

## The identification of gram-negative anaerobic bacilli isolated from clinical infections

BY B. I. DUERDEN

*Department of Medical Microbiology,  
University of Sheffield Medical School,  
Beech Hill Road, Sheffield, S10 2RX*

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### SUMMARY

Gram-negative anaerobic bacilli isolated from specimens submitted to the routine diagnostic bacteriology laboratory and regarded as significant pathogens were identified by conventional bacteriological tests; 399 strains isolated from 356 specimens submitted from 332 patients were studied and most were readily identified by the results of a combined set of morphological, biochemical, tolerance and antibiotic disk resistance tests. *B. fragilis* has particular pathogenic potential and was the commonest species isolated, accounting for > 50% of strains. The next commonest was *B. asaccharolyticus* with 55 strains, and 16 other species or groups were represented by smaller numbers. Many (68%) were from infections related to the gastro-intestinal tract, but there were significant numbers from infections of the male and female genito-urinary tracts, the head, neck and central nervous system and from a variety of soft tissue infections. Most infections were mixed, and a pure culture of a *Bacteroides* sp. was obtained from only 26% of infections; two or more strains of *Bacteroides* were recovered from 55 infections. The specific identification of *Bacteroides* may help the bacteriologist to judge the significance of laboratory findings, influence the patient's management and prognosis and help determine the source of infection.

### INTRODUCTION

Gram-negative non-sporing anaerobic bacilli of the *Bacteroides-Fusobacterium* group are important causes of infection in hospital practice and have been isolated from a wide variety of clinical conditions. *Bacteroides* spp. are a major component of the normal flora of the lower gastro-intestinal tract (Drasar, Shiner & McLeod, 1969; Drasar & Hill, 1974; Holdeman, Good & Moore, 1976), mouth (Gibbons *et al.* 1963; Socransky & Manganiello, 1971) and vagina (Gorbach *et al.* 1973; Sanders *et al.* 1975), and most infections follow surgical or accidental injury related to these sites, particularly in debilitated patients. The reported incidence of anaerobic infections varies with the interest of the investigators in anaerobic bacteriology. Martin (1971) recovered anaerobes from 35% of specimens, and these were 49.3% of all bacteria isolated; in a later study, anaerobes were isolated

from 49% of culture-positive specimens (Martin, 1974). Holland, Hill & Altemeier (1977) isolated anaerobes from 48.8% of specimens, and *Bacteroides* spp. were found in 70% of anaerobe-positive cultures. The extensive literature on bacteroides infection has been reviewed by Balows *et al.* (1974), Phillips & Sussman (1974), Finegold (1977) and Willis (1977).

Improvements in anaerobic techniques (Collee, Rutter & Watt, 1971; Holdeman & Moore, 1973; Watt, 1973; Watt, Hoare & Collee, 1973; Watt, Collee & Brown, 1974) have provided laboratories with reliable methods for the isolation of bacteroides organisms, but precise identification is rarely attempted in the routine laboratory. The classification of gram-negative anaerobic bacilli has been confused, and in most laboratories methods have not been available for accurate identification. Many workers have reported their strains as '*Bacteroides* spp.', or '*B. fragilis*', '*B. melaninogenicus*' and 'fusobacteria'. Organisms of the *B. fragilis* group are the commonest species isolated from infections in general, and when specific identification has been undertaken *B. fragilis* (formerly *B. fragilis* spp. *fragilis*) has been the commonest species, with *B. thetaiotaomicron* isolated regularly but much less frequently (Werner & Pulverer, 1971; Werner, 1974; Holland *et al.* 1977; Tally & Gorbach, 1979). '*B. melaninogenicus*' is the only other species that has been identified consistently in bacteroides infections, but it has been found much less frequently than *B. fragilis* (Burdon, 1928; Heinrich & Pulverer, 1960).

In the present study, *Bacteroides* spp. isolated from clinical specimens in the routine diagnostic laboratory were identified by a combined set of tolerance tests, antibiotic disk resistance tests, morphological and biochemical tests. This investigation was part of the evaluation of a scheme for the identification of clinically important gram-negative anaerobic bacilli by conventional bacteriological tests (Duerden *et al.* 1976; Duerden *et al.* in the Press).

