THE ISOLATION AND IDENTIFICATION OF *BACTEROIDES* SPP. FROM THE NORMAL HUMAN VAGINAL FLORA

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THE GRAM-NEGATIVE anaerobic bacilli that form part of the normal commensal flora of the vagina and cervix have been the subject of several investigations, but the results have been conflicting. The presence of *Bacteroides* in the vagina was first reported by Burdon (1928) who isolated *B. melaninogenicus* from 28 out of 35 normal women. Mead and Louria (1969) and Suzuki and Ueno (1971, cited by Finegold 1977) found that *Bacteroides* were commonly present in the normal vaginal flora. Gorbach *et al.* (1973) isolated *Bacteroides* spp. from the cervical flora of 57% of 30 healthy women and Sanders *et al.* (1975) isolated *Bacteroides* spp. from endocervical cultures from 65% of 26 healthy women. However, Neary *et al.* (1973) recovered *Bacteroides* spp. from only 8.6% of vaginal swabs from 246 pre-operative gynaecological patients and Leigh, Kershaw and Simmons (quoted by Leigh, 1976) isolated *Bacteroides* from only 4.6% of 500 women attending a family-planning clinic and 5% of 200 patients attending a gynaecological outpatient clinic. Hurley *et al.* (1974) collected specimens of vaginal secretions from the posterior fornix of 280 unselected pregnant women and found *Bacteroides* spp. in only 15 (5.4%) and Werner *et al.* (1978) isolated *Bacteroides* spp. from vaginal swabs from four out of 100 healthy pregnant women. In a quantitative study, Lindner, Plantema and Hoogkamp-Korstanje (1978) found that the mean log₁₀ number of viable *Bacteroides* spp. per ml of vaginal secretions was 7–8 but they isolated *Bacteroides* spp. from only 4% of normal women, 1% of pregnant women and 28% of women with cervicitis.

The differences may reflect different methods of investigation. The vagina is not a single environment; the flora of the lower vagina is related to that of the perineum and introitus and differs from that of the cervix and fornices. Moreover, the physiological state of the vaginal mucosa and secretions changes with age, with the menstrual cycle and with pregnancy (Hurley *et al.*, 1974).

The *Bacteroides* spp. isolated in most studies have not been identified and the relationship of these isolates to those from other sites is not clear. The present study was undertaken to determine the carriage of *Bacteroides* and identify the strains isolated from 20 normal healthy women attending a family-planning clinic.

MATERIALS AND METHODS

One hundred and twenty strains isolated from 20 vaginal specimens were studied.

Anaerobic incubation. The anaerobic procedure of Collee *et al.* (1972) was followed in all essential aspects; incubation was at 37°C in an atmosphere of H₂ 90% and CO₂ 10%. A slope of Simmons citrate medium seeded with *Pseudomonas aeruginosa* was included in each jar as a control.

Specimens. High vaginal swabs were collected from 20 normal healthy women and the swabs were immediately broken off into a vial of VMG II transport medium (Möller, 1966). The swabs were collected from the cervix and posterior fornix under direct vision during examination with a Sim's speculum and before manual examination. The subjects were women aged 20–40 years attending a family-planning clinic for routine vaginal examination and cervical cytology. Samples were not accepted from women who had any vaginal pathology or who were receiving antibiotic therapy. The specimens were processed on the day that they were collected.

Isolation of Bacteroides. Each swab was used to seed a sector of a plate of pre-reduced BM medium made selective for *Bacteroides* by the addition of kanamycin 75 µg/ml and vancomycin 2.5 µg/ml (see Duerden, 1980a). The plates were examined after incubation for 48 h. All colony types were noted and five representative colonies were subcultured on to fresh BM agar without antibiotics. After re-incubation for a further 48 h, additional colony types were noted and another five representative colonies were subcultured. The colony types were selected in approximate proportion to their comparative numbers on the primary isolation plate. When possible, 10 colonies were collected from each specimen; but this was not always possible because there was no growth of *Bacteroides* from seven of the specimens and fewer than 10 colonies were isolated from two specimens. The isolates were suspended in a freezing medium of Nutrient Broth No. 2 (Oxoid) with inactivated Horse Serum (Wellcome) 10% and glucose 1% and held at –70°C in a liquid-nitrogen container.

