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Oxygen Tolerance of Fresh Clinical Anaerobic Bacteria

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The oxygen tolerance and sensitivity of 57 freshly isolated anaerobic bacteria from clinical specimens was studied. All the organisms tolerated 8 h or more of exposure to oxygen in room air. Growth of the isolates in increasing oxygen concentrations demonstrated that the 57 isolates varied in oxygen sensitivity from strict to aerotolerant anaerobes. Comparison of the oxygen tolerance and sensitivity showed that the most tolerant organisms (best survival after prolonged exposure) included anaerobes capable of growth at only 0.4% or less O_2 (strict) as well as those able to grow in as much as 10% O_2. The least tolerant were predominately strict anaerobes. Decrease in the inoculum size from a concentration of 10^8 to 10^6 colony-forming units per ml had only a minor effect. The data indicate that the brief oxygen exposure with bench techniques in clinical laboratories would not be deleterious to the anaerobic bacteria present in clinical specimens.

Recent advances in microbiology have rekindled an interest in anaerobic bacteria and an increased awareness of their importance in human infections (4, 5). The deleterious effects of oxygen exposure on these organisms has been discussed in numerous articles but there is a dearth of experimental data. In 1969, Loesche studied the effect of varying concentrations of oxygen and varying periods of exposure to atmospheric oxygen on a miscellany of anaerobes which he then classified as strict, moderate, and aerotolerant (8). However, the strains tested in this study were derived from stock cultures. Ueno, in a limited study, showed a significant difference in the oxygen tolerance of fresh cultures compared to stock cultures of *Bacteroides fragilis* (18). The oxygen tolerance of fresh isolates is an important factor in the selection of the most advantageous method for the isolation, identification, and antimicrobial susceptibility testing of anaerobes in the clinical laboratory. The object of this study is to define the oxygen tolerance and sensitivity of various anaerobic bacteria recently isolated from clinical specimens.

MATERIALS AND METHODS

Clinical specimens were collected by a member of the Infectious Disease Section in a manner which avoided contamination by normal flora of the skin or mucous membranes. The material was immediately placed in an anaerobic transport tube (2) and processed within 1 h in an anaerobic chamber (1). All the isolation procedures were carried out in the chamber in an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide.

After isolation and confirmation of anaerobiosis, the organisms were grown for 18 h in prereduced brain heart infusion broth with peptic digest of sheep blood 5% and vitamin K_1 (0.1 μg/ml). The inoculum was adjusted to the turbidity equivalent to one-half of a no. 1 McFarland standard (approximately 10^8 colony-forming units [CFU]/ml). Twenty blood agar plates (Brucella agar with 5% defibrinated sheep blood and vitamin K_1 [10 μg/ml]) were reduced for 48 h in the chamber and inoculated with a Steers replicator, with approximately 10^5 CFU per spot applied to the agar surface (15). The inoculum of eight of the isolates was further diluted 10^{-2} for a comparison of the effect of different inoculum size and replicated in parallel with the standard inoculum (final inoculum, 10^3 CFU per spot).

The anaerobic control plate was sealed in an anaerobic jar with a palladium catalyst and methyl blue indicator while still in the chamber. Nineteen plates were removed from the chamber; one was used as an aerobic control. Eleven plates were exposed to room air in individual unsealed vented anaerobic jars to determine oxygen tolerance. The exposure periods were 10, 20, 30, and 45 min and 1, 2, 4, 8, 24, 48, and 72 h. At each time interval, the jars were sealed with an indicator and palladium catalysts, and anaerobiosis was established by five evacuations and exchanges, with a final atmosphere of 80% N_2, 10% H_2, and 10% CO_2. Seven plates were placed in separate vented jars without indicator or catalyst to determine oxygen sensitivity. The jars were evacuated and exchanged

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four times with N_2; a fifth evacuation to 25 inches (approximately 64 cm) Hg was followed by a fill consisting of a mixture of 80% N_2, 10% CO_2, and varying amounts of room air by the method of Loesche (8). The oxygen concentrations were calculated to obtain 0.4, 0.8, 1.6, 2.5, 5, 7.5, and 10%. No direct measurements of O_2 concentrations were taken. All jars were incubated at 35 C for 48 h. End points were determined when there were 10 discrete colonies or less.

The definition of oxygen tolerance was the time the isolates survived on exposure to room air. Oxygen sensitivity was defined as the greatest concentration of oxygen in which the organisms grew: strict anaerobes, $\leq$0.4%; moderate anaerobes, 0.8 to 2.5%; and aerotolerant, $\geq$5%.

RESULTS

Twenty-one clinical specimens were included in the study: transtracheal aspiration (10), intraabdominal abscesses (3), subcutaneous abscesses (6), and surgically excised tissue (3). Fifty-seven anaerobic organisms were isolated, including *B. fragilis* (9), *B. oralis* (4), *B. melaninogenicus* (7), *Bacteroides* sp. (3), various *Fusobacterium* species (6), gram-negative cocci (4), *Peptococcus* (8), *Peptostreptococcus* (6), various *Clostridium* species (5), and gram-positive non-spore-forming bacilli (4).