#### MATERIALS AND METHODS

##### *Organisms*

Three hundred and ninety-nine strains of gram-negative anaerobic bacilli isolated from specimens submitted to the four diagnostic bacteriology laboratories of the Southern District of the Sheffield Area Health Authority (Teaching) were studied. All were regarded as significant and were reported to the clinicians. The *Bacteroides* strains were present in pure culture in only a minority of the specimens but they were usually predominant or, at least, present in sufficient numbers relative to any other organisms to justify the view that they were significant.

##### *Patients and specimens*

The 399 strains were isolated from 356 specimens submitted from 332 patients. Details of the clinical condition of each patient were recorded and the sites from which the specimens were obtained are shown in Table 1. The specimens were pus, exudates or swabs sent routinely to the laboratories from patients with clinically apparent infections. The clinicians were encouraged to send specimens

Table 1. *The sources of specimens that yielded a significant growth of gram-negative anaerobic bacilli*

Site of infection	Number of specimens that yielded <i>Bacteroides</i> spp. (total in group of infections)
Infections related to the gastro-intestinal tract	(241)
Upper g.i. tract	40
Appendix	65
Colon and rectum	87
Perianal infections	49
Gynaecological infections	22
Genito-urinary tract (male)	12
Facial and dental infections	6
Central nervous system	5
Bone and joint infections	8
Miscellaneous soft-tissue infections	28
Ear, nose and throat infections	3
Respiratory tract	2
Bacteraemia	3
Unknown	26
Total	356

of pus, where possible, and to avoid delay in transport of specimens; these were routine requests, and no special measures were taken to obtain special specimens for the purpose of this investigation or to provide special transport facilities.

#### *Isolation methods*

All cultures, sub-cultures and other manipulations were carried out on the open laboratory bench. In the early stages of the investigation, specimens submitted to three laboratories were seeded on horse-blood agar and neomycin-horse-blood agar; later, the selective medium was changed to kanamycin-menadione-lysed blood agar (Loesche, Hockett & Syed, 1971; Holbrook, Ogston & Ross, 1978). The media were pre-reduced in an atmosphere of H<sub>2</sub> at room temperature overnight and then held in an atmosphere of CO<sub>2</sub> until seeded, when they were again held under CO<sub>2</sub> until the jar was sealed and evacuated. The plates were incubated in BTL anaerobic jars in an atmosphere of 80% N<sub>2</sub>/10% H<sub>2</sub>/10% CO<sub>2</sub> (BOC Special Gases); the jar was filled with the gas mixture and then re-evacuated to a vacuum of -630 mmHg before filling with the final incubation atmosphere. Anaerobiosis was monitored by including a nutrient agar plate seeded with *Pseudomonas aeruginosa* in each jar. All plates were examined after overnight incubation and then reincubated anaerobically for a further 48 h. Specimens submitted to the fourth laboratory were seeded on horse-blood agar and neomycin-blood agar, and the anaerobic procedure of Collee *et al.* (1972) was followed; the plates were examined after overnight incubation and after a further 48 h.

*Identification of gram-negative anaerobic bacilli*

The strains were identified by the combined set of cultural, biochemical, tolerance and antibiotic disk resistance tests described by Duerden *et al.* (1976 and in the Press). The tests were: colony morphology after incubation for 48 h 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 & Holbrook, 1978); pigment production on BM agar; haemolysis on human-blood agar; motility in BM broth; antibiotic disk resistance tests with neomycin 1000 µg, kanamycin 1000 µg, penicillin 2 units and rifampicin 15 µg disks; 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; fermentation tests with glucose, rhamnose, trehalose, mannitol and xylose, with tests for fermentation of lactose and sucrose added when appropriate. All tests were incubated at 37 °C in an atmosphere of H<sub>2</sub> 90% and CO<sub>2</sub> 10% (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 *P. aeruginosa* was included in each jar as a control.

## RESULTS

A total of 399 strains of gram-negative anaerobic bacilli isolated from 356 specimens were included in the study. Strains from more than one specimen from a single patient were included if they were different, or if the same species was isolated from different sites, or during different episodes of infection.

*Identification of strains*

The identity of the 399 strains is shown in Table 2. *B. fragilis* was the commonest species and accounted for > 50% of all strains; the next commonest species was *B. asaccharolyticus* (formerly *B. melaninogenicus* ssp. *asaccharolyticus*) with 55 strains.

