JOURNAL OF CLINICAL MICROBIOLOGY, June 1977, p. 564-569
Vol. 5, No. 6

Printed in U.S.A.

Recovery of Anaerobic, Facultative, and Aerobic Bacteria from Clinical Specimens in Three Anaerobic Transport Systems

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Received for publication 6 January 1977

With aspirated specimens from clinical infections, we evaluated the recovery of anaerobic, aerobic, and facultative bacteria in three widely used transport systems: (i) aspirated fluid in a gassed-out tube (FGT), (ii) swab in modified Cary and Blair transport medium (SCB), and (iii) swab in a gassed-out tube (SGT). Transport tubes were held at 25°C and semiquantitatively sampled at 0, 2, 24, and 48 h. Twenty-five clinical specimens yielded 75 anaerobic strains and 43 isolates of facultative and 3 of aerobic bacteria. Only one anaerobic isolate was not recovered in the first 24 h, and then, only in the SGT. At 48 h, 73 anaerobic strains (97%) were recovered in the FGT, 69 (92%) in the SCB, and 64 (85%) in the SGT. Two problems hindered the recovery of anaerobes in the SCB and SGT systems: first, die-off of organisms, as evidenced by a decrease in colony-forming units of 20 strains (27%) in the SCB and 25 strains (33%) in the SGT, as compared with 7 strains (9%) in the FGT, over 48 h; and second, overgrowth of facultative bacteria, more frequent with SCB and SGT. The FGT method was clearly superior at 48 h to the SCB and SGT systems in this study and is recommended as the preferred method for transporting specimens for anaerobic culture.

The frequent occurrence and serious nature of anaerobic infection have been amply documented in recent years, in great measure due to improved laboratory techniques for the isolation and identification of anaerobic bacteria (7, 9, 10, 12, 14-17). There has been considerable concern about how to maintain the viability of anaerobes within clinical specimens during transit to the laboratory. The use of gassed-out tubes (1, 5, 7-9, 11, 12, 17, 18), swabs in Cary and Blair transport medium (8, 9, 18), and swabs in gassed-out tubes (12) have been recommended and are widely used in many clinical laboratories. Several of these and other transport systems have been studied using stock cultures under conditions simulating clinical use (1, 2, 3, 5, 6, 20, 21); however, little information is available regarding their performance with clinical specimens. This study was undertaken to evaluate the ability of three common transport systems to maintain the viability of anaerobic bacteria in specimens from a wide variety of clinical infections, over a 48-h period. In addition, the influence of each system on associated aerobic and facultative bacteria in the specimens was studied.

(This paper was presented in part at the annual meeting of the American Society for Microbiology held in Atlantic City, 5 May 1976.)

MATERIALS AND METHODS

Specimen collection. Clinical specimens selected for study were limited to properly collected materials from deep infections with a high likelihood of yielding pathogenic bacteria. In each case, fluid was aspirated with a needle and syringe from the infected site by one of us. Stringent precautions were taken to minimize contamination from contiguous areas harboring normal flora. In nearly every instance, the specimen was hand carried directly to the laboratory, and the maximum time interval between collection and laboratory processing was 30 min.

Transport systems. Three transport systems were comparatively studied: (i) fluid in a gassed-out tube (FGT), (ii) swab in Cary and Blair medium (SCB), and (iii) swab in a gassed-out tube (SGT). The FGT system consisted of a screw-capped tube (20 by 125 mm) containing 1 ml of a 4% salts solution with resazurin. Tubes were placed in an anaerobic glove box with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. When the resazurin turned colorless, tubes were stoppered with recessed butyl rubber stoppers, fitted with screw caps, autoclaved, and thereafter held at room temperature until used.

Cary and Blair medium was prepared prereduced

with resazurin, and 15-ml amounts were dispensed in tubes (18 by 142 mm) fitted with butyl rubber stoppers. Tubes were stored at room temperature until used.

Cotton-tipped swabs in gassed-out tubes were prepared according to directions provided in the VPI *Anaerobe Laboratory Manual* (12). Two rubber-stoppered tubes containing 2 or 3 drops of 4% salts solution with resazurin were gassed with oxygen-free CO_2. A swab was attached to the rubber stopper in one tube. After gassing, the tubes were tightly sealed with the rubber stoppers, autoclaved, and stored at room temperature until used. The maximum time of storage was 1 month.

The protocol for inoculating the three transport systems is shown in Fig. 1. The clinical specimen was first injected from the collection syringe into a gassed-out tube. From the FGT, 1.5 ml was withdrawn and transferred into a sterile cotton-stoppered tube (13 by 100 mm). Four swabs were gently swirled in the fluid and immediately placed in four tubes of Cary and Blair medium. To inoculate SGT, a two-tube system was used. The rubber stopper with a swab attached was removed from the first tube, gently swirled in the 1.5 ml of fluid, and quickly inserted into the second tube. Care was taken to keep the second tube upright to prevent the CO_2 from spilling out. This procedure was also done in quadruplicate for serial sampling.

Sampling from the three transport systems. Portions were taken from each transport system at 0, 2, 24, and 48 h for semiquantitative culturing. For FGT, 0.5 ml of the fluid was transferred into a sterile tube. One drop of the specimen was used to make a direct smear, and a calibrated loopful (0.01 ml) was plated onto each of two anaerobic media: (i) prereduced brain heart infusion agar with 7.5% defibrinated sheep blood, supplemented with hemin and menadione, and (ii) prereduced laked sheep blood agar containing kanamycin and vancomycin. Two aerobic plates, which included veal infusion agar with 7.5% sheep blood and litmus lactose agar were inoculated in the same manner.

A modified, but equivalent, procedure was employed for the two transport systems containing swabs. The swab was transferred into a tube containing 1 ml of prereduced peptone-yeast-glucose broth and shaken on a Vortex mixer for 20 s. One drop of broth was used to make a direct smear, and 2 drops of broth were used to inoculate each of the two primary anaerobic and two aerobic media. Two drops approximated the 0.01 ml of original specimen delivered by the calibrated loop. Inoculation of the transport systems, sampling, and streaking of plates were done in room atmosphere.

Aerobic plates were incubated in 10% CO_2 at 35°C for 48 h. Each colony type was quantitated on the basis of $<10^3$, 10^3 to 10^4, or $>10^4$ colony-forming units (CFU) per ml of specimen. Aerobic and facultative bacteria were identified by standard methods. Anaerobic plates were incubated in GasPak jars at 35°C for 5 days and then examined for anaerobic growth. A representative colony of each colony type present was selected for subculture to an aerobic sheep blood agar plate and prereduced chopped meat glucose broth. Anaerobic bacteria recovered were enumerated as described for aerobic and facultative bacteria and identified by using the methods of the Virginia Polytechnic Institute (12).

RESULTS

Twenty-five clinical specimens yielded a total of 75 anaerobic and 46 aerobic or facultative

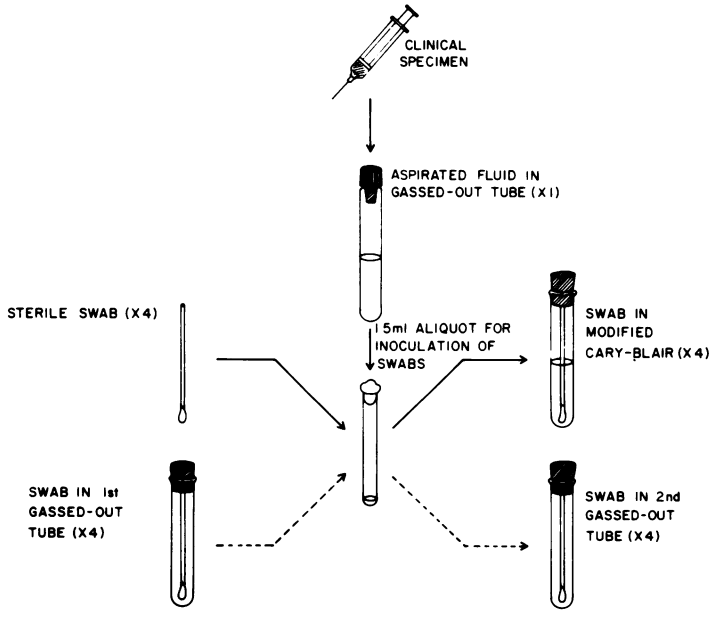

FIG. 1. *Procedure for inoculating clinical specimens into the three anaerobic transport systems.*

isolates (Table 1). About two-thirds of the specimens and bacteria isolated were from suppurative pleuropulmonary or intra-abdominal infections. Nineteen specimens contained a mixed population of anaerobic and facultative or aerobic bacteria. Five specimens produced no anaerobes, and one specimen no aerobic or facultative bacteria. The 20 specimens yielding anaerobic bacteria contained from one to seven species (mean, four) per specimen. Of the 24 specimens that produced aerobic or facultative bacteria, the number ranged from one to four strains, averaging two per specimen.

Table 2 lists the species and number of strains of anaerobic bacteria recovered from the 25 specimens. Thirty-nine strains of gram-negative bacilli, 23 gram-positive cocci, and 13 gram-positive bacilli were isolated. The infrequent recovery of *Propionibacterium acnes* (one strain) probably reflects the careful method of specimen collection, which avoided contamination from normal flora.

Facultative and aerobic bacteria isolated are shown in Table 3. Forty-six strains were recovered, including 43 facultative and 3 aerobic strains. The latter comprised three strains of *Pseudomonas aeruginosa*. Fifty percent of the strains were *Enterobacteriaceae*; 11 of the 23 were *Escherichia coli*. Fifteen strains of streptococci were recovered; eight belonged to the enterococcus group.

Table 4 summarizes the recovery of the 75 anaerobic strains from three transport systems over the 48-h period of study. All 75 strains were recovered at zero time and at 2 h in all three systems. At 24 h, both the FGT and SCB gave 100% recovery, but one strain of *Peptostreptococcus anaerobius* was not recovered in the SGT. After 48 h, 97% of the anaerobes initially present were recovered from the FGT, 92% from the SCB, and 85% from the SGT. One strain of *Bacteroides* species and one *Lactobacillus* species were not recovered from any of the three systems at 48 h. An additional 4 anaerobic strains were not recovered from the SCB at 48 h, and 10 strains could not be recovered from the SGT at 48 h. All original isolates of *Bacteroides fragilis, Bacteroides melaninogenicus, Bacteroides ruminicola* subsp. *brevis, Clostridium* species, *Peptococcus* species, and 10 of the 11 *Peptostreptococcus* species were recovered from all three transport systems at 48 h.

The number of anaerobic strains that showed a 10-fold or greater decrease in CFU in the three systems over 48 h is shown in Table 5. This includes organisms recovered in decreased numbers, as well as strains that no longer could be recovered. A total of 7 strains (9%) of anaerobes decreased in number in the FGT; 20

TABLE 1. *Sources of clinical specimens studied*

Site of infection	No. of infections	No. of anaerobic isolates	No. of aerobic and facultative isolates
Empyema	6	21	11
Intra-abdominal abscess	4	14	9
Pelvic abscess	2	11	2
Subphrenic abscess	2	6	6
Surgical wound	2	6	2
Septic compound fracture	2	3	4
Lung abscess	1	5	3
Para-esophageal abscess	1	5	1
Perineal phlegmon	1	3	2
Peritonitis	1	1	3
Perineal fistula	1	0	1
Perirectal abscess	1	0	1
Perinephric abscess	1	0	1

TABLE 2. *Anaerobic bacteria isolated from 25 clinical specimens*

Organism	No. of strains
Bacteroides melaninogenicus	10
B. fragilis	9
B. ruminicola subsp. *brevis*	7
Other *Bacteroides* species	6
Fusobacterium naviforme	3
F. nucleatum	2
F. varium	1
F. gonidiaformans	1
Peptostreptococcus anaerobius	4
Other *Peptostreptococcus* species	7
Peptococcus species	12
Eubacterium lentum	3
Eubacterium species	3
Clostridium bifermentans	2
C. perfringens	1
C. oroticum	1
Propionibacterium acnes	1
Lactobacillus species	2

TABLE 3. *Aerobic and facultative bacteria isolated from 25 clinical specimens*

Organism	No. of strains
Pseudomonas aeruginosa	3
Eikenella corrodens	3
Escherichia coli	11
Klebsiella pneumoniae	3
Proteus morganii	4
P. mirabilis	4
Citrobacter diversus	1
Enterococcus	8
Alpha streptococci	5
Beta streptococcus group F	2
Staphylococcus aureus	1
Corynebacterium species	1

TABLE 4. *Recovery rate of anaerobic bacteria from clinical specimens in three transport systems over 48 h*

Time (h)	Transport system					
	FGT		SCB		SGT	
	No. of strains recovered (%)	Strains not recovered (no.)	No. of strains recovered (%)	Strains not recovered (no.)	No. of strains recovered (%)	Strains not recovered (no.)
0	75 (100)		75 (100)		75 (100)	
2	75 (100)		75 (100)		75 (100)	
24	75 (100)		75 (100)		74 (99)	*Peptostreptococcus anaerobius* (1)
48	73 (97)	*Bacteroides* species (1) *Lactobacillus* species (1)	69 (92)	*Bacteroides* species (2) *Fusobacterium varium* (1) *Eubacterium lentum* (1) *Lactobacillus* species (1) *Peptostreptococcus* species (1)	64 (85)	*Bacteroides* species (1) *Fusobacterium* species (5) *Eubacterium* species (2) *Lactobacillus* species (1) *Peptostreptococcus* species (1)

strains (27%) decreased in number in SCB, and 25 strains (33%) decreased in number in SGT. Few strains demonstrated a decrease in number of CFU over the first 24 h of study (FGT, 0%; SCB and SGT, 7% each). Strains of *Bacteroides melaninogenicus, Fusobacterium, Eubacterium, Peptococcus*, and *Peptostreptococcus* most frequently lost viability.

A major factor influencing the recovery of anaerobes was overgrowth of facultative bacteria on anaerobic culture plates. Overgrowth greatly hampered or prevented isolation of anaerobes. The problem of overgrowth occurred at 48 h in one specimen in the FGT, in four specimens in the SCB, and in five specimens in the SGT.

Recovery of aerobic and facultative bacteria in the three transport systems is shown in Table 6. Of the 46 total isolates recovered, none of the three systems yielded the entire number at any one time of culture. *Proteus mirabilis* from one specimen was isolated at zero time from the SGT, but less than 10^3 CFU were present. This strain apparently grew in all three transport systems and was eventually recovered from each. An enterococcus in specimen no. 9 was isolated from all three systems at 48 h but not before that time. *E. coli* from specimen no. 16 was recovered at zero time only in the FGT, and the quantitation showed less than 10^3 CFU/ml; at 2 h, it was also isolated from the SCB and SGT transport systems, with a count of $<10^3$ CFU/ml. By 48 h, this *E. coli* had reached a concentration of $>10^4$ CFU/ml in the SCB. One strain of *Eikenella corrodens* and two strains of group F streptococcus were lost at 48 h in all three systems.

Ten facultative strains demonstrated an increase in CFU of 1 log or greater at room temperature over 48 h; five showed the increase within 24 h, and 5 only demonstrated such an increase at 48 h. Included were three strains of *E. coli*, one of *Klebsiella pneumoniae*, one of *Proteus mirabilis*, one of *Citrobacter diversus*, and four of enterococcus. Four strains had a 1 log or greater increase in the FGT, nine in SCB, and nine in SGT.

TABLE 5. *Number of strains of anaerobic bacteria demonstrating a decrease in CFU of 1 log or greater in each transport system*

Time (h)	No. of strains (%) in transport system:		
	FGT	SCB	SGT
2	1 (1)	1 (1)	2 (3)
24	0 (0)	5 (7)	5 (7)
48	6 (8)	14 (19)	18 (24)

DISCUSSION

The isolation of anaerobic bacteria involved in clinical infections is dependent on proper methods of collection, transport, and culture of specimens (2, 6–8, 10, 11–15, 18). Anaerobes vary widely in their tolerance of atmospheric oxygen (10, 13, 19), but it is generally considered important to maintain maximal anaerobiosis during transport of the specimen to the laboratory. Most of the early transport media devised for clinical specimens were not directed toward maintaining the viability of anaerobic pathogens. A number of systems have been advocated for transport of specimens that might contain anaerobes; these include an evacuated syringe (8, 9, 11, 18), a miniature jar (1, 8) and a complex tube (20) kept anaerobic by

TABLE 6. *Recovery rate of aerobic and facultative bacteria from clinical specimens in three anaerobic transport systems*[a]

Time (h)	Transport system					
	FGT		SCB		SGT	
	No. of strains recovered (%)	Strains not recovered (no.)	No. of strains recovered (%)	Strains not recovered (no.)	No. of strains recovered (%)	Strains not recovered (no.)
0	44 (96)	*Proteus mirabilis* (1) Enterococcus (1)	42 (91)	*Proteus mirabilis* (1) Enterococcus (1) *E. coli* (2)		Enterococcus (1) *E. coli* (1)
2	44 (96)	*P. mirabilis* (1) Enterococcus	43 (93)	*P. mirabilis* (1) Enterococcus (1) *E. coli* (1)		*P. mirabilis* (1) Enterococcus (1) *E. coli* (1)
24	45 (98)	Enterococcus (1)	45 (98)	Enterococcus (1)		Enterococcus (1)
48	41 (89)	*Eikenella corrodens* (1) *P. mirabilis* (1) Enterococcus (1) Group F streptococcus (2)	43 (93)	*Eikenella corrodens* (1) Group F streptococcus (2)		*Eikenella corrodens* (1) Group F streptococcus (2)

[a] Total number of strains isolated was 46.

chemical means, a gassed-out vial or tube with or without prereduced saline or medium (5, 7-9, 11, 12, 14, 15, 17, 18), and a swab in gassed-out tubes (12). Although a number of these systems have been well studied using stock cultures (1, 2, 3, 5, 6, 20, 21), the oxygen tolerance and growth of anaerobic pathogens within purulent exudate, which usually contains a complex mixture of bacterial species, almost certainly differ substantially from those properties of pure cultures in artificial media. Published studies of anaerobic transport systems using clinical specimens from different types of anaerobic infections are nonexistent.

This study evaluated the ability of three of the most widely used transport systems to maintain viability of all types of bacteria in specimens from a wide variety of major anaerobic infections (Table 1). All three systems gave excellent results, affording 98 to 100% recovery of anaerobes as well as excellent recovery of facultative and aerobic bacteria at 24 h, with little change in concentration (Tables 4 to 6). Only 1 of 75 anaerobic strains was not recovered at 24 h and, in that instance, it was lost only in the SGT. This strain may have lost viability, or our inability to recover it may have been due to technical error or limitations of the experimental methods. After 48 h, the FGT was clearly superior to the SCB and SGT in maintaining the viability of anaerobes. Only 2 strains (a *Bacteroides* sp. and a *Lactobacillus* sp.) were not recovered in FGT, as compared with 6 in SCB and 11 in the SGT.

It was apparent that certain facultative organisms such as enterococcus and some strains of *Enterobacteriaceae* grew significantly (>1 log) in all three systems. This occurred most frequently in the SGT and SCB, in which five and four strains multiplied, respectively; only one aerobic strain grew in the FGT over 24 h. Three facultative strains were lost in 48 h in all three systems, one of *Eikenella corrodens* and two of group F streptococcus.

Of the three systems, the FGT was the easiest to use, because the specimen could be directly streaked on plates. Swabs generally must first be immersed in broth and shaken to elute and disperse microorganisms. Only about 3 to 5% of the bacteria from a clinical specimen on a swab are recovered when the swab is inoculated directly onto solid medium (6). The use of a Vortex mixer to drive organisms into the broth results in an approximately 78% recovery (21).

Anaerobic cultures of specimens collected with swabs are widely regarded as inferior to those collected by needle aspiration (7, 9, 14, 18). Hallander et al. (11), in a study of peritonsillar abscesses, reported a wide disparity between anaerobes isolated from specimens collected with a needle and syringe as opposed to those collected with swabs. It was found that the use of swabs resulted in significant contamination and overgrowth by pharyngeal flora. All of our specimens were collected by needle aspiration, and the problem of contamination by normal flora was successfully obviated, considering the paucity of anaerobic commensals such as *Propionibacterium* sp. that were recovered. However, we also were unable to assess the effect of contamination in various transport systems.

Recently Bartlett et al. (4) studied the viabil-

ity of anaerobic bacteria within clinical specimens after exposure to atmospheric oxygen at room temperature for varying periods up to 24 h. They found that most anaerobes survived relatively well in purulent exudate, although 4 (15%) of 26 strains could not be recovered at 24 h (3 strains of fusobacteria and 1 of *Peptostreptococcus*). In our study only 1 (1%) of 75 anaerobic strains was lost over this time period in SGT, and none was lost (0%) in the FGT and SCB systems. Laboratory evaluations of anaerobic transport systems that used stock cultures generally found heavy die-off of anaerobes in controls exposed to ambient air for 4 to 24 h, and much less in transport systems that were designed to maintain anaerobiosis (1, 3, 5, 6, 15, 20, 21). And Hallander et al. (11), studying specimens from peritonsillar abscesses, encountered marked loss of anaerobic strains in two aerobic transport media, but excellent recovery in evacuated syringes and in gassed-out tubes containing prereduced salt solution. Thus, although brief exposure of clinical specimens to room air probably does not significantly compromise isolation of many anaerobic pathogens, the use of an anaerobic transport system as FGT clearly affords enhanced and maximal recovery.

We elected to study recovery of bacteria at 48 h. Most laboratories obviously do not encounter such a prolonged delay before processing specimens, but such information is valuable to laboratories such as ours, which process a large number of mailed specimens, both primary clinical cultures and isolates for identification. Virtually all limitations of the three transport systems became apparent after 24 h.

In conclusion, this study with clinical specimens found that the FGT was clearly superior to the other two transport methods evaluated when a prolonged delay occurs before specimens are cultured for anaerobic bacteria. Anaerobic strains remained viable more uniformly and for longer periods, up to 48 h, and overgrowth by facultative bacteria was minimal. Obtaining specimens by aspiration implicitly circumvents contamination by normal flora, which is common when specimens are collected with swabs. Furthermore, gassed-out tubes or vials are simple to use, economical, and relatively safe. We believe the aspirated specimen in a gassed-out tube or vial should be considered as the preferred method for transporting fluid specimens for anaerobic culture.

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