The results of the oxygen tolerance study revealed that all the 57 anaerobes survived 8 h or more of exposure to atmospheric oxygen (Table 1). Forty-four of the 57 isolates tolerated prolonged exposure (48 to 72 h), including 10 *B. fragilis*, 3 *Bacteroides* species, 4 gram-negative cocci, and the 5 clostridia. *B. oralis*, *B. melaninogenicus*, fusobacteria, and the gram-positive cocci showed oxygen tolerances varying from 8 to 72 h. The four nonsporing gram-positive bacilli tolerated 24 h of oxygen exposure. Growth of the isolates at the designated intervals on the test plates was similar to the control plates, with abrupt inhibition in isolates tolerating less than 72 h of exposure.

The oxygen sensitivity of the 57 anaerobic clinical isolates varied from strict to aerotolerant (Table 2). *B. oralis* and the nonsporing gram-positive bacilli were strict anaerobes (grew at 0.4% O_2 or less). Nine of the *B. fragilis* isolates were strict, and one was a moderate anaerobe. The isolates of *B. melaninogenicus*, *Bacteroides* sp., *Fusobacterium*, gram-negative cocci, and peptococci were divided into strict to moderate anaerobes. Peptostreptococci were classified as strict, moderate, and aerotolerant (tolerating >2.5% O_2). An isolate of *Peptostreptococcus anaerobius* grew in 10% O_2. The five clostridia were classified as moderate to aerotolerant, a *Clostridium sordellii* grew in 7.5%, and

TABLE 1. *Oxygen tolerance of anaerobes*

Organism	No. isolates tested	Time (h)[a]			
		8	24	48	72
Bacteroides fragilis	10			1	9
Bacteroides oralis	4	1[b]	1	1	1
Bacteroides melaninogenicus	7		2	2	3
Bacteroides sp	3			1	2
Fusobacterium sp.	6	1	1	1	3
Gram-negative cocci	4				4
Peptococcus sp.	8		1	1	6
Peptostreptococcus sp.	6	2		1	3
Clostridium sp.	5				5
Non-sporing gram-positive bacilli	4		4		

[a] Period of time isolates survived exposure to room air (21% O_2) before being incubated anaerobically.
[b] Number of strains surviving for each time period.

TABLE 2. *Oxygen sensitivity of anaerobes*

Organisms	No. isolates tested	Strict[a]		Moderate			Aerotolerant	
		<0.4[b]	0.4	0.8	1.6	2.5	7.5	10
Bacteroides fragilis	10	4[c]	5	1				
Bacteroides oralis	4	4						
Bacteroides melaninogenicus	7	4	1		1	1		
Bacteroides sp.	3		2	1				
Fusobacterium sp.	6	1	1	3		1		
Gram-negative cocci	4	2		1		1		
Peptococcus sp.	8	3	2	1	1	1		
Peptostreptococcus sp.	6	2	1	1	1			1
Clostridium sp.	5				1	2	1	1
Non-sporing gram-positive bacilli	4	2	2					

[a] Classification of oxygen sensitivity based on criteria of Loesche (8).
[b] The percentage of oxygen concentration in incubation atmosphere.
[c] Number of strains which grew at indicated oxygen concentration.

a *C. putrificum* grew in 10% O_2. Growth of organisms at higher concentrations of oxygen was usually somewhat less than on the control plates.

Comparing the results of the oxygen tolerance and sensitivity tests indicates that the most tolerant organisms ranged widely in sensitivity from strict to aerotolerant anaerobes (Fig. 1). The less tolerant isolates (8 and 24 h) were predominately strict anaerobes. One isolate which survived 24 h of room air exposure was a moderate anaerobe.

Use of smaller inoculum size did not markedly alter the results of the oxygen tolerance or sensitivity tests (Table 3). *F. necrophorum* decreased in tolerance from 24 to 8 h with an inoculum of 10^6 CFU/ml. This organism and an isolate of *Veillonella parvula* became more oxygen sensitive when the lower inoculum was used. Otherwise, test results were unchanged by decreases in the inoculum size.

TABLE 3. *Effect of inoculum size in oxygen tolerance and sensitivity*

Isolates	Tolerance[a]		Sensitivity[a]	
	10^{6b}	10^3	10^6	10^3
Bacteroides fragilis	72[c]	72	<0.4[d]	<0.4
Bacteroides sp.	48	48	0.8	0.8
Bacteroides sp.	72	72	0.4	0.4
Fusobacterium necrophorum	24	8	0.8	0.4
Veillonella alcalescens	72	72	2.5	2.5
Veillonella parvula	72	72	2.5	0.8
Peptococcus prevotii	72	72	<0.4	<0.4
Peptococcus variabilis	72	72	0.4	0.4

[a] See Materials and Methods for definitions.
[b] Inoculum size (CFU/ml).
[c] Duration of survival in room air (hours).
[d] Incubation atmosphere, percent oxygen concentration.