Identification of isolates. The strains were identified by the methods of Duerden *et al.* (1976, 1980). The tests were: colony morphology after 48-h incubation on blood agar; cell morphology in gram-stained smears from 48-h cultures on blood agar and in BM broth with cooked-meat particles (see Deacon, Duerden and Holbrook, 1978); pigment production on BM agar; haemolysis on human blood agar; motility in BM broth; antibiotic-disk resistance tests with disks separately containing neomycin 1000 µg, kanamycin 1000 µg, penicillin 2 units and rifampicin 15 µg; tolerance tests with taurocholate, deoxycholate, Victoria blue 4R and ethyl violet; biochemical tests for the production of indole, digestion of gelatin and hydrolysis of aesculin; and fermentation tests with glucose, lactose, sucrose, rhamnose, trehalose, mannitol and xylose.

RESULTS

Gram-negative, non-sporing, non-motile obligately anaerobic bacilli of the *Bacteroides* group were isolated from 13 (65%) subjects and 120 isolates were selected for identification; 10 colonies were selected from each of the 11 primary plates that yielded a moderate or heavy growth of *Bacteroides*; eight colonies were obtained from one specimen and only two colonies from another. Complete sets of results were obtained with 113 isolates; seven isolates failed to grow on subculture from the preserved cultures. One hundred and two isolates representing 11 recognised species and subspecies were identified and the numbers of isolates allocated to each species are shown in table I. Six isolates were allocated to the *B. fragilis* group, 88 to the *B. melaninogenicus/oralis* group, 16 were non-saccharolytic, including nine pigmented *B. asaccharolyticus* isolates, and three isolates could not be allocated to a recognised species.

TABLE I
Identification of vaginal isolates from 13 subjects

Species	Number of isolates (and group total)
<i>B. fragilis</i> group	(6)
<i>B. distasonis</i>	1
<i>B. vulgatus</i>	3
<i>B. thetaiotaomicon</i>	1
<i>B. splanchnicus</i>	1
<i>B. melaninogenicus/oralis</i> group	(88)
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i>	14
ss. <i>intermedius</i>	19
<i>B. bivius</i>	16
<i>B. bivius/disiens</i>	21
<i>B. oralis</i> group (unidentified)	1
<i>B. oralis</i>	10
<i>B. ruminicola</i>	7
Asaccharolytic group	(16)
<i>B. asaccharolyticus</i>	9
Non-pigmented non-saccharolytic spp.	7
<i>Bacteroides</i> spp. (unidentified)	(3)
TOTAL	113

B. fragilis group

All the *B. fragilis*-group isolates except the *B. distasonis* strain were from one subject. The patterns of results obtained with the isolates were typical of the designated species except that the *B. splanchnicus* strain was tolerant of ethyl violet and two identical isolates of *B. vulgatus* were tolerant of deoxycholate.

B. melaninogenicus/oralis group

The most common species of *Bacteroides* isolated from the normal vaginal flora were members of the *B. melaninogenicus/oralis* group. The 88 isolates allocated to this group accounted for 78% of the *Bacteroides* isolated and identified in this study. All were inhibited by taurocholate, deoxycholate and ethyl violet, resistant to kanamycin and sensitive to rifampicin. Seventeen isolates were tolerant of Victoria blue 4R, 24 were resistant to penicillin and 12 isolates of *B. melaninogenicus* were resistant to the neomycin 1000 µg-disk.

