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Numbers and Types of Anaerobic Bacteria Isolated from Clinical Specimens Since 1960

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Between 1960 and 1974, 826 specimens, excluding stool, urine, sputum, and blood, yielded 689 (83%) positive cultures, of which 403 (58.5%) contained anaerobic bacteria. This represents 48.8% of the total specimens cultured. Isolates from 153 specimens obtained and stocked from 1965 to 1974 were reidentified by current criteria. Gram-negative bacilli, primarily *Bacteroides*, were the most frequently isolated anaerobes, being found in 70% of 153 anaerobe-positive specimens and accounting for 42% of the total anaerobes isolated. Gram-positive cocci were second in occurrence, being found in 66% of 153 specimens and accounting for 40% of the total isolates. *Bacteroides fragilis* was by far the most frequently isolated species. Comparison of 14 years of cumulative data with data from current studies covering 1- to 2-year periods indicated that the anaerobes isolated from clinical material have not changed significantly in type or relative numbers.

Anaerobic bacteria are the most primitive and prevalent organisms of the microflora (15), outnumbering the aerobes 1,000:1 in the lower intestine and 10:1 or more on the skin and in the mouth (22). Evidence of their involvement in infections, such as tubo-ovarian abscesses (3) and peritonitis secondary to acute perforative appendicitis (2, 4), has been reported and accumulated since the early 1900s. These reports lay practically dormant and few bacteriology laboratories concerned themselves with anaerobes, even though some infections involving these bacteria were found to be fatal when undiagnosed (5). Today, through the efforts of a few workers to modify and improve collection, culture, and identification procedures, more laboratories have become aware that anaerobic bacteriology is possible and necessary. This awareness has led to renewed interest in the earlier literature and to recent surveys that strongly implicate anaerobic bacteria in an almost endless number and variety of infections.

The results reported here show that the percentage of clinical specimens yielding anaerobes over the 14-year period from 1960 to 1974 compares well with values reported from the 1970s. Reidentification of isolates stocked since 1965 allows a comparison of the numbers and types of anaerobes isolated from clinical specimens over a 9-year period with values from current studies covering 1- to 2-year periods. Such comparisons indicate that the types and numbers of anaerobic bacteria found in clinical specimens have not changed significantly with time.

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MATERIALS AND METHODS

Clinical specimens. The number of specimens included in this study was relatively small because this laboratory is primarily a research facility and does not receive the large quantity of specimens processed by a hospital bacteriology laboratory. Most specimens were obtained from patients on the surgical services at the Cincinnati General Hospital and occasionally from surrounding area hospitals. In most cases, the laboratory was consulted before obtaining specimens so that collection and transportation instructions could be given. Specimens were transported in several ways, depending on the specimen type. When possible, pus and fluids were collected and transported in syringes. Large volumes of pus were sometimes placed in sterile tubes. Tissues were transported in sterile aerobic tubes, in essentially oxygen-free gassed-out tubes, or in small volumes of thioglycolate broth. Swab cultures were placed in transport media or in aerobic or gassed-out tubes. When possible, specimens were hand delivered by medical or laboratory personnel to avoid delays encountered in normal hospital delivery procedures. Some specimens were processed at the bedside or in the operating area by inoculating the appropriate media (listed below) and immediately placing the media in an anaerobic jar. All other specimens were processed in the laboratory by inoculating phenylethyl alcohol blood agar (BBL), blood agar base (BBL) containing 2 to 5% defibrinated rabbit blood, and blood agar base (containing 2 to 5% defibrinated sheep blood) with and without menadione (0.5 μg/ml, final concentration) and/or kanamy-

cin (100 μg/ml, final concentration). Liquid media such as chopped-meat medium and thioglycolate medium without indicator (BBL) were inoculated as backup cultures. Blood, phenylethyl alcohol, and eosin-methylene blue agar plates (BBL) were inoculated and incubated aerobically for isolation of aerobic and facultative organisms (aerobes). Direct Gram stains were made on each specimen plated. The specimens listed in Table 1 include all clinical material cultured by this procedure since 1960 except stool, urine, sputum, and blood. The anaerobic isolates from many of the specimens cultured since 1965 were stocked at −70°C. Of these specimens, 153 had sufficient source information to allow them to be placed in one of the following specimen categories: intra-abdominal, pleuropulmonary, subcutaneous and soft tissue, cutaneous, perirectal, fluid, brain, or bone (Table 3). Isolates from these specimens were reidentified and are included in Tables 2 through 5.

