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Anaerobes Survive in Clinical Specimens Despite Delayed Processing

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Quantitative cultures were performed on 11 purulent specimens of at least 2 ml from mixed aerobic-anaerobic infections to determine the effect of prolonged exposure to air on the recovery of anaerobes. The specimens were processed immediately and after air exposure for periods of 10 min and 1, 4, and 24 h. There were a total of 37 anaerobic and 36 aerobic strains recovered from these specimens. Of the anaerobes, 26 were isolated with the initial processing and 22 were still present after air exposure for 24 h. The numerical concentrations of anaerobes showed little change with the sequential samplings. Eleven anaerobic strains were not detected in the initial culture but appeared sporadically in subsequent cultures. Using the types of specimens and method of processing employed in this study, most pathogenic anaerobes survived in purulent exudate despite extended periods of air exposure. The major cause of discrepent results with periodic cultures was attributed to vagaries in sampling.

According to conventional wisdom, clinical specimens from anaerobic infections require special care in laboratory processing. Loss of oxygen-sensitive forms is frequently ascribed to excessive air exposure between the time of collection and laboratory handling. Thus, rapid transport or the use of specialized transport devices are generally advocated. Recent studies indicate that the anaerobes most often isolated in clinical specimens are relatively aerotolerant (7, 10), but the actual duration of allowable air exposure is unknown. The purpose of this study was to determine the qualitative and quantitative loss of anaerobic bacteria when a specimen is exposed to air for extended periods.

MATERIALS AND METHODS

Inclusion criteria. The following criteria were required for inclusion in the study. (i) The infection was judged likely to involve anaerobic bacteria on the basis of clinical observations. (ii) Specimens from the infected site were available for culture prior to the institution of antimicrobial therapy. (iii) There were at least 2 ml of exudate for bacteriological processing.

Specimen collection and processing. Exudate from the infected site was collected by syringe aspiration in a manner that avoided contamination by the normal flora of mucocutaneous surfaces. The specimen was transported immediately to the laboratory in a closed syringe or in transport containers without indicator or other fluid. In each instance, the duration of air exposure from the time of collection to placement in the anaerobic chamber was restricted to 5 min or less.

Upon arrival in the laboratory, a 2-ml aliquot of the specimen was immediately placed in the anaerobic glove box, transferred to a sterile screw-capped vial (18 by 40 mm), and vortexed. (The column height of the specimen in this vial was approximately 1 cm). A 0.1-ml sample was then aspirated, and serial dilutions were prepared with prereduced Virginia Polytechnic Institute dilution salts to give final concentrations of 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}. A 0.1-ml sample from each dilution was plated on each of six media. For anaerobes, these included brucella-base agar with 5% sheep blood and 10 μg of menadione per ml (BMB), BMB containing 100 μg of neomycin sulfate per ml and prereduced laked blood agar containing 75 μg of kanamycin and 7.5 μg of vancomycin per ml. These media were previously stored in the anaerobic chamber and had a shelf life of less than 7 days. The following media were employed for aerobic and facultative isolates: blood agar plates, MacConkey agar, and Pfizer Selective Enterococcus Agar.

After the initial processing, the original specimen was transferred out of the chamber, stirred gently in air, and left loosely capped in the 18- by 40-mm vial on the bench. A 0.1-ml sample was aspirated at the following intervals of air exposure: 10 min, 1 h, 4 h, and 24 h. Each of these samples was obtained after stirring with a tuberculin syringe and then aspirating from the central portion. The sequential samplings were reintroduced into the anaerobic chamber and processed as previously described.

The three anaerobic media were retained in the anaerobic chamber for incubation at 37 C. Aerobic

media were incubated at 37 C in 10% CO_2 (blood agar plates) or air. After incubation, colonial types of aerobes and anaerobes were enumerated, isolated, and identified. Aerobic plates were analyzed at 24 h, whereas anaerobic plates were analyzed at 48 h, 5 days, and 7 days. When multiple plates from the same sample yielded identical organisms, the plate indicating the highest concentration was used for final quantitation.

