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Preparing Inoculum for Susceptibility Testing of Anaerobes

JANA M. SWENSON* AND CLYDE THORNSBERRY

Antimicrobics and Infection Mechanisms Branch, Hospital Infections Program, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

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We studied six methods for preparation of inoculum to use in susceptibility tests of anaerobic bacteria to determine viable counts of 15 different species of anaerobes. We counted viable bacteria for each method-organism combination. Methods studied included those used for the more routine tests (broth microdilution and broth-disk elution) and for the National Committee for Clinical Laboratory Standards standard reference agar method. Inocula prepared from broth suspensions of organisms from 24- and 48-h anaerobe blood agar plates and adjusted to the turbidity of a 0.5 McFarland standard gave the most consistent counts for all organisms and also the highest numbers. Counts from these suspensions were higher than those from overnight growth in thioglycolate or Schaedler broth when all were adjusted against the same turbidity standard. Preparing inoculum directly from agar plates may also speed up the sometimes lengthy process of susceptibility testing of anaerobes and thus make results more clinically useful.

The only standard method for testing the susceptibility of anaerobic bacteria is the reference agar dilution method developed by the National Committee for Clinical Laboratory Standards (NCCLS) (6), but it is not usually used for routine tests. Procedures used routinely for anaerobe susceptibility testing include several broth-disk elution methods (3, 4, 13) and broth microdilution (7, 8, 12), a method now being used more often for testing anaerobes. Jones et al. (2) determined that susceptibility testing of anaerobes can be reliably performed by broth microdilution with supplemented brain heart infusion or Wilkins-Chalgren broth, since it compares favorably to the NCCLS reference agar procedure. Rosenblatt et al. (7) showed that broth microdilution procedures with many of the commonly employed anaerobe broths and the Kurzynski thioglycolate (THIO) broth disk elution procedure (4) compared favorably with the reference agar procedure. Although these methods have been shown to give comparable results, variables such as inoculum preparation within each of the procedures have not been standardized, and, unfortunately, most of these methods, which are summarized in Table 1, differ in the procedure used for preparing the inoculum.

To investigate the effects of various inoculum preparations and to confirm our belief that suspensions prepared directly from growth on an agar surface give viable counts comparable to other methods used more routinely for anaerobe inoculum preparation, we chose to compare some of the different methods that have been used. The differences in the methods occur not only in the kind of medium used but also in the way the inocula are prepared and adjusted. Overnight broth cultures are the most frequently used inocula in anaerobe susceptibility tests. We chose to study inoculum prepared from suspensions of growth from an agar plate because it had the advantage of saving time, the plates could be examined for purity, and we had shown that this method works well for facultatively anaerobic organisms (1). To determine whether differences might exist between these methods for anaerobes, we compared several of them by performing colony counts on the inoculum used in the various tests.

* Corresponding author.

MATERIALS AND METHODS

The strains of organisms tested were selected from our frozen culture collection to include a representative of those species often associated with infection, some of which also might be considered poor growers. Some of them are organisms recommended for quality control of anaerobe susceptibility tests by the NCCLS (6). One strain of each of the following was used in the study (the American Type Culture Collection number is given if applicable): *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, *Bacteroides vulgatus* ATCC 29327, *Bacteroides distasonis*, *Bacteroides asacharolyticus*, *Clostridium perfringens* ATCC 13124, *Clostridium ramosum*, *Peptococcus magnus* ATCC 29328, *Peptococcus variabilis* ATCC 14956, *Peptococcus asacharolyticus* ATCC 29743, *Peptostreptococcus anaerobius*, *Fusobacterium nucleatum*, *Fusobacterium mortiferum*, *Propionibacterium acnes*, and *Eubacterium lentum*. After the organisms were removed from frozen storage, they were thawed, inoculated into chopped meat broth (CM), and kept at room temperature in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.). For all tests, the isolates were subcultured twice from storage before use.