Anaerobiosis. Rosenthal's chromium-sulfuric acid (21) with sodium carbonate (17) was the most successful early method for obtaining anaerobic conditions evaluated in this laboratory and was the primary (but not the only) system used before the hydrogen-carbon dioxide generator packet (GasPak, BBL) became commercially available.

Identification media and procedures. Clinical isolates were stocked by freezing 4-ml volumes of thioglycolate culture at −70°C in 6-ml plastic tubes. Frozen stocks were thawed at room temperatures and streaked on modified McClung-Toabe egg yolk agar plates (7) and blood agar plates (blood agar base containing 2 to 5% defibrinated rabbit or sheep blood) incubated aerobically, anaerobically, and in candle jars. After 4 days, colonies were described and picked to thioglycolate medium without indicator (BBL) to be used as test medium inoculum. Glucose, fructose, lactose, sucrose, maltose, starch, salicin, glycerol, mannitol, xylose, arabinose, rhamnose, and trehalose in the concentrations recommended by the Virginia Polytechnic Institute (VPI) (11) were filter sterilized and added to sterile thioglycolate medium without dextrose or indicator (BBL). Esculin broth was prepared as recommended by the Center for Disease Control (7). Gelatin, nitrate, and indole were prepared by using Thiogel (BBL) and indole-nitrite (BBL) media as directed. Pyruvate and lactate utilization medium, threonine conversion medium, salts solution, peptone-yeast extract-glucose (PYG) broth, PYG agar, and sulfide-indole-motility medium were prepared as described by VPI (11) except that resazurin indicator was not included and media were not prereduced anaerobically sterilized. Bile medium was made by adding 0.50 ml of 40% oxgall (Matheson, Coleman, and Bell) to 10 ml of PYG broth. Milk medium was made by adding 0.5 g of L-cysteine to 1,000 ml of skim milk. When required, media were supplemented by adding Tween 80, bile, or hemin and menadione (11).

Immediately before use, all liquid media were heated to 100°C for 10 min and rapidly cooled. All inoculated media were incubated at 37°C. Indole, PYG, PYG with bile, and milk media were incubated anaerobically in GasPak jars (BBL). Tests were read after 10 days (gelatin up to 30 days). Fermentation reactions were checked by adding a few drops of bromothymol blue solution to each tube. Other test reagents and procedures, and fatty acid extraction methods, were those described by VPI (11) except that we chromatographed 5-μl rather than 14-μl volumes of fatty acid extracts. A Perkin-Elmer model 3920 gas chromatograph with dual-flame ionization detectors and Resoflex (11)-packed columns was used for most of this work.

These identification procedures were tested in our laboratory on a blind basis by reidentifying American Type Culture Collection cultures and cultures previously identified by the Center for Disease Control. These cultures included 31 strains of anaerobic bacilli. All 31 strains correlated to the genus level, and 29 (94%) of the 31 strains correlated to the species level.

RESULTS

In this laboratory, 826 specimens from 562 patients were cultured between 1960 and 1974 (Table 1). These figures exclude stool, urine, sputum, and blood specimens. Of these 826 specimens, 689 (83%) yielded bacterial growth, and 403 (58.5%) of these positive cultures contained anaerobic bacteria. This represented 48.8% of the total specimens. Further breakdown of the data from the 689 positive specimens showed that anaerobes alone were found in 11.8% and anaerobes mixed with aerobes were found in 46.7%.

