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Recovery of Anaerobic Microorganisms from Clinical Specimens in Prereduced Media Versus Recovery by Routine Clinical Laboratory Methods[1]

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Prereduced anaerobically sterilized culture media, used with rigid adherence to the cultivation techniques described by Moore and his associates, were capable of recovering more than twice the number of anaerobic bacteria from clinical specimens than could be recovered by the conventional use of fluid thioglycolate medium and of blood-agar plates incubated anaerobically with hydrogen generation packets. No loss of clinical isolates was encountered with the more sensitive methods; however many of the isolates recovered only in prereduced media would not grow when placed into thioglycolate medium. A representative anaerobic isolate placed into aerobic transport broth was unable to survive beyond 30 min. Methods employing prereduced media were not difficult to master and were feasible for clinical laboratory use. Evidence implicating the gingival crevice flora as an important possible source of anaerobic bacteria that become involved in systemic infections was considered.

There is ample evidence in the literature that anaerobic bacteria form a distinctive part of the flora of many types of lesions (1, 6, 20, 21).

Severe and fatal infections attributed to bacteroides septicemia (12) and fusospirochetal infections of the lungs (3, 19) are among the anaerobic infections for which present hospital laboratory culture procedures may frequently be inadequate.

Whereas the pathogenicity of indigenous anaerobic bacteria is still in need of additional study, improved ability to isolate and distinguish anaerobes and to determine their sensitivity to antibiotics, when the need is indicated, could greatly assist treatment of disease processes in which they are involved.

Hungate (10) developed new techniques for recovery of anaerobic bacteria from rumen specimens based on preventing oxygen contamination of culture medium from the time of its initial preparation. The Hungate procedures were recently adapted to anaerobic media preparation, culture inoculation procedures (4, 14), and improvements in classification (4, 13, 20) that should be applicable to diagnostic microbiology laboratories.

[1] Taken in part from a thesis submitted by M. T. McMinn in partial fulfillment of the requirements for a master of science degree from the University of North Carolina.

Difficulty in utilizing these more sensitive techniques for cultivation of anaerobic bacteria resides mainly in the preparation of prereduced anaerobically sterilized media. The present commercial availability of these media should make widespread uses of these media feasible for hospital laboratories.

This present study was conducted to compare the feasibility and sensitivity of the newer prereduced medium techniques with conventional thioglycolate medium and the Brewer-jar techniques for the cultivation of anaerobic microorganisms from clinical specimens. An experiment was performed to determine the recovery of a common oxygen-sensitive anaerobe from a simulated clinical specimen handled without protection from air.

MATERIALS AND METHODS

Selection of specimens. Clinical specimens were obtained from patients of the University of North Carolina Dental School and the Clinical Microbiology Laboratory of the North Carolina Memorial Hospital at Chapel Hill, N. C.

Specimens included exudates from deep lesions, pleural fluids, fresh urines, joint fluids, exudates from pockets of inflammation in the throat and dental material from the gingival margin and crevices. Scrapings from skin and open lesions, bandage material and nasal and throat swabs were excluded, al-

though some of these types of specimens contain anaerobic organisms. Most specimens were submitted aerobically.

To determine the degree of resistance a strict anaerobic bacterial strain had to atmospheric oxygen, an experiment was conducted with sterile dry cotton swabs placed in Trypticase Soy Broth. Cotton swabs containing *Propionibacterium propionicum* isolated from fresh pleural fluid in prereduced media were placed in ordinary culture tubes containing 4.0 ml of the broth prepared 6 days prior to the experiment. Swabs were removed at various times and inoculated into prereduced media for incubation at 37 C.

Media. For comparison purposes, standard 5% sheep blood-agar plates, deoxycholate-agar plates, and thioglycolate broth without indicator but with 1% soluble starch were inoculated and incubated aerobically. Sheep blood-agar plates for anaerobic incubation were also inoculated.

Most of the methods for preparation and inoculation of pre-reduced anaerobically sterilized media (PRAS) have been described by Hungate (10), Moore and Cato (14) and Moore (13).