The 19 isolates of *B. melaninogenicus* ss. *intermedius* readily produced pigmented colonies; they produced indole and fermented glucose but 11 isolates, including six identical isolates from one subject and three from another, were resistant to neomycin and 10 of them fermented glucose weakly after 4–7 days. Most of the 14 *B. melaninogenicus* ss. *melaninogenicus* isolates were typical of the subspecies but one isolate was resistant to the neomycin disk and six identical isolates from one subject were tolerant of Victoria blue 4R, resistant to penicillin and produced brown-pigmented colonies only slowly.

The 37 isolates allocated to the *B. bivius/disiens* complex formed the largest group of vaginal isolates. The two species are distinguished by the results of tests for the fermentation of lactose but the significance of this test was not appreciated until the study was in progress and it was not included in the set of tests until the later stages of the investigation. All the isolates gave patterns of results typical of *B. bivius* and *B. disiens*; 10 isolates were tolerant of Victoria blue 4R and 18 were resistant to penicillin. Tests for the fermentation of lactose and sucrose were performed on 16 isolates in the later stages of the investigation; all fermented lactose but not sucrose and were, therefore, identified as *B. bivius*.

The 10 *B. oralis* isolates gave patterns of results that were generally typical of the species; seven strains were resistant to penicillin and two identical isolates from one subject fermented mannitol. One isolate gave an intermediate pattern of results within the group and could not be identified. It fermented glucose, lactose and sucrose, like *B. oralis*, but did not hydrolyse aesculin. The seven isolates identified as *B. ruminicola* hydrolysed aesculin and fermented glucose and xylose. In addition two isolates fermented rhamnose and two identical isolates from one subject fermented rhamnose, trehalose and mannitol.

Asaccharolytic group

The 16 non-saccharolytic isolates included nine pigmented strains of *B. asaccharolyticus*; eight isolates gave patterns of results typical of the species but one did not produce indole. The seven non-pigmented non-saccharolytic strains gave three distinct patterns of results. Three isolates were similar to *B. asaccharolyticus* but did not produce pigment. A pair of isolates from one subject were inhibited in tolerance tests, resistant only to kanamycin and digested gelatin, but hydrolysed aesculin and did not produce indole. The remaining two isolates, from different subjects, were inhibited in the tolerance tests, sensitive to the four antibiotic disks and unreactive in other tests except for the digestion of gelatin.

Three fermentative isolates could not be identified; two identical isolates from one subject gave the tolerance and antibiotic-disk resistance patterns of the *B. fragilis* group, but they did not produce indole or hydrolyse aesculin and fermented only glucose. The third isolate gave the tolerance and antibiotic disk resistance patterns of the *B. melaninogenicus/oralis* group but it produced indole, hydrolysed aesculin and fermented glucose and mannitol.

Identification of isolates from individual subjects

When identical isolates from one subject were treated as a single strain, 56 strains were isolated from the 13 subjects and the identity of these strains is shown in table II. The distribution of species amongst the 13 subjects is shown in table III. The mean number of strains isolated from subjects for whom complete sets of results were obtained with more than six isolates was 4.6

TABLE II
Strains of Bacteroides spp. isolated from 13 subjects

Species	Number of strains isolated (and group total)
<i>B. fragilis</i> group	(5)
<i>B. distasonis</i>	1
<i>B. vulgatus</i>	2
<i>B. thetaiotaomicron</i>	1
<i>B. splanchnicus</i>	1
<i>B. melaninogenicus/oralis</i> group	(38)
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i>	5
ss. <i>intermedius</i>	7
<i>B. bivius</i>	4
<i>B. bivius/disiens</i>	11
<i>B. oralis</i> group (unidentified)	1
<i>B. oralis</i>	5
<i>B. ruminicola</i>	5
Asaccharolytic group	(11)
<i>B. asaccharolyticus</i>	6
Non-pigmented non-saccharolytic spp.	5
<i>Bacteroides</i> spp. (unidentified)	(2)
TOTAL	56

(range 2–8). The subject that yielded eight strains was unusual because the eight strains included all except one of the *B. fragilis*-group isolates and only one isolate from the *B. melaninogenicus/oralis* group. At least one strain of the *B. melaninogenicus/oralis* group was isolated from each subject and one member of the group was often predominant but strains of the *B. fragilis* and asaccharolytic groups were detected in specimens from only a few subjects.