The types of anaerobes obtained since 1965 from 153 anaerobe-positive specimens whose isolates were stocked are expressed in Table 2. The source distribution of these specimens is shown in Table 3. Duplicate species or subspecies from a single specimen were counted as one isolate. Anaerobic gram-negative bacilli were found in 70% of the specimens and were the only anaerobes recovered from 14% of the specimens. *Bacteroides fragilis* strains were present

TABLE 1. *Summary of the bacterial isolates obtained from 826 specimens cultured aerobically and anaerobically since 1960 (excluding blood, urine, sputum, and stool specimens)*

Category	Specimens		
	No.	% of total	% of positives
Patients	562		
Total specimens	826	100.0	
Positive specimens	689	83.4	100.0
Positive for aerobes	608	73.6	88.2
Positive for anaerobes	403	48.8	58.5
Mixed aerobes-anaerobes	322	38.9	46.7
Aerobes only	286	34.6	41.5
Anaerobes only	81	9.8	11.8
Negative specimens	137	16.6	

in at least 52% and were the only anaerobes isolated from 10% of the specimens. Tables 3 and 4 list all anaerobes recovered and the species most frequently recovered from 153 specimens. The gram-negative bacilli comprised the largest single group of organisms isolated and accounted for 173 (42%) of the 408 anaerobic isolates. *Bacteroides* accounted for 164 (95%) of the 173 gram-negative bacilli isolated. The *Bacteroides* group contained 99 (60%) *B. fragilis*, 29 (18%) *B. melaninogenicus*, 12 (7%) other species, and 24 (15%) isolates that could not be identified as to species. The strains not identified as to species were often biochemically nonreactive even when growth was heavy. This pattern was not significantly altered by addition of bile and/or hemin and menadione. These nonreactive bacteria were variable in their sensitivity to 2-unit penicillin disks. The subspecies of *B. fragilis* isolates are shown in Table 5.

Table 6 shows the current identification of isolates originally identified as *Bacteroides* or *Sphaerophorus* on the basis of Gram stain morphology. Of 208 isolates identified as *Bacteroides* on the basis of morphology, 197 (95%) were *Bacteroides* when reidentified. One hundred and twenty-two isolates originally identified as *Sphaerophorus* yielded 111 (91%) *B. fragilis*, 4 other *Bacteroides* species, 4 *Fusobacterium* species, and 3 *Clostridium ramosum* strains.

Fusobacterium nucleatum accounted for nearly one-half of the *Fusobacterium* species isolated. The remaining isolates were divided between other recognized species and strains that could not be identified as to species. No *F. necrophorum* strains were identified from human clinical material.

Gram-positive cocci were the second most isolated anaerobic bacteria. Of 153 anaerobe-positive specimens, 66% had anaerobic gram-positive cocci and 15% had these bacteria as the only anaerobic strains isolated (Table 2). The anaerobic gram-positive cocci accounted for 163 (40%) of 408 anaerobic isolates from 90 patients

TABLE 2. *Types of anaerobic bacteria isolated from 153 anaerobe-positive specimens obtained from 90 patients between 1965 and 1974*

Organisms	Specimens with A, B, C, D, or E (%)	Specimens with A, B, C, D, or E as the only anaerobe (%)
A. Gram-negative bacilli	70	14
B. Gram-positive bacilli (nonsporing)	24	9
C. Gram-positive cocci	66	15
D. Clostridia	16	2
E. *Bacteroides fragilis*	52	10

TABLE 3. *Relative incidence of anaerobes isolated from 153 specimens obtained from 90 patients since 1965 and the source distribution of these specimens*