Six different procedures or modifications of procedures for preparing inoculum were studied (Table 1). They were used in one or more of the methods listed in Table 1 and are as follows: overnight growth in THIO (BBL Microbiology Systems, Cockeysville, Md.) enriched with 5 μg of hemin per ml, 0.1 μg of vitamin K_1 per ml, and 1 μg of sodium bicarbonate per ml; overnight growth in CM (Nolan Biological Labs, Inc., Atlanta, Ga.); overnight growth in chopped meat-glucose broth (CMG) (Nolan Biological); 18- to 20-h growth in Schaedler broth (SCH) (BBL) supplemented with 0.1 μg of vitamin K_1 per ml; and suspensions made from 24- and 48-h Centers for Disease Control anaerobe blood agar plates (BAP24 and BAP48) (Nolan Biological). All media were reduced in an anaerobic chamber for at least 4 h before inoculation.

The broth media were inoculated in room air from a BAP48 as described in the NCCLS anaerobe standard (6). All broth cultures were vortexed well inside the anaerobic chamber before being incubated. The BAP24 and BAP48 suspensions were made in 5 ml of freshly reduced brucella

TABLE 1. Methods for preparing inoculum for anaerobe susceptibility tests

Method	Medium	Adjustment	Final inoculum (CFU)	Reference
Agar dilution				
NCCLS	THIO (overnight)	McFarland[a] (0.5)	10^5 per spot	6
Broth microdilution				
Rotilie et al.	SCH (18 h)	Dilution[b] (1:10–1:100)	10^5–10^7/ml	8
Thornsberry-Swenson	BAP (24–48 h)	McFarland[c] (0.5)	10^5/ml	12
Rosenblatt et al.	THIO or SCH (24–36 h)	McFarland (0.5)	10^5/ml	7
Jones et al.	SCH (overnight)	Dilution (1:100)	5×10^5/ml	2
Broth-disk elution or category				
Wilkins-Thiel	CM (overnight)	BHI[d] (1 drop/5 ml)	10^6–10^7/ml	13
Kurzynski et al.	CMG (overnight)	BHI (2 drops/5 ml)	NG[e]	4
Jorgensen et al.	SCH (overnight)	SCH (1 drop/4 ml)	NG	3
Stalons-Thornsberry	SCH (overnight)	Spectrophotometric	10^6/ml	10

[a] Achieved by adjusting the broth to equal the turbidity of a 0.5 McFarland standard.
[b] Depending on subjective determination of turbidity.
[c] Achieved by suspending the growth from agar plate to equal the turbidity of a 0.5 McFarland standard.
[d] BHI, Brain heart infusion.
[e] NG, Not given.

broth or SCH with sterile cotton swabs. Cultures of the overnight THIO and SCH and the suspensions made from the BAP24 and BAP48 were adjusted by diluting with brucella broth or SCH to match a 0.5 McFarland standard. For the CM and CMG, adjustment was made by adding 1 drop for CM and 2 drops for CMG with a Pasteur pipette to 5 ml of brain heart infusion broth.

For all the counts, an initial 1:100 dilution was made by adding 0.1 ml of culture to 9.9 ml of phosphate-buffered gelatin diluent (pH 7.0); further 1:10 dilutions were made by adding 1.1 ml of diluted culture to 9.9 ml of diluent. Appropriate dilutions of the culture were subcultured by transferring 0.1 ml to a BAP. All manipulations of broth cultures, suspension of the BAP24 and BAP48, and the dilutions were done inside the anaerobic chamber. Plating of the diluted cultures was done in room air. Each method was tested in triplicate for each of the organisms. To assure maximum survival of organisms, all plates were reduced in an anaerobic chamber and kept in a holding jar immediately after being inoculated. Inoculated plates were returned to the anaerobic chamber within 15 min for incubation at 35°C, and individual colonies were counted after 3 to 5 days.