PRAS media used in this study were prepared commercially (Robbin Laboratories, Inc., Chapel Hill, N.C.) in tubes closed with solid black rubber stoppers by the method of Cato et al. (4). Some media were also prepared in this laboratory including PRAS milk, bile, gelatin, nitrate, peptone-yeast, glucose, and glycerol. The *p*H of the media did not change appreciably when the tubes were stoppered under oxygen-free carbon dioxide, although nitrogen is recommended (4).

Commercial PRAS fluid "E" medium was used for primary isolation in most instances, which we dispensed under oxygen-free CO_2 in 3-ml volumes in 5-ml serum vials with elongated narrow necks (5 mm, internal diameter). Since the recommended butyl rubber stoppers (4) were not available, the vials were closed with stoppers of red rubber. This reduced shelf life to 6 or 8 weeks before oxidation, so the vials were used almost immediately after their preparation, which would not be practical for routine use.

PRAS milk was prepared from commercial homogenized pasteurized whole milk by the method of Cato et al. (4). The *p*H was adjusted to 6.9 to 7.2. Powdered skim milk formed undesirable precipitates when autoclaved.

Culture methodology. All inoculation needles used in this study were of stainless steel. Cleaning wires supplied with 5.5-inch (14.0 cm) 18-gauge filling needles (B-D; Becton, Dickinson and Co., Rutherford, N.J.) were satisfactory. Other metal needles tended to oxidize the media.

Oxygen-free gas used for media preparation and for inoculations was obtained by passing commercial carbon dioxide through a glass tube (5 by 30 cm) filled with copper turnings maintained at 350 to 400 C. All connections were made with high quality gum rubber surgical tubing, completed with long 16-gauge stainless-steel filling needles preceded by a short length of glass tubing filled with sterile cotton. The needles were flamed and inserted into the tubes to deliver a steady stream of gas into the culture tubes while their stoppers were removed for inoculating or media-dispensing purposes.

A mixture of commercial N_2 or CO_2 (97%) and H_2 (3%) can be purified of oxygen by passing the gas through a catalytic gas purifier ("Deoxo" Hydrogen Purifier, Fisher Scientific Co., Pittsburgh, Pa.). The purifier will only reduce gas containing at least 3% hydrogen. The CO_2-H_2 mixture is preferred (4). PRAS agar slants and PRAS agar roll tubes were found to be satisfactory for streak isolation of mixed cultures. A device to facilitate inoculation of roll tubes was obtained from Robbin Laboratories, Inc., Chapel Hill, N.C.

Material from the deepest part of liquid specimens was drawn into a syringe and inoculated into 5-ml serum vials containing 2.4 to 3.0 ml of "E" media or, occasionally, tubes of PRAS chopped meat. Fresh material from cotton swabs in small volumes of liquid media was mixed well and drawn into a syringe flushed with "E" medium for inoculation into the vials. At all times, great care was maintained to prevent as much contamination with the air as possible.

All specimens were examined microscopically. Notation was made of material containing several different morphological types of organisms.

For comparison all specimens were also placed in fluid thioglycolate medium and on blood-agar plates and deoxycholate-agar plates in Brewer jars. Disposable hydrogen-carbon dioxide generators ("Gaspak," BBL) were used to produce anaerobic conditions in the Brewer jars similar to those in the clinical microbiology laboratory (2). Blood-agar plates and deoxycholate-agar plates were inoculated with the specimens and incubated aerobically to help screen out aerobic and facultative species.

Specimens were incubated at 37 C and checked each day for turbidity. Microscopic examinations were made at the time of noticing turbidity, or at the end of 72 hr and at the end of 10 days. Negative specimens were discarded at 3 weeks after microscopic observation. Specimens on agar plates were examined at 48 hr under a dissecting microscope.

Specimens exhibiting growth in the vials of "E" media were inoculated into chopped-meat media, and the morphology of the organisms present was noted.

Anaerobes that grew in the prereduced recovery medium were reinoculated into thioglycolate broth to determine their subsequent ability to grow in that medium.