Genus	Isolates		Source									
	No.	%	Intra-abdominal	Pleuropulmonary	Subcutaneous and soft tissue			Cutaneous	Perirectal	Fluid[a]	Brain	Bone
					Leg and foot	Arm	Trunk					
Bacteroides	164	40.2	80	11	8	3	16	9	25	2	2	8
Peptococcus	95	23.3	39	8	7	2	16	3	9	3	2	6
Peptostreptococcus ..	30	7.4	15	5	2	1	2	2	2	0	1	0
Propionibacterium ..	26	6.4	8	5	1	2	6	1	0	2	0	1
Streptococcus	25	6.1	10	6	0	0	4	0	1	0	3	1
Clostridium	25	6.1	9	3	0	0	2	3	8	0	0	0
Eubacterium	10	2.5	4	1	0	0	2	1	1	0	1	0
Fusobacterium	9	2.2	4	0	1	0	2	0	1	0	1	0
Bifidobacterium	3	0.7	3	0	0	0	0	0	0	0	0	0
Lactobacillus	2	0.5	0	0	2	0	0	0	0	0	0	0
Veillonella	2	0.5	0	1	0	0	1	0	0	0	0	0
Megasphaera	1	0.2	1	0	0	0	0	0	0	0	0	0
Unidentified GPC[b] .	13	3.2	5	2	0	0	1	0	1	0	1	3
Unidentified anaerobes	3	0.7	0	1	0	0	2	0	0	0	0	0
Total isolates	408	100.0	178	43	21	8	54	19	48	7	11	19
Total specimens ..			69	17	11	3	16	6	16	3	6	6

[a] Synovial fluids.
[b] GPC, Gram-positive cocci.

TABLE 4. *Top 10 species of anaerobes isolated from 153 specimens obtained from 90 patients since 1965*

Species	No.	%
Bacteroides fragilis	99	24.3
Peptococcus magnus	48	11.8
Bacteroides melaninogenicus	29	7.1
Peptococcus asaccharolyticus	23	5.6
Propionibacterium acnes	22	5.4
Peptococcus prevotii	21	5.1
Peptostreptococcus anaerobius	17	4.2
Streptococcus intermedius	17	4.2
Clostridium perfringens	10	2.5
Eubacterium lentum	8	2.0
Totals	294	72.2

TABLE 5. *Subspecies of Bacteroides fragilis isolated from 153 specimens obtained between 1965 and 1974*

B. fragilis subspecies	No.
B. fragilis	44
B. thetaiotaomicron	27
B. distasonis	10
B. vulgatus	4
B. ovatus	1
No good fit	13

(Table 3). Ninety-five (58%) of these isolates were *Peptococcus* species, 30 (18%) were *Peptostreptococcus* species, 25 (15%) were *Streptococcus* species, and 13 (8%) could not be identified. This is in contrast to earlier data obtained basing identification on hydrogen peroxide degradation and microscopic examination.

Peptococcus magnus was the most commonly isolated coccus, followed by *P. asaccharolyticus, P. prevotii, Peptostreptococcus anaerobius*, and *Streptococcus intermedius* (Table 4). Many of the gram-positive cocci appeared gram variable to gram negative after 4 days of incubation. *Peptococcus magnus* had a tendency to become pleomorphic as well. True gram-negative anaerobic cocci were rarely encountered.

The gram-positive nonsporeforming bacilli represented 10% of the total anaerobic isolates and were found in 24% of the specimens. They were the only anaerobes recovered from 9% of the 153 anaerobe-positive specimens. Approximately two-thirds (26 of 41) of the nonsporing bacilli were *Propionibacterium* species, of which 22 (85%) were *P. acnes*. The most frequently isolated nonsporing gram-positive bacillus other than *P. acnes* was *Eubacterium lentum*, which accounted for 80% of the *Eubacterium* species isolated. *Eubacterium* and *Bifidobacterium* species were isolated primarily from the abdominal cavity. *Arachnia, Actinomyces, Lachnospira*, and *Lactobacillus* species were detected rarely or not at all.

Clostridia were recovered from 16% of the anaerobe-positive specimens, were the only anaerobes recovered from 2%, and comprised 6% of the total anaerobic isolates (Tables 2 and 3). Forty percent (10 of 25) of the *Clostridium* strains were *C. perfringens*.