*B. fragilis* group. Two hundred and sixty-one strains were members of the *fragilis* group and 204 (78%) were *B. fragilis*. Most (184) strains allocated to this species gave the same pattern of results as the reference strains, and the others differed in only minor respects. Most atypical results occurred in the tolerance tests and the antibiotic disk resistance tests, but nine strains did not ferment xylose. The next commonest species in the group was *B. thetaiotaomicron* with 35 strains; the other species accounted for only a few strains each. The strains of *B. vulgatus*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis* and *B. splanchnicus* gave patterns of results that were typical, in general, of those species with only a few atypical results. *B. variabilis* and *B. eggerthii* are separated only by the result of the test for sucrose fermentation, and they were not distinguished in the early part of the study when the two strains were isolated.

*B. melaninogenicus/oralis* group. Fifty-seven strains belonged to species in this group; 38 were pigmented strains of *B. melaninogenicus* ssp. *melaninogenicus* (13) or ssp. *intermedius* (25) and the remainder were non-pigmented species. All



abscesses, peritonitis and wound infections following surgery. Most infections were characterized by tissue necrosis, poor vascularity and the production of foul-smelling pus.

*Infections related to the gastro-intestinal tract.* The species isolated from infections related to the gastro-intestinal tract are shown in Table 3. The infections related to the upper gastro-intestinal tract followed surgery to the oesophagus, stomach, small intestine and biliary tract and included two infected pancreatic pseudocysts. *B. fragilis* accounted for > 50% of the strains but it is significant that four strains of *B. melaninogenicus* ssp. *melaninogenicus* and two strains of ssp. *intermedius*, which are essentially oral organisms, were isolated from wound infections following surgery to the oesophagus and stomach. Seventy-two strains were from 65 cases of gangrenous appendix, appendix abscess, peritonitis or wound infection following appendicectomy. *B. fragilis* was isolated from 68% of these infections. Ninety-seven strains were isolated from 87 infections related to the colon and rectum, which included wound infections, peritonitis and intra-abdominal abscesses following surgery to or perforation of the large intestine. *B. fragilis* was found in 62% of the specimens that yielded a significant growth of gram-negative anaerobic bacilli and *B. asaccharolyticus* and *B. thetaiotaomicron* were also isolated regularly. Fifty-seven strains were isolated from 49 specimens of pus from perianal abscesses. *B. fragilis* was isolated from 50% of these abscesses and there were nine strains of *B. asaccharolyticus* and five strains of *B. corrodens*.

Only specimens that yielded a significant growth of gram-negative anaerobic bacilli have been included in this analysis; the figures given of the proportions of isolations of individual species from groups of specimens relate only to these selected specimens. However, there were very few specimens from infections related to the appendix, colon and rectum, or from perianal abscesses that did not yield a significant growth of one or more *Bacteroides* spp., and most infections related to those sites that occurred during the study are included.

*Gynaecological infections.* The 25 strains from 22 specimens from infections related to the female genital tract are shown in Table 4. *B. fragilis* was isolated from 16 infections that included post-partum pelvic abscesses, Bartholin's abscesses, and wound infections following gynaecological surgery. One of the *B. melaninogenicus* ssp. *intermedius* strains was from a pelvic abscess and the others were from cervical pus.

*Genito-urinary infections (male).* Gram-negative anaerobic bacilli were isolated as significant pathogens from 12 infections related to the male genito-urinary tract (Table 4). One strain of *B. fragilis* was from a wound infection following removal of a renal calculus, but the others were all from abscesses or post-operative wound infections of the scrotum (7), penis (2) or groin (2).

*Infections of the head and neck.* Seven strains of *Bacteroides* were isolated from six infections of the mouth and face and eight strains from four patients with infections of the central nervous system. *B. melaninogenicus* ssp. *melaninogenicus* was isolated from two dental abscesses and a compound fracture of the mandible; *B. fragilis* and *B. asaccharolyticus* were isolated from a facial abscess, and *B. corrodens* was recovered in large numbers from an actinomycotic abscess.