RESULTS

The geometric mean of the triplicate values was determined for each of the organism-method combinations in Table 2. When any of the triplicate counts varied by more than 1 $\log_{10}$ step, that organism-method combination was repeated again in triplicate until the results were within a reasonable range of each other (usually within 1/2 $\log_{10}$ step of each other). Those combinations requiring repeat testing were *P. asacharolyticus*-THIO, *P. magnus*-CM, *C. ramosum*-CM, *F. nucleatum*-CMG, *C. ramosum*-CMG, *B. fragilis*-SCH, *B. vulgatus*-SCH, *B. asacharolyticus*-SCH, *E. lentum*-SCH, and *C. ramosum*-SCH. None of the counts

TABLE 2. Geometric means of viable counts for inoculum prepared by six different methods for 15 anaerobes

	BAP24[a]	BAP48[a]	THIO[b]	SCH[b]	CM[c]	CMG[c]
Bacteroides fragilis	4.9×10^8	5.4×10^8	1.2×10^8	1.1×10^8	1.6×10^7	4.2×10^7
Bacteroides thetaiotaomicron	2.4×10^8	3.7×10^8	8.8×10^7	7.4×10^7	2.0×10^7	5.6×10^7
Bacteroides vulgatus	1.9×10^8	1.3×10^8	3.9×10^7	2.4×10^7	1.3×10^6	2.4×10^7
Bacteroides distasonis	1.3×10^8	2.0×10^8	6.2×10^7	1.3×10^7	8.4×10^6	4.0×10^7
Bacteroides asacharolyticus	1.3×10^8	3.0×10^8	1.5×10^8	1.3×10^8	1.7×10^7	1.2×10^7
Peptococcus magnus	2.7×10^7	1.4×10^7	3.3×10^6	1.0×10^7	1.6×10^6	3.2×10^6
Peptococcus variabilis	1.8×10^7	1.3×10^7	3.5×10^6	1.4×10^7	9.6×10^5	8.4×10^6
Peptococcus asacharolyticus	NS[d]	7.5×10^7	7.5×10^7	1.5×10^7	5.0×10^5	5.1×10^5
Peptostreptococcus anaerobius	1.6×10^7	9.5×10^6	1.8×10^7	4.4×10^6	1.7×10^6	2.5×10^6
Fusobacterium nucleatum	NS	8.9×10^7	2.9×10^7	5.7×10^7	1.8×10^6	2.6×10^6
Fusobacterium mortiferum	3.5×10^7	7.0×10^7	3.1×10^7	6.8×10^7	1.5×10^6	1.6×10^7
Propionibacterium acnes	NS	9.0×10^7	4.4×10^7	4.3×10^7	2.9×10^6	1.9×10^6
Eubacterium lentum	5.2×10^8	2.9×10^8	3.5×10^7	–[e]	2.1×10^7	3.8×10^7
Clostridium perfringens	2.2×10^7	3.6×10^7	1.9×10^7	2.0×10^7	1.3×10^6	5.4×10^6
Clostridium ramosum	1.7×10^8	1.7×10^8	9.3×10^6	4.2×10^4	1.1×10^5	8.6×10^6

[a] Inoculum prepared by suspending growth from a BAP24 or BAP48 and adjusting against a 0.5 McFarland turbidity standard.
[b] Inoculum prepared from overnight growth in THIO or SCH adjusted against a 0.5 McFarland.
[c] Inoculum prepared by adding 1 and 2 drops, respectively, of CM and CMG to 5 ml of brain heart infusion broth.
[d] NS, Growth not sufficient to prepare suspension.
[e] –, Counts too variable to include.

done from BAP suspensions required repeat testing because of variability within the triplicate values. One organism-method combination (*E. lentum*-SCH) failed to give results within a reasonable range after several repeat testings. Results for that combination are not given in Table 2 or Fig. 1.