TABLE III
Species of Bacteroides isolated from vaginal swabs from 13 subjects

Species	Isolation of the given species from subject number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>B. distasonis</i>								+					
<i>B. vulgatus</i>							+						
<i>B. thetaiotaomicron</i>							+						
<i>B. splanchnicus</i>							+						
<i>B. melaninogenicus</i> ss. <i>melaninogenicus</i>		+	+						+			+	
ss. <i>intermedius</i>	+	+	+								+		+
<i>B. bivius</i>					+	+							
<i>B. bivius/disiens</i>	+			+			+	+	+	+	+		+
<i>B. oralis</i> group (unidentified)				+									
<i>B. oralis</i>						+			+		+		
<i>B. ruminicola</i>			+	+								+	+
<i>B. asaccharolyticus</i>				+			+				+		+
Non-pigmented non-saccharolytic spp.		+					+	+		+			
<i>Bacteroides</i> spp. (unidentified)		+									+		

+ Denotes isolation.

Colony and cell morphology

Colony size and morphology were of little value in the identification of vaginal isolates except for the characteristic pigmentation of *B. melaninogenicus* and *B. asaccharolyticus* strains. These species grew better on BM agar than on blood agar; colonies were larger and pigmentation developed earlier. *B. melaninogenicus* ss. *melaninogenicus* strains often produced brown rather than black colonies with a paler periphery. There were no consistent differences between the small, pale colonies of the non-pigmented species in the *B. melaninogenicus/oralis* group but the non-pigmented non-saccharolytic strains produced very small (<0.5 mm diam.) colonies even after prolonged incubation.

Bacterial-cell morphology was of little discriminatory value. Many strains from the *B. melaninogenicus/oralis* group appeared similar to *B. fragilis*-group strains but a little more than half of them were predominantly coccobacillary and a few strains were highly pleomorphic with many distorted and bizarre forms. The asaccharolytic strains were predominantly coccobacillary.

DISCUSSION

Gram-negative anaerobic bacilli of the *Bacteroides* group were isolated from swabs taken from the cervix and vaginal fornices of 65% of the women examined; a heavy growth was obtained from 11 specimens but only a few colonies were isolated from two specimens. This carriage rate is similar to the 65% found by Sanders *et al.* (1975) and the 57% found by Gorbach *et al.* (1973); it is much greater than the 4% quoted by Leigh (1976) in a similar population of women attending a family planning clinic, the 4% in normal women and 1% in pregnant women found by Lindner *et al.* (1978), and the 5.4% and 4% found by Hurley *et al.* (1974) and Werner *et al.* (1978) in pregnant women. These differences in carriage rates may reflect differences between the study populations, such as between pregnant and non-pregnant subjects, or differences in the anaerobic methods of the investigators. In the quantitative study (Lindner *et al.*, 1978), the viable count of *Bacteroides* spp. in many women may have been below the lower limit of sensitivity of their methods. Vaginal carriage of *Bacteroides* spp. may vary with the menstrual cycle and could be related to the presence of altered blood (Hurley *et al.*, 1974). The stage of the cycle in the subjects in the present study was not recorded and this aspect of vaginal colonisation could not be assessed. Moreover, the vagina is not a single environment and the differences may in part reflect the sites sampled and the methods of sampling. In the present study, the flora of the cervix and vaginal fornices were considered to represent the high or "true" vaginal flora.