Careful comparison was made between the microscopic and colonial morphology of the aerobic organisms that grew on the aerobically incubated agar plates and organisms that grew in the PRAS media so that facultative organisms growing in the PRAS media could be distinguished from true anaerobes. An additional check consisted of inoculating organisms from the PRAS media onto aerobically incubated agar plates. Roll tubes were examined with the aid of a dissecting microscope for careful differentiating and picking of colonies.

Identification was based on morphology and on the biochemical reactions described by Cato et al. (4), Hare (8), and Smith and Holdeman (20). These included final *p*H obtained from growth in peptone

yeast broth, glucose, fructose, esculin, lactose, maltose, sucrose, and glycerol broth, motility, nitrate reduction, gelatin liquefaction, catalase production, reactions in milk, and oxygen tolerance. Ambiguous results indicated the presence of more than one organism in every instance in this study.

More critical identification of the species by gas chromatographic analysis of metabolic products as specified by Cato et al. (4) was not utilized in this initial study.

RESULTS

Recovery of anaerobic bacteria. The recovery of 49 organisms from 38 specimens collected by routine methods and inoculated into PRAS media and thioglycolate broth and onto agar plates incubated in anaerobic jars is shown in Table 1.

TABLE 1. *Recovery rate of 49 anaerobic bacteria from 38 clinical specimens inoculated into various media*[a]

Inoculation	Media[b]			
	1	2	3	Both 2 and 3
Initial inoculation	100%	6.1%	8.2%	28.6%
Subsequent inoculation from original PRAS culture	100%	10.2%	24.5%	28.6%

[a] Columns 2 through 4 present recovery results obtained by the usual anaerobic culture techniques compared with total recovery of anaerobes in PRAS media in column 1.

[b] Media: 1, PRAS media; 2, thioglycolate medium; 3, agar plates in Brewer jar.

Time required for the anaerobic organisms to appear in the PRAS broth media ranged from 24 hr to 7 days. All cultures were maintained at 37 C for 3 weeks, but no recovery was observed after 7 days, although there were several instances of increased turbidity with time. There were no instances of organisms being recovered from any clinical specimen in thioglycollate broth or in the anaerobic jars that did not demonstrate better growth in the PRAS media. All recovered anaerobes that did not appear in culture tubes in 24 hr eventually developed a recognizable turbidity in the PRAS media before demonstrating any growth in the thioglycolate broth.

Inoculations of agar plates and thioglycolate broth were occasionally made from vials of PRAS "E" media that had been inoculated a few hours prior to subculture. These vials had been stored at refrigerator temperature, and there was seldom any suggestion of bacterial growth at the time of subculture.

All bacteria that had not originally grown in thioglycolate broth or on anaerobically incubated agar plates were subsequently inoculated onto these media from viable anaerobic cultures maintained in PRAS media. These cultures were usually taken from tubes of PRAS chopped-meat media or PRAS "E" media and rarely from PRAS peptone-yeast media. There was no significant difference in subsequent growth from the inocula of 0.1 ml on agar plates and 1.0 ml into thioglycolate broth from the three different PRAS media. The increase in organisms subsequently growing in thioglycolate broth and in the anaerobic jars after initial recovery and culture in PRAS media can also be seen in Table 1.

TABLE 2. *Organisms isolated and identified from 38 various clinical specimens*

Organisms	Pleural fluid	Abscess		Urine	Blood culture	Wound	Surgical drain	Knee fluid	Prostatic fluid
		Sputum	Ulcer						
Bacteroides melaninogenicus	4	1	1	1				1	
Propionibacterium propionicum	2	1	1						
Peptostreptococcus anaerobius	1	3	2	1					
P. intermedius			1	2			1		1
Fusobacterium fusiforme	1	2		1			1		
Bacteroides oralis	1					1			
B. fragilis				2	1	1			
Veillonella alcalescens	2	1	2						
Peptococcus sp. (unable to identify)								1	
P. asaccharolyticus	1								
P. magnus			2	1					
Propionibacterium acnes						1			
Eubacterium ventriosum						1			
Catenabacterium filamentosum		1							
Propionibacterium anaerobium			1						