RESULTS

Eleven specimens from as many patients were included in the study. The sources of purulent exudate were intraabdominal abscesses (8), neck abscesses (2), and lung abscesses (1). Ten specimens yielded a mixed aerobic-anaerobic flora, whereas one yielded only anaerobes. In the entire series there were 73 bacterial strains, including 37 anaerobes and 36 aerobic or facultative strains (Table 1). The types of organisms encountered were similar to those reported in previous clinical studies of anaerobic infections, which employed optimal bacteriological techniques (4, 8–11). Principal anaerobic isolates were *Bacteroides fragilis, B. melaninogenicus,* fusobacteria, peptostreptococci, peptococci, and clostridia. Major aerobic and facultative isolates were *Escherichia coli, Proteus* sp., *Klebsiella* sp., and *Streptococcus faecalis.*

TABLE 1. *Bacteriological results*

Isolate	No. isolated
Anaerobes	
Gram-negative	
B. fragilis	8
B. melaninogenicus	5
B. oralis	1
B. corrodens	1
F. nucleatum	2
F. varium	1
Gram-positive	
Peptostreptococcus	6
Peptococcus	3
Actinomyces naeslundii	2
Eubacteria	2
Clostridium perfringens	2
C. ramosum	1
Clostridium sp.	1
Unidentified coccus	1
Unidentified nonsporulating bacillus	1
Aerobes and facultative	
Gram-negative	
E. coli	7
Proteus sp.	6
Klebsiella sp.	4
Citrobacter sp.	2
Enterobacter sp.	2
P. aeruginosa	1
Gram-positive	
Streptococcus faecalis	5
Streptococcus (other)	8
S. aureus	1

Qualitative analysis of results at the various intervals showed that 26 of the 37 anaerobic strains were recovered in the initial processing (Table 2). Each of the samplings at 10 min, 1 h, and 4 h yielded 31 anaerobic isolates. At 24 h there were 25 anaerobes. Thus, the recovery was incomplete at all sampling intervals.

Four of the 26 anaerobic strains isolated in the first processing were lost in subsequent cultures. Each of these four organisms were initially present in concentrations of 10^7/ml or greater. One was a *Fusobacterium nucleatum,* which was no longer present at 10 min or at any later sampling. A strain of *Peptostreptococcus* was recovered in the 10-min and 1-h culture but could not be cultivated after 4 h or 24 h of air exposure. Two additional strains of fusobacteria were detected in all samples up to 4 h but were not recovered in the 24-h culture. Thus, four anaerobic strains isolated initially were lost when processing was delayed up to 24 h.

Eleven anaerobic organisms were not detected in the initial processing but were recovered at other time intervals. These included peptostreptococci (3 strains), eubacteria (2 strains), clostridia (2 strains), *Actinomyces naeslundii* (1 strain), *B. melaninogenicus* (1 strain), an unidentified gram-positive coccus (1 strain), and an unidentified gram-positive bacillus (1 strain). With five culture attempts and 11 bacteria there were 55 possible recoveries. However, these organisms were detected on just 26 occasions, and in each instance isolation was erratic; i.e., negative cultures were interspersed with positives in random fashion. Mean counts of these 11 isolates when recovered were 7×10^6/ml.

The 22 remaining anaerobes were present initially, still cultivatable after the specimen was exposed to air for 24 h, and rather consistently present throughout the experiment. Of 110 possible recoveries (22 organisms × 5 samples) they were recovered a total of 106 times.

An analysis of the 36 aerobic and facultative strains showed that 35 were detected with the

TABLE 2. *Total number of strains recovered at each sampling interval*

Strain	No. of strains					
	0[a]	10 min	1 h	4 h	24 h	Total
Anaerobes	26	31	31	31	25	37
Aerobes	35	32	31	31	29	36

[a] Sampling interval.

first sampling; 32 at 10 min, 31 at 1 h, 31 at 4 h, and 29 at 24 h. Organisms inconsistently present included streptococci (7 strains), *Klebsiella* sp. (1 strain), and *Citrobacter freundi* (1 strain).

Quantitative analysis showed that the mean concentration of anaerobes recovered at the initial processing was 10^7/ml (Table 3). Mean counts of anaerobes after air exposure for periods of 10 min to 24 h ranged from 4×10^6 to 8×10^6. Mean concentrations of aerobic and facultative strains at the various samplings were similar, 5×10^6 to 8×10^6/ml. These results indicate no substantial quantitative changes in total bacterial populations of either anaerobes or aerobes with delayed processing of the specimen for periods up to 24 h. Analysis of individual strains showed that five anaerobes had a decrease of greater than 1 log/ml during sequential samplings. These included *B. melaninogenicus* (decrease of 8×10^4/ml at 24 h compared to 0 time), *Clostridium perfringens* (5×10^4/ml), *B. oralis* (5×10^2/ml), *B. fragilis* (5×10^2/ml), and *B. melaninogenicus* (5×10^1/ml).

TABLE 3. *Concentrations of bacteria with delayed processing*

Sampling time	Mean concn for strains recovered ($\log_{10} \pm 2$ standard errors)	
	Anaerobes	Aerobes
0 (initial sampling)	7.0 ± 0.7	6.9 ± 0.5
10 min	6.6 ± 0.6	6.7 ± 0.7
1 h	6.6 ± 0.6	6.8 ± 0.7
4 h	6.8 ± 0.6	6.9 ± 0.6
24 h	6.9 ± 0.7	6.9 ± 0.7

DISCUSSION

Isolation rates of anaerobic bacteria from clinical material are highly variable. Some laboratories recover these organisms in as many as 25 to 50% of all specimens appropriate for anaerobic culture (8, 9, 11). Others isolate anaerobes only on rare occasions. Such divergent results have been often ascribed, in part, to differences in specimen transport.