Table 3. *Bacteroides* spp. isolated from infections related to the gastro-intestinal tract

Species	Number of isolates from infections related to				Total
	Upper g.i. tract	Appendix	Colon and rectum	Perianal abscesses	
<i>B. fragilis</i> group	(33)	(55)	(75)	(36)	(199)
<i>B. fragilis</i>	24	44	54	29	151
<i>B. vulgatus</i>	1	1	3	2	7
<i>B. distasonis</i>	1	1	2	1	5
<i>B. ovatus</i>	—	—	1	—	1
<i>B. thetaiotaomicron</i>	5	9	15	3	32
<i>B. eggerthii/variabilis</i>	2	—	—	—	2
<i>B. splanchnicus</i>	—	—	—	1	1
<i>B. melaninogenicus/oralis</i> group	(10)	(7)	(3)	(4)	(24)
<i>B. melaninogenicus</i>					
ssp. <i>melaninogenicus</i>	4	—	—	1	5
ssp. <i>intermedius</i>	2	7	1	2	12
<i>B. oralis</i>	—	—	—	1	1
<i>B. bivius/disiens</i>	2	—	—	—	2
<i>B. ruminicola</i>	2	—	2	—	4
Asaccharolytic group	(3)	(9)	(18)	(15)	(45)
<i>B. asaccharolyticus</i>	3	7	17	9	36
<i>B. corrodens</i>	—	—	—	5	5
Non-pigmented non-saccharolytic spp.	—	2	1	1	4
<i>Fusobacterium</i> group	—	1	1	2	4
Total	46	72	97	57	272

Table 4. *Bacteroides* spp. isolated from infections related to the genito-urinary tract

Species	Number of isolates from infections of		Total
	Female g.u. tract	Male g.u. tract	
<i>B. fragilis</i> group	(17)	(6)	(23)
<i>B. fragilis</i>	16	5	21
<i>B. thetaiotaomicron</i>	1	—	1
<i>B. uniformis</i>	—	1	1
<i>B. melaninogenicus/oralis</i> group	(7)	(2)	(9)
<i>B. melaninogenicus</i>			
ssp. <i>melaninogenicus</i>	—	2	2
ssp. <i>intermedius</i>	4	—	4
<i>B. oralis</i>	1	—	1
<i>B. bivius/disiens</i>	1	—	1
<i>B. disiens</i>	1	—	1
Asaccharolytic group	(1)	(4)	(5)
<i>B. asaccharolyticus</i>	1	1	2
<i>B. corrodens</i>	—	3	3
Total	25	12	37

A mixture of *B. fragilis* and *B. asaccharolyticus* was recovered from a frontal lobe abscess secondary to a penetrating injury to the orbit and frontal sinus and four strains (*B. melaninogenicus* ssp. *melaninogenicus*, ssp. *intermedius*, and two separate strains of *B. ruminicola*) were isolated from the cerebrospinal fluid and the pus from an abscess of a cervical vertebral body with secondary meningitis. *B. fragilis* and *B. oralis* were isolated from specimens of pus from separate brain abscesses.

*Miscellaneous infections.* The 34 strains from bone, joint and miscellaneous soft tissue infections are shown in Table 5. Six strains of *B. fragilis* and one each of *B. distasonis* and *B. asaccharolyticus* were from cases of osteomyelitis or deep infections following bone and joint surgery. The others were recovered in large numbers from the depths of infected decubitus ulcers, varicose ulcers, diabetic gangrene, paronychia and two breast abscesses. Strains of *B. thetaiotomicron*, *B. asaccharolyticus* and *B. melaninogenicus* ssp. *intermedius* were isolated from three cases of chronic otitis media, and two strains of fusobacteria were from a lung abscess and an empyema. Only three strains of *Bacteroides* were isolated from blood cultures during the study; *B. fragilis* was isolated from the blood of two patients following abdomino-perineal resection of the colon and gastro-jejunostomy, and *B. bivius* was from a patient with a pelvic abscess.

#### *Monobacterial and mixed infections*

A pure culture of a *Bacteroides* spp., or a heavy growth with only a light admixture of coagulase-negative staphylococci, diphtheroids or coliforms was obtained from only 92 infections; *B. fragilis* was the sole pathogenic strain isolated in 60 of these infections and other species were found in pure culture on a few occasions only. *B. asaccharolyticus* was the second commonest species isolated overall but only four of the 55 strains were recovered in pure culture.

More than one species of potentially pathogenic bacteria were isolated in significant numbers from 246 infections for which full details were obtained. The number of species and their identity varied with the site of infection. The most common additional species were coliform organisms, particularly in infections related to the lower gastro-intestinal tract; 148 specimens yielded a heavy growth of a coliform organism. Streptococci were also implicated in a large number of mixed infections; a variety of streptococci, including  $\alpha$ - and  $\beta$ -haemolytic streptococci, enterococci and anaerobic cocci were isolated in potentially significant numbers from 121 specimens.