TABLE 3. *Analysis of clinical specimens with anaerobic bacteria in mixed populations*

Specimen	Anaerobic organisms recovered	Aerobic organisms recovered
Vaginal abscess	*Propionibacterium propionicum*	Alpha-hemolytic streptococcus
Decubital ulcer	*Bacteroides melaninogenicus*	*Alcaligenes-Klebsiella* group
	Peptococcus magnus	
Knee fluid	*Peptococcus* sp.	None
	Bacteroides melaninogenicus	
Inguinal drain	*Peptostreptococcus intermedius*	*Escherichia coli*
		Proteus sp.
Pleural fluid	*Bacteroides melaninogenicus*	None
	Fusobacterium fusiforme	
Urine	*Peptococcus magnus*	*Escherichia coli*
Neck lesion exudate	*Peptostreptococcus anaerobius*	None
	Bacteroides oralis	
Urine	*B. fragilis*	*Alcaligenes-Klebsiella* group
	Peptostreptococcus intermedius	
Neck lesion exudate	*Bacteroides fragilis*	None
	Veillonella alcalescens	
Sputum	*Treponema* sp.[a]	Numerous alpha-hemolytic streptococci
	Trichomonas sp.[a]	*Neisseria* sp.
	Leptospira sp.[a]	
	Borrelia sp.[a]	
	Bacteroides melaninogenicus	
	Peptostreptococcus anaerobius	
	Fusobacterium fusiforme	
	Propionibacterium propionicum	
Tracheal secretion	*Veillonella alcalescens*	None
	Peptostreptococcus anaerobius	
Ear	*Veillonella alcalescens*	Nonhemolytic staphylococcus
Skin lesion	*Peptostreptococcus intermedius*	Hemolytic staphylococci
		Alpha-hemolytic streptococcus
Pleural fluid	*Bacteroides melaninogenicus*	Alpha-hemolytic streptococcus
Sputum	*Fusobacterium fusiforme*	*Alcaligenes-Klebsiella* group
	Peptostreptococcus anaerobius	
	Catenabacterium filamentosum	

[a] Identified by phase microscopy study of morphology of both fresh specimen and 24-hr culture in chopped-meat PRAS broth.

Organisms from various specimens. The 15 different species of anaerobic bacteria isolated in these experiments are shown in Table 2, together with the type of clinical specimens from which they were obtained. Approximately as many more routine specimens collected by aerobic methods were inoculated into PRAS media in the laboratory and were negative for anaerobes. Many of these were positive for aerobic bacteria.

Anaerobes in mixed culture. Tabulation of isolates from specimens containing anaerobes in mixed populations is presented in Table 3. Mixed cultures were observed in morphology and culture studies. When there appeared to be more than one organism present in these studies or when biochemical reactions were ambiguous, careful observations of PRAS-agar slant cultures were made with a stereoscopic microscope. Colonies with good separation and different colonial morphology were studied microscopically, and subsequent cultures were made in PRAS media and on aerobically incubated agar plates. Extensive detailed identification was not attempted for the aerobic bacteria observed.

In one instance spirochetal forms were observed in a sputum sample. No attempt was made to classify these organisms beyond morphological observations. The spirochetal forms remained viable and motile in the PRAS chopped-meat broth for 48 hr and in PRAS "E" media for more than 24 hr but less than 48 hr. No recovery of the spirochetal forms occurred in the thioglycolate broth. A motile *Trichomonas* sp. was observed in this same specimen and remained active for 48 hr. Subcultures of these cultures were not made.

A summary of the specimens yielding mixed anaerobic and aerobic cultures is presented in Table 4.