Since oxygen is by definition toxic for anaerobic bacteria, a number of devices have been developed to minimize air exposure of specimens. These include transport tubes containing oxygen-free gas, specialized transport media, and even portable anaerobic chambers (1, 2, 4, 6, 9). Although such precautions appear meritorious, there are no data available to document efficacy except with specimens transported on swabs. With swabs, however, bacterial adherence to fibers is an important variable independent of oxygen toxicity (3). In actual fact, the duration of air exposure that will permit recovery of clinically significant anaerobes has not been defined.

The results of this study indicate that there is minimal loss of anaerobic bacteria when large-volume purulent specimens are exposed to air for extended periods. Twenty-four of 26 anaerobic strains could be cultivated after specimens were left in loosely capped vials on the bench for 4 h. After 24 h, all but four of the initial isolates could still be recovered. Eleven other anaerobes were not cultured in the initial specimen but emerged sporadically in later samples. There were no important changes in total concentrations of anaerobes or in the relative concentrations of anaerobes to aerobes with sequential samplings. Thus, delayed processing had little impact on the yield of anaerobes by both qualitative and quantitative parameters.

One possible explanation for these observations is that purulent exudate is itself a good transport medium. In this regard it is important to note that all specimens contained at least 2 ml of grossly purulent exudate from untreated patients. It is conceivable that smaller volumes, less purulence, or concurrent antimicrobial therapy would have produced quite different results.

Another explanation for the prolonged survival of anaerobes is that the species recovered were relatively aerotolerant. Support for this hypothesis comes from previous studies by Loesche (7) and Tally et al. (10). Loesche classified "strict anaerobes" as those that showed no surface growth at a pO_2 greater than 0.5% and had complete inhibition of growth after 60 to 100 min of exposure to air. These included *Succinivibrio dextrinosolvens, Butyrivibrio fibrisolvens, Treponema macrodentium, Treponema denticola, Treponema oralis, Clostridium haemolyticum, Selenomonas ruminatium,* and *Lachnospira multiparus*. Such organisms are commonly present in normal flora but are seldom encountered in infected sites, even when utilizing optimal culture techniques. "Moderate anaerobes" were capable of surface growth in oxygen concentrations of 2 to 8% without appreciable loss of viability after air exposure for 80 min. Organisms in this category were common clinical isolates such as *B. fragilis, B. melaninogenicus, B. oralis, F. nucleatum,* and *Peptostreptococcus elsdenii*. These observations have been extended by Tally et al. in oxygen tolerance studies of anaerobic isolates from clinical sources (10). Subcultures of 57 strains were plated and exposed to air for various time periods before reincuba-

tion in anaerobic conditions. All 57 strains survived 8 h of air exposure, and 36 isolates continued to survive for 72 h. These studies document marked variations in oxygen tolerance between different anaerobic species. Those that normally colonize mucosal surfaces are often extremely oxygen sensitive (5), whereas anaerobes commonly recovered from infected sites are relatively aerotolerant. In both investigations oxygen tolerance was evaluated with pure cultures obtained by subculturing the original isolate. In contrast, we designed our study to test viability of anaerobes in purulent specimens in an effort to simulate conditions encountered in clinical practice. This distinction is important since oxygen tolerance of anaerobes may change with subculturing. Furthermore, it is conceivable that significant dynamic changes might occur within a purulent exudate containing a complex mixture of aerobes and anaerobes.

The major differences in recovering various organisms from sequential cultures appeared to be due to vagaries in sampling. Although precautions were taken by mixing the specimen prior to each sampling and by careful subculturing of all colony types, nevertheless, 11 of the 37 anaerobic isolates appeared sporadically in serial cultures. These inconsistencies could not be attributed to oxygen sensitivity, since none of the 11 strains were detected in the initial sampling. Furthermore, they could not be ascribed to low concentrations, since the mean counts of these organisms, when detected, were similar to those of other isolates. It is curious that 10 of these 11 anaerobic strains were gram-positive organisms. Similarly, seven of the nine facultative bacteria that appeared inconsistently in periodic samplings were gram positive. Thus, although less than one-half of all isolates were gram positive, these organisms accounted for 17 of the 20 strains detected erratically. It would appear that the irregular distribution of gram-positive bacteria in purulent specimens was a greater cause of discrepent culture results than was delayed processing.

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