Two or more strains of *Bacteroides* were isolated from 55 infections of which 43 were related to the gastro-intestinal tract. *B. fragilis* was usually one of the species isolated and in many instances the second species was *B. asaccharolyticus*.

#### DISCUSSION

These studies have shown that a wide range of clinically important gram-negative anaerobic bacilli can be isolated in the diagnostic bacteriology laboratory by the careful application of routine bench methods with a controlled and standardized anaerobic jar technique. Most strains can be identified by a relatively





of the opportunity given by faecal soiling, the proportions of the different species isolated from infections would reflect their proportions in faeces. It is clear that *B. fragilis* has particular pathogenic potential; this appears to be related to cell surface properties and the presence of a polysaccharide capsule (Kasper, 1976*a, b*) but *B. fragilis* also produces a number of extracellular and membrane-associated enzymes that may contribute to virulence (Gesner & Jenkin, 1961; Müller & Werner, 1970; Rudek & Haque, 1976).

Other workers found that *B. thetaiotaomicron* was the second most common species isolated from clinical specimens (Werner & Pulverer, 1971; Werner, 1974; Holland *et al.* 1977). *B. thetaiotaomicron* was the second most common species in the fragilis group in the present studies but there were almost twice as many isolates of the pigmented, non-fermentative species *B. asaccharolyticus* (formerly *B. melaninogenicus* ssp. *asaccharolyticus*; Finegold & Barnes, 1977). This species is a commensal of the lower gastro-intestinal tract (Werner, Pulverer & Reichertz, 1971; Duerden *a*, in the Press) and some clinical strains isolated were from infections related to the appendix, colon and rectum and the perianal area, where it was usually recovered in mixed culture with *B. fragilis*. *B. asaccharolyticus* was also isolated from nine soft-tissue infections where it appeared to be the most significant pathogen in lesions such as diabetic and varicose ulcers, a relationship recognized by Peromet *et al.* (1973), Rissing *et al.* (1974) and Willis (1977).

The proportions of the different *Bacteroides* spp. isolated varied with the site of infection. Many of the melaninogenicus/oralis group isolated reflected the normal habitat of these species in the mouth. However, *B. fragilis* is not a common member of the normal vaginal flora (Duerden *b*, in the Press) but it was isolated from 73% of gynaecological infections where *Bacteroides* spp. were implicated. This emphasizes the specific pathogenicity of the species and casts doubt upon the assumption that bacteroides infections of the female genital tract are caused by *Bacteroides* spp. present in the normal vaginal flora. The isolation of several *Bacteroides* spp. from cerebral abscesses supports the findings of Ingham, Selkon & Roxby (1977) that *Bacteroides* spp. are important pathogens in brain abscesses that follow infections or penetrating injuries of the middle ear and mastoid, paranasal or posterior pharyngeal wall.

*Bacteroides* spp. were isolated in pure culture from only 26% of infections in this study, and it is generally recognized that many anaerobic infections yield a mixture of organisms (Gorbach & Bartlett, 1974). Most mixed infections originate in areas where colonization with a variety of species generally occurs. Many of the organisms in these mixtures were potential pathogens, but there is some evidence that the anaerobes are the more significant pathogens. Willis *et al.* (1975, 1976, 1977) showed that treatment of the anaerobic component of infections related to the appendix, colon, rectum and female genital tract with metronidazole controlled the infections, and Jones, Willis & Ferguson (1978) obtained healing of infected decubitus ulcers with topical metronidazole. However, the facultative and aerobic species in the mixtures should not be ignored; there may be synergy between components of the mixed flora (Tally & Gorbach, 1979) or the common

occurrence of facultative organisms, e.g. coliforms, may provide suitable conditions of Eh and growth factors for the anaerobes to cause the tissue damage and necrosis characteristic of these infections.

Analysis of the incidence of different species of *Bacteroides* in clinical infections and comparison with their incidence in the normal flora shows that certain species have particular pathogenic potential. The identification of *Bacteroides* strains may, therefore, help to assess the significance of laboratory findings, may influence the patient's management and prognosis, and may also help to determine the source of infection when this is not immediately apparent.

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