TABLE 4. *Occurrence of 49 anaerobic bacteria in pure and mixed cultures*

Bacteria	Occurrence
Anaerobe in pure culture.....	45.0
Mixed, two anaerobes.....	20.4
Mixed, an aerobe with an anaerobe...	12.2
Mixed, aerobes and anaerobes.....	22.4

TABLE 5. *Recovery of Propionibacterium propionicum from aerobic media*

Trypticase Soy Broth tube	Time in min before inoculation of PRAS media	Growth at 24 hr[a]	Growth at 48 hr[a]
1	0	4+	4+
2	5	4+	4+
3	10	4+	4+
4	15	4+	4+
5	20	3+	4+
6	25	2+	4+
7	30	1+	2+
8	35	?	NG
9	45	?	NG
10	50	NG[b]	NG
11	60	NG	NG

[a] Key: 1+, bacteria detected only microscopically; 2+, barely visible turbidity; 3+, marked turbidity; 4+, dense turbidity with sediment.

[b] No growth.

Inhibitory effects of aerobic media. Many specimens with requests for anaerobic studies were received at the laboratory on cotton swabs in regular culture tubes containing small volumes of well-aerated Trypticase Soy Broth. Only one anaerobic species was ever recovered from a swab specimen collected in this manner.

An experiment was designed to determine what effect such aerobic transport and storage would have on the ability to recover an anaerobic bacterium. A sheep blood-agar plate was covered with 0.5 ml of a viable culture of *Propionibacterium propionicum* originally isolated from a deep tube of pleural fluid and maintained in PRAS chopped-meat media. Cotton swabs were used to pick up organisms from different areas of the agar surface and placed in 3 ml of Trypticase Soy Broth that had been stored under cotton plugs for 1 week. Results of growth obtained from the inoculated swabs removed at different times and used to inoculate PRAS "E" media are shown in Table 5. No recovery of this anaerobe was obtained after it had been in plain aerobic broth for 30 min.

Cultures of oral specimens. Specimens obtained from the oral cavity of four individuals were cultured in PRAS media. Two of the specimens were from diseased gingiva and the other two were from healthy oral surfaces. Isolating pure cultures of individual species from the original extremely mixed population posed remarkable difficulties. Several attempts at colony separation on PRAS agar slants with the aid of a dissecting microscope were required before a good separation could be achieved. The extent to which selective media and serial dilutions may lessen these difficulties was not determined.

Table 6 shows the anaerobes isolated from these oral specimens.

A distinctively heavier population of similar organisms in the cultures from diseased gingival tissues was observed.

TABLE 6. *Anaerobes isolated and identified from oral specimens*[a]

Patient	Anaerobes observed
17-year-old female with clinically diagnosed acute periodontal disease	*Fusobacterium fusiforme* *Veillonella parvula* *Peptococcus magnus* *Peptococcus prevotii* *Propionibacterium propionicum* *Trichomonas* sp.[b] *Treponema* sp.[b] *Borrelia* sp.[b]
47-year-old male with clinically diagnosed periodontal disease	*Bacteroides melaninogenicus* *Fusobacterium fusiforme* *Veillonella alcalescens* *Peptostreptococcus intermedius* *Propionibacterium propionicum* *Treponema* sp.[b] *Borrelia* sp.[b]
10-year-old male with excellent oral health	*Fusobacterium fusiforme* *Bacteroides melaninogenicus* *Veillonella alcalescens* *Peptostreptococcus intermedius* *Borrelia* sp.[b]
33-year-old male with good oral health	*Bacteroides melaninogenicus* *Bacteroides fragilis* *Propionibacterium anaerobium* *Treponema* sp.[b] *Borrelia* sp.[b]

[a] All specimens contained numerous aerobic bacteria which were not identified.

[b] Tentative identification on the basis of morphological studies alone.

The spirochetal forms were obviously viable and extremely active 48 hr after inoculation into PRAS media. No differences were noted in morphological studies of fresh specimens from the periodontal patients and in the 48-hr PRAS cultures. There was no recovery of viable spirochetal or trichomonal forms when either fresh specimens of diseased tissue or viable PRAS cultures were inoculated into thioglycolate broth.

DISCUSSION

Prereduced media and the anaerobic culture procedures involved in their utilization were found to be feasible for use in the clinical laboratory. These procedures were at least twice as effective for detecting anaerobic species in the clinical specimens studied as fluid thioglycolate medium or aerobically stored agar plates incubated in a Brewer jar made anaerobic by use of a commercial hydrogen-generating packet. Increased recovery outweighed any inconvenience in passing a stream of oxygen-free gas into each tube while inoculating or transferring cultures in the prereduced media. Technical mastery of the procedures was not difficult to achieve.