DISCUSSION

The results of this study indicate that fresh clinical isolates of anaerobic bacteria can tolerate oxygen exposure for at least 8 h and frequently for more prolonged periods. This data supports the concept that after primary isolation of anaerobes from clinical specimens, rapid method for identification and antimicrobial susceptibility testing can be safely performed on the bench in room air (15, 17). Previous studies have demonstrated the efficacy of the anaerobic jar in isolating anaerobes from clinical specimens (10, 13). Rosenblatt et al., in their direct comparison of bench technique to chamber and roll tube techniques, demonstrated that bench techniques are suitable for primary isolation (13).

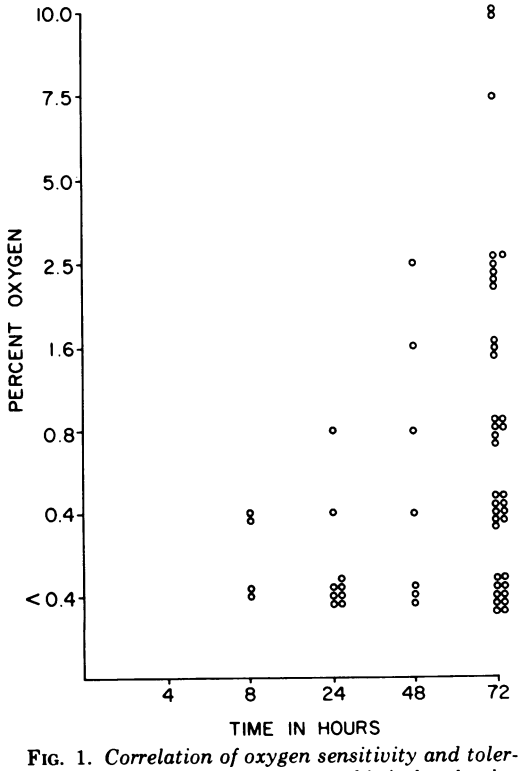

FIG. 1. *Correlation of oxygen sensitivity and tolerance. Each* (O) *represents an anaerobic isolate having that oxygen sensitivity shown on the oridinate and the corresponding tolerance on the abscissa.*

Previous normal flora studies utilizing chamber and roll tube techniques have significantly increased the number and total count of anaerobes isolated (1, 11). Extremely oxygen sensitive (EOS) organisms may account for a significant proportion of the total counts in certain normal flora studies (3). A conspicious absence of EOS bacteria was noted in our study of clinical isolates despite the use of chamber methods. Rosenblatt et al. in their comparative study, also failed to isolate any EOS organisms from clinical material (13). This phenomenon has recently been observed in an experimental intraabdominal abscess model in rats (12). The inoculum was pooled cecal contents known to contain EOS type organisms, but no EOS isolates were recovered from the experimental

abscesses. The dichotomy in the oxygen tolerance of isolates from infected sites versus that of normal flora may be due to the selective pressure of tissue oxygen concentrations, in addition to other cryptic factors.

Recent work by Hill and Osterhout has documented the protective effect of catalase (contained in blood agar plates) on the survival of clostridia and non-sporeforming gram-negative bacilli exposed to hyperbaric oxygen (6, 7). The catalase in our blood agar plates may have protected our isolates, but because of the differences in their experimental conditions, in which organisms were exposed to 100% O_2 at 3 atmospheres pressure at 37 C, no direct correlation can be established. Despite the catalase, EOS organisms on blood agar succumbed to brief exposures to O_2 in Loesche (8) and Attebery et al. (3) studies. Indeed, we may be looking at completely different phenomena of survival, growth and death, as suggested by Smith's definition of anaerobiosis (14).

The oxygen sensitivity test demonstrated that although the isolates survived prolonged oxygen exposure, 36 strains were strict anaerobes. Intraspecies variation in the sensitivity of the bacteria was evident, with isolates of the same species growing at varying oxygen concentrations. A plausible explanation of this phenomenon may be found in an enzyme theory of anaerobiosis proposed by McCord et al., based on the function of superoxide dismutase (9). This enzyme catalyses the conversion to toxic O_2^- radicals to H_2O_2. Dismutase was not detected in several anaerobes found primarily in ruminants, whereas human clinical isolates were not studied. If this theory is valid, it is possible that the clinical isolates in our study which tolerated oxygen exposure and grew in low O_2 concentrations contain reduced amounts of this enzyme. Further studies are planned to investigate this question.

This study was an attempt to test the oxygen tolerance and sensitivity of fresh clinical anaerobic isolates with a minimum number of subcultures. Further work is needed to determine the oxygen tolerance of anaerobic bacteria in the original clinical specimen and to investigate whether or not anaerobic organisms become more oxygen tolerant on repeated transfers. Hopefully, further studies of the molecular basis of anaerobiosis will help elucidate the specific mechanisms of oxygen's toxic effect on anaerobes.

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