DISCUSSION

The percentage of positive clinical specimens yielding anaerobic bacteria varies from 10.5% (23) to 66% (11) in the literature reviewed. Zabransky (26) and Martin (14) reported values of 39.5 and 49%, respectively. Over a 14-year period, 58.5% of our positive cultures (excluding blood, urine, sputum, and stool specimens) have yielded anaerobes. This high percentage was not due to increased numbers of anaerobe-positive cultures in recent years. Nearly 60% of our total of 689 positive specimens were cultured before 1970, and 58.4% of these contained anaerobes. The discrepancies in reported values can be explained by differences in specimen selection practices, delivery and plating techniques, systems of anaerobiosis, media, and work time available per specimen. Even though values differ, they are all sufficient to demonstrate the abundance of anaerobic bacteria in clinical specimens.

There is general agreement that the nonsporulating gram-negative bacilli are the most frequently isolated anaerobes from clinical material and that *B. fragilis* is the most commonly isolated species (14, 16, 24, 26). Our results agree with these findings. Forty-two percent of the anaerobes isolated from 153 specimens were gram-negative bacilli (Table 3), and 70% of the anaerobe-positive specimens contained these bacteria. *B. fragilis* was found in more specimens (52%) and comprised a greater percentage of the total anaerobic isolates (24%) than any other species. Holdeman and Moore (10) state that gram-negative anaerobic bacilli will probably be found in 55 to 60% of the anaerobe-positive specimens (if adequate anaerobic tech-

TABLE 6. *Current identification of some clinical isolates originally identified and stocked as Bacteroides or Sphaerophorus species*

Original identification	No.	Current identification	No.	%
Bacteroides sp.	208	*B. fragilis*	127	61.1
		Bacteroides (other than *fragilis*)	70	33.6
		Fusobacterium sp.	3	1.4
		Clostridium ramosum	1	0.5
		Other	7	3.4
Sphaerophorus sp.	122	*B. fragilis*	111	91.0
		Bacteroides (other than *fragilis*)	4	3.3
		Fusobacterium sp.	4	3.3
		C. ramosum	3	2.5

niques are used), that 85 to 90% of the bacilli will be *Bacteroides*, and that 50 to 60% of the *Bacteroides* will be *B. fragilis*. We found that 70% of our anaerobe-positive specimens contained anaerobic gram-negative bacilli, that 95% were *Bacteroides*, and that 60% of the *Bacteroides* were *B. fragilis*. Further characterization of *B. fragilis* isolates to the subspecies level (Table 5) shows that the specimens cultured between 1965 and 1974 yielded the same subspecies as specimens currently being cultured.

Clinical concern about the gram-positive cocci is emphasized by the finding that 66% of the anaerobe-positive specimens contained these bacteria and that 15% contained them as the only anaerobes present (Table 2). These cocci accounted for 40% of the total anaerobic bacteria isolated over the 9-year period (Table 3). Other reported values are 21 (16), 25 (18), 25 (14), 28.6 (26), and 48% (23). In this laboratory, cocci were frequently isolated from wound infection and abscess specimens. Two brain abscesses yielded *Streptococcus intermedius* in pure culture.

The regularity with which the gram-positive cocci were isolated from clinical material, their isolation in pure culture such as *S. intermedius* from two brain abscesses, and numerous reports in the literature (19, 22, 25) combine to document the pathogenicity of the anaerobic cocci. Our attempts to establish pure culture infections in laboratory animals, however, have generally been unfruitful. Although this is not uncommon or even unexpected, it is unfortunate because the pathogenic mechanisms of the cocci and the nonsporeforming anaerobes in general remain unresolved.

Peptostreptococcus species have been reported as the causative agents in several infectious processes and have been implicated in many others, whereas *Peptococcus* species have received less attention. Much of the concern about *Peptostreptococcus* has been based on a man-made system of taxonomy that has undergone many changes. All anaerobic gram-positive cocci isolated in this laboratory were originally identified on the basis of Gram stains and the ability to decompose hydrogen peroxide. Using these criteria, 78% of the stocked anaerobic cocci isolated from clinical specimens were classified as *Peptostreptococcus* species. The same isolates reidentified by VPI criteria yielded only 31% *Peptostreptococcus* species. Of 408 isolates stocked since 1965 from 90 patients, 103 (25%) were *Peptococcus* species and 47 (11%) were *Peptostreptococcus* species. These figures are in fair agreement with those of Pien et al. (18) and Martin (14). *Peptococcus morbillorum*, *Peptococcus constellatus*, and *Peptostreptococcus intermedius* are now placed in the genus *Streptococcus* (12); however, for comparison with other studies, these species are discussed here as members of their former genera.