Inability of an anaerobic isolate, *P. propionicum*, to survive beyond 30 min in a tube of aerobic transport broth supported the assertion that more anaerobic strains would be recovered from clinical specimens if they were placed directly into prereduced media at the time of their collection, as described by Cato et al. (4).

Although the data presented were obtained mainly from routine specimens collected and transported to the laboratory by aerobic means, use of prereduced media under those limiting circumstances still markedly increased the yield of anaerobic strains over that obtained by the other commonly used methods.

Each specimen that yielded growth of anaerobic species produced noticeably more growth in a shorter period of time in prereduced media than in the other media. Microscopic examination of thioglycolate cultures that appeared negative often revealed the presence of anaerobic bacteria. All gave rise to rapid heavy growth when transferred to prereduced media. No difficulty was encountered in retaining viability of the anaerobic strains for 1 to 2 weeks in "E" medium at incubator or room temperature, whereas in thioglycolate medium few turbid cultures survived for up to a week.

The superiority of PRAS media is attributable to the reduced state of the medium components and an oxidation reduction potential of -100 mv (15). In contrast to the common method of using the Brewer jar with a chemical hydrogen-generating packet, more effective procedures can be employed (4). These include use of agar plates stored anaerobically and repeated flushing of the tank with oxygen-free gas. The equipment needed is relatively immobile, and the specimen inoculum must still be exposed to some oxygen. These disadvantages can be avoided by the direct inoculation of prereduced rubber-stoppered tubes at the patient's bedside with only a syringe and needle. A small lecture tank of 3% H_2 in CO_2 with a deoxo catalyst can provide a mobile source of gas for use in placing swabs or solid material directly into prereduced tubes. In the laboratory, direct streaking of specimens on solid media in prereduced roll tubes should improve recovery of anaerobes further by avoiding overgrowth of anaerobes by facultative organisms. More detail about these procedures is available in the manual published by the Virginia Polytechnic Institute Anaerobe Laboratory (4).

Incidence of detectable anaerobic species in clinical specimens of blood and exudates cannot be estimated from the present study, which was concerned only with comparing methods of cultivation. However, the data support the suggestions of other workers that anaerobic bacteria are much more commonly involved in infectious processes than past experience would indicate (15, 21). Studies of patients' specific immune response to anaerobic isolates should reveal more about their pathogenic involvement.

Recent evidence indicates that anaerobic isolates have varied profiles of antibiotic sensitivities and some are resistant to broad-spectrum antibiotics (15). Improved ability to cultivate such anaerobes that become involved in persistent infections, and to determine their sensitivity to specific drugs should help to establish a scientific basis for treatment of such infections. In acute anaerobic infections such as bacteriodes septicemia (12) early diagnosis and treatment would appear essential and require use of sensitive and reliable culture techniques.

The origin of anaerobic organisms found in infections is also of clinical concern, and pertains to the data presented. It was recently shown that many of the anaerobic organisms isolated from infected tissues are common to the normal gastrointestinal tract and may derive from lesions of the gastrointestinal mucosa (15). The present investigation and literature on the oral flora (3, 18) reveal that the vast majority of anaerobic species isolated in prereduced media from systemic lesions are also common to comparatively healthy and diseased mouths. A number of studies have indicated that bacteremias commonly result from minor, even daily, manipulations of the oral gingival tissues (5, 7, 9, 11, 16, 17).

Chronic disease of the gingival tissues associated with large numbers of anaerobic bacteria is considered ubiquitous among adults (3), and should not be overlooked as a possible source of anaerobic organisms in systemic infections (9, 19).

The results presented indicate the significantly greater sensitivity of prereduced media for cultivation of anaerobic microorganisms from clinical specimens. They support the findings of other workers that use of prereduced media can provide a useful clinical and research tool to aid diagnosis of infections, to investigate the involvement of anaerobic organisms in many disease processes, and to facilitate improved approaches to their treatment.

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