supplementary feeds of Cow and Gate premium milk.

Isolation of bacteroides. The specimens were dispersed in the transport medium by vortex mixing (Thermolyne Maxi Mix, Clandon Scientific, Thermolyne Corp., Dubuque, Iowa, U.S.A.) for 3 min. One loopful (0·01 ml) of the suspension was plated on pre-reduced BM-kanamycin-vancomycin agar (Holbrook, Ogston & Ross, 1978)–a selective medium for *Bacteroides* spp.–by the standard plating method of Gillies & Dodds (1976) for semi-quantitative assessment of growth on a scale 0–5$^+$ in which 1$^+$ = *c*. 10^5 organisms/ml and 5$^+$ = >10^{10} organisms/ml in the faecal suspension (Rotimi & Duerden, 1981). One 10-fold dilution of the suspension was made in (cooled) pre-steamed BM broth and one loopful of the dilution was similarly plated out on a second plate of BM-kanamycin-vancomycin agar. The plates were incubated anaerobically at 37 °C in an atmosphere of 90 % H$_2$ and 10 % CO$_2$ (BOC Special Gases). The anaerobic procedure of Collee *et al.* (1972) was followed in all essential aspects and a slope of Simmons Citrate Medium seeded with *Pseudomonas aeruginosa* was included in each jar as a control. The plates were examined after continuous incubation for 48 h and all colony-types were noted: six representative colonies were subcultured from each plate onto fresh plain BM agar with a metronidazole disk (5 μg) on each plate. The primary isolation plates were reincubated anaerobically for a further 48 h and another six representative colonies were subcultured. Each selected colony was also plated on blood agar and incubated in air+10 % CO$_2$ to check the purity of the anaerobic culture. Gram-negative bacilli that grew only anaerobically and were sensitive to metronidazole were selected for identification. A total of 12 colonies were studied from each specimen. The numbers of each colony-type were selected in approximate proportion to their comparative numbers on the primary isolation plates.

Tests for the identification of isolates. The strains were identified by the following combined set of cultural, tolerance, antibiotic resistance, biochemical and fermentation tests (Duerden *et al.* 1980); most of the tests were performed by the rapid methods developed in this laboratory (Rotimi *et al.* 1980). The tests were: colony morphology after incubation for 48 h on blood agar and BM agar; pigment production on BM agar; motility in BM broth; antibiotic disk resistance tests with separate disks containing neomycin 1000 μg, kanamycin 1000 μg, penicillin 2 units, and rifampicin 15 μg; tolerance tests with taurocholate, Victoria blue 4R and gentian violet; biochemical test for the production of indole and hydrolysis of aesculin; fermentation tests with glucose, lactose, sucrose, rhamnose, trehalose, mannitol and xylose.

RESULTS

A heavy growth (4$^+$–5$^+$) of *Bacteroides* spp. was obtained from 12 of the faecal specimens obtained from the 16 normal healthy neonates studied. Complete sets of results were obtained with 144 anaerobic gram-negative, non-motile, non-sporing isolates from the 12 faecal samples. The number of strains allocated to each species are shown in the Table; 141 belonged to the *B. fragilis* group and three to the *B. melaninogenicus/oralis* group.

B. fragilis group. The 141 isolates allocated to this group were non-pigmented. They were resistant to kanamycin, neomycin and penicillin, and sensitive to rifampicin; most were tolerant of taurocholate and Victoria blue 4R, and all were inhibited by gentian violet. All fermented glucose, lactose and xylose. Eight species were represented within the *B. fragilis* group and the majority of the isolates gave patterns of results that were generally typical of the recognized species (Duerden *et al.* 1980). The 18 *B. fragilis* isolates fermented glucose, lactose, sucrose and xylose, and hydrolysed aesculin but did not produce indole. The patterns of results obtained with the 66 *B. vulgatus* isolates were typical of the species. All fermented glucose, lactose, sucrose, rhamnose and xylose; they did not produce indole and 11 of them did not hydrolyse aseculin; 62 isolates were tolerant of taurocholate. All 10 isolates of *B. distasonis* and 42 isolates of *B. thetaiotaomicron* gave results consistent with these species, although one strain of *B. distasonis* did not ferment rhamnose. The two isolates of *B. uniformis* gave typical sets of results; they produced indole, hydrolysed aesculin, and fermented glucose, lactose, sucrose, trehalose, and xylose but not rhamnose or mannitol. The pattern of results obtained with the two strains of *B. variabilis* were identical with the typical pattern for this species; they fermented all the test sugars except trehalose and mannitol, produced indole and hydrolysed aesculin. Only one strain of *B. eggerthii* was isolated and it gave a pattern of results identical with the reference strain of this species; it hydrolysed aesculin, produced indole and fermented glucose, lactose, sucrose, rhamnose and xylose but not trehalose or mannitol.

B. melaninogenicus-oralis group. The three isolates allocated to this group were non-pigmented; two isolates were *B. ruminicola* and one was *B. oralis*. The *B. ruminicola* strains were resistant only to kanamycin and inhibited by taurocholate, Victoria blue 4R and gentian violet; they did not produce indole but hydrolysed aesculin, fermented glucose, lactose, sucrose, rhamnose and xylose but not trehalose or mannitol. The *B. oralis* strain was sensitive to neomycin and rifampicin but resistant to kanamycin and penicillin, and inhibited by Victoria blue 4R, gentian violet and taurocholate; it hydrolysed aesculin but did not produce indole, and fermented glucose and lactose only.