The triplicate counts for 3 of the 86 organism-method combinations were slightly greater than 1/2 $\log_{10}$ step apart (*B. thetaiotaomicron*-SCH, *F. nucleatum*-THIO, and *P. acnes*-SCH), but all others were less than 1/2 $\log_{10}$ step apart. The data in Table 2 show that the number of viable cells in these inocula depended on the species of anaerobe and the method. The number of gram-positive cells was generally lower than the number of gram-negative cells. The inocula prepared from BAP tended to have more organisms; those prepared from chopped meat, with and without glucose, contained fewer.

To better show the differences between the methods, we plotted the geometric means on logarithm graphs (Fig. 1 and 2). In the figures, the organisms were grouped in four groups as follows: *Bacteroides* spp.; gram-positive cocci; *Fusobacterium* spp., *P. acnes*, *E. lentum*, and *C. ramosum*; and *C. perfringens*. Figure 1 includes the methods in which turbidity was adjusted by a 0.5 McFarland standard. Figure 2 shows the two procedures in which the turbidity was not adjusted to match a standard, but instead the inoculum was prepared by using drops of overnight broth into a second broth.

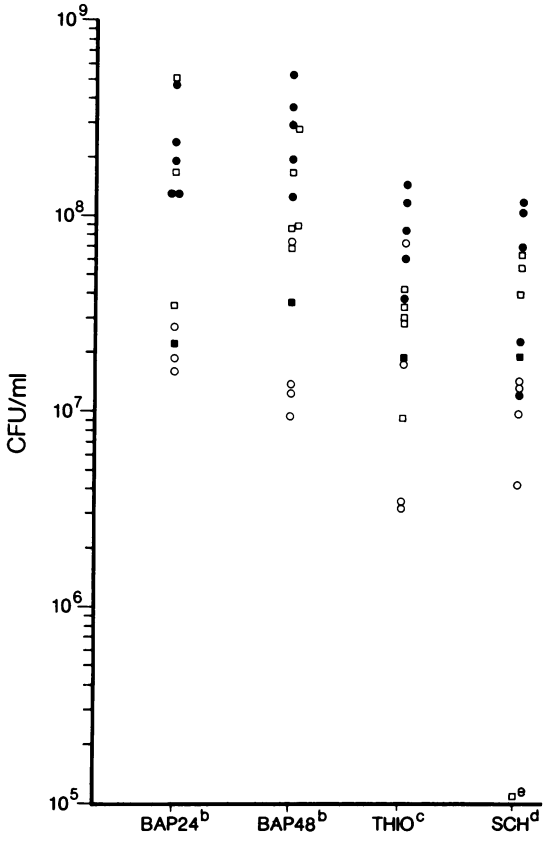

FIG. 1. Geometric means of viable counts for 15 different species of anaerobic bacteria obtained by four methods of inoculum preparation with visual adjustment of turbidity against a 0.5 McFarland standard. Symbols: ●, *Bacteroides* spp.; ○, gram-positive cocci; □, *Fusobacterium* spp., *P. acnes*, *E. lentum*, and *C. ramosum*; ■, *C. perfringens*. (b) Suspensions made from 24- to 48-h growth on agar and adjusted to equal a 0.5 McFarland standard. Three organisms (*P. asacharolyticus*, *F. nucleatum*, and *P. acnes*) did not grow well enough at 24 h to prepare suspensions. (c) Overnight growth in enriched THIO adjusted to equal a 0.5 McFarland standard. (d) Overnight growth in enriched SCH adjusted to equal a 0.5 McFarland standard. Results for *E. lentum* are not included because of too much variability. (e) A total of 4.2×10^4 CFU/ml (*C. ramosum*).