The predominant *Bacteroides* spp. in the normal vaginal flora in the present study were members of the *B. melaninogenicus/oralis* group; 78% of isolates belonged to this group. The commonest subgroup of species was the *B. bivius/disiens* complex; 42% of the *B. melaninogenicus/oralis*-group isolates

belonged to this complex and all that were identified fully proved to be *B. bivius*. *B. melaninogenicus* ss. *melaninogenicus* (16%) and ss. *intermedius* (22%) were the other common species in the group. Only 5% of the isolates belonged to the *B. fragilis* group and all except one of these isolates were from a single subject. This indicates that the *B. fragilis* group are not part of the normal commensal flora of the vagina. *Fusobacterium* spp. were not detected, but studies on the gingival flora (Duerden, 1980b) showed that they could be isolated on the selective medium under the same conditions of culture. Asaccharolytic strains, including *B. asaccharolyticus* were isolated in smaller numbers than the *B. melaninogenicus/oralis*-group strains; they comprised 12% of the isolates but were isolated from 54% of subjects.

The *Bacteroides* spp. reported in most other studies have not been identified. Lindner *et al.* (1978) identified their isolates as *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus* and *B. vulgatus* but did not detect strains of the *B. melaninogenicus/oralis* group. This may reflect differences in the anaerobic procedures or the selective medium used; the medium contained neomycin sulphate 1000 mg/litre and results in antibiotic-disk resistance tests have shown that the *B. melaninogenicus/oralis* group is less resistant to neomycin than the *B. fragilis* group (Duerden *et al.*, 1976; Holbrook, Duerden and Deacon, 1977; Duerden *et al.*, 1980). *B. fragilis*, *B. thetaiotaomicron*, *B. melaninogenicus* and *B. oralis* were the four strains isolated by Werner *et al.* (1978) from normal pregnant women but *B. oralis* was the predominant anaerobic species isolated from the uterine cavity, cervical canal or vagina of puerperal women; 42% of these patients harboured clinically important anaerobes.

The predominance of the *B. melaninogenicus/oralis* group amongst the *Bacteroides* spp. in the normal vaginal flora and the absence of significant numbers of *B. fragilis*-group isolates contrasts with the preponderance of the *B. fragilis* group in the faeces where the *B. melaninogenicus/oralis* group is not commonly part of the normal flora. (Duerden, 1980a).

The common vaginal species were, however, similar to the oral species isolated from subgingival plaque; in a parallel study (Duerden, 1980b) the commonest oral species were *B. oralis*, *B. melaninogenicus* ss. *melaninogenicus* and ss. *intermedius*. The similarity in the identity of isolates from the two sites may reflect similarities in the physiological environments. A comparison of saliva with cervical secretions shows that the pH, osmolarity, carbohydrate and enzyme content, in particular the amylase content, are similar (Jordan and Singer, 1976; Mason and Chisolm, 1975).

Pigmented strains of *B. melaninogenicus* and *B. asaccharolyticus* were isolated from nine (45%) of the 20 subjects in the present series. This is a lower carriage rate than Burdon (1928) found; he isolated "*B. melaninogenicus*" from 28 (80%) of the 35 vaginal specimens from normal women.

The present study has shown that gram-negative anaerobic bacilli are common but not universal commensals of the cervix and vaginal fornices. The common species found in the normal vaginal flora were members of the *B. melaninogenicus/oralis* group and these species were readily isolated and identified by the conventional methods used in this study.

SUMMARY

The occurrence of gram-negative anaerobic bacilli in the normal vaginal flora was studied in 20 normal healthy women attending a family-planning clinic. A swab was taken from the cervix and posterior fornix and *Bacteroides* spp. were isolated on a selective medium from 13 (65%) subjects. A heavy growth of *Bacteroides* was obtained from 11 specimens but only a few colonies were isolated from two specimens. Where possible, 10 representative colonies from each subject were studied and 113 isolates were identified by conventional bacteriological tests. Most isolates (78%) belonged to the *B. melaninogenicus/oralis* group. The commonest species identified in this group were the *B. bivius/disiens* complex (42%), *B. melaninogenicus* ss. *intermedius* (22%) and ss. *melaninogenicus* (16%). Asaccharolytic strains were isolated in smaller numbers from 54% of subjects, but only five strains of the *B. fragilis* group were isolated from two subjects; fusobacteria were not detected.

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