Isolates from individual subjects. When identical isolates from individual neonates were regarded as single strains the overall distribution of isolates between the species was not distorted by the presence of large blocks of identical isolates from individual subjects (Table). *B. vulgatus* was isolated from all 12 neonates from whom gram-negative anaerobic bacilli were isolated; *B. thetaiotaomicron* was isolated from 11 subjects, *B. fragilis* from 9, *B. distasonis* from 3, *B. uniformis*, *B. variabilis* and *B. ruminicola* from 2, and *B. eggerthii* and *B. oralis* from one each. The mean number of isolates of *B. vulgatus* from each neonate was 5·5 (range 2–7). *B. thetaiotaomicron* accounted for a smaller proportion of isolates with a mean number of isolates of 3·8 (range 1–6). The other species were recovered in small numbers from individual neonates.

Table 1. *Number of isolates of each Bacteroides species from neonates*

Species	No. of isolates of the given spp. from subject no.												Total number of isolates
	1	2	3	4	5	6	7	8	9	10	11	12	
B. fragilis	7	—	1	1	2	—	1	1	—	1	1	3	18
B. vulgatus	5	5	5	7	2	7	7	5	7	6	5	5	66
B. distasonis	—	2	—	—	6	—	—	—	2	—	—	—	10
B. thetaiotaomicron	—	1	6	4	1	3	3	6	3	5	6	4	42
B. uniformis	—	—	—	—	1	1	—	—	—	—	—	—	2
B. variabilis	—	1	—	—	—	1	—	—	—	—	—	—	2
B. eggerthii	—	1	—	—	—	—	—	—	—	—	—	—	1
B. ruminicola	—	1	—	—	—	—	1	—	—	—	—	—	2
B. oralis	—	1	—	—	—	—	—	—	—	—	—	—	1
Total	12	12	12	12	12	12	12	12	12	12	12	12	144

DISCUSSION

In the present study, anaerobic bacteriological studies were carried out by conventional bench handling combined with a carefully controlled and standardized anaerobic jar technique and using fresh or pre-reduced media. Other workers have shown that these methods are perfectly adequate for quantitative recovery of gram-negative anaerobic bacilli from human faeces and give results comparable with those obtained with an anaerobic cabinet (Watt, Collee & Brown, 1974).

The *B. fragilis* group are the commonest *Bacteroides* spp. found in the adult human faecal flora and they have also been isolated from 78 % of breast-fed neonates receiving supplementary bottle feeds with premium milk in one series (Rotimi & Duerden, 1981) and from 61 % of bottle-fed infants in another (Long & Swenson, 1977). However, the contribution of the individual species with the *B. fragilis* group to the normal neonatal faecal flora was not determined.

In the present study all the babies were breast-fed and had supplementary bottle feeds with Cow and Gate premium milk. A very heavy growth (score 4–5⁺) of *Bacteroides* spp. was obtained from the 12 specimens that yielded gram-negative anaerobic bacilli. This correlates with the scores of 4–5⁺ and viable counts of 10^{11} colony forming units (c.f.u.) of *Bacteroides* per g of faeces reported in adults (Drasar, 1967; Duerden, 1980*a*; Finegold, Attebery & Sutter, 1974). The *B. fragilis* group accounted for c. 98 % of the total *Bacteroides* isolated from the neonatal faeces. This is a higher figure than that found by Duerden (1980*a*) in the adult faecal flora, but the trend is similar. The commonest species isolated was *B. vulgatus* which formed 46·8 % of the *B. fragilis* group and 45·8 % of all the *Bacteroides* spp. isolated. *B. thetaiotaomicron* was the next commonest (29·8 %) of the *B. fragilis* group whereas *B. fragilis* (12·8 %) and *B. distasonis* (7·1 %) represented a smaller proportion. The high incidence *B. vulgatus* and *B. thetaiotaomicron* in the neonatal faecal flora is in broad agreement with the findings of other investigators in adults (Moore & Holdeman, 1974; Watt *et al.* 1974; Finegold *et al.* 1975; Holdeman, Good & Moore, 1976; Duerden, 1980*a*). *B. distasonis* is found in a relatively high proportion of adult faeces (Duerden, 1980*a*) but formed only a small proportion of the *B. fragilis* group in the neonatal faecal flora. *B. fragilis* is the type-species of the *B. fragilis* group, and is the species most commonly isolated from clinical infections (Holland, Hill & Attemeier, 1977; Duerden, 1980*b*) but it forms only a small proportion of the adult faecal flora (Duerden, 1980*a*; Moore & Holdeman, 1974). Similarly it formed only a small proportion of the *B. fragilis* group in the present study in neonates. The other members of the *B. fragilis* group – *B. uniformis*, *B. variabilis* and *B. eggerthii* – were not well represented, whereas Duerden (1980*a*) found that the *B. eggerthii/variabilis* complex formed a relatively large proportion of the adult faecal flora.

There were only three isolates representing two species of the *B. melaninogenicus/oralis* group in this study. Two strains were *B. ruminicola* and one was *B. oralis*. Similarly this group forms only a small proportion of the adult faecal flora.

No asaccharolytic *Bacteroides*, pigmented or non-pigmented, no strains of *B. melaninogenicus* and no fusobacteria were isolated from the neonates. This indicates that the *B. melaninogenicus/oralis* group, the asaccharolyticus *Bacteroides* and the fusobacteria are not part of the normal flora of the lower gastro-intestinal

tract and faeces in newborn infants. These findings are similar to those of Duerden (1980*a*) in adults except for the absence of *B. asaccharolyticus*.

Most of the isolates in this study were identified by the rapid methods developed in this laboratory (Rotimi *et al.* 1980) and by conventional methods (Duerden *et al.* 1980); there were no difficulties experienced with the rapid methods and no abberent results in these tests which confirmed that the rapid methods are as reliable as the conventional methods for the identification of the *B. fragilis* group of *Bacteroides*.

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