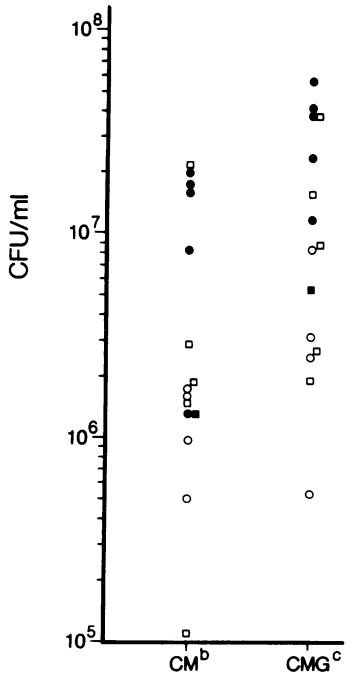

FIG. 2. Geometric means of viable counts for 15 different species (see the legend to Fig. 1) of anaerobic bacteria prepared by direct inoculation of overnight cultures into broth. (b) One drop of overnight CM culture into 5 ml of brain heart infusion broth. (c) Two drops of overnight CMG culture into 5 ml of brain heart infusion broth.

The inoculum intended for use in the dilution tests (BAP24 or BAP48, THIO, and SCH) is meant to be diluted in some fashion to reach a predetermined final concentration of organisms, and, consequently, manipulation of the culture to achieve that concentration of organisms would vary by the system or method used. The inoculum intended for use in the broth-disk elution procedures (CM and CMG) is meant to be used directly in the tests, and no further dilution is called for.

For those methods with adjustments against a standard (Fig. 1), counts were slightly higher for the suspensions made directly from an agar plate when compared with counts made from overnight THIO and SCH. Counts from overnight CMG were slightly higher in general than those from overnight CM (Fig. 2), a difference that might be explained in part by the use of 2 drops (CMG) instead of 1 drop (CM) into 5 ml of broth, but for several organisms there was about a 1 $\log_{10}$ increase in CFU in counts from CMG over CM (*B. vulgatus*, *P. variabilis*, and *F. mortiferum*).

All counts were consistently higher for the *Bacteroides* spp. than for the gram-positive cocci and the *C. perfringens*. The other strains varied by method with the most striking difference occurring with *C. ramosum* in SCH. For that organism, counts done from the SCH varied by more than 3 $\log_{10}$ steps from those done from the BAP48 suspension.

DISCUSSION

The methods for preparing inoculum for aerobic and facultatively anaerobic organisms have varied little for different susceptibility methods, probably because most of these organisms grow rapidly and well enough in Mueller-Hinton or Trypticase soy broth in a reasonable time to be used for testing. Unfortunately, this has not been true for obligate anaerobes. Stalons et al. showed that the time required to reach stationary phase in SCH varied from 6 h for *C. perfringens* to 12 h for *B. fragilis* to more than 36 h for *Eubacterium alactolyticum* (11). The number of CFU per milliliter at stationary phase also varied considerably by organism in their study.

In our study of these different methods for preparing inoculum, we found the BAP24 and BAP48 methods to have certain advantages. By preparing inoculum directly from a 24-h agar plate, tests could be set up sooner than if a broth had to be inoculated and incubated. With that method, the purity of the culture could be confirmed at the same time the test was set up. Cultures prepared from growth on an agar plate gave the highest numbers of viable organisms and also showed the most consistency within triplicate values. This was also true for a 24-h culture if growth was adequate; three strains did not have sufficient growth in 24 h to prepare a suspension, but they did have sufficient growth at 48 h.

Although the counts done from the overnight THIO and SCH showed viable numbers to be slightly less in general than those from suspensions from BAP, they appear to be adequate, except for *C. ramosum* and *E. lentum* in SCH. For these two species, counts were also less variable in THIO than in SCH. A possible explanation for the failure of SCH to give uniformly consistent results that necessitated repeat testing of some of the strains (see above) and for the lowered counts for the *C. ramosum* strain may be the relatively high glucose content of SCH, which results in more acid that could cause faster death of organisms and possibly aberrant forms.