Anaerobic gram-positive bacilli were isolated from 40% of 153 specimens, but their significance in clinical material was in most cases difficult to assess. With the exception of *Propionibacterium acnes*, these bacteria were usually found in mixed-culture infections. In most cases, *P. acnes* was considered to be a contaminant from the skin; however, occasional reports of bacterial endocarditis (8), chronic meningitis (9), and other infections involving *P. acnes* indicate that this anaerobe cannot always be dismissed as a contaminant.

Clostridium perfringens accounted for 2.5% of our total isolates. This figure is lower than the 5.3% (14) and 8.5% (26) found by others. Since *C. perfringens*, like *P. acnes*, is considered to be a contaminant in many cases, these differences seem insignificant. *C. innocuum* and *C. ramosum* were the next most frequently isolated clostridia. Their clinical significance has been discussed by others (19, 22). Table 6 shows that some *C. ramosum* strains were originally identified as *Bacteroides* or *Sphaerophorus* species. *C. septicum* was only isolated from three patients in this study, but two of the three had known malignancies. This was not surprising in light of Alpern and Dowell's (1) finding that 23 of 27 patients from whom *C. septicum* was isolated also had malignancies.

Eubacterium lentum was the most commonly encountered gram-positive bacillus other than *P. acnes* and *C. perfringens*. The occasional isolation of this anaerobe from specimens such as brain, liver, and pleural abscesses indicates that it may have a role in some infections.

The growing concern over anaerobes today is viewed by some as a reflection of renewed interest in endogenous infections and our indigenous microflora and is not equated to increased anaerobe numbers or increased pathogenicity of any particular organism. Support for this view can be found by comparing results obtained from different time periods. Martin (14), in 1971 and 1972, found that 10,998 anaerobes from 27,588 unselected specimens were identified as follows: gram-negative bacilli, 30%; cocci, 27%; gram-positive nonsporulating bacilli, 27%; and clostridia, 7%. Our cumulative data agreed fairly well in that we found 42% gram-negative bacilli, 40% cocci, 10% gram-positive nonsporulating bacilli, and 6% clostridia. The greatest difference was seen in the numbers of gram-positive nonsporeforming bacilli, a group significantly influenced by specimen selection.

Bacteroides fragilis comprised 23% of the 10,998 anaerobes isolated in 1971 and 1972 as compared with 24% in this study. Values of 8 and 17% (18) and 9 and 17% (14) have been reported for *Peptostreptococcus* and *Peptococcus*, respectively. These values are near the 11 and 25% reported here.

The comparison of our data with those reported from other laboratories shows that the species (and subspecies) being isolated more recently are the same as those isolated between 1965 and 1974 and that they are being found in similar relative percentages. Additionally, since 1960, 58.5% of our positive cultures have yielded anaerobes. This figure compares favorably with the values 39.5 (26), 49 (14), and 66% (11) reported since 1970. These data suggest that the greater numbers of anaerobes being reported today are not due to an increasing presence of these bacteria but are a reflection of the growing awareness of anaerobic bacteria and greater care in collection, transportation, and cultivation. The data also show that simple anaerobic-jar techniques are sufficient for the isolation of anaerobic bacteria from clinical specimens. This is not to say that we should be satisfied with these techniques, or that other methods could not result in greater recovery. These data and comparative studies (6, 13, 20) of anaerobic systems should indicate to many clinical laboratories that some of the more sophisticated anaerobic techniques and equipment now available are not a necessity and that good anaerobic bacteriology can be done with simple equipment and attention to a few basic factors such as collection and transportation procedures, use of the Gram stain, and technician orientation.

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