There appears to be slightly more variability in the inoculum prepared in CM and CMG (Fig. 2) that are used in the broth-disk elution procedures (4, 13) than in that used for the other procedures. This is particularly important since in these procedures there is no final adjustment of the turbidity to that of a standard. Viable counts for some of the *Bacteroides* spp. in CMG showed the number of CFU to approach 10^8/ml, which in our opinion is too high for use as a final inoculum in susceptibility tests. Perhaps using 1 drop when a known *Bacteroides* sp. is being tested instead of 2 drops would eliminate or alleviate the potential problem of increased inoculum. Since the inoculum preparation used for the other methods is intended to be diluted, depending on the procedure used, the number of organisms in the inoculum can be controlled for dilution tests by visual adjustment of turbidity or further dilution.

Several investigators have concluded from their studies that measuring turbidity nephelometrically is not an accurate way to estimate CFU of anaerobes in broth culture and have reported that different organisms have different counts when turbidity measurements are equal (9, 11). Our data tend to support this conclusion. If one is aware, however, that counts for gram-positive cocci and the *C. perfringens* are about 1 $\log_{10}$ step lower than *Bacteroides* spp. when adjusted to the same standard, our study suggests that CFU per milliliter could be estimated with the 0.5 McFarland standard, since, for the suspensions made from the BAP48, counts for the organisms other than the gram-positive cocci and the *C. perfringens* were within the range of 7×10^7 to 5×10^8 CFU/ml.

The optimal number of organisms in the final inoculum to be used in anaerobe susceptibility tests is a point still in question, but the NCCLS recommends 10^5 CFU per spot for the reference agar dilution test (6), 10-fold higher than the inoculum recommended for agar dilution tests on aerobic bacteria (5). If the same recommendation is made for inocula to be used in broth dilution tests, the final inoculum would be 10^6 CFU/ml instead of 10^5 CFU/ml, as is used in broth dilution tests for aerobic bacteria. We have found, however, that using this inoculum does create some problems. If one assumes that a culture adjusted to the 0.5 McFarland turbidity standard contains ca. 10^8 CFU/ml and then dilutes the culture to have a final inoculum of 10^6 CFU/ml after inoculation into broth microdilution plates, endpoints for some organisms are very difficult to read because of trailing (unpublished data). We know from our data that *Bacteroides* spp. may approach viable counts of 10^9 CFU/ml when a suspension from a BAP is adjusted visually against a 0.5 McFarland turbidity standard, so the final inoculum could approach 10^7 CFU/ml instead of the intended 10^6 CFU/ml. This increase might explain the trailing endpoints we sometimes see. A possible solution to this problem would be to further dilute the initial suspensions of organisms other than the gram-positive cocci 1:5 for use in the broth microdilution procedure. Although this dilution would result in lowered counts for some organisms (i.e., *F. mortiferum*), the initial inoculum concentration for most of the organisms would be about the same as would be achieved with THIO in the reference agar method.

Of the methods studied, we prefer the BAP suspension for several reasons. Most clinically significant anaerobes (*Bacteroides* spp. and *Clostridium* spp.) will grow enough in 24 h to be tested by this method. The consistency was best with the BAP; i.e., no organism-method combination had to be retested because of variability in the triplicate counts. The numbers of CFU were higher, ranging from 1.6×10^7 to 5.2×10^8 for BAP24. In general, the gram-positive cocci had the lowest counts, and the gram-negative rods had the highest.

In summary, we have investigated the preparation of inocula for anaerobe susceptibility tests from various media with 15 species of anaerobic bacteria. Counts prepared from suspensions of bacteria taken from 24- or 48-h agar cultures were less variable and higher than counts for other media. Counts from CM or CMG were consistently lower but were not intended to be further diluted. THIO and SCH cultures yielded adequate counts, in general, but THIO was generally more consistent. We prefer the use of inocula prepared from suspensions of organisms of 24- or 48-h agar cultures for susceptibility testing of anaerobic bacteria. However, because of the higher numbers of organisms, the initial inoculum may need to be diluted when suspensions are made from BAP. This dilution is not necessary when overnight thioglycolate cultures are used as